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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Can *Helicobacter pylori* infection influence human reproduction?

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Abstract

Helicobacter pylori (*H. pylori*) infection could be associated with extra-digestive diseases. Here, we report the evidences concerning the decrease in reproductive potential occurring in individuals infected by *H. pylori*, especially by strains expressing CagA. This infection is more prevalent in individuals with fertility disorders. Infected women have anti-*H. pylori* antibodies in cervical mucus and follicular fluid that may decrease sperm motility and cross react immunologically with spermatozoa, conceivably hampering the oocyte/sperm fusion. Infection by CagA positive organisms enhances the risk of preeclampsia, which is a main cause of foetus death. These findings are supported by the results of experimental infections of pregnant mice, which may cause reabsorption of a high number of foetuses and alter the balance between Th1 and Th2 cell response. Infected men have decreased sperm motility, viability and numbers of normally shaped sperm and augmented systemic levels of inflammatory cytokines, such as TNF-α, which may damage spermatozoa. In countries where parasitic infestation is endemic, detrimental effects of infection upon spermatozoa may not occur, because the immune response to parasites could determine a switch from a predominant Th1 type to Th2 type lymphocytes, with production of anti-inflammatory cytokines. In conclusion, the evidences gathered until now should be taken into consideration for future studies aiming to explore the possible role of *H. pylori* infection on human reproduction.

Key words: Antigenic mimicry; *Helicobacter pylori* infection; Human sperm; Inflammatory cytokines; Preeclampsia; Reproductive disorders

Core tip: The evidences that *Helicobacter pylori* (*H. pylori*) infection may have a role in decreasing human reproductive potential are steadily increasing. Sperm quality of men infected by *H. pylori* strains expressing CagA is reduced. Infected women have specific antibodies in cervical mucus, which decrease sperm motility, as well as in follicular fluids, which may cross react with sperm. In women with polycystic ovary syndrome and preeclampsia the prevalence of *H. pylori* infection is increased. The putative pathogenic mechanisms that account for these observations include elevated inflammatory cytokine levels in infected individuals and phenomena of antigenic mimicry between bacterial antigens and human proteins.

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INTRODUCTION

The bacterium *Helicobacter pylori* (*H. pylori*), a microaerophilic, Gram-negative and spiral-shaped organism, is a member and the type species of the genus *Helicobacter* and is specialised to infect humans at the level of the gastroduodenal tract[1]. Despite its small genome of only 1667 kb, its restricted niche and the expression of a small number of metabolic activities, *H. pylori* has been capable of setting up an efficacious adaptive evolutionary machinery due to the possession of particular virulence determinants[2].

The infection is commonly acquired during the infancy and the first contact of susceptible individuals with this organism results in an acute gastritis[3]. In most cases, the infection becomes chronic and lasts throughout the entire patients' life. Bacterial factors, together with a remarkable local and systemic immune response, are substantial determinants of peptic ulceration and important factors for the development of gastric tumours (carcinoma and non-Hodgkin's lymphoma associated with mucosal lymphoid tissue)[4-7]. Not all strains have the same virulence; those harbouring the insertion named *cag* pathogenicity island in their chromosome are endowed with an increased inflammatory and carcinogenetic potential[8]. The terminal gene of this insertion, the *cagA* gene, encodes for an immunodominant protein called CagA, which is considered a marker for the presence of *cag* in the organisms and that induces the production of serum antibodies detectable by simple serological tests.

The possible outcomes of *H. pylori* infection are numerous because they may not be restricted to the gastroduodenal tract. In the nineties, epidemiological studies suggested that *H. pylori* infection might be associated with extra-digestive diseases that may affect heart and vessels; soon afterwards, the list of disorders related to this infection steadily increased and now encompass skin, oropharynx and various systems, such as the endocrine, respiratory, haemopoietic, immune and central nervous systems *etc.*[9,10]. If we exclude iron deficiency anaemia and idiopathic thrombocytopenic purpura, for which the results of different studies are consistent, most investigations on associated diseases have been given contradictory results. It is however possible that many diseases escape this association because their signs and symptoms are overlooked most of the time[9,10]. The most common extra-digestive diseases associated with *H. pylori* infection are reported in Table 1. Rising evidences in the literature suggest that even the reproductive sphere seems to be negatively influenced by *H. pylori* infectious status. Thus, the aim of the present mini-review is to deal with the possible role of *H. pylori* infection in reducing the reproductive potential of both women and men.

H. PYLORI INFECTION AND FEMALE REPRODUCTION

The hypothesis that *H. pylori* infection might increase the risk of infertility in women was first considered by our group in 2002, when we reported an increased prevalence of *H. pylori* infection in female patients with fertility disorders respect to controls (44.8% *vs* 29.7%, $P = 0.033$)[11]. After a few years, such observation was supported by a Japanese retrospective survey including 204 female patients[12]; in this study, seropositivity for *H. pylori* infection in the group of women with idiopathic infertility was twice higher than in patients with one or more known causes of infertility (38.09% *vs* 20.2% respectively, OR = 2.16). In addition, the majority of infected patients had antibodies to *H. pylori* at significant titres in the follicular fluids, supporting our previous observation that specific antibodies were detected in all the six analyzed follicular fluids from infected women (and in no samples from five uninfected patients)[11]. Recently, anti-*H. pylori* antibodies have been detected in the cervical mucus of infected women with unexplained infertility[13] and these antibodies inhibited in vitro the sperm progression. It is likely that the presence of specific antibodies in the different districts of the female genital apparatus may have pathogenetic meaning, as they could interfere with sperm motility and sperm capacitation, a pivotal step required for acquiring fertilization ability. This immune cross reactivity could be explained on the basis of an antigenic mimicry between sperm and *H. pylori* antigens since we have shown the existence of a partial linear homology between *H. pylori* peptides and tubulin, an important constituent of sperm flagella[11], and some enzymes of glycolytic process and Krebs cycle[14], all putatively involved in sperm motility.

A further possible mechanism that may interfere with fertility could be based on an observation made by Kellerman and Hunter[15], who identified a receptor on the vitelline membrane of bovine oocytes that recognized the Fc fragment of immunoglobulin G; these authors hypothesize that the adhesion of IgG to this receptor could play a role in sperm/egg interaction. As follicular fluids of infected women contain antibodies against *H. pylori*, we may hypothesize that the surface of oocytes could bind IgG through the Fc fragments impairing the interaction of egg with spermatozoa; alternatively, the free Fab fragments of adhered IgG might react immunologically with spermatozoa, thus hampering fertilization. This immune reaction could take place for a putative mechanism of antigenic mimicry, as we showed that antibodies obtained by immunizing animals with *H. pylori* or present in serum samples and other fluids of infected individuals could cross-react with spermatozoa[11].

Even though the number of investigations is scanty, studies showing a role of *H. pylori* in the development of endocrinopathies[16] suggest that this infection may influence reproduction. One of the most common endocrine disorders causing infertility is polycystic ovary syndrome (PCOS) and recently, Yavasoglu *et al*[17] reported an increase prevalence of *H. pylori* seropositivity in women suffering from PCOS respect to controls (40% *vs* 20%, $P = 0.007$).

The interference of *H. pylori* infection with human

Table 1 Most common extra-digestive diseases and syndromes associated with *Helicobacter pylori* infection

Organ, region, system	Association
Haemopoietic system[51,52]	Iron deficiency anemia, idiopathic thrombocytopenic purpura, extra-gastric mucosal lymphoid tissue lymphoma
Cardiovascular or vascular system[53-55]	Atherosclerosis (coronary heart disease, strock, aneurism), migraine, idiopathic arrhythmia
Endocrine system[56,57]	Autoimmune thyroid disorders, resistance to insulin/diabetes, post-prandial hypoglycemia
Respiratory system[57-59]	Chronic obstructive pulmonary disease, bronchiectasis, asthma, chronic otitis media with effusion
Skin[60-64]	Rosacea, primitive chronic urticaria, pruritus cutaneus, Sweet's syndrome, angioedema, alopecia areata, prurigo nodularis, lichen planus
Eye[65,66]	Glaucoma, blepharitis
Rheumatologic diseases[67-70]	Systemic sclerosis, Raynaud's syndrome, Sjögren syndrome, Behçet disease
Central nervous system[71-74]	Parkinson's and Alzheimer's disease, Guillain-Barre syndrome, hepatic encephalopathy
Reproductive system[11-21]	Reduced fertility
Miscellanea[75-78]	Sudden infant death syndrome, hyperemesis gravidarum, halitosis, growth delay

reproduction may also occur after oocyte fertilization, especially in case of preeclampsia (PE), one of the main causes of foetal (and maternal) mortality and morbidity. PE affects 2%-7% of pregnant women in the western world and much more in underdeveloped nations; it is characterized by high blood pressure and significant proteinuria in previously healthy women, generally after the 20th wk of gestation[18]; coagulative disorders are also very common. Despite the exact pathogenic mechanisms of PE are still obscure, some researchers have hypothesized that this syndrome may be caused by infectious agents[19].

Following the observation that *H. pylori* infection may cause fetal intrauterine growth restriction among Australian women suffering from PE[20], Ponzetto *et al*[21] examined 47 consecutive women with PE for *H. pylori* infection and anti-CagA serum antibodies and observed an increased prevalence of the infection in patients respect to that in women with uncomplicated pregnancy enrolled as controls (51.0% *vs* 31.9%, $P = 0.033$, OR = 2.6). Interestingly enough, the vast majority of patients had serum antibodies to the CagA protein, while only a few controls were CagA seropositive ($P < 0.001$, OR = 26.0). The presence of *H. pylori* DNA was also explored in placenta samples by PCR, but in no case it was positive. The association of PE with *H. pylori* infection was confirmed by a Turkish study[22] whose results showed increased TNF-α and CRP levels in the blood of infected patients respect to controls (normotensive pregnant women); the CagA status was not verified. Later on, Cardaropoli *et al*[23] observed that CagA positive *H. pylori* infection exclusively caused or contributed to PE complicated by foetal growth retardation (FGR), but not to PE without foeto-placental impairment. The results of their study prompted them to consider PE complicated by FGR and "pure FGR" as different pathologies and to classify PE as placental (with feto-placental involvement) or maternal (without feto-placental impairment), both with early or late onsets.

Now, it is well known that PE is a disease in which the systemic indices of inflammation are increased, together with elevated blood pressure and significant proteinuria[24]. The reasons of biological plausibility that link *H. pylori* infection to PE development are all fulfilled: acute phase reactants, such as CRP and polymorphs, are increased either in *H. pylori* infection and PE[22]; similarly, levels of pro-inflammatory cytokines, such as IL-6, macrophage migration inhibitory factor, TNF-α *etc*., and indices of a pro-coagulative status are augmented in both diseases[24-28]. Due to these observations, it is possible that PE can be associated with CagA positive *H. pylori* infection: strains expressing such immunodominant antigen are endowed with an increased inflammatory potential and infections caused by these organisms may contribute to the development of many digestive and extra-digestive diseases based on inflammation. Along infections by CagA positive *H. pylori* organisms, many vasoactive substances and cellular mediators, such as TNF-α and other cytokines, are produced locally in response to the infection and may diffuse in the bloodstream; in addition, chemokines may also be secreted systemically by immunocytes stimulated during the gastric circulation. All these phenomena may promote an inflammatory response in organs distant from the stomach.

The putative role of infection by *H. pylori* harbouring *cagA* in the development of PE has been suggested also by a recent study of Franceschi *et al*[29]. They started from a double order of observations: (1) endothelial dysfunctions may damage trophoblasts and are known to play a key role in the development of PE; and (2) anti-CagA antibodies may recognize immunologically antigens expressed by endothelial cells[30]. Since trophoblast cells have an endothelial origin, these researchers verified whether anti-CagA antibodies might also recognize antigens present on the surface of trophoblasts. This hypothesis was confirmed, as anti-CagA antibodies cross-reacted with β-actin of cytotrophoblast cells, whose invasiveness ability was diminished[29].

The risk of PE after intra-cytoplasmic sperm injection (ICSI) following oocyte donation is particularly high (up to 30%). Also the possibility of abortion is relatively increased after ICSI. The results of a recent Iranian study showed that precocious abortions after ICSI occurred far more frequently in women infected by CagA positive *H. pylori* strains[31]: 150 infertile women underwent ICSI and serological diagnosis of *H. pylori* infec-

tion; 58 patients were infected and 92 women were not. Interestingly, six out of seven infected women seropositive for CagA presented spontaneous abortion, *vs* only one out of 16 infected women seronegative for CagA ($P = 0.0004$, OR = 90.0, Fisher exact test). Despite this difference is highly significant, the infection by itself did not seem to affect the pregnancy rates: 23/58 infected women (39.6%) *vs* 27/92 uninfected patients (29.3%) became pregnant ($P = 0.26$).

The meaning of such observations is supported by the results of a study performed in animals experimentally infected. Female CD1 mice were infected before mating with a type I *H. pylori* strain (the cytotoxic and *cagA* positive *H. pylori* strain SPM326), isolated by one of our group (NF) from a patient with moderate, non-active, non-atrophic chronic gastritis. Afterwards, the animals were evaluated throughout the pregnancy for embryo/foetus characteristics and histopathological changes of the endometrium[32]. The endometrial lining of infected animals presented lymphocyte infiltration, epithelial transcytosis and cellular necrosis; the uterine glands showed loss of architecture and infiltration of inflammatory cells within the lamina propria. These pathological phenomena were accompanied by macrophage activation, increased endometrial infiltration of CD4+ and CD8+ lymphocytes and augmented expression of interferon-γ and major histocompatibility class Ⅱ complex. Syncytiotrophoblastic cells of infected mice showed a substantial decrease of annexin V levels. Even more important were the observations that infected mice showed significantly increased numbers of resorbed foetuses and that the foetal weights were lower than those of non-infected controls. It is well known that *H. pylori* infection induces preferentially a Th1-type cell response[33]. Thus, the prevalent hypothesis explaining these findings is that, in infected pregnant mice, the immune response triggered by *H. pylori* infection could modify the natural immunosuppressive mechanisms of pregnancy by altering the systemic Th1/Th2 cell balance. The increased foetal resorption and the reduced foetal weight observed in infected mice could be related to a diminished expression of annexin V in syncytiotrophoblastic cells, since the loss of this molecule has been reported to be associated with placental disorders, including foetal resorption[34]. Should the results of future clinical studies confirm these findings, it would be reasonable to determine the *H. pylori* and the CagA status of women that desire to get pregnant.

H. PYLORI INFECTION AND MALE REPRODUCTION

Spermatozoa are the only flagellated human cells; sperm flagella may therefore share a linear homology with the bacterial flagella because structures with the same function are generally conserved during evolution. This idea prompted us to investigate the possible role of *H. pylori* in determining alterations of the male reproductive sphere. We found that the prevalence of *H. pylori* infection in men with fertility problems was higher than in controls (50.8% *vs* 36.1%, $P = 0.003$, OR = 1.83); we also detected anti-*H. pylori* antibodies in seminal plasma of 58% of infected men and in no samples of 11 seronegative patients. Immunocytochemical studies highlighted that anti-*H. pylori* hyperimmune sera, as well as serum samples from infected men, reacted immunologically with the flagellum (particularly rich in tubulin) and the equatorial segment of human ejaculated spermatozoa[11]. In addition, a partial linear homology was observed between human tubulin, the main constituent of sperm flagellum, and *H. pylori* proteins (flagellin, CagA and VacA), suggesting that mechanisms of antigenic mimicry may stimulate cross-reactive antibodies.

Further investigations, carried out in a group of idiopathic infertile patients, showed that infected men, especially those with serum antibodies to CagA, showed reduced sperm motility and a greater number of necrotic and apoptotic sperm in their ejaculates[25]. Concomitantly, increased systemic levels of tumour necrosis factor-alpha (TNF-α), a proinflammatory cytokine that may cause sperm damage, were observed in the group of idiopathic infertile males infected by *H. pylori* strains expressing CagA.

The influence of *H. pylori* strains expressing CagA on semen quality was further explored in a very recent study in which we examined 87 infected males for the possible relationship between infection by CagA positive *H. pylori* strains and sperm parameters evaluated following WHO guidelines[35]. In the CagA positive subjects, sperm motility (18% *vs* 32%, $P < 0.01$), sperm vitality (35% *vs* 48%, $P < 0.01$) and the percentage of sperm with normal morphology (18% *vs* 22%, $P < 0.05$) were significantly reduced compared with those measured in the CagA negative infected subjects[14].

In addition we recently observed that this infection may influence the semen concentrations of some hormones such as ghrelin and obestatin, peptides encoded by the same gene, mainly produced by the stomach and involved in energy balance and reproduction[36]. The relationship between this hormone and *H. pylori* infection resides in the fact that most circulating ghrelin is produced in the gastric oxyntic area and that ghrelin levels may decrease in patients infected by strains expressing CagA as result of mucosal atrophy[37]. Recently, both peptides were detected in human semen[38,39] at increased concentrations respect to serum levels. Afterwards, we detected significantly augmented ghrelin levels in the semen of patients infected by *H. pylori* strains expressing CagA, and we considered this finding as a possible response to a negative effect of infection upon the semen quality[40]. Also obestatin semen concentrations were increased in this kind of patients, but in non-significant manner.

There are only a limited number of studies on this subject. Should the reported results be confirmed by conclusions of other investigations, we believe that physicians should consider the eventuality of routinely test-

ing men, who suffer from idiopathic fertility disorders, for *H. pylori* infection and the CagA status.

WHY IS THE BIRTH RATE STEADILY INCREASING IN THE DEVELOPING COUNTRIES? THE AFRICAN ENIGMA

Assuming that the infection increases the risk of infertility, why is the birth rate steadily increasing in the developing countries, where almost everyone is infected by *H. pylori*? The answer may be part of the paradox called "African enigma"[41]. Gastric *H. pylori* infection is common, almost ubiquitous in Africa, but the pattern of infection, age of acquisition, environmental, dietary, and genetic influences are different from those in West Countries. These different features may alter the pathological role and clinical relevance of the organism in Africa, where, apart from gastritis, there is no established correlation between *H. pylori* infection and upper gastrointestinal disease. These divergences could be applied to the possible consequences of infection upon the reproductive sphere in developing countries. Mitchell *et al*[42] provided evidence that the host immune response to *H. pylori* infection in an African population differs from that observed in subjects living in developed countries; for instance, IgG1/IgG2 predominant response was observed in 81% of Sowetan adults and 90% of children compared with 4.7% of Australians and 4.4% of Germans.

The most convincing explanation of such apparent paradox comes from the study of the cell immune response to *H. pylori* infection in Western population (who have a very low rate of parasitic infestation) and in developing countries (where parasitic infestation is very common). The results of these studies have shown that co-infestation with parasites might alter the expression of the infection and result in a change in the pattern and/or severity of gastritis and thus in the prevalence and clinical outcome of disease. One constant consequence of *H. pylori* infection is the production of reactive oxygen species (ROS) by immunocytes infiltrating the gastric mucosa and circulating in the blood stream. If the infecting strains contain the *cagA* gene, particularly high amounts of ROS are generated. In an Egyptian study, it was shown that patients with a concurrent *Schistosoma mansoni* infestation had a modified inflammatory response to gastric *H. pylori* infection while ROS were dramatically reduced. The authors concluded that co-infestation with helminths may have a protective effect against the possible progression of *H. pylori*-induced gastritis towards gastric carcinoma[43]. Due to the high prevalence of parasitic infestation in developing countries, differences with Western population may also be extended to the cell immune response to *H. pylori* infection. In Western population, *H. pylori* causes a predominant and prolonged Th1 type response that is unable to eliminate the organism, may damages the mucosa and lead to gastroduodenal disease and systemic, inflammatory-mediated involvement[44,45]. Data from Mitchell and colleagues have supported the hypothesis that a Th2 polarised response to *H. pylori* is more common in Africa while a Th1 polarised response is more common in Europe and Australia[42]. Such a particular behaviour is also common in underdeveloped Centre American Countries: the results of a study performed in Colombian children suggest that intestinal helminthiasis promotes Th2-polarizing responses to *H. pylori*, may reduce the mucosal damage caused by inflammation and therefore affect the progression of gastritis to gastric atrophy, dysplasia, and cancer[46]. Another study confirmed that in Africa *H. pylori* infection does not appear to elicit the range of host mucosal response commonly observed in developed countries: despite the high ratio of anti-CagA seropositivity among infected Gambian adults (93%), histological findings of gastric atrophy and intestinal metaplasia were very rare; in addition, there was no difference in mucosal response between CagA positive and CagA negative individuals[47].

The observation that helminths induce a more polarized Th2 response and theoretically would help decrease the histological lesions induced by *H. pylori* infection, has been confirmed in the mouse animal model: co-infestation with helminths and *H. felis* resulted in reduced epithelial damage and atrophy compared with animals infected exclusively with *H. felis*[48]; in particular, the Th-cell immune response switched from a predominantly Th1-type to a Th2-type response, which is characterized by the release of the non-inflammatory cytokines IL-4, IL-5 and IL-10[49,50]. In case of co-infestation with helminths, in areas where parasitosis is endemic, such as Africa and Central America, Th2 response may protect the gastric mucosa from the damage caused by the microorganisms and determine the releasing of anti-inflammatory cytokines, which might partially protect the gastric mucosa and concur to explain the African enigma[44]. These observations could also be applied to the reproductive sphere. In other terms, the production of anti-inflammatory cytokines and a predominant Th2 response, both triggered by parasitic infestation could also protect various organs and fluids, in addition to the gastric epithelium, from the damage caused by the presence of *H. pylori* organisms.

CONCLUSION

There are evidences that *H. pylori* infection, especially by strains expressing CagA, may influence negatively the human reproductive potential. It is generally accepted that one of the mechanisms that may explain the association of *H. pylori* infection with extra-digestive diseases, could reside in the overproduction of inflammatory cytokines, which is a distinctive feature of strains expressing CagA. It is also assumed that the effects of local inflammation may not be confined to the digestive tract, but they can spread to involve extra-gastroduodenal organs and

systems. Antigenic mimicry between CagA and other bacterial antigens and human peptides could also play a relevant role. Before reproductive disorders could be considered an additional manifestation of extra-digestive diseases associated with *H. pylori* infection, it is necessary to carry out other studies concerning the prevalence of *H. pylori* infection in patients and controls, and in particular to perform study dealing with evaluation of reproductive potential after eradication of *H. pylori* infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori: A chameleon-like approach to life

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Abstract

Helicobacter pylori (*H. pylori*) is widely adaptable for colonization in human stomachs in more than half of the world's population. The microorganism is characterized by an unusual capability of arranging itself in both genotypic and phenotypic ways. Stressing conditions, including antimicrobial agents in sub-inhibitory concentrations, facilitate entering the viable but non-culturable state in which bacterial cells acquire the coccoid form. This morphotype represents an important strategy for bacterial survival in unsuitable conditions and also allows escape from the immune system. *H. pylori* is capable of forming biofilm outside and inside the host. For the bacterial population, the sessile growth mode represents an ideal environment for gene rearrangement, as it allows the acquiring of important tools aimed to improve bacterial "fitness" and species preservation. Biofilm formation in *H. pylori* in the human host also leads to recalcitrance to antibiotic treatment, thus hampering eradication. These lifestyle changes of *H. pylori* allow for a "safe haven" for its survival and persistence according to different ecological niches, and strongly emphasize the need for careful *H. pylori* surveillance to improve management of the infection.

Key words: *Helicobacter pylori*; Viable But Non Culturable state; Route of transmission; Biofilm *in vitro*; Biofilm *in vivo*

Core tip: *Helicobacter pylori* (*H. pylori*) is a Gram negative bacterium that colonizes the human stomach early in the life of the host and tends to persist. The present review is focused on the general phenomenon of the fickleness in *H. pylori* and analyses the significance and role of this "chameleon-like" approach to life in the persistence of this fastidious bacterium outside and inside the host.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram negative bacterium that colonizes the human stomach early in the life of the host and tends to persist. It is estimated that the bacterium is present in the gastric mucosa of half of the world population, but disease only occurs in about 15% of colonized individuals[1].

Today, *H. pylori* is recognized as the most common cause of gastritis, peptic, and duodenal ulcers, and is responsible for an increasing incidence of gastric cancer[2-4]. The natural habitat of the microorganism is the mucus layer of the stomach, but it may also need to survive in other environments to become a life-long infection threat[5]. In fact, a large number of studies support evidence of the microorganism in dental plaque (detected by culture and PCR techniques), in houseflies, in human and animal feces[6-10], and in natural environmental waters[11-17]. Therefore, water supplies contaminated by sewage containing fluids or feces from infected people have been considered as a potential route of *H. pylori*

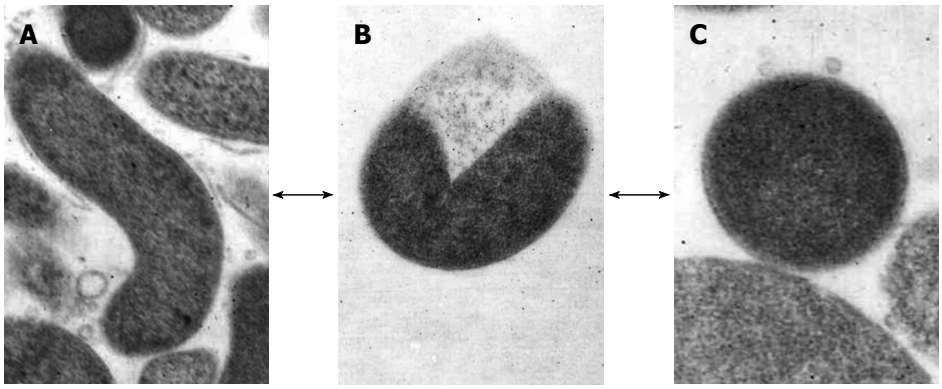

Figure 1 Morphological appearance of *Helicobacter pylori*. A: Rod-shaped; B: U-shaped; C: Coccoid form. Arrows show the hypothetical alternative pathway between the different forms. Transmission electron microscope: original magnification × 20000.

transmission[13,14,18,19].

H. pylori is able to overcome environmental stressed conditions, such as sub-inhibitory concentrations of drugs or non-permissive atmosphere, by entering the viable but nonculturable (VBNC) state, in which the microorganism modifies its morphology from a spiral to coccoid (spherical) form with a loss of cultivability[20,21]. This important strategy of survival is emphasized when bacterial cells organize themselves into microbial communities, establishing a sort of "free multicellularity" forming biofilm[22-26].

Moreover, as a species, *H. pylori* possesses one of the most fluid genomes within the prokaryotic kingdom[27,28], with many investigators asserting that *H. pylori* polymorphisms reflect human phylogeography and historical migrations[29-31], as it is virtually impossible to find two identical DNA patterns in microorganisms isolated from different hosts[28,32,33]. Furthermore, a host individual can harbor more than one isolate, which can derive either from a micro-evolutionary change among strains coming from a unique host microorganism or from a multi-strain infection. This typology of colonization may offer a condition for a more efficacious bacterium-host association during long-term harboring colonization[33,34].

The present review is focused on the general phenomenon of the fickleness in *H. pylori* and analyzes the significance and role of this "chameleon-like" approach to life in the persistence of this fastidious bacterium outside and inside the host.

VBNC STATE: A GENERAL VIEW

Bacteria, when subjected to inauspicious environmental conditions such as an insufficient supply of nutrients, non-permissive temperature, oxygen, or pH conditions, irradiation, or toxic chemicals, can survive by entering the VBNC state[35-37]. In this "survival state" that is well-documented in both Gram negative and Gram positive bacteria (including those of medical interest and widely-recognized in aquatic environments), the bacteria are not detectable by conventional culture techniques, and can undergo changes in morphology[38], cell wall composition[39], gene expression[36], and protein synthesis[40].

This protective condition, first described by the Rita Colwel group[35], represents a viable survival strategy in unsuitable situations that has contributed to the formation of environmental reservoirs of non sporulating bacteria[39].

It has been demonstrated that bacteria in the VBNC state are able to maintain their metabolic activity and pathogenicity, as well as, in some cases, the ability to revert to active re-growth conditions[41-44].

The broad distribution of VBNC cells among bacterial species underlines their significance in the ability to cope with stresses, and also draws attention to their potential risk for human health[37].

VBNC STATE IN *H. PYLORI*

H. pylori, which can be defined "a master of adaptation", is able to overcome stressed conditions, such as sub-inhibitory drug concentrations or non-permissive atmospheres which occur outside and inside the host, by entering the VBNC state[45]. This protective state occurs when the microorganism modifies its elongated, spiral morphology to transform into the coccoid morphotype through a U-shaped intermediate form (Figure 1), which results in it becoming unculturable[20]. Thus, *H. pylori* essentially displays three different cellular types: the spiral cells which grow under optimum conditions for replication and are both virulent and capable of inducing inflammation in experimental models; the viable coccoid forms that are unable to grow on solid media and are characteristically more persistent in the host and environment; and the degenerative spiral and coccal dead forms[21].

The conversion into the VBNC state represents an active process in which the microorganism switches on their adaptive machinery as a protection mechanism. In support of this, a study by Costa *et al*[46] demonstrated that the change in shape in *H. pylori* was related to its more resistant condition, due to a significant modification in the cell wall which resembled those of endospores. In fact, the peptidoglycan of *H. pylori* coccoid cells was similar to those of sporulating *Bacillus sphaericus*.

In another work, Chaput *et al*[47] demonstrated the ability of *H. pylori* coccoid cells to escape detection by the immune system because of a significant modification of the cell wall peptidoglycan which had no IL-8 stimulatory activity in gastric epithelial cells. Thus, *H. pylori* in the VBNC state may be able to escape or modulate the host response and thereby persist in the human stomach.

The capability of viable *H. pylori* coccoid cells to be persistent outside the host has been demonstrated in many works. In a study by Shahamat *et al*[48], it was demonstrated that VBNC *H. pylori* cells could be present for up to 1 year in fresh water. In another work, the authors[49] verified the entrance of *H. pylori* into a VBNC state as the cells aged in a natural freshwater environment by using viability assays, also confirming that coccoid viable cells continued to transcribe several genes, including those responsible for its virulence.

Regarding this last aspect, Wang *et al*[50] obtained a coccoid *H. pylori* population by exposure to sub-inhibitory concentration of antibiotics, with the target fragment of the *cagA* gene of these cells being amplified and cloned into a plasmid, and then transformed in *Escherichia coli*. By sequence analysis, the authors demonstrated that coccoid *H. pylori* contained a completed *cagA* gene that displayed a homology with the reported original sequence of vegetative forms of *H. pylori* (99.7%), thus supporting speculation about the pathogenicity of these cells.

The contamination of drinking water by human feces has been suggested as one of the possible routes of *H. pylori* transmission, and it has been demonstrated that the microorganism is present in the VBNC state in this unsuitable environment[51], meaning that their role in fecal-oral transmission *via* contaminated water sources cannot be disregarded.

In our study[19], we demonstrated, by Nested-PCR, the presence of *H. pylori* DNA in seawater both free and bound to zooplanktonic organisms, such as copepod and cladocerans. Considering that the intensive activity of enzymes produced by prokaryotic and eukaryotic cells in seawater favors the instability and degradation of nucleic acids[52], we assumed that the detected nucleic acids were part of viable resistant coccal cells able to survive in marine environments. Indeed, *H. pylori* was isolated by culture from marine zooplankton, supporting speculation about the potential role of zooplankton in *H. pylori* survival and transmission[15]. This isolated microorganism, named *H. pylori* MDC1, harbored a genotype coding for the most important virulence markers and, in particular, contained cagPAI, which could both exert a role in adapting to the marine environment and also be acquired by different species. In this regard, a *cagA* like gene of *H. pylori* was found to be present in environmental isolates of *Aeromonas* spp. from different water samples in India[53].

All of these considerations strongly underline that the morphological fickleness of *H. pylori* is in response to external stimuli entering the VBNC state, and representing, during the lifespan of the microorganism, a powerful response to improve bacterial "fitness" and species preservation.

MICROBIAL BIOFILM: A GENERAL VIEW

Bacterial biofilms may be considered an ancestral selective event used by prokaryotes to adapt to the environment. In this way, microorganisms are organized in communities that settle and proliferate on biotic and abiotic surfaces embedded in a highly hydrated self-produced matrix constituted of extracellular polymeric substances[54-57].

Bacteria aggregated in biofilm represent a complex dynamic system that could be considered the best program of survival in unsuitable conditions[57]. Many bacterial species match their lifecycle to the human host and environment, and thus change their regulatory processes to adapt to this new niche[54].

It is widely recognized that an ever increasing number of infections arise from biofilm-producing microorganisms that are extremely difficult to eradicate. Infections caused by sessile bacteria are characterized both by a strong tolerance to antimicrobial/biocidal agents and by an extraordinary resistance to phagocytosis, which allows them to evade the hosts' defenses[58,59]. These processes are thought to be the major contributors to the etiology and the persistence of infectious diseases.

Biofilm growing bacteria represent a major cause of exacerbating chronic infections with persistent inflammation and damage of tissue[60].

Many signals and gene products are involved in biofilm development under a cyclic and dynamic process depending on different bacteria and surfaces[61,62]. Into these microbial communities, bacteria may convey their presence to one another by producing, detecting, and responding to small diffusible signal molecules referred to as autoinducers, which carry out the Quorum-Sensing[63].

Moreover, bacteria organized in a biofilm can find a protected environment to facilitate horizontal gene transfer, thus providing a bacterial population with newly-modified genomes[64].

The biofilm represents an ideal environment for gene rearrangement and also for the horizontal bacteriophage and plasmid transfer that contributes significantly to strain variability and adaptability[65].

BIOFILM FORMATION IN *H. PYLORI*

It is well known that *H. pylori* is capable of forming biofilm both outside and inside the human host, which likely provides greater protection under stressful conditions.

The first evidence of biofilm formation in *H. pylori* was provided by Stark *et al*[66] in 1999, which characterized the water-insoluble biofilm accumulated at the air/liquid interface of a continuous culture of *H. pylori* NCTC 11637 by gas chromatography and mass spec-

trometry. Fucose, glucose, galactose, glycero-manno-heptose, N-acetylglucosamine, and N-acetylmuramic acid were identified, suggesting their role in enhancing resistance to host defense factors, antibiotics, and micro-environmental pH homeostasis, which facilitates the growth and survival of *H. pylori in vivo*.

When studied on abiotic surfaces, *H. pylori* forms a structured biofilm with an extracellular polymeric substance (EPS) matrix in which are mixed exogenous DNA fragments (eDNA)[67]. This extracellular DNA (eDNA), detected in the 2 d-old EPS biofilm matrix of *H. pylori* strains, showed some remarkable differences when compared by RAPD-PCR analysis to the intracellular DNA (iDNA). The different profiles of eDNA and iDNA indicated that lysed cells were not the primary source of eDNA release, which suggested a role in the active dynamic flow of information, such as recombination processes (*via* transformation), and contributing to the wide genomic variability of this microorganism that has been defined as a "quasi-species". Moreover, promotion of genetic transfer was studied by our group[27] between two clinical *H. pylori* strains when grown in the biofilm mode. Two co-cultured *H. pylori* clinical strains were analyzed for their cooperative/competitive behavior and selected clones, coming from their mixed mature biofilm, were compared through DNA fingerprinting and main virulence factors analysis.

Biofilms developed by mixed *H. pylori* strains were well-structured, with a higher amount of EPS matrix and viable cells than those detected by the parental strains. Finally, genetic analysis by both RAPD-PCR and *cagA* (EPIYA motifs)/*vacA* virulence genes of 45 clones showed a high number of recombinant clones together with the generation of more virulent strains. Thus, these recombinant clones might provide an advantage to the bacterial population by promoting the development of a more adhesive and stable biofilm. These data demonstrated that the biofilms developed by multiple *H. pylori* strains were more complex and structured than the ones associated with single strains. Such conditions might promote the genetic exchange favored by the protected environment and explain the development, in a single host, of more virulent and difficult to eradicate strains.

In an *in vitro* study, Cole *et al*[68] demonstrated the negative effect of mucin on *H. pylori* biofilm formation, suggesting that in the mucus-rich stomach, *H. pylori* planktonic growth is favored over biofilm formation. Moreover, these authors found, in the *H. pylori luxS* mutant, that biofilm formation was affected by overproduction of LuxS, as was observed in a *Streptococcus mutans luxS* mutant by Merritt *et al*[69]. In this study, Cole indicated the relative importance of the Quorum-Sensing gene, *luxS*, and also the *cagE* type Ⅳ secretion gene to the production of biofilms by *H. pylori*.

However, biofilm production and its characterization are strongly influenced by the different methods and media used for biofilm culture[70]. In a recent study by Bessa *et al*[24], the authors reported on the important influence of culture media on *H. pylori* growth, both in its free-living and biofilm growth modes. In particular, they suggested that the adherence and ability of *H. pylori* to form biofilm were not accomplished by the same mechanism in different media.

Finally, they demonstrated that sub-Minimal Inhibitory Concentration (MIC) values of amoxicillin and clarithromycin could increase biofilm biomass. The sub-MIC drug influence on *H. pylori* biofilm-forming capability may have clinical consequences, as during any antibiotic treatment focused to a particular infection, *H. pylori* bacteria can be exposed to sub-MICs of antibiotics, which constitute a condition that can stimulate the switching from planktonic to sessile cells forming biofilm, and consequently lead to recalcitrance to antibiotic treatment, and thus hampering eradication.

Similar results were obtained by Yonezawa *et al*[71] that displayed the increasing of biofilm biomass after various concentration of clarithromycin treatment. They also demonstrated that biofilm-forming capability in *H. pylori* affects the generation of clarithromycin resistance with the presence of a point mutation at positions 2142 and 2143 in the domain V loop of the *23 r*RNA gene more frequently detected in sessile cells than their planktonic counterparts.

These conclusions strongly underline that biofilm formation can affect the generation of antibiotic resistance mutations in *H. pylori*.

The first evidence of an *ex vivo H. pylori* biofilm was raised by the Carron group[25,26]. They showed, *via* Scanning Electron Microscopy (SEM) analysis, the presence of dense, mature *H. pylori* biofilm detectable in urease *H. pylori* positive biopsy specimens that were absent in urease-negative controls. Of the patients who tested urease positive for *H. pylori*, the average percentage of total surface area covered by biofilms was 97.3%. Those testing negative had average surface area coverage of only 1.64%. This study demonstrated that, compared with controls, urease-positive specimens have significant biofilm formation, whereas urease-negative specimens have little to none. This was reflected in the significantly-increased biofilm surface density in urease positive specimens compared with urease-negative controls.

The dynamic behavior of *H. pylori* in the colonization of human gastric mucosa was investigated in patients previously treated for *H. pylori* infection by us[23]. In our study, biopsy samples were taken and analyzed for *H. pylori* detection by cultural, molecular, and ultra-structural methods. Viable *H. pylori* cells were isolated in 33% of performed cultures, whereas the expression of the *glmM* constitutive gene and the Quorum-Sensing related l*uxS* gene were detected in 90% of the analyzed biopsies. In these positive cases, the analysis of *glmM* and *luxS* sequences confirmed *H. pylori* identity. The SEM analysis of biopsies coming from patients harboring culturable bacteria revealed a prevalent "S-shape" *H. pylori* morphotype co-existent with coccoid aggregated bacteria embedded in abundant matrix; samples coming from *H.*

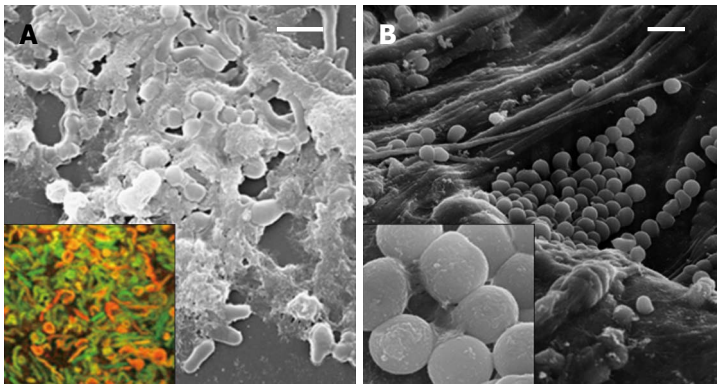

Figure 2 Biofilm of *Helicobacter pylori*. A: Scanning electron micrograph of mature biofilm on a polystyrene surface with rod shaped and coccoid cells embedded in an abundant matrix. Insert: Confocal laser scanning microscopy image of mature *in vitro* biofilm with viable (green) and dead (red) cells, live/dead staining; B: Clusters of coccoid *Helicobacter pylori* cells in the gastric mucosa also embedded in a matrix (insert). Bars represent 5 μm.

pylori positive patients showed clustered coccoid bacteria arranged in a microbial biofilm only through molecular method (Figure 2).

The undoubted clinical significance of coccoid *H. pylori* cells in epithelial gastric cells[72], also described in cases of adenocarcinoma[73], alone or grouped in clusters, underlines the need for planning of more efficacious testing protocols, such as RT-PCR methodology, to avoid underestimating *H. pylori* colonization by identifying camouflaged and protected clustered bacteria, and taking into account this serious microbial problem in medicine in the recommendation of therapeutic regimens.

CONCLUSION

H. pylori, more than other microorganisms, displays an amazing adaptive ability when confronted with stress conditions.

The viable coccoid morphotypes able to retain virulence factors and the aggregative behavior among *H. pylori* cells growing as a biofilm suggest a long-term survival of these bacterial communities outside and inside the host, enabling bacterial transmission with important clinical repercussions. In particular, these new living conditions, consisting of new self-organized populations, guarantee persistence, genetic variability, and antimicrobial resistance, as well as prolonging protection. For successful therapy, it may be essential not only to eliminate the bacillary forms but also to rapidly suppress and/or destroy the coccoid forms that are clustered in biofilm as well[74,75].

A recent study suggested a new effective treatment for the demolition of *H. pylori* biofilm which includes in the therapeutic regimen N-acetylcysteine (NAC), a mucolytic agent used in medical practice for the treatment of patients with chronic respiratory diseases[76]. In a clinical trial[77], the authors obtained a significantly higher percentage of *H. pylori* eradication (65% *vs* 20%) in patients with at least 4 treatment eradication failures by using NAC pretreatment prior to a culture guided antibiotic regimen. N-acetylcysteine may act by disrupting the biofilm agent and favoring the planktonic growth mode of *H. pylori*, thus overcoming the tolerance phenomenon described for bacterial biofilms[78].

Novel therapeutic regimens including plant extracts[79] or substances capable of inhibiting or destabilizing the formation of *H. pylori* biofilm should be explored to improve management of the infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Immune responses to *Helicobacter pylori* infection

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Abstract

Helicobacter pylori (*H. pylori*) infection is one of the most common infections in human beings worldwide. *H. pylori* express lipopolysaccharides and flagellin that do not activate efficiently Toll-like receptors and express dedicated effectors, such as γ-glutamyl transpeptidase, vacuolating cytotoxin (vacA), arginase, that actively induce tolerogenic signals. In this perspective, *H. pylori* can be considered as a commensal bacteria belonging to the stomach microbiota. However, when present in the stomach, *H. pylori* reduce the overall diversity of the gastric microbiota and promote gastric inflammation by inducing Nod1-dependent pro-inflammatory program and by activating neutrophils through the production of a neutrophil activating protein. The maintenance of a chronic inflammation in the gastric mucosa and the direct action of virulence factors (vacA and cytotoxin-associated gene A) confer pro-carcinogenic activities to *H. pylori*. Hence, *H. pylori* cannot be considered as symbiotic bacteria but rather as part of the pathobiont. The development of a *H. pylori* vaccine will bring health benefits for individuals infected with antibiotic resistant *H. pylori* strains and population of underdeveloped countries.

Key words: *Helicobacter pylori*; Vaccine; Immune response; Peptic ulcer; Gastric cancer

Core tip: *Helicobacter pylori* (*H. pylori*) infection is one of the most common infections in human beings worldwide. *H. pylori* actively induce tolerogenic signals and can be considered as a commensal bacteria belonging to the stomach microbiota. However, *H. pylori* also promote a chronic inflammation in the gastric mucosa and the direct action of virulence factors confers pro-carcinogenic activities to *H. pylori*. Hence, *H. pylori* cannot be considered as symbiotic bacteria but rather as part of the pathobiont. The development of a *H. pylori* vaccine will bring health benefits for individuals infected with antibiotic resistant *H. pylori* strains and population of underdeveloped countries.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is one of the most common infections in human beings worldwide[1]. After entering the stomach, this spiral, Gram-negative, microaerophilic bacterium penetrates the mucus gastric layer[2] but does not traverse the epithelial barrier[3], and therefore it is considered as a non-invasive bacteria. Most of *H. pylori* organisms are free living in the mucus layer, but some organisms attach to the apical surface of gastric epithelial cells[3] and small numbers have been shown to invade epithelial cells[4]. Humans carry an estimated of 10^4 to 10^7 *H. pylori* CFU per gram of gastric mucus[5].

Upon infection, *H. pylori* uses urease and α-carbonic anhydrase to generate ammonia and HCO_3^{2-} which mitigate the effects of low pH[6,7]. Moreover, thanks to its flagella and shape, *H. pylori* penetrate the mucus layer. *H. pylori* null mutant defective in production of flagella are unable to colonize gnotobiotic piglets[8]. Once established in the inner mucus layer, several outer membrane proteins, including BabA, SabA, AlpA, AlpB and HopZ can mediate bacterial adherence to gastric epithelial cells. Once attached, bacterial effector molecules, both secreted [vacuolating cytotoxin (VacA) and cytotoxin-associated gene A (CagA)] or attached [components of the type Ⅳ secretion system (CagL)], modulate gastric epithelial cell behaviour leading to loss of cell polarity, release of nutrients and chemokines [*e.g.*, interleukin (IL)-8], and regulation of acid secretion *via* control of gastrin and H^+/ K^+ ATPase[9,10].

The infections are acquired during childhood; frequent clonal transmission of *H. pylori* between first degree relatives demonstrates intra-familial transmission of *H. pylori* in developed countries. In developing world, members of the same family can be infected with widely diverse strains, and multiple infections were common arguing for horizontal transmission of *H. pylori* infection[11]. After ingestion, there is a period of intense bacterial proliferation and gastric inflammation. Concomitant with the intense gastritis is hypochlorhydria. Fecal shedding of *H. pylori* is maximal during this period, facilitating transmission to new hosts. Ultimately, the inflammatory response is reduced to a low-level stable state, normal gastric pH is restored, and most of the infected person becomes asymptomatic[12]. This outcome persists for years or decades and appears to predominate in the population. Depending on *H. pylori* virulence factors, environmental factors and the host response to bacterial infection, *H. pylori* infection can be associated with several clinical complications such as gastritis, peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma[13-15]. *H. pylori* eradication therapies have revolutionised the natural course of peptic ulcer disease[13]. Antibiotic treatment of *H. pylori* infection is relatively successful, with the organism being eradicated from around 80% of patients[16].

IMMUNE RESPONSE TO *H. PYLORI* INFECTION

Immune responses to *H. pylori* infection have been studied in twenty adult volunteers experimentally infected with *H. pylori*[17]. Gastric biopsies performed 2 wk after infection showed infiltration of lymphocytes and monocytes, along with significantly increased expression of IL-1, IL-8, and IL-6 in the gastric antrum[17]. Anti-*H. pylori* immunoglobulin (Ig)M and IgG responses were detected in the serum of infected individuals. In addition, 4 wk after infection, the numbers of gastric $CD4^+$ and $CD8^+$ T cells were increased compared to preinfection levels[18]. These data provide evidence that gastric and systemic immune responses develops within a short period of time after *H. pylori* infection.

Gastric mucosal biopsies from humans persistently infected with *H. pylori* reveal an increased infiltration of various types of leukocytes compared to biopsies from uninfected humans[19]. Lymphocytes (T and B cells), monocytes, eosinophils, macrophages, neutrophils, mast cells and dendritic cells are usually present[19,20]. B cells and $CD4^+$ T cells together with dendritic cells (DC) sometimes organize into lymphoid follicles[21] reflecting ongoing antigen presentation and chronic immune responses. *H. pylori*-specific $CD4^+$ T cells are detectable in the gastric mucosa and peripheral blood of infected-individuals but not uninfected humans[22]. Levels of cytokines [interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-1, IL-6, IL-7, IL-8, IL-10, and IL-18] are increased in the stomach of *H. pylori*-infected humans compared to uninfected humans[23]. IL-4 has not been detected in the gastric mucosa of most *H. pylori*-infected individuals[24]. Therefore, it has been concluded that *H. pylori* infection leads to a T helper cell (Th)1-polarized response. *H. pylori* infection has also been associated with upregulation of IL-17A expression in the gastric mucosa[25]. IL-17A is the most widely studied member of the IL-17 family of cytokines (IL-17A-F), and is produced by Th17 $CD4^+$ T cells as well as other subsets of immune cells[26]. Extracellular bacterial and fungal infections elicit strong IL-17A responses that stimulate stromal and epithelial cells to release pro-inflammatory cytokines and chemokines, *e.g.*, TNF-α, IL-1β, IL-6, CXCL1, CXCL2, CCL2, CCL7, CCL20, which recruit neutrophils, macrophages and lymphocytes to the site of infection[27]. Furthermore, it has been described that *H. pylori* infection also leads to the generation of regulatory T cells (Treg)[28-30]. Depletion of Treg through injection of anti-CD25 antibodies to mice before *H. pylori* infection promoted gastritis and reduced bacterial load[31]. Very elegant studies originated from the group of PD Smith clearly showed that in children[30,32], *H. pylori* infection is associated with low Th17 and Th1 responses, high Treg response and reduced gastritis as compared with adults, suggesting that *H. pylori* specific Treg play key roles in bacterial persistence.

Associated with cellular responses, a humoral immune response is elicited in nearly all *H. pylori*-infected humans[33]. Serum IgA and IgG antibodies in chronically infected persons are directed toward many different *H. pylori* antigens[33]. A local antibody response directed toward *H. pylori* antigens is also detectable with chronic *H. pylori* infection. These subjects have remarkably higher frequencies of total IgA- and IgM-secreting cells than the noninfected subjects, while the frequencies of IgG-secreting cells were virtually the same in the different groups[34]. Notably, *H. pylori* infection induces autoantibodies reactive with gastric epithelial cells, which could drive gastritis[35]. These autoantibodies could be directly cytolytic to epithelial cells through activation of complement, inducing apoptosis or triggering an antibody-

dependent cellular cytotoxicity reaction leading to the tissue destruction.

GASTRO-INTESTINAL TRACT IMMUNE DEFENCES

H. pylori colonizes the gastro-intestinal tract, thus there is a need to study the immune responses directed toward *H. pylori* in the context of the general functioning of the gastro-intestinal tract immune defences. In the following paragraph, we will briefly summarize our current understanding of the functioning of the mucosal immune responses.

The mucosal defences are multiple and might be physical, chemical and immune-mediated. The mucosal epithelium blocks invasion by pathogenic and commensal bacteria by forming multiple layers of physical (tight junctions), chemical nitric oxide and immune protection (local secretion of defensins, anti- and/or pro-inflammatory chemokines/cytokines and IgA/IgG/IgM transport). In addition, numerous bone marrow-derived cells belonging to the innate or adaptive immune systems colonized the intestinal mucosa to fight the invaders, but at steady state the same cells have to tolerate commensals.

IgA response

A major defensive mechanism that excludes commensals and pathogens from the mucosal surface involves IgA[36]. Mucosal IgA comprises antibodies that recognize antigens with high- and low-affinity binding modes. In general, high-affinity IgA neutralizes microbial toxins and invasive pathogens, whereas low-affinity IgA confines commensals in the intestinal lumen. High-affinity IgA is thought to emerge in Peyer's Patches (PPs) and mesenteric lymph nodes (MLNs) from follicular B cells stimulated *via* T cell-dependent pathways, whereas low-affinity IgA likely emerges in PPs, MLNs and lamina propria from B cells stimulated *via* T cell-independent pathways[36]. IgA response is powerfully induced by the presence of commensal microbes in the intestine[37,38] and has been shown to promote the maintenance of appropriate bacterial communities in specific intestinal segments[39]. In contrast to the lungs, vagina and most of the gastrointestinal tract, the healthy mammalian stomach produces very low level of polymeric immunoglobulin receptor (pIgR)[40,41], the receptor mediating IgA transport into the gastrointestinal lumen. Studies in *H. pylori*-infected humans have shown that baseline pIgR expression by the gastric epithelium can be up-regulated in response to gastric inflammation[42] due to increased local IFN-γ production[43]. However, despite significantly increased pIgR expression and IgA plasma cell infiltration in response to *H. pylori* infection[44] there is no concomitant increase in IgA secretion into the stomach; and it is non-secretory monomeric IgA which predominates in the stomach of *H. pylori*-infected individuals[45]. Hence, the IgA that is present in the gastric lumen would be unstable, susceptible to degradation by proteases. These observations suggest that, the stomach anti-*H. pylori* IgA responses do not play similar biological roles as compared with anti-commensal or anti-pathogen IgA response taking place in the intestine.

IgG response

In unmanipulated specific pathogen-free animals it has been showed that there was no specific serum IgG response detectable directed against commensal bacteria[46]. In pathogen-free mice, the systemic immune system appeared to remain ignorant of the commensal microbes. However, in human, a certain degree of systemic exposure to gut commensal bacteria and the associated priming of systemic immune response seems to be well tolerated, harmless and common in healthy humans since systemic antibody responses against live gut commensal bacteria and fungi can be detected[47]. Most of the *H. pylori* infected individuals develop systemic anti-*H. pylori* IgG responses[18]. Recently, Ben Suleiman *et al*[48] detected the expression of neonatal Fc receptor in gastric epithelial cells, this receptor was shown to transport IgG into gastric secretion. These results indicate that systemic anti-*H. pylori* IgG response might gain access to the gastric mucosa and exert some anti-bacterial and/or pro-inflammatory activities.

$CD4^+$ T cell responses

Since *H. pylori* is an extra-cellular bacteria, anti-*H. pylori* specific $CD8^+$ T cell responses are inadequate to protect the host against such pathogen. Hence, in this review we will only describe the priming of $CD4^+$ T cell response. As discussed above for IgA response, $CD4^+$ T cell responses are initiated within the PPs and MLNs. DC capture, process and present antigens to naive T cells in PPs and MLNs. In the stomach, DCs are penetrating the mucosa[49] to sample luminal antigens and migrate to the stomach lymph node[50].

At steady state, mucosal $CD4^+$ T cells are tolerant to microbiota-derived antigens[51]. Remarkably, systemic $CD4^+$ T cells are not tolerant to microbiota-derived antigens and conserved a naïve state to these antigens[52]. It has recently been suggested that antigen-specific intestinal IgA play a critical role in inhibiting the systemic $CD4^+$ T-cell responses to commensal antigens by providing immune exclusion[51].

At mucosal surfaces, DCs maintain homeostasis by dampening inflammatory Th1 and Th17 cell responses[53]. Mucosal DCs are particularly skilled in eliciting these anti-inflammatory responses because they receive conditioning signals from intestinal epithelial cells (IECs)[54,55]. One of these signals is provided by thymic stromal lymphopoietin (TSLP), that shifts the Th1/Th2 balance toward Th2 polarization by attenuating DC production of IL-12 but not of IL-10[56]. In addition to TSLP, IECs release transforming growth factor (TGF)-β and retinoic acid, which stimulate the development of $CD103^+$ DCs[53]. These DCs promote the formation of

Treg cells *via* TGF-β and retinoic acid and suppress the development of inflammatory Th1 and Th17 cells[53].

In addition to initiating responses that create an overall tolerant state towards harmless intestinal antigens, mucosal DCs are also implicated in the generation of protective immune responses aimed at the clearance of enteric pathogens. A fundamental difference between the steady state and a state of infection may lie in the greater propensity of pathogens to invade and penetrate beneath the epithelial-cell layer. Invasion of IECs would allow for the activation of cytosolic pattern-recognition receptors, TLRs and both quantitative and qualitative changes in the secretion of pro-inflammatory cytokines and chemokines. Consistent with this, IECs produce CXC-chemokine ligand 8 (CXCL8) when infected with strains of *Salmonella* spp. that are both invasive and flagellated[57]. CXCL8 may serve to attract neutrophils to the site of infection, furthering the inflammatory milieu. As a result, the rate of blood-borne DC precursors migrating into the tissues and becoming DCs will increase. These cells will not have been subjected to IECs conditioning and can be directly activated by a combination of pathogens that have breached the epithelial-cell barrier and the pro-inflammatory cytokine milieu. Experimental data support this scenario; human monocyte-derived DCs conditioned with IEC supernatants are impaired in their ability to secrete IL-12 and drive Th1-cell responses following exposure to pathogenic *Salmonella* spp[56] but can drive Th1-cell responses if they encounter bacteria before conditioning by IEC-derived factors. One other possible route for the generation of protective immunity to pathogens may be the uptake of pathogenic species by DCs that are normally resident in the MLNs. In this respect, $CD103^-$ MLN DCs have been shown to produce higher levels of pro-inflammatory cytokines than their intestinal-derived $CD103^+$ counterparts and drive IFNγ and IL-17 production by $CD4^+$ T cells[53].

Collectively, since *H. pylori* is mostly a non-invasive bacteria living within the stomach mucosa, these observations suggest that, the $CD4^+$ T cells responses directed against *H. pylori*, initiated within PPs, MLNs and stomach draining lymph node, might be naturally more tolerogenic than pro-inflammatory. This assumption is corroborate by the recent demonstration that in children, *H. pylori* infection is associated with low Th17 and Th1 responses and high Treg response[32]. However, the detection of *H. pylori* specific Th17/Th1 in chronically infected individuals[24,25] shows that the initial tolerogenic response is progressively lost, showing that with time the mucosal immune system identified *H. pylori* as a pathogen.

Intra-epithelial lymphocytes, innate immune cells and others

Intra-epithelial cells[58], innate immune cells[59], natural killer cells[60], neutrophils[61], mast cells[62], eosinophils[63], macrophages[64], monocytes[64], suppressive myeloid cells[65] are playing roles in the functioning of the mucosal immune system, however due to space limitation we will not discuss their roles in the context of *H. pylori* infection.

STOMACH MICROBIOTA

It was previously admitted that the stomach was a sterile organ and that pH values < 4, peristalsis and high bile concentration were able to sterilize the stomach, but in the past 30 years with the discovery of *H. pylori* it is now known that the stomach supports a bacterial community with hundreds of phylotypes[66-68] Although, the stomach, along with the esophagus and the duodenum, are the least colonized regions of the gastro-intestinal (GI) tract, in contrast to the high bacterial counts (10^{10} to 10^{12} CFU/g) observed in the colon. While it has been postulated that the indigenous stomach microbiota might be a reflection of transient bacteria from the mouth and esophagus, three separate studies demonstrated that in spite of high inter-subject variability, the gastric microbiota were distinguishable from microbiota found in the mouth, nose, and distal GI tract[69]. The most abundant phyla in *H. pylori* positive subjects are *Proteobacteria*, *Firmicutes* and *Actinobacteria*. In the absence of *H. pylori*, the most abundant phyla are *Firmicutes*, *Bacteroidetes* and *Actinobacteria*[69].

In the gastro-intestinal tract, the microbiota has a major impact on the functioning of the mucosal immune system and vice versa. Germ-free mice have small size of PPs, decrease number of lamina propria IgA secreting-plasmocytes, low levels of serum immunoglobulin and demonstrate no Th17/Th1 in the intestine[70]. The composition of the intestinal flora modulates the functioning of the immune system, for instance, the presence of *Segmented filamentous bacteria* (*SFB*) in the microbiota is associated with the development of Th17 in the intestinal lamina propria[71]. The presence of some *Clostridia* strains within the human intestinal microbiota has been recently associated with the development of intestinal Treg[72]. In addition, some commensal bacteria and microbiota-derived metabolites like short-chain fatty acids have been shown to inhibit inflammatory reactions at intestinal levels and promote pathogen clearance[59,73,74]. Inversely, defects in antibody response lead to a modification of the bacterial composition of the intestinal flora[39]. Collectively, these observations suggest that the colonization of the stomach mucosa by *H. pylori* and/or the associated microbiota might also impact the functioning of the immune system of the host and vice versa.

TOLEROGENIC ACTIVITIES OF *H. PYLORI*

Studies indicate that *H. pylori*-derived factors are capable to inhibit T-cell proliferation. Using normal T cells and Jurkat cells, a human T-cell line, it was demonstrated that VacA interfered with calcium-signaling events inside the cell and prevented activation of the calcium-

dependent phosphatase calcineurin[75,76]. The subsequent dephosphorylation of nuclear factor of activated T cells (NFAT), a transcription factor that regulates immune responses, was suppressed resulting in inhibition of IL-2 expression and proliferation of T cells. Similar anti-proliferative effect on T cells was reported for the γ-glutamyl transpeptidase (γ-GGT), this immunosuppressive factor inhibits T-cell proliferation by induction of a cell cycle arrest in the G(1) phase[77]. In addition to VacA and γ-GGT, *H. pylori* arginase can impair T-cell function during infection. Using Jurkat T cells and human normal lymphocytes, it was found that a wild type *H. pylori* strain, but not an arginase mutant strain, inhibited T-cell proliferation, depleted L-arginine, and reduced the expression of the CD3 chain of the T-cell receptor[78]. Most (80%-90%) *H. pylori* strains display Lewis blood-group antigens on their LPS, and these are similar to the Lewis blood-group antigens that are expressed on the mucosal surface of the human stomach[79]. Lewis positive *H. pylori* variants are able to bind to the C-type lectin DC-SIGN and present on gastric DCs, and demonstrate that this interaction blocks Th1 development[80].

In addition to suppress T cell activation, *H. pylori* has been demonstrated to decrease the functioning of the innate immune system. For instance, efficient phagocytosis and killing of *H. pylori* is prevented by the presence of the *cag* pathogenicity island[81,82] and *H. pylori* induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity[83]. Importantly, at the opposite to the LPS and flagellins of others gram-negative bacteria, the LPS and flagellins of *H. pylori* do not adequately activate the antigen presenting cells *via* the Toll-like receptors[84,85].

Collectively, *H. pylori* counteract innate and T cell responses and clearly exhibited tolerogenic activities on the immune system. It can be suggested that these tolerogenic activities participate to the *H. pylori* persistence within the stomach mucosa.

VACCINE-INDUCED PROTECTIVE IMMUNE RESPONSES

H. pylori infection is the main cause of gastritis, peptic ulcers, and gastric adenocarcinoma. It is believed that *H. pylori* contributes to gastric cancer development by direct action of its virulence factors and indirectly by initiation and maintenance of a chronic inflammation in the gastric mucosa[86]. Hence, gastroenterologists use a combination of anti-secretory and antimicrobial agents to eradicate *H. pylori*[16]. Similar to other antimicrobial treatments, the therapy may select resistant *H. pylori* strains[16]. Therefore, alternative therapies to eradicate *H. pylori* infection have been evaluated like the development of a vaccine against *H. pylori*.

In the seminal work reported in 1990 by Lee *et al*[87] demonstrated the feasibility to study different aspects of the pathology and the immune response induced by *Helicobacter* species in mice. These investigators using germ-free mice and *H. felis*, a bacteria that naturally infects cats and dogs, achieved successful long-term colonization and associated gastritis in these mice. This model became very popular and a large number of immunization studies were performed in *H. felis* infected mice. This was made possible by the fact that vaccine candidate antigens are shared between *H. felis* and *H. pylori* species (*i.e.*, urease and heat shock proteins). Thereafter, *H. pylori* strains have been adapted to the mouse stomach and this experimental model reproduces several aspects of the human infection[88-90]. Successful colonization with *H. pylori* has been reported in rats, guinea pigs, Mongolian gerbils, Gnotobiotic pigs, cats and Beagle dogs[90]. *H. pylori* naturally infects some species of nonhuman primates, with pathological changes in the stomach resulting from *H. pylori* infection being very similar to those observed in humans[91].

Numerous studies in animals suggested that T cells, mast cells and neutrophils are of prime importance for protection, while B cells (antibodies) are dispensable for protection[61,62,92,93]. However some studies suggested that antibodies can also participate to the vaccine-induced *H. pylori* clearance in some circumstances[94-98]. Indeed, vaccination-induced protection against *H. pylori* in mice requires major histocompatibility complex class II-restricted $CD4^+$ T cells[90,92], Th-1, Th-2 and/or Th-17 $CD4^+$ T cell responses and the α4β7 integrin-mediated homing process[99] have been implicated in protection[100-103]. Recently, the production of IL-17, by Th17 cells, has been clearly identified as a key player in the vaccine-induced *H. pylori* clearance. IL-17 has also been linked to neutrophil recruitment and activation through the induction of granulocyte-stimulating factor and IL-8[104] and to resistance against extracellular microbial infections[105], leading to the conclusion that IL-17 production by *H. pylori* specific Th17 cells can mediate the vaccine-induced *H. pylori* clearance (Figure 1).

Collectively, it was clearly demonstrated that *H. pylori* infections could be substantially prevented, reduced or even eliminated by prophylactic and therapeutic mucosal and systemic vaccinations[106-110]. This result is of great interest not only for the development of *H. pylori* vaccine but also for vaccine strategy aimed at clearing commensal bacteria with genotoxic and mutagenic activities[111].

CONCLUSION

H. pylori can be considered as a commensal bacteria belonging to stomach microbiota. Indeed, *H. pylori* promote the generation of *H. pylori* specific Treg. The tolerogenic environment created by *H. pylori* might explain that *H. pylori* seropositivity was inversely related to recent wheezing, allergic rhinitis, dermatitis, eczema, asthma or rash[112]. Very elegant pre-clinical studies conducted by the group of A Müller recently gave support to this assumption by showing that *H. pylori* infection during the neonatal period promote the development of Treg responses that protect adult mice from asthma[113]. Hence,

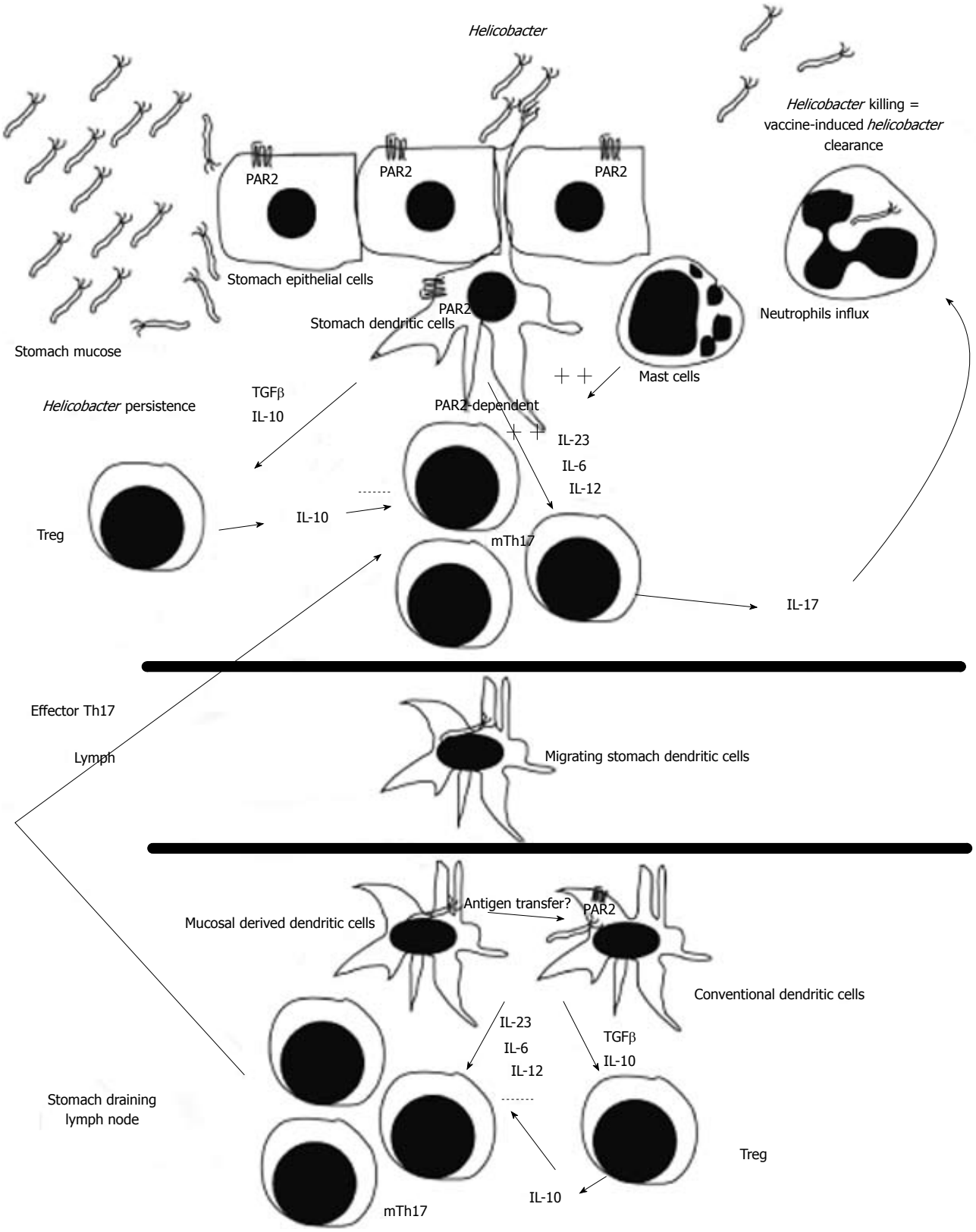

Figure 1 Schematic representation of the vaccine-induced *Helicobacter pylori* clearance. During *Helicobacter* infection of vaccinated hosts, memory T helper (Th)17 cells (mTh17) are primed by protease-activated receptor (PAR)2-dependent dendritic cell (DC)[126] directly in the stomach and/or in the stomach draining lymph nodes (conventional DCs). Effector memory Th17 cells originated from the stomach and/or from the stomach draining lymph nodes will produce high levels of interleukin (IL)-17 leading to recruitment of neutrophils and to *Helicobacter* clearance. In naïve hosts, DCs mainly prime regulatory T cells (Treg), leading to *Helicobacter* persistence.

H. pylori, like other commensal bacteria such as *SFB*[71], *Calibacterium prausnitzii*[74], *Lactobacillus reuteri*[59], *Lactobacillus Acidophilus*[59], and *Clostridia*[72] profoundly impact the functioning of the immune system of the colonized host.

Although *H. pylori* infection can be beneficial for the host, when present in the stomach, *H. pylori* reduce the overall diversity of the gastric microbiota[114] and promote gastritis. The modification of the stomach microbiota might, independently or not of the presence of *H. pylori*, modulate the susceptibility of the host to immune-mediated diseases. The *H. pylori*-induced gastritis is most probably cause by the type Ⅳ secretion apparatus-dependent introduction of muropeptides into epithelial cells, that promote Nod1-dependent induction of a proinflammatory program[115]. In addition *H. pylori* promote gastric inflammation through the production of a neutrophil activating protein[116]. In spite of the natural tolerogenic environment provided by the stomach mucosa and the tolerogenic activities of *H. pylori*, these pro-inflammatory signals initiating systemic and local pro-inflammatory Th1/Th17 responses[22-24].

Since *H. pylori* possess pro-carcinogenic activities *via* maintenance of a chronic inflammation in the gastric mucosa and by direct action of its virulence factors (vacA and cagA), *H. pylori* cannot be considered as symbiotic bacteria but rather as part of the pathobiont[117]. Hence, *H. pylori* has to be eliminated when individuals are prone to develop duodenal and stomach ulcers[118,119] to prevent further major diseases development like MALT lymphoma and stomach adenocarcinoma. Although, the design of a vaccine directed *H. pylori* is challenging since it has to overcome the natural tolerogenic environment provided by the stomach mucosa and the tolerogenic activities of *H. pylori*, its development will bring health benefits for individuals infected with antibiotic resistant *H. pylori* strains and population of underdeveloped countries[120-125].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Overview of the phytomedicine approaches against *Helicobacter pylori*

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Abstract

Helicobacter pylori (*H. pylori*) successfully colonizes the human stomach of the majority of the human population. This infection always causes chronic gastritis, but may evolve to serious outcomes, such as peptic ulcer, gastric carcinoma or mucosa-associated lymphoid tissue lymphoma. *H. pylori* first line therapy recommended by the Maastricht-4 Consensus Report comprises the use of two antibiotics and a proton-pomp inhibitor, but in some regions failure associated with this treatment is already undesirable high. Indeed, treatment failure is one of the major problems associated with *H. pylori* infection and is mainly associated with bacterial antibiotic resistance. In order to counteract this situation, some effort has been allocated during the last years in the investigation of therapeutic alternatives beyond antibiotics. These include vaccines, probiotics, photodynamic inactivation and phage therapy, which are briefly revisited in this review. A particular focus on phytomedicine, also described as herbal therapy and botanical therapy, which consists in the use of plant extracts for medicinal purposes, is specifically addressed, namely considering its history, category of performed studies, tested compounds, active principle and mode of action. The herbs already experienced are highly diverse and usually selected from products with a long history of employment against diseases associated with *H. pylori* infection from each country own folk medicine. The studies demonstrated that many phytomedicine products have an anti-*H. pylori* activity and gastroprotective action. Although the mechanism of action is far from being completely understood, current knowledge correlates the beneficial action of herbs with inhibition of essential *H. pylori* enzymes, modulation of the host immune system and with attenuation of inflammation.

Key words: *Helicobacter pylori*; Alternative treatment; Phytomedicine; Herbal medicine; Phytotherapy; Botanical therapy; Herb medicine; Probiotics; Antibiotic resistance

Core tip: Considering the worldwide spread of *Helicobacter pylori* (*H. pylori*) antibiotic resistance, therapeutic alternatives beyond antibiotics have been investigated during the last years, including vaccines, probiotics, photodynamic inactivation, phage therapy and phytomedicine, which are reviewed in the present paper, giving particular attention to phytomedicine. The manuscript offers an extensively referenced text about the effect of herbal medicines on *H. pylori*, describing the first applications of herbal medicine, passing by the category of performed studies, enumerating the tested compounds, identifying the active principle and the mode of action, and concluding with the limitations and promises of this old made new therapy.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infects more than half of the human population worldwide. Is the etiologic agent of peptic ulcer disease in 10%-20% of the infected individuals, while 1%-2% are at risk of developing gastric carcinoma or mucosa-associated lymphoid tissue (MALT) lymphoma[1]. On a global scale the burden of disease due to *H. pylori* is huge; elimination of these bacteria would have a major impact on present and future world health.

Currently, the standard first line clarithromycin-based therapy presents undesirable cure rates, and the recent guidelines for *H. pylori* eradication from the Maastricht-4 Consensus Report do not recommend this therapy in regions with high prevalence of clarithromycin resistance[2]. Current treatments are therefore not an effective strategy for worldwide eradication and public health measures improving living conditions may help to reduce the transmission of this infection in selected areas, but will have only a limited effect on infected individuals.

In alternative, infection may be dribbled by the use of new treatment approaches, based on ancient alternative medicines. This paper addresses the problem of *H. pylori* infection, the disease-associated spectrum and the antibiotic resistance against the current treatment regimens, and alternative therapeutic options against resistant strains, with special emphasis on phytotherapy approaches.

H. PYLORI BIOLOGY

The human stomach mucosa is the known ecological niche of *H. pylori*, a pathogenic spiral-shaped, microaerophilic, Gram-negative bacterium, which is unique in its ability to persist and establish a chronic infection. During colonization, propelled by its flagella and resisting to gastric acidity through urease activity, *H. pylori* crosses the gastric mucus layer and adheres to mucins and cells' surface-receptors of the gastric epithelium. Once here, it delivers its virulence factors into the host cells' cytoplasm both through the type-IV secretion system and/or by releasing outer membrane vesicles. The cytotoxin-associated gene A (CagA) and the vacuolating cytotoxin (VacA), are among the best studied translocated proteins (reviewed in[3]).

In addition to its set of colonization and virulence factors, *H. pylori* has adapted itself *via* complex strategies to maintain an inflammation of the gastric epithelium while limiting the extent of the immune response in order to prevent its elimination, through reduced recognition by immune sensors, downregulation of immune cells and escape from immune effectors (reviewed in[4]).

Another unique feature of this bacterium is its tremendous genetic variability, with each strain of these hypermutable bacteria acting as a quasispecies[5,6]. This genome plasticity is mainly due the bacterium natural competence for transformation and for conjugative transfer of genomic islands, resulting in extensive polymorphic genes and in differences in gene content among strains[7]. Moreover, *H. pylori* displays a high frequency of recombination, which in addition to the small size of the recombined fragments results in a mosaic gene structure[8]. Intragenomic recombination has also been reported to occur in *H. pylori*, especially between members of the large family of paralogous outer membrane proteins (OMP) encoding genes or between repetitive sequences, leading to variation even in the absence of mixed colonization[9-11].

Occurrence of point mutations is another mechanism of genetic diversity in *H. pylori*, involved for example in the development of antibiotic resistance[12]. It is likely that the high rate of mutation in *H. pylori* is due to a relative deficiency in DNA repair systems, since many of these systems appear to be absent in this organism[13].

H. PYLORI DISEASES AND TREATMENT OPTIONS

In a similar fashion *H. pylori* is worldwide spread, this bug is implicated in a broad spectrum of diseases, considering its restrict niche. *H. pylori* infection of the human stomach, usually occurring in the childhood, will always elicit an acute immune response. However, if left untreated, infection and inflammation (gastritis) persist. Although often asymptomatic, gastritis may cause dyspeptic symptoms, or it may further progress, causing peptic ulcer disease, distal adenocarcinoma and gastric mucosal lympho-proliferative diseases such as MALT lymphoma in 10%-15% and 2% of adult patients, respectively[1]. *H. pylori* infection has been linked to diseases localized outside of the stomach as well, with the strongest evidences linking infection with cardiovascular diseases, lung diseases[14], hematologic diseases, such as idiopathic thrombocytopenic purpura[15], neurological diseases[16] and Diabetes Mellitus, although more studies are required to clarify such proposed causal links (reviewed in[17]). In addition, the relationship between bacterial CagA positivity and coronary heart disease has been reportedly emphasized[18,19]. In contrast, the beneficial effects of *H. pylori* concerning allergic diseases[20] and obesity appear clear, while the association with gastroesophageal reflux disease is still controversial[21,22].

H. pylori eradication aims mostly to cure functional-associated disease, such as peptic ulcer, but is also a strategy to prevent gastric cancer[23].

In an era in which no anti-*H. pylori* vaccine is yet available, the treatment relies on the use of antimicrobials. Currently, the first-line treatment of *H. pylori* infection consists of two antimicrobials, being the standard combination the use of amoxicillin with clarithromycin or metronidazole, plus a proton-pump inhibitor (PPI). In al-

ternative, levofloxacin can replace clarithromycin in first-line therapy, with apparently higher cure rates[24]. Moreover, an alternative empiric strategy is mandatory when local clarithromycin resistance is higher that 20%[2]. When the triple schemes fail, a quadruple second-line therapy is recommended. The most popular quadruple therapy is still the one containing bismuth, consisting of a combination of bismuth salts, tetracycline and metronidazole, which is now available in 3-in-1 pill, plus a PPI[25,26]. The non bismuth-based quadruple therapy comprises several combinations of antibiotics, administered in a sequential or concomitant way. An example is the recent combination of levofloxacin, nitazoxanide and doxycycline plus the PPI omeprazole, which showed eradication rates of around 90%[27].

After failure of second-line treatment, treatment should be guided by antimicrobial susceptibility testing whenever possible.

TREATMENT FAILURE

Treatment failure is one of the major problems associated with *H. pylori* infection and is mainly associated with bacterial antibiotic resistance but also because bacteria may be in a protective environment like the stomach mucus layer or even inside the epithelial cells[28]. Failure in therapy may also occur because of the lack of patient compliance due to non negligible side effects.

Among the most used antibiotics against *H. pylori*, clarithromycin is the one that poses higher concerns since resistance to this antibiotic decreases the rate of success of the standard therapy to 20%, against 90% when the strain is susceptible[29]. Currently, *H. pylori* resistance to antibiotics is uneven distributed worldwide, with higher rates reported in developed countries than in developing countries in agreement with prescription frequency. Accordingly, in Europe clarithromycin resistance rate has doubled in a 10 years period, from 9.9% in 1998 to 17.5% in 2008-2009, and it was significantly correlated with the outpatient consumption of long-acting macrolides[30,31]. The consumption varied greatly among European countries and thus the rate of *H. pylori* resistant strains was also highly heterogeneous. Indeed, the rate of resistance strains was found to be significantly higher in Western/Central and Southern Europe (> 20%) than in Northern European countries (< 10%)[31].

Levofloxacin is the other antibiotic for which resistance is also of concern, since success of PPI-amoxicillin-levofloxacin regimen decreases radically if the *H. pylori* strain is resistant to levofloxacin compared with a susceptible strain[32]. Similarly to clarithromycin, the higher the consumption of fluoroquinolones in the community, the higher the *H. pylori* resistance rate to levofloxacin[31].

A high rate of *H. pylori* resistant strains to these two antibiotics has also been reported in other geographies, such as Japan[33], Korea[34], Vietnam[35], China[36] and Iran[37], as well as in South America, such as Mexico[38] and Brazil[39], mostly for levofloxacin, while there are little data concerning US.

Concerning the other antibiotics used to treat *H. pylori* infection, such as amoxicillin, tetracycline and rifampicins, the resistance is still rare, probably because the implicated point mutations have a high biological cost to the bacterium.

As regard to metronidazole, resistance to this antibiotic involves complex mechanisms and although it can contribute to, it is not directly correlated with treatment failure, being overcome in the majority of the situations by changing the associated antibiotics as well as the dosage and length of treatment[40].

ALTERNATIVE THERAPIES

In light of the current situation of a worldwide spread of *H. pylori* antibiotic resistance, therapeutic alternatives beyond antibiotics have been investigated during the last years, including vaccines, probiotics, photodynamic inactivation, phage therapy and phytomedicine. This latest further explored below.

Immunization is one of the most cost-effective and successful public health achievements of the 20th century to prevent infectious diseases. Similarly, a prophylactic vaccine against *H. pylori* infection would prevent gastric diseases associated with this infection, in particular gastric cancer. Pioneering work in the early 1990s provided evidence that vaccination against *H. pylori* infection was possible, based on murine models. The feasibility of a preventive vaccination against *H. pylori* infection has since been proven in other animal models, such as dogs, and vaccine candidates against *H. pylori* infection have been tested in humans (reviewed in[41]). The antigens previously used in attempts to develop a vaccine against *H. pylori* infection were mostly secreted proteins (such as urease or VacA) rather than antigens associated with the cell envelope. *H. pylori* possesses an unusual set of OMPs reflecting its adaptation to the unique gastric environment[9]. In this context, effort should be taken in the evaluation of OMPs of *H. pylori* as target antigens for a DNA multivalent vaccine construct.

Probiotics are live organisms or produced substances that are orally administered to promote health[42]. In the case of *H. pylori* infection, their use could be attractive mostly to prevent antibiotic side effects, such as diarrhea, as well as improve eradication rates. Indeed, probiotics can act in several ways in the gut microbiota, for instance by direct antagonism to pathogens through the production of inhibitory substances, competition for adhesion or nutrients, host immune modulation or inhibition of toxins[43,44]. Various probiotics have shown favorable effects in animal models of *H. pylori* infection, by reducing colonization and alleviating the inflammation of the stomach[45,46]. Most of the studies in humans, using combinations of antibiotics and probiotics showed an overall improvement of *H. pylori* gastritis and an increase in *H. pylori* eradication, as well as attenuation of total side effects after administration of probiotics[47,48]. However,

no study could demonstrate complete eradication of *H. pylori* infection by probiotic treatment. Finally, long-term intakes of products containing probiotic strains may have a favorable effect on *H. pylori* infection, particularly by reducing the risk of developing gastric inflammation-associated disorders.

More unconventional alternative anti-*H. pylori* treatments have revisited some "old" technologies, such as photodynamic inactivation and phage therapy, both dating long before the golden era of antibiotics. Photodynamic therapy (PDT) uses a photosensitizer and light sources of specific wavelengths to treat malignant tumors or localized infectious diseases. The reactive oxygen species generated by the photodynamic reaction will induce damage to multiple cellular structures, with bactericidal effects[49]. The bactericidal effect of PDT is well known against Gram-positive bacteria but usually inactive against Gram-negative bacteria. However, *H. pylori* displays two characteristics that turn it susceptible to PDA: its natural ability to accumulate photoactive porphyrins and lack of genes to repair phototoxicity-induced DNA damage[50]. Therefore, efficient *H. pylori* killing is possible just by low fluency of broad-spectrum conventional white endoscopic light[51]. Moreover, the localization of the infection in the gastric mucosa facilitates the endoscopic access for light delivery. A recent study showed that the bactericidal activity of PDT against *H. pylori* involved cell membrane injury[52].

Phage therapy consists of the use of lytic bacteriophages to treat infectious diseases[53]. The description of phages in *H. pylori* is still limited, although is a growing field, prompted by the recent description of a temperate phage of *H. pylori*, induced by UV[54], and with the sequences of complete[55-57] and remnant prophages provided by whole genome sequencing of *H. pylori* strains[58]. Nevertheless, there is no information on the nature of the life cycle of the described *H. pylori* phages, and therefore of their potential usage in phage therapy. An alternative would be the use of phage lytic proteins, such as a lysin, which is responsible for the lysis of host bacterial cell wall. However, lysins would have to be modified in order to overcome the limitation of crossing the Gram-negative outer membrane, as it was described for another bacterial species[59].

PHYTOTHERAPY

Phytotherapy, also described as herbal therapy or botanical therapy, consists in the use of plants or plant extracts for medicinal purposes[60]. Herbal products include raw or processed parts of plants, such as leaves, stems, flowers, roots, and seeds. According to legislation herbs are considered dietary supplements that can be marketed without previous demonstration of safety and efficacy[61]. Western medicine typically employs an active principal, often of synthetic origin, for therapy proposes. On the opposite, in phytotherapy applications rarely the active principle is either identified or administrated solely. Instead herbs are complex mixtures of organic chemicals. Herbal medicine origins are based on empirical knowledge, and scientific validation of these products is still very limited[60]. This lack of knowledge and evidence indicating the efficacy of herbal medicine makes it suspicious for western physicians and researchers. The risks and benefits of herbal medicine are incomplete, complex, and confusing. There is a need for further controlled clinical trials addressing the potential efficacy of herbal medicine, together with understanding the mode of action and implementation of legislation to maximize their safety and quality[62].

The whole plants and plant extracts used are very diverse and typically belong to the natural flora of a specific world's area. For this reason the use of search motors can easily miss publications owned to the dispersion of key words selected by authors. Our search was done on Pubmed and ISI web of knowledge, from 1983 to 2013, using the keywords "herbal *H. pylori*", "herbs *H. pylori*", "phytomedicine *H. pylori*", "botanical medicine *H. pylori*", "dietary supplement *H. pylori* not probiotics" and "functional food *H. pylori*" to find any *in vitro* and *in vivo* studies evaluating single or compound herbal preparations in the management of *H. pylori* infection. While the first four keywords correctly identify the use of plants or plant extracts for the eradication of *H. pylori*, the last two terms identify mainly the use of vitamins for eradication or slow of disease progression.

History: A therapy older than H. pylori discover

Phytotherapy is as old as human civilization and for that reason telling its early years, that occurred sooner than written history, should always lead to an incomplete report. The ancient use of plants was based on experience, since the cause of illness and the mode of cure was not understood. Until the application of chemistry to medicine in the 16th century, herbs were the source of treatment and prophylaxis. Then the use of herbs gradually diminished being replaced by synthetic drugs. In the last three decades there was another inversion, owing to the increasing of resistance of microorganisms to drugs[63].

Even long before the identification of *H. pylori* in the beginning of the early 1980s[64,65] herbs have been used to deal with diseases that today are known to be associated with *H. pylori* infection[66,67]. This is the case of the use of *Symphitum officinalis* and *Calendula officinalis* to treat a group of patients with duodenal ulcer or gastroduodenitis. In this trial, a group of patients received the herbs and an antiacid, while the control group just received the herbs. The pains disappear in both groups, but earlier in the group that received the antiacid[67]. In fact, the reduction of acid production was central in the therapy of peptic ulcer. Several drugs that act as anticholinergic or antimuscarinic, that reduce gastric acid secretion, were used in an attempt to replace parietal cell vagotomy, in which the resection of the vagus nerve led to the reduction of the production of acid by the parietal cells of the stomach[68], including the use of herbs, such as belladonna (*Atropa belladonna* L. or its variety acuminata Royle ex Lindl)[69].

Presently, three decades after the discovery of *H. pylori*, herbs are still being used for stomach diseases but not all of them have been tested either *in vitro* or *in vivo* for their anti-*H. pylori* activity yet. For instance this is the case of African São Tomé plants, such as *Leonotis nepetifolia* (L.) W. T. Ainton var. *nepetifolia* (gastric indisposition), *Solenostenom monastachyus* (*P.* Beauv.) Briq. subsp. *monostachyus* (stomach pain), *Piper umbellatum* L. (stomach problems), *Bertiera racemosa* (G. Don) K. Shum var. *elephantina* N. Hallé (stomach pain), *Allophyllus grandifolius* (Baker) Radlk (gastric affection), and *Solanum gilo* Raddi (stomach pain)[70].

Category of performed studies

The study of phytotherapy products is typically subdivided in two groups, one based on *in vitro* testing using *H. pylori* pure cultures obtained from clinical isolates or reference strains; another based on *in vivo* tests, in which the herbal products are administered to animal models or used in clinical trials involving humans. The first studies are more abundant in the literature namely because of their simplicity, cost, legislation demands and to early years of studding herbal products in a similar way to western medicine products.

Concerning preparation of plants extracts, these are prepared usually by drying and reduce to fine powder which is then dissolved in a solvent, such aqueous ethanol or methanol, sonicated, filtered or centrifuged and the solvent evaporated. The herbal residue is dissolved in dimethyl sulfoxide (DMSO)[71,72]. Different concentration of plant extracts are mixed with a bacterial suspension of *H. pylori* for 1 h and plated in standard *H. pylori* medium. The minimum bactericidal concentration corresponds to the test sample at which there was no visible growth[71]. Alternatively, wells can be punched on the plates and the herbal extract introduced; extract embedded paper discs are another option. The inhibitory action is evaluated by determination of the clear zone around each well or disc[72]. For the *in vitro* test, the 96-well micro-titer plates cultured micro-aerobically can also be used[73]. Regarding the negative and the positive control, DMSO may be used as negative control[71,72], while standard antibiotic agents can be applied as positive control[74].

Other *in vitro* assays include the use of gastric epithelial cells, such as AGS cells[75,76] or macrophage cells, like RAW264.7[75,77], or HeLA cells[78]. In this assays eukaryotic cells are treated with herbal extracts followed by infection with *H. pylori* (multiplicity of infection 1:100), for instance during 6 h. Then several parameters of infection can be determined to understand if the herbal plants interfere with their concentration. These parameters include nuclear factor κB (NF-κb) and cytokines, such as interleukin-8, tumor necrosis factor-α, nitric oxide (NO) production and expression levels of inflammation related proteins inducible NO synthase and cyclooxygenase[74]. The effect of the herbal compound on bacterial adhesion and invasion of epithelial cells may also be determined[74,79]. The effect of herbal extracts on cell-adhesion is determined by removing unbound bacteria using a series of washes in phosphate-buffered saline, followed by cell lysis with distilled water. The lysates are then platted on *H. pylori* appropriate medium and colony forming units determined. To verify the effect on the number of viable intracellular bacteria, infected epithelial cells are treated with the membrane impermeable antibiotic gentamicin in order to eliminate external bacteria. Then the same procedure is applied and the colony forming units determined. Appropriate controls without herbal extracts and without gentamicin should be performed, so that these may be considered as total adhesion or invasion[79]. The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) viability assay may be used to measure the cytotoxicity of tested agents[75].

Animal models may also be used to understand the action of herbal medicine on gastric colonization by *H. pylori* and gastric pathology. In those, such as the Mongolian gerbil[80-83], the specific-pathogen-free mice[78,84,85], or the Wistar albino rat[86,87], animals are infected with *H. pylori* strains and treated with different doses of plant extracts. After sacrifice the eradication or decrease in the number of *H. pylori* colonies may be determined. Further histopathological analysis can also be performed in sections of the stomach fixed in formalin and embedded in paraffin.

A summary of clinical trials using different plant extracts is presented in Table 1. In these trials a plant extract is tested in opposition to a placebo or, more recently, in addition to conventional triple therapy. In these studies there is no evidence of statistical significant improvement in eradication when herbs are used. Nonetheless these studies are still few, involving a small number of patients and moreover applied as a supplement to antibiotic triple therapy that is known to eradicate *H. pylori* in the great majority of the cases.

From Table 1 only the study of Puram *et al*[88] uses herbal medicine (GutGard) alone against a control group receiving placebo. Although the eradication rate is evident in the group receiving GutGard (56%) against 4% in the placebo group, it is still lower to the eradication obtained using triple therapy. Nevertheless, it should be emphasized that the treatment with GutGard was found to be 3.73 times more effective than placebo.

Detail attention should be given to data on clinical trials. For instance in the study of Salem *et al*[89], two recruited patients were positive for *H. pylori* after two consecutive triple therapy courses, but they changed to negative after receiving *N. sativa* treatment in a dose of 3 g/d along with 40 mg omeprazole for four weeks, *H. pylori* status evaluated by stool antigen test.

A systematic review of the use of traditional Chinese medicine against *H. pylori*[90] analyzed 16 randomized clinical trials using several different herbs with proton pump inhibitor or colloidal bismuth subcitrate based triple therapy as controls. The heterogeneity of the studies did not allow a meta-analysis. Overall, conventional triple therapy originated higher eradication rate than Chinese medicine,

Table 1 Examples of clinical trials using herbal medicines

Herb	Study design	Sample	Experimental intervention	Control	Outcome	Difference between experimental and control group		Ref.
Garlic oil	Blind non-randomized trial	20 dyspeptic patients	275 mg garlic oil 3 times a day for 14 d	Same plus 20 mg omeprazole	Negative for histology and urease test	No	Symptom score (8.7 ± 1.70 *vs* 8.5 ± 1.51) and *H. pylori* density (2.0 ± 0.82 *vs* 2.1 ± 0.74) did not significantly changed	[111]
Fresh garlic or jalapeno peppers	Open non-randomized trial	12 healthy patients with *H. pylori*	10 cloves fresh garlic or 6 jalapeno peppers with 3 meals per test day	Bismuth subsalicylate with 3 meals per test day or no intervention	Reduction in urea breath test counts		Garlic and jalapeno add no effect (P > 0.8), but significant reduction after bismuth (P < 0.001)	[112]
Cinnamon	Blinding placebo-controlled	23 patients undergoing gastroscopy	40 mg cinnamon extract twice a day for 4 wk	Placebo	Reduction in urea breath test counts	No	Mean urea breath test reading (23.9 *vs* 25.9) did not significantly changed	[109]
Lycopene	Quasi-control trial	54 patients with *H. pylori*	Metronidazole 500 mg/*bd*, amoxicillin 1g/*bd*, omeprazole 20 mg/*bd*, bismuth 240 mg/*bd*, and lycopene 30 mg/daily	Metronidazole 500 mg/*bd*, Amoxicillin 1 g/*bd*, Omeprazole 20 mg/*bd*, Bismuth 240 mg/*bd*	Slight increased eradication rate with lycopene (no statistical difference) evaluated by urease rapid test	No statistical difference		[113]
Nigella sativa (*N. sativa*)	Randomized trial	88 dyspeptic patients	Triple therapy (TT: clarithromycin 500 mg twice daily, amoxicillin 1g twice daily, omeprazole 40 mg once daily) and 1, 2 or 3 g *N. sativa*	Clarithromycin 500 mg twice daily, amoxicillin 1g twice daily, omeprazole 40 mg once daily	2 g/d and TT no statistical difference 1 g/d and 3 g/d significantly less effective than TT by stool antigen test	No	Eradication rates with 2 g *N. sativa* and TT with no statistical difference; eradication rate with 1g or 3 g *N. sativa* was significantly less than that with TT (P < 0.05)	[89]
Green propolis	Non-randomized clinical trial	18 patients infected with *H. pylori*	20 drops of alcoholic preparation of Brazilian green propolis 3 times a day for 7 d	No	One patient negative for *H. pylori* 40 d after treatment	Not applicable		[108]
Glycyrrhiza glabra	Randomized double blind placebo controlled trial	107 patients infected with *H. pylori*	55 patients - 150 mg of GutGard (root extract of *G. glabra*) once daily for 60 d	52 patients - placebo once daily for 60 d	56% of patients receiving GutGard eradicate *H. pylori* *vs* 4% on placebo	Yes	A significant interaction effect between group and time (P = 0.00)	[88]
Chinese patent medicine wenweishu /yangweishu	Randomized, controlled and multicenter trial	642 patients infected with *H. pylori*	PCM plus wenweishu group (n = 196); and PCM plus yangweishu group (n = 224)	PCM group (n = 222, pantoprazole 40 mg twice a day, clarithromycin 500 mg twice a day, metronidazole 400 mg twice a day, for 7 d)	Higher healing rate in PCM plus wenweishu; Higher rates of symptom relief in PCM plus wenweishu and PCM plus yangweishu; Eradication rate between PMC group and PMC plus wenweishu or PMC plus yangweishu group was not significantly different (P = 0.108, 0.532, respectively)	Yes	Healing rate in PCM plus wenweishu groups was significantly higher than the rate in PCM group (P = 0.004) Symptom relief rates in PCM plus wenweishu groups and PCM plus yangweishu were significantly higher than the rate in PCM group (both P < 0.01)	[110]

PCM: Pantoprazole, clarithromycin, metronidazole; *H. pylori*: *Helicobacter pylori*.

and the opposite is observed for secondary effects, favoring Chinese medicine.

From the clinical trials analysis it is not possible to completely understand the efficacy of the herbs used, namely because of the poor quality of the trials[90]. Only the extension of the requirements of evidence-based medicine to phytomedicine clinical trials would allow assessing with accuracy the efficacy of the herbal extracts.

Table 2 Herbal medicines tested against *Helicobacter pylori*

Herb	Study type	Result	Observation	Active principle	Mechanism of action	Ref.
Garlic	*In vivo* clinical trial	No improved eradication (consult Table 1)	NA	NA	NA	[111,112]
Pelargonium sidoides roots (eps) 7630	*In vitro* using AGS cells and in situ using biopsies	Inhibit *H. pylori* growth and cell adhesion	South African herbal remedy	NA	Anti-adhesive activity	[114,115]
Cranberry juice	*In vitro* using immobilized human mucus and erythrocytes	Inhibit *H. pylori* cell adhesion	NA	NA	Anti-adhesive activity (sialic acid-specific adhesion)	[116,117]
Oregano and cranberry	*In vitro* agar difusion assay	Inhibition zones on agar plate	NA	Phenolic compounds	Urease inhibition; disruption of energy production inhibiting proline dehydrogenase at the plasma membrane	[93]
Magnolia officinalis Rehd. Et Wils. (*Magnoliaceae*) and *Cassia obtusifolia* L. (*Leguminosae*)	Compounds tested against *Jack bean* urease	Inhibit urease	Chinese medicinal herbs	Hydroxamic acids, phosphoramidates, urea derivatives, quinones, and heterocyclic compounds	Inhibit urease	[118]
Camellia sinensis	*In vitro* test against *H. pylori*, urease activity assay	Inhibit urease; reduction of *H. pylori* population	Tea leaves	Polyphenolic compounds and catechin contents (epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate)	Inhibit urease	[94]
Apple peel polyphenols	Compounds tested against Jack bean urease; *in vitro* test against *H. pylori*; *in vitro* test using hela cells; *in vivo* test using C57BL6/J mice	Inhibit urease; prevented vacuolation in hela cells; antiadhesive effect; anti-inflammatory effect	NA	Polyphenols	Inhibit urease; anti-adhesive activity	[78,95]
Calophyllum brasiliense Camb. (*Clusiaceae*)	*In vitro* disk diffusion; *in vivo* using Wistar rats infected with *H. pylori*	Dose-dependent reduction of ulcerated area; decreased number of urease-positive animals; partial anti-*H. pylori* inhibition	Large tree widely distributed in Latin America known in Brazil as "guanandi"	Mixture of chromanone acids	Inhibit urease; modulation of endogenous antioxidant systems	[86,87]
Mouriri elliptica Martius (*Melastomataceae*)	*In vivo* Swiss albino mice and male Wistar albino rats animal	Gastric protective action without antisecretory effect; anti-*H. pylori* action	Brazilian fruit-bearing plant of known as “coroa-de-frade”	Acid derivatives, acylglycoflavonoids and condensed tannins	Inhibit NO production by macrophages; stimulating proliferation factors (PCNA), COX-2	[119]
Hancornia speciosa Gomez (Mangaba)	*In vivo* Swiss albino mice and male Wistar albino rats animal	Antiulcer activity	Medium-sized tree (3–10 m) from central Brazil, known as “mangaba”, “mangabeira” or “mangava”	Polymeric proanthocyanidins	Increase pH and decrease acid output of gastric juice, stimulate mucus synthesis and produce antisecretory effect	[120]
Byrsonima fagifolia Nied. (*Malpighiaceae*)	*In vivo* Swiss albino mice and male Wistar albino rats animal *In vitro* disc diffusion technique	Gastric protective action; anti-inflammatory effect; anti-*H. pylori* action	Brazilian herb known as "murici" or "murici-do-mato"	Phenolic compounds, flavonoids, gallic acid derivatives	Antioxidant properties	[97]
Alchornea triplinervia	*In vivo* mouse model	Antisecretory property; anti-*H. pylori* effect; gastroprotective action	Medicinal plant from Brazil	Flavonoids	Antisecretory action, increase of gastric mucosa prostaglandin E(2) levels	[96]
Amphipterygium adstringens (Schltdl.) Standl. (*Anacardiaceae*)	*In vitro* killing assay	Exhibits potent dose-dependent anti-*H. pylori* activity	Mexican folk medicine	Anacardic acids mixture	NA	[121]

Extract of Japanese rice also	*In vivo* Mongolian gerbil model	Anti-*H. pylori* activity; anti-inflammatory effect	NA	NA	NA	[122]
16 Mexican plants[1]	*In vitro* broth microdilution method	Anti-*H. pylori* activity	Mexican folk medicine	NA	NA	[123]
Bacopa monniera	*In vitro* broth microdilution method	Anti-*H. pylori* activity	In Ayurveda, (ancient medicine of India) known as *medhya rasayana* or "brahmi"	NA	Augmentation of defensive mucosal: mucin secretion, life span of mucosal cells, and gastric antioxidant	[124]
Propolis	*In vitro* test against *H. pylori* Test against Recombinant protein (peptide deformylase)	Anti-*H. pylori* activity	Resinous hive product collected by honeybees	Phenolic compounds, mainly Caffeic acid phenethyl ester (CAPE)	CAPE is a competitive inhibitor of *H. pylori* peptide deformylase	[107,125]
Gosyuyu (Wu-Chu-Yu) and Psoralen (extract from *Psoralea corylifolia*)	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Chinese herbal medicine, namely "buguzhi"	Alkyl methyl quinolone alkaloids	NA	[126]
Amu-ru 7, a	*In vitro* test against *H. pylori* *In vivo* Mongolian gerbil	Anti-*H. pylori* activity Did not cure Mongolian gerbil, but colonization rate diminish	Mongolian folk medicine	Alkyl methyl quinolone alkaloids *Rhei rhizome* is the most effective component	Inhibited biofilm formation by *H. pylori* partial inhibition of urease	[127]
Agrimonia eupatoria, *Hydrastis canadensis*, *Filipendula ulmaria*, and *Salvia officinalis*	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Western herbal medicine	NA	NA	[128]
Curcumin diferuloylmethane	*In vitro* test against *H. pylori* *In vitro* using AGS cells *In vivo* C57BL/6 mice and Male Sprague-Dawley rats	*In vitro* anti-*H. pylori* activity; effective in eradication of *H. pylori* from infected mice and in restoration of *H. pylori*-induced gastric damage	The major yellow pigment present in the rhizome of turmeric (the perennial herb *Curcuma longa*)	Diferuloylmethane	Suppressing secretion of metalloproteinases 3 and 9 by gastric cells noncompetitive inhibitor of *H. pylori* shikimate dehydrogenase, among others decrease nuclear factor-kB (NF-κB) p65	[76,106,129]
Nigella sativa (*Ranunculaceae*)	*In vivo* adult patients	Administrated with omeprazole had a eradication rate similar to triple therapy (consult Table 1)	Grows in the Middle East, Eastern Europe, and Eastern and Middle Asia	Thymoquinone, dihydrothymoquinone and terpenes	Disrupting the lipid structure of the cell membrane	[89]
Methanol extract of the leaf of *Allium ascalonicum*	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Known as garlic	Alkaloids, cardiac glycosides and saponins	Decrease urease activity	[130]
Leaves of *Piper carpunya* Ruiz and Pav. (syn *Piper lenticellosum* C.D.C.) (*Piperaceae*)	*In vitro* test against *H. pylori* *In vitro* against rat peritoneal leukocytes	Anti-inflammatory; anti-ulcer action	Widely used in folk medicine in tropical and subtropical South American	Flavonoids	Inhibition of H^+, K^+-ATPase activity	[98,99]
Apium graveolens seeds	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Used in Ayurvedic medicine	Compound with anti-*Helicobacter* activity, an asymmetric phthalide dimer	Induces morphologic changes in *H. pylori* and inhibits protein and DNA synthesis	[131]
Davilla elliptica and Davilla nitida	*In vivo* Swiss albino mice and male Wistar albino rats animal *In vitro* disc diffusion technique	Anti-*H. pylori* activity; gastric protection action	Brazil folk medicine	Phenolic acid derivatives, acylglycoflavonoids, and condensed tannins	Stimulats moderate levels of H_2O_2, trigger moderation of the oxidative burst and consequently the immune response	[132]
Resveratrol	*In vitro* test against *H. pylori* *In vitro* using MKN-45 cells	Inhibit urease, anti-inflammatory and anti-cancer activity cardioprotective and neuroprotective properties	highly abundant in red grapes	Polyphenol	Modulation of interleukin (IL)-6, NF-κB, and mitogen-activated protein kinases; modulatory effects on *H. pylori*-induced IL-8 secretion, reactive oxygen species production, and morphological changes	[102,103,133, 134-136]

Anisomeles indica	*In vitro* test against *H. pylori* *In vitro* using AGS cells	Anti-*H. pylori* activity; anti-inflammatory properties	From Southeast Asia and Australia	Ovatodiolide	Attenuated the inflammatory response by decreasing NF-κB activation and IL-8 secretion, inhibit lipopolysaccharide-induced inflammation in macrophages (including the secretion of the pro-inflammatory cytokine tumor necrosis factor-α, and nitric oxide (NO) production, and protein expressions of inducible NO synthase and cyclooxygenase-2 (COX-2)	[75]
Glycyrrhiza glabra	*In vitro* test against *H. pylori* *In vitro* using AGS cells *In vivo* adult patients	Anti-*H. pylori* activity; anti-inflammatory properties (consult Table 1)	Legume known as licorice from southern Europe and parts of Asia	Flavenoid, main component glycyrrhetinic acid	Inhibition *H. pylori* of DNA gyrase, protein synthesis and dihydrofolate reductase enzyme; anti-inflammatory activity likely through inhibition of COX and lipoxygenase pathways	[88,100]
Cistus laurifolius	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Folk medicine in Anatolia	Flavenoid, most active is quercetin 3-methyl ether (isorhamnetin)	NA	[137]
Sclerocarya birrea	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Medicinal plant used by Zulus, Vhavendas, Xhosas and Sothos of South Africa	Essential oils: terpinen-4-ol (35.83%), pyrrolidine (32.15%), aromadendrene (13.63%) and α-gurjunene (8.77%)	NA	[138]
Phyllanthus urinaria	*In vitro* test against *H. pylori* *In vitro* using AGS cells	Anti-*H. pylori* activity; anti-inflammatory properties	Tropical and subtropical countries (Taiwan)	Phyllanthin, phyltetralin, trimethyl-3,4-dehydrochebulate, methyl gallate, rhamnocitrin, methyl brevifolincarboxylate, β-sitosterol-3-O-β-d-glucopyranoside, quercitrin and rutin	Inhibits AGS cells adhesion and invasion; decreases NF-κB activation and IL-8 secretion	[139,140]
Artemisia douglasiana Besser (*Asteraceae*)	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Folk medicine in Argentina known as "matico"	Dehydroleucodine, a sesquiterpene lactone of the guaianolide type	Potent inhibitors of the transcription factor NF-κB	[141,142]
Geranium wilfordii	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Herb from China	Corilagin (1), and 1,2,3,6-tetra-O-galloyl-β-D-glucose	NA	[143]
HZJW, composed of 12 medicinal herbs	*In vitro* test against *H. pylori* *In vivo* Balb/c mice	Anti-*H. pylori* activity; reduction of ulcerative lesion; eradicate *H. pylori* in mice	Chinese herbal formula composed of 12 herbs listed in (91)	Protoberberine alkaloids palmatine, coptisine and aporphine alkaloid of magnoflorine	NA	[91]
Cratoxylum arborescens (Vahl) Blume	*In vitro* test against *H. pylori* *In vivo* Balb/c mice	Anti-*H. pylori* activity, anti-inflammatory activity; reduced ulcer area, higher mucus content	Asian herbal medicine	α-mangostin (AM), is a prenylated xanthone	Anti-COX-2 and anti-NO activities	[144]
Chenopodium ambrosioides L. And *Adina pilulifera*. *Chenopodium ambrosioides* L.	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Jinghua Weikang Capsule (Chinese patent drug for peptic ulcer	NA	NA	[145]
Momordica cochinchinensis Springer (*Cucurbitaceae*)	*In vivo* mice	Gastroprotective effect		Momordica saponin Ⅰ	NA	[146]

Sangre de grado (*Croton lecheleri* and *Croton palanostigma*)	*In vitro* test against *H. pylori* *In vivo* C57BL/6 mice	Anti-*H. pylori* activity No bactericidal effect in mice	Sangre de grado is a red, viscous latex from the cortex of trees used in Peruvian medicine	NA	Mice with higher hepatic metallothionein levels	[147]
Polygonum tinctorium Lour	*In vitro* test against *H. pylori* *In vivo* Mongolian gerbil	Anti-*H. pylori* activity; anti-inflammatory effect; decreased bacterial load in Mongolian gerbil	Known as indigo	Tryptanthrin and kaempferol (flavenoid)	Inhibition of nitric oxide production, and the transcription of cyclooxygenase	[80]
Artocarpus obtusus Jarret	*In vitro* test against *H. pylori* *In vivo* Mongolian gerbil	Anti-*H. pylori* activity; gastroprotective effect; increased mucus content	Endemic species of Borneo known as "pala tupai"	Pyranocycloartobiloxanthone A, a xanthone	Free radical scavenging effect, induction of HSP70, via anti-apoptotic property (down regulate bax gene), inhibits Cox-2 enzyme	[148]
Punica granatum and *Juglans regia*	*In vitro* test against *H. pylori*	Anti-*H. pylori* activity	Iranian plants	NA	NA	[149]

[1]*Castella tortuosa, Amphipterygium adstringens, Ibervillea sonorae, Pscalium decompositum, Krameria erecta, Selaginella lepidophylla, Pimpinella anisum, Marrubium vulgare, Ambrosia confertiflora, Couterea latiflora, Byophyllum pinnatum, Tecoma stans linnaeus, Kohleria deppena, Jatropha cuneata, Chenopodium ambrosoides*, and *Taxodium macronatum*. NA: Not applicable; *H. pylori*: *Helicobacter pylori*.

Tested compounds

There is a high diversity of tested compounds (Table 2) against *H. pylori* using diverse experimental approaches. The era of blind screening of compounds come to an end, the natural resources screening being no exception, so it is rational to use folk medicine plants. The majority of the studies report the use of herbs from China, given that the traditional Chinese medicine is a common practice in this country. Latin American countries come in second place, another continent with a rich history in medicinal plants usage. Usually each country studies its own herbs from folk medicine. So it is understandable the diversity of medicinal plants already tested or currently being tested.

The access to the information is not always an easy task. Effectively, many papers have only the abstract in English language, which difficult the access to information by the global scientific community.

Considering the high diversity of herbal medicines used, finding all papers reporting their use is not straightforward. In fact, the keywords associated with each study not always include general terms, but the name of the species used or its active compound. We suggest that studies analyzing the efficacy of plant extracts include the keywords phytomedicine, phytotherapy, herbal medicine or herb medicine, in order to turn papers' identification easier.

Most studies report the *in vitro* efficacy of the herbal therapy against *H. pylori*, but this isn't always followed by an effective eradication of the bacterium in animal models and/or clinical trials (Table 2). The clearance of *H. pylori* from the stomach of infected patients occurs by direct topical activity of the ingested drugs at the gastric mucosal epithelium, and specially by the systemic therapeutic activity, which result from the back secretion and re-entry of the absorbed active principle from the basal to the apical side of the gastric epithelium[91]. The inefficiency of the herbal product in an *in vivo* test after proved efficient in an *in vitro* test against *H. pylori* may be due to the inability of the compound to resist to the acidic medium of the stomach, inability to reach the bacteria trough the mucus layer secreted by the gastric mucosa epithelial cells (the thickness of the mucus layer or its impermeability to herbs at the site of infection), use of insufficient dose or to its inability to reach the bacteria *via* systemic circulation.

Active principle and mode of action

The active ingredient is not always identified; sometimes the group of compounds, but not the exact formula, is identified. The most common active principle identified belong to the group of flavonoids (Table 2). Flavonoids are widely distributed in plants and are recognized as the pigments responsible for the colours of leaves, especially in autumn (yellow). Flavonoids have low molecular weight and are composed of a three-ring structure with various substitutions. The flavonoids are recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities. The flavonoids are phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers[92]. These properties are again evidenced in the studies present in Table 2.

The mode of action of the herbs can be through the inhibition of essential bacterial enzymes. Some examples are given. Considering *H. pylori*, some flavonoids have also demonstrated inhibitory effects on *bacterium* growth[78,80,88,93-100], on *H. pylori* DNA gyrase[88,100] and urease[78,93,95,99], and vacuolation activity[99]. *H. pylori* induces gastric epithelial cell apoptosis *via* secreted mediators such as the VacA cytotoxin and lipopolysacccharides, damaging epithelial acid-secreting parietal cells[101]. Several flavonoids may inhibit the apoptotic signaling induced by *H. pylori* VacA toxin[99]. Since urease of *H. pylori* is es-

sential for its colonization, the inhibition of this enzyme explains partly the anti-*H. pylori* activity[83]. Resveratrol, which inhibits *H. pylori in vitro* and is present in grapes and red wine[102], inhibits urease enzyme as well[103]. Resveratrol also targets bacterial ATPases, which protect *H. pylori* from low pH levels by maintaining a proton gradient across membranes[104]. These results suggest that the consumption of grape extracts and wine constituents, in addition to triple therapy, might be useful in the treatment of *H. pylori* infection[105].

The *H. pylori* shikimate dehydrogenase, present in the shikimate pathway is essential for the synthesis of important metabolites, such as aromatic amino acids, folic acid, and ubiquinone. Curcumin is a competitive inhibitor of shikimate dehydrogenase[76]. Besides this action, it was shown that curcumin administration diminish the expression of NF-κB p65 in *H. pylori*-infected mice. Gastric inflammation is associated with increased NF-κB activation, which appears to be attenuated by curcumin[106]. Curcumin also suppresses the expression of, the matrix metalloproteinase-3 and -9 inflammatory molecules associated to the pathogenesis of *H. pylori* infection[76].

Some compounds with a known mechanism of action[107], like propolis (Table 2) are active *in vitro* but a not randomized clinical trial (Table 1) show that propolis was not efficient in eradicating *H. pylori*, which might be related to an insufficient dosage[108]. Briefly, caffeic acid phenethyl ester, the propolis active compound, is a competitive inhibitor of *H. pylori* peptide deformylase that catalyzes the removal of formyl group from the N-terminus of nascent polypeptide chains, which is essential for *H. pylori* survival[107]. Nevertheless, for the majority of the compounds the active component and the molecular mechanism of action (inhibition) against *H. pylori* remain unknown.

Limitations and promises

Adverse effects are typically minor than the ones that patients taking antibiotics have. The comparative study of *Nigella sativa* (*N. sativa*) and triple therapy revealed that adverse effects in patients taking *N. sativa* were minor than in patients taking antibiotics[89]. Side effects using cinnamon[109] and GutGard[110] were minor as well. Also, adverse reactions to flavonoids in humans appear to be rare[92].

Even considering that herbs are commonly perceived as natural products and thus safe, there is a need to test the biological active constituents of herbs, side effects caused by contaminants and drug-herb interactions. The safety of herbs could be obtained by requiring manufacturers to register with the FDA (or similar), to proceed with mandatory safety tests similar to those required for drugs, to require registering all health claims, and to assure that product labels provide an accurate list of all ingredients[61].

CONCLUSION

There is a huge multiplicity of phytotherapy studies; the majority of them done *in vitro* by exposing *H. pylori* cultures to the herbs. Some of these herbs appear very promising for fighting *H. pylori* antibiotic resistant strains. However, the mode of action, the active principle and the design of accurate clinical trials of promising herbal products should be addressed in future studies. Most of these phytotherapy approaches uses folk medicine products, especially from Asia (China) and Latin America, although other herbs are being tested from countries all over the world. For the herbs for which the mechanism of action is known, the anti-*H. pylori* activity appears to include inhibition of essential bacterial enzymes, while the gastroprotective action appears to be related with the modulation of the host immune system and/or attenuation of inflammation.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Factors that mediate colonization of the human stomach by *Helicobacter pylori*

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Abstract

Helicobacter pylori (*H. pylori*) colonizes the stomach of humans and causes chronic infection. The majority of bacteria live in the mucus layer overlying the gastric epithelial cells and only a small proportion of bacteria are found interacting with the epithelial cells. The bacteria living in the gastric mucus may act as a reservoir of infection for the underlying cells which is essential for the development of disease. Colonization of gastric mucus is likely to be key to the establishment of chronic infection. How *H. pylori* manages to colonise and survive in the hostile environment of the human stomach and avoid removal by mucus flow and killing by gastric acid is the subject of this review. We also discuss how bacterial and host factors may together go some way to explaining the susceptibility to colonization and the outcome of infection in different individuals. *H. pylori* infection of the gastric mucosa has become a paradigm for chronic infection. Understanding of why *H. pylori* is such a successful pathogen may help us understand how other bacterial species colonise mucosal surfaces and cause disease.

Key words: *Helicobacter pylori*; Colonization; Infection; Gastric mucosa; Urease; Flagella; Polymorphisms; Adhesins; CagA; Type Ⅳ secretion system

Core tip: Colonization of gastric mucus by *Helicobacter pylori* (*H. pylori*) key to the establishment of chronic infection. How *H. pylori* manages to colonise and survive in the hostile environment of the human stomach and avoid removal by "mucus flow" and killing by gastric acid is the subject of this review. We also discuss how bacterial and host factors may together go some way to explaining the susceptibility to colonization and the outcome of infection in different individuals. Understanding of how *H. pylori* causes chronic infection will likely serve as a valuable reference system for how other bacteria colonise mucosal surfaces.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram negative, microaerophillic, spiral shaped bacterium, which colonizes the human stomach. *H. pylori* is one of the most common infections in the world, persistently colonizing the gastric mucosa of over 50% of the global population.

Colonization with *H. pylori* induces a chronic gastritis in all infected individuals[1]. The majority of infections are asymptomatic, however long-term infection increases the risk of developing site-specific disease. 10%-15% of infections result in the development of peptic ulcer disease and *H. pylori* is associated with 95% of duodenal ulcers and 80% of gastric ulcers[2]. Infection with *H. pylori* is a significant risk factor for the development of gastric cancer and 1%-3% of infected individuals develop the disease[3,4]. As a result, *H. pylori* is classified as a group 1 carcinogen by the World Health Organisation[5]. Infection is associated in particular with intestinal-type (approximately 90% of patients) rather than diffuse-type gastric cancers (approximately 32% of patients)[6]. The risk of developing gastric cancer is reduced in patients with duodenal ulcers[7]. The gastric mucosa does not normally contain any mucosa-associated lymphoid tissue (MALT), however the pan-gastric inflammation induced by *H. pylori* infection results in the development of MALT. In < 0.1% of infected individuals this develops into B cell MALT lymphoma[3], however at early stages the lymphoma can be cured by eradication of *H. pylori*[8,9].

H. pylori exhibit a very strict tissue tropism. It colonizes the gastric mucosa of humans and is only found colonizing other sites in the body where gastric metaplasia occurs[10]. Within the gastric mucosa the majority of organisms are found living in gastric mucus[11] and we suggest that these organisms act as a reservoir of infection for the underlying epithelial cells. Only a small percentage of the organisms colonizing are found in association with epithelial cells but the interaction of the bacteria with the cells is essential for the development of disease. How *H. pylori* causes chronic infection, which can persist for the lifetime of the host, in the hostile acidic environment of the stomach while avoiding removal by both mucus, which is constantly "turning over", and the immune response is not completely understood. Evasion of the host innate immune response by *H. pylori* has recently been covered in two excellent reviews[12,13]. This review will focus on what we know about other specific bacterial and host factors that promote *H. pylori* survival in the stomach and colonization of the gastric mucosa, causing disease in some individuals but asymptomatic infection in others.

UREASE AND MOTILITY: TWO KEY ESSENTIAL COLONIZATION FACTORS

Urease

H. pylori is not an acidophile and key to its ability to overcome the acidic conditions of the gastric lumen is the production of a very potent urease enzyme. The expression of urease[14] and its activation[15] is essential for colonization of the gastric mucosa. *H. pylori* survives at a pH range between 4.0 and 8.0 in the absence of urea[16]. However, in the presence of urea the organism can survive at a pH as low as 2.5. The urease enzyme of *H. pylori* hydrolyses urea to NH_3 and CO_2 and has a Km value for urea of 0.8 mmol/L[17], meaning that it displays a much higher affinity for its substrate than that of ureases produced by other bacterial species. This allows for the utilization of the limited amounts of urea (5 mmol/L) present in the human stomach. The generation of NH_3 provides both acid-neutralising and acid buffering capabilities, enabling *H. pylori* to raise the pH in its microenvironment and periplasm thus maintaining the proton motive force. The biosynthesis of urease is controlled by a seven gene cluster. The urease enzyme, estimated to be approximately 600 kDa in size[18], consists of a complex of 12 UreA and UreB subunits[19], which individually are 30 and 62 kDa in size[18]. The protein is originally produced as an immature apoenzyme, and activation takes place when four chaperone proteins, UreE, UreF, UreG and UreH assemble the catalytic site of the protein[20]. The insertion of 24 nickel ions into the enzyme is essential for complete activation of the protein, as well as GTP hydrolysis[19]. The genes encoding UreAB are located in one operon[21], while the *ureEFGH* genes are found on another[22]. Recent studies have shown that the *ureAB* operon can yield a 2.7 kB transcript that produces a functional enzyme and also a 1.4 kB transcript, the product of which exhibits much lower urease activity and is generated by cleavage of the 3' *ureB* region. The expression of this smaller transcript was shown to be influenced by pH, the presence of the histidine kinase ArsS and the phosphorylation state of the response regulator ArsR, illustrating the influence that pH has on the expression of an active urease[23]. Urease is found in both the cytoplasm of *H. pylori* and on the surface of the bacteria due to the lysis of some organisms[24-26]. Intracellular urease, regulated by external pH, acts to increase the pH of the periplasm and increase membrane potential thereby allowing protein synthesis at low pH[27,28]. A proton-gated channel, UreI, which regulates the uptake of urea[29], is only active at acidic pH and therefore does not allow for the transport of urea into the bacterial cell at neutral pH, thus preventing lethal alkalinisation of the cytoplasm[30]. Buffering of the periplasm also occurs through the conversion of CO_2 to HCO_3^- by the periplasmic α-carbonic anhydrase[31]. HCO_3^- acts in conjunction with NH_3 to buffer the periplasmic and cytoplasmic pH, generating neutral conditions. This acid acclimation feature of the bacteria, maintaining an intracellular neutral pH while the pH of the environment is acidic, is unique to *H. pylori*, and is critical to survival of the organism in the stomach. The data above is strong evidence that the urease enzyme of *H. pylori* is a factor absolutely essential for survival of the organism in the human stomach. Indeed it may be a factor that explains why *H. pylori* is not found readily at other sites in the body, as production of ammonia by the urease enzyme at sites that are not acidic could increase the local pH above pH 7.0 and it has been shown that *H. pylori* is sensitive to alkaline conditions[30].

Motility

Another key colonization factor shown to be absolutely essential for colonization is the possession of polar flagella which confer motility on *H. pylori*[32]. Non-motile mutants lacking flagella are unable to establish persistent infection in animal models[33,34]. Studies focusing on the motor protein MotB, have shown that it is the motility conferred on the organism by the flagella that contributes to colonization and the presence of flagella alone is not sufficient[35]. *H. pylori* possesses two to six sheathed unipolar flagella. The structures extend 3-5 μm from the bacterial surface, with bulb-like structures often seen at the tip of the filaments[32]. The sheath consists of both proteins and lipopolysaccharide, and is thought to be an extension of the bacterial outer membrane that protects the flagellar filaments from acid in the stomach[36]. Expression of the two major flagellar proteins, FlaA and FlaB, are required for full motility of the bacteria. FlaA mutants exhibit a greater decrease in motility than that of FlaB mutants[37]. Other components of the flagellar structure which have also been shown to be essential for motility and colonization include the hook protein FlgE[38] and FliD, which functions as a hook-associated protein[39].

In keeping with flagella being an essential colonization factor for *H. pylori* flagellar biogenesis is a very well regulated process, dysfunction of which can have a strong impact on motility and infection. For example, deletion of the regulator FlhA, which controls expression of *flaA, flaB* and *flgE*, leads to the generation of non-motile mutants[40]. Of interest FlhA also regulates urease expression, and deletion of this membrane protein leads to a decrease in colonization rates[41]. Thus flagellar biosynthesis and urease activity, two key essential colonization factors of *H. pylori*, are linked. The response regulator FlgR, which is part of the two-component FlgRS system, controls expression of RpoN-regulated genes, including *flgE* and *flaB*, and deletion of this regulator abolishes expression of its target genes[42]. *H. pylori* utilises the histidine kinase FlgS to detect changes in its environment which in turn activates FlgR to modify transcription of flagellar genes. Interestingly, it does so independently of pH, so there must be other environmental triggers that activate transcription of flagellar genes[43]. More recently, production of auto-inducer 2 (AI-2) protein, a product of the *luxS* gene, has been shown to regulate expression of flagellar genes. Auto-inducers play a role in quorum sensing, and so it is possible that *H. pylori* regulates expression of its flagellar genes in response to bacterial numbers and it's environment through this system[44,45]. This is supported by evidence that mutants deficient in *luxS* exhibit reduced motility and infectivity rates compared to the wild-type[46]. Thus efficient and controlled expression and synthesis of the flagellar components is essential for successful motility and colonization.

Modifications to the flagella that impact on motility and colonization can also occur at a post-transcriptional level. The glycosylation state of the structure has been shown to impact on both, with *H. pylori* mutants deficient in a deglycosylase HP0518, exhibiting increased levels of the pseudaminic acid on FlaA and displaying a hypermotile phenotype. Infection studies showed that the mutants are able to associate more closely with epithelial cells and induce rapid activation of NF-κB, and also exhibit increased *in vivo* colonization rates. Thus increased levels of flagellin glycosylation in turn seem to increase the ability of *H. pylori* to colonize[47]. Other genes, including HP0326B and HP0178, involved in the biosynthesis of the precursors to pseudaminic acid, have been shown to have a similar role to HP0518. Inactivation of these also affects motility and colonization[48]. As well as modifying sugars found directly on flagellin proteins, changes in bacterial peptidoglycan by transglycosylases affects the functioning of the flagella by disturbing the localisation of MotB, thus preventing the flagellum from moving[49]. Thus, like the urease enzyme, motility is an essential colonization factor for *H. pylori*. The structure of the flagella with an outer sheath to protect against the effect of acid, stringent regulation of flagellar biogenesis and post translational modifications to flagella which result in enhanced motility and ability to colonise all underline the pivotal role of motility and flagella in *H. pylori* colonization.

COLONIZATION OF GASTRIC MUCUS BY *H. PYLORI*

The mucus layer that overlies the epithelial cells in the gastrointestinal tract is a physical barrier which acts to prevent pathogens from colonizing and interacting with the underlying epithelium. Pathogens which infect mucosal surfaces share two main goals: (1) to overcome the mucus barrier; and (2) to interact with the underlying epithelial cells which results in disease. The majority (approximately 80%) of infecting *H. pylori* are found living in gastric mucus rather than in contact with the underlying epithelium[11].

Penetration of gastric mucus

The entire epithelial surface of the gastrointestinal tract is covered in a thick layer of secreted mucus[50]. The mucus layer in the stomach is approximately 300 μm thick[50], and *H. pylori* has to penetrate it in order to colonise and gain access to the underlying epithelium. A pH gradient exists across the gastric mucus layer with the pH being approximately neutral at the epithelium but very acidic (pH 1-2) close to the lumen. *H. pylori* can lose motility rapidly when it encounters acidic conditions[51], therefore it is imperative that it penetrates the gastric mucus quickly and establish persistent colonization in an area close to the epithelium[52].

H. pylori is able to alter mucus structure which may aid movement through the viscoelastic mucus gel layer. A thioredoxin system that specifically reduces interchain disulphide bonds of mucins has been identified[53]. This reduces the gel-forming capabilities of mucins and therefore the viscoelastic properties of mucus aid-

ing travel of the bacterium through mucus. In addition the rheology of gastric mucin exhibits a reversible pH-dependent transition. At a strongly acidic pH the viscoelastic properties of mucus increase whereas the mucus becomes less gel-like as the pH increases above approximately 4.0[54]. The ability of *H. pylori* to utilise urea to raise the pH in its microenvironment modifies mucus so that it is less gel-like, enabling the bacterium to move quickly through it[55]. *H. pylori* is usually found close to the gastric epithelium[56]. When the pH gradient that exists across the gastric mucus layer was disrupted in Mongolian gerbils *H. pylori* were no longer found close to the epithelium but were scattered throughout the mucus layer, suggesting that pH plays an important role in maintaining the particular localization of *H. pylori*[56]. The characteristic helical shape of *H. pylori* is also thought to play a role in mediating penetration of gastric mucus. Alterations in the cross linking of peptidoglycan in the outer membrane has been shown to modify the shape of *H. pylori.* Some cell shape mutants are unable to colonise as efficiently as helical shaped bacteria despite displaying similar motility to wildtype bacteria *in vitro*[57]. However mutants which exhibited the most dramatic changes in cell shape had reduced motility compared to that of the wild-type and other mutants, and they exhibited further reduced ability to colonise mice compared to mutants that retained wild type motility. This led to the hypothesis that the characteristic helical shape of *H. pylori* allows the bacteria to penetrate gastric mucus in a corkscrew like motion[58].

Interaction of H. pylori with gastric mucus

H. pylori has been shown to form microcolonies within the mucus secreted by the surface epithelium of the gastric mucosa[59]. MUC5AC is the predominant secreted gel forming mucin expressed by gastric surface epithelial cells and *H. pylori* has been shown to co-localise with MUC5AC *in vivo*[60]. Lower mucus neck cells found in the antral glands produce MUC6. α1,4-linked N-acetyl-glucosamine capped O-linked glycans expressed on the surface of MUC6 have anti-microbial activity towards *H. pylori,* inhibiting the synthesis of cholesteryl-α-D-glucopyranoside a vital cell wall component[61]. Aberrant expression of MUC2 occurs occasionally in the stomach in areas of intestinal metaplasia, however *H. pylori* is not found in association with areas of complete intestinal metaplasia, although it is sometimes found in areas of incomplete metaplasia[62,63]. This suggests that MUC5AC or a molecule co-expressed with it may explain the preference of *H. pylori* for gastric mucus.

H. pylori has been shown to interact with the Lewis^b blood group antigen structure found on the surface of MUC5AC in gastric mucus and this interaction, which has been extensively studied, is mediated through the bacterial outer membrane protein BabA[64]. *H. pylori* can also interact with sialylated structures found on mucins *via* the sialic-acid binding adhesin (SabA). This 66 kDa outer membrane protein is a major adhesin of *H. pylori* and mediates binding to sialyl-Lewis^x and sialyl-Lewis^a glycoconjugates[65]. During chronic infection by *H. pylori* there is an increase in the proportion of sialylated structures present in the gastric mucosa, which is attributed to mucosal inflammation and transformation[66], and so SabA is thought to play a role in promoting chronic infection.

H. pylori can also interact with the membrane bound mucin MUC1 and this has been shown to be mediated *via* both BabA and SabA outer membrane adhesins. A Δ*babA* strain of *H. pylori* displayed significantly reduced adherence to MUC1 from two independent sources[67]. In addition, mutants lacking SabA, also displayed some reduction in binding suggesting that binding to sialylated structures play a role in the interaction of *H. pylori* with MUC1 *in vivo*[67]. MUC1 has been shown to be important in limiting infection by Helicobacter in a mouse model of infection[68]. Over expression of MUC1 was shown to counter regulate *H. pylori* induced gastric inflammation[69]. The interaction of *H. pylori* with MUC1 blocks *H. pylori* stimulated β-catenin nuclear translocation and attenuates IL-8 production and neutrophil induced gastric inflammation[70]. Speculation that augmented expression of MUC1 could be used for treatment of *H. pylori* gastritis highlights the need for model systems that express adherent mucus layers and appropriate mucins in order to test such hypotheses. Using an *in vitro* infection model we recently showed a clear interaction between *H. pylori* and the membrane bound mucin MUC1 present on the apical surface of the mucus secreting cell line HT29-MTX-E12[71] suggesting that this might be a useful model system to study the interaction of *H. pylori* with mucus and mucins.

Infection of mice with *H. pylori* demonstrated a reduction in the rate of mucin turnover and decreased levels of MUC1 in infected animals, thus creating a more stable niche for the bacteria to colonise and increasing the ability of the organisms to interact with the epithelial cells[72]. The bacterial factors which signal to the cells to modulate mucin synthesis and turnover have not yet been elucidated.

Gastric mucins have been shown to promote the proliferation of *H. pylori* and to alter gene expression[73]. We looked at the interaction of *H. pylori* with purified native mucins from different animals that were printed on a microarray slide. Three strains of *H. pylori* were examined, strains J99, G27 and 26695. Strain 26695 is known not to express active BabA or SabA proteins and is therefore unable to bind to Lewis^b or Sialyl Lewis^x. Surprisingly all three strains bound to porcine gastric mucins equally well. This finding suggests that in addition to BabA and SabA other bacterial adhesins must exist which can mediate the binding of *H. pylori* strains such as strain 26695 to mucin.

While the interaction of *H. pylori* with gastric mucins has been well characterized the interaction of the organism with other non-mucin components of mucus has not received as much attention. In addition to mucin *H.*

pylori has recently been shown to interact with mucus bound glycolipids[74] and we have previously shown that *H. pylori* interacts directly with TFF1, a member of the trefoil peptide family of proteins which is co-expressed with MUC5AC in the stomach, and this interaction is mediated by the core oligosaccharide portion of *H. pylori* LPS[75,76]. The optimum pH for *H. pylori* binding to TFF1 was 5.0-6.0[76]. The pH dependence of this interaction indicates that binding of *H. pylori* to TFF1 in the stomach could promote colonization of the mucus layer adjacent to the gastric epithelial surface. The interaction of *H. pylori* with TFF1 may explain the distinct tropism that the organism exhibits for gastric tissue and specifically for the gastric mucin MUC5AC. A role for TFF1 in colonization of mucosal surfaces by *H. pylori* was confirmed in a study which showed that *H. pylori* mutants expressing a truncated core oligosaccharide unable to interact with TFF1 have a reduced ability to colonise the mucus layer produced by the mucus secreting HT29-MTX-E12 cell line compared to wildtype strains[71]. In summary *H. pylori* interacts with both secreted and membrane bound mucins in the intestinal tract. These interactions are mediated *via* the well described outer membrane adhesins BabA and SabA interacting with fucosylated and sialylated glycans found on mucins and possibly other as yet undescribed adhesins. *H. pylori* has also been shown to interact with TFF1 a small protein found co-expressed with MUC5AC in the stomach. This suggests that the interaction of bacteria with non mucin components of mucus warrants further investigation.

INTERACTION OF *H. PYLORI* WITH THE GASTRIC EPITHELIAL CELLS

While the majority of *H. pylori* organisms live in gastric mucus and only a small percentage of infecting organisms interact with gastric epithelial cells, it is widely accepted that the organisms in contact with the epithelial cells cause disease. A number of bacterial outer membrane proteins have been identified that can act as adhesins. Following binding of *H. pylori* to epithelial cells the organism signals to the cells to subvert host cell function and this results in the development of pathology.

BabA

BabA together with SabA, both already mentioned above, are the two best characterized adhesins of *H. pylori*. Binding of *H. pylori* to fucosylated structures, including the H-1 type and Lewis^b blood group antigen[77] is mediated *via* BabA, a 78 kDa outer membrane protein[64]. Two genes encode for a BabA protein, *babA1* and *babA2*. Only the protein encoded by *babA2* is functionally active, due to the presence of a 10 bp insert in the gene that encodes for a translational initiation codon[64].

A high level of heterogeneity is found in the BabA protein amongst strains, with various polymorphisms being identified, and different levels of Lewis^b binding observed[78]. There is also a high level of allelic variation in *bab* genes. *H. pylori* possesses a closely related gene to *babA*, *babB*, and both proteins are highly similar in their N- and C-terminus regions, but vary quite significantly in their central region. The BabB protein does not bind Lewis^b, indicating that the central region of the two proteins confers unique functions[79].

Geographic location can also influence the binding specificities of BabA, with strains from specific regions often exhibiting a specialist phenotype in which they only bind to certain blood group antigens, whereas strains from other regions are more generalised in their BabA-mediated binding and interact with numerous different blood-group antigens. This differential binding is thought to be a result of a selective pressure that led to evolution of the BabA protein, with the predominant blood group of a specific region influencing the binding specificity of the BabA adhesin[80]. Furthermore, strains have been identified which possess an active *babA2* gene, but do not produce a functional protein[81].

BabA expression is extremely dynamic *in vivo*. Experimental infection of Rhesus macaques showed a loss of babA expression post-infection, caused by either a change in the number of CT dinucleotide repeats in the 5' region of *babA*, or replacement of *babA* by the uncharacterised *babB*. Both events yielded a non-functional BabA protein that exhibited no Lewis^b binding[82]. Another study showed loss of BabA expression six months after infection of Mongolian gerbils, and this was attributed to nucleotide changes that introduced a stop codon into the sequence, which in turn yielded a truncated BabA protein[83]. When these results were elaborated on, experiments showed that modification in six amino acids eliminated binding of *H. pylori* BabA to Lewis^b following infection of Rhesus macaques. The *babA2* gene underwent gene conversion in which a portion of the gene was replaced by a portion of the non-functioning *babA1* gene. While the strains still expressed BabA, these six amino acid changes were enough to eliminate BabA binding[84]. A recent study reported inter-micro-niche variation in *H. pylori* infected patients, with isolates of the same strain exhibiting differences in *babA* and *babB* copy number and gene location, as well as BabA/B chimeras[85]. BabA is thus a highly variable protein that is easily susceptible to change at both a genetic and protein level.

SabA

H. pylori binds to sialylated structures present on gastric mucin and on epithelial cells *via* the sialic-acid binding adhesin, SabA. SabA also binds to sialylated receptors on neutrophils, which leads to nonopsonic activation of the neutrophils, phagocytosis of the bacteria and induction of the oxidative burst response[86]. Furthermore, the adhesin exhibits haemagglutinating activity, binding to gangliosides on erythrocytes in mucosal blood vessels. Differences between strains in their ability to bind to sialylated carbohydrates has been seen, and such differences may allow for the pathogen to adapt to changing

glycosylation patterns in the host during infection[87]. Similar to BabA there is a high degree of genetic diversity in SabA. The gene has a number of poly-T tracts in its promoter region and a stretch of CT dinucleotide repeats in its coding region[88]. Phase variation can occur at these sites, and leads to allelic variation in the SabA locus.

Differences in the length of the CT dinucleotide repeats found in the coding regions are often seen, giving rise to various alleles. The number of repeats differs among strains, and was originally thought to determine the functionality of the *sabA* gene, with seven repeats yielding a functional gene, while six or eight repeats leads to the gene being turned off[89]. However, later findings speculated that other sequences in the gene rather than the number of repeats were playing a role in the functionality of SabA. The sequence following the CT repeat region has been shown to play a role in expression, with strains possessing seven CT repeats having both in frame and out of frame *sabA* genes depending on the nucleotide sequence following the repeat region[88].

The poly-T tracts which are found upstream of the *sabA* gene also undergo phase variation. Recently it was shown that the length of these tracts can vary between strains, and that the length can influence the promoter activity of the *sabA* gene. This variation also arises through slip-strand mispairing, similar to the CT repeats[90]. This variation could be very beneficial to *H. pylori*, for example when it is exposed to acidic conditions. SabA is regulated by the acid-responsive two-component ArsRS system[91]. Expression of the gene correlates inversely with the acid secretion in the stomach[92]. Therefore, when acidic conditions prevail, there is low SabA expression. The ArsRS system itself appears to repress expression of SabA, with a major increase in adherence dependent on SabA seen in mutants lacking the histidine kinase ArsS. However, this repressive effect on SabA expression only occurs in strains that have an in-frame SabA allele[93]. This highlights the role that phase variation plays in infection, as it may allow *H. pylori* to alter its adhesin expression in response to a change in environmental conditions.

Another mechanism that *H. pylori* utilises to modify *SabA* expression is gene duplication. It was recently shown that an increase in the copy number of *sabA* leads to greater production of the protein, which in turn leads to greater adherence by the bacteria *in vitro*. It is thought this gene conversion event occurs due to natural uptake of the DNA by competent bacteria[94]. The *sabA* locus is therefore a highly heterogenic one, enabling *H. pylori* to alter its adhesin profile depending on the microenvironment and what host defences it comes in contact with.

AlpA and AlpB

AlpA and AlpB are two closely related proteins carried on the same operon in the *H. pylori* genome[95]. Loss of these proteins was found to influence the ability of *H. pylori* to colonise the guinea pig stomach[96] and to bind to gastric tissue, indicating a role in adhesion of the bacteria[97]. Virtually all strains express AlpA and AlpB, indicating they have an essential function[98]. Recently, these two proteins were shown to contribute to the ability of *H. pylori* to bind host laminin[99]. While their role in colonization is established, the effect AlpA/B has on infection remains unclear.

HopZ

Helicobacter expresses up to 33 outer membrane proteins (OMPs) or Hops (Helicobacter outer membrane porins). HopZ was identified as an OMP of *H. pylori* that plays a role in colonization[100]. Two allelic variants of the *hopZ* gene were identified, with a 20 amino acid region present in only one allele. The sequence of *hopZ* consists of a number of dinucleotide repeats in the signal peptide. These have also been found in other outer membrane proteins of *H. pylori*, and it is thought that they allow for slip-strand mispairing, which in turn generates phenotypic variation between strains. The number of repeats varies among strains, and determines functionality of the protein, with 7 or 10 dinucleotides allowing for expression of an intact protein[100]. The role of HopZ in infection has been largely unexplored. There is strong selection *in vivo* for HopZ expression as *hopZ* ON variants were recovered from volunteers challenged with a *hopZ* OFF strain, BCS 100[101]. Transmission of *H. pylori* within families has also been associated with a status change of *hopZ*. In contrast, *hopZ* sequences obtained from 26 sets of sequential isolates from chronically infected individuals showed no changes of status, suggesting that the *hopZ* status selected during early infection is subsequently stable[101].

CagA and cag PAI

Bacterial factors that increase the virulence of certain strains are responsible in part for the outcome of infection with *H. pylori*. The best characterized virulence factor is the product of the cytotoxin-associated gene A, CagA[102]. Strains expressing CagA are associated with more severe forms of disease and expression is closely related to that of the vacuolating cytotoxin, VacA[103]. Following binding of *H. pylori* to epithelial cells strains that express the CagA protein can inject it into the cells *via* a Type IV secretion system (T4SS) encoded by genes present on a pathogenicity island termed the *cag*PAI. Upon translocation into host cells CagA can be phosphorylated by host cell kinases and act to subvert host cell signaling mechanisms[104].

The *cagPAI* contains approximately 30 genes, many of which are involved in synthesis of the T4SS[105]. CagL is a highly conserved protein amongst *H. pylori* strains that forms the tip of the pilus of the T4SS, allowing for CagA translocation into host cells. It possesses an arginine-glycine-aspartate (RGD) motif, which allows the bacteria to bind to the $\alpha_5\beta_1$ integrin on the surface of target cells[106]. A closely related protein, CagI, has also

been shown to be an essential part of the T4SS, and the expression of both proteins is influenced by the expression of other *cag*PAI products, indicating that their expression requires partial assembly of the T4SS[107]. CagI however is not required for transport of CagA from the cytoplasm to the bacterial membrane[108]. The recently annotated transmembrane spanning CagM protein also appears to contribute to CagA translocation, with mutants deficient in the protein exhibiting little to no CagA translocation upon contact with host cells[109]. Non-CagPAI proteins have also been shown to be involved in the translocation process. For example the outer membrane protein HopQ is required for CagA injection as well as the intracellular responses induced by CagA[110]. While not essential, the iron-regulator Fur was also shown to influence CagA expression and cellular phenotypic changes induced upon CagA injection using adherence assays with AGS cells[111]. CagA positive strains of *H. pylori* which possess a functional BabA along with VacA are thought to be more virulent in the disease type they induce[112]. BabA-mediated adherence contributes to the pathogenesis of disease by inducing proinflammatory cytokines and precancerous related factors (such as CDX2), a phenotype which is dependent upon expression of the T4SS[113]. While BabA appears to promote CagA mediated pathogenesis, in contrast, binding of *H. pylori via* the AlpAB outer membrane proteins may signal to restrict the amount of CagA protein injected into the host cell[114]. AlpAB also seems to influence signalling cascades and immune responses upon infection, with differences seen between strains of different origins[115].

EPIYA motifs present on CagA are phosphorylated and the 145 kDa phosphorylated protein induces a strong immune response[116]. The number of motifs varies amongst strains, and strains with a higher number of motifs are more biologically active and hence more virulent, potentially playing a role in the development of gastric carcinoma[117]. Each EPIYA motif can be classified into a certain type, namely EPIYA-A,-B,-C and -D motifs[118]. Studies have shown that host kinases can be specific in the EPIYA motif that they target, with c-Src kinases exhibiting preference for EPIYA-C and EPIYA-D motifs, while c-Abl is more general and phosphorylates all four motifs[119].

CagA also exhibits phosphorylation-independent effects, many of which remain elusive. A conserved-motif in the C-terminus of the non-phosphorylated protein has recently been identified, and was shown to interact with the host hepatocyte growth factor receptor Met, which contributes to cellular proliferation and inflammation *via* the Akt signalling pathway, which activates NF-κB and β-catenin[120]. CagA also disrupts Par1b kinase activation, a protein involved in maintaining cell polarity and adherens junctions, independently of tyrosine phosphorylation[121]. The complex formed between E-cadherin and β-catenin in the adherens junctions of cells is also targeted by *H. pylori* in a CagA phosphorylation-independent manner. A physical interaction between CagA and E-cadherin disrupts the complex, and leads to a cytoplasmic accumulation of β-catenin, a protein which, when deregulated, has been shown to contribute to carcinogenesis[122]. Taken together the above suggests that binding of *H. pylori* to epithelial cells is a dynamic process and bacteria can alter the expression of adhesins depending on the availability of receptors. Close association of the bacteria with epithelial cells enables the translocation of the bacterial CagA protein across the cell membrane where it can be phosphorylated and lead to subversion of cell signaling. CagA can also cause disruption of epithelial cell junctions in a phosphorylation independent manner. CagA is thus a key virulence factor of *H. pylori*. Figure 1 illustrates *H. pylori* colonization of gastric mucus, the interaction of bacteria with host cells and the events triggered by that interaction.

HOST FACTORS THAT PLAY A ROLE IN THE DEVELOPMENT OF *H. PYLORI* ASSOCIATED DISEASE

The ability of *H. pylori* to cause disease depends not just on bacterial factors but also on environmental and host factors. A number of host gene polymorphisms have been identified which are thought to increase *H. pylori* colonization and increase susceptibility to disease. Some polymorphisms have been identified as risk factors for *H. pylori* associated gastric cancer. The *IL-1* gene cluster located on chromosome 2q is composed of three genes which encode the pro-inflammatory cytokines IL-1α and IL-1β as well as their endogenous receptor antagonist IL-1ra. IL-1β is a potent inhibitor of gastric acid secretion *in vivo*[123,124]. *H. pylori* infection results in an upregulation of IL-1β, which plays an important role in initiating and amplifying the immune response[125,126]. Three diallelic polymorphisms in the *IL-1β* gene have been identified all of which are C-T substitutions at positions -511, -31 and +3954 from the transcriptional start site. Polymorphisms at position -511 and -31 are associated with increased IL-1β secretion and subsequent hypochlorhydria in the presence of *H. pylori*[127], symptoms which have been implicated in the development of gastric cancer. A recent study in Venezuela has reported that infection with the more virulent *cagA*+ *H. pylori* strains is associated with individuals harbouring the +3954 polymorphism[128].

Polymorphisms in *IFN-γ* were associated with infection by *cag* positive strains and polymorphism in *TNF-α* were associated with development of peptic ulcer disease while polymorphisms in *IL-10* favoured the development of intestinal metaplasia and non-cardia gastric cancer[129]. Polymorphisms which effect cytokine function may explain the highly variable outcomes of *H. pylori* infection, highlighting the importance of genetic background of the host in disease progression and susceptibility to infection by specific *H. pylori* strains.

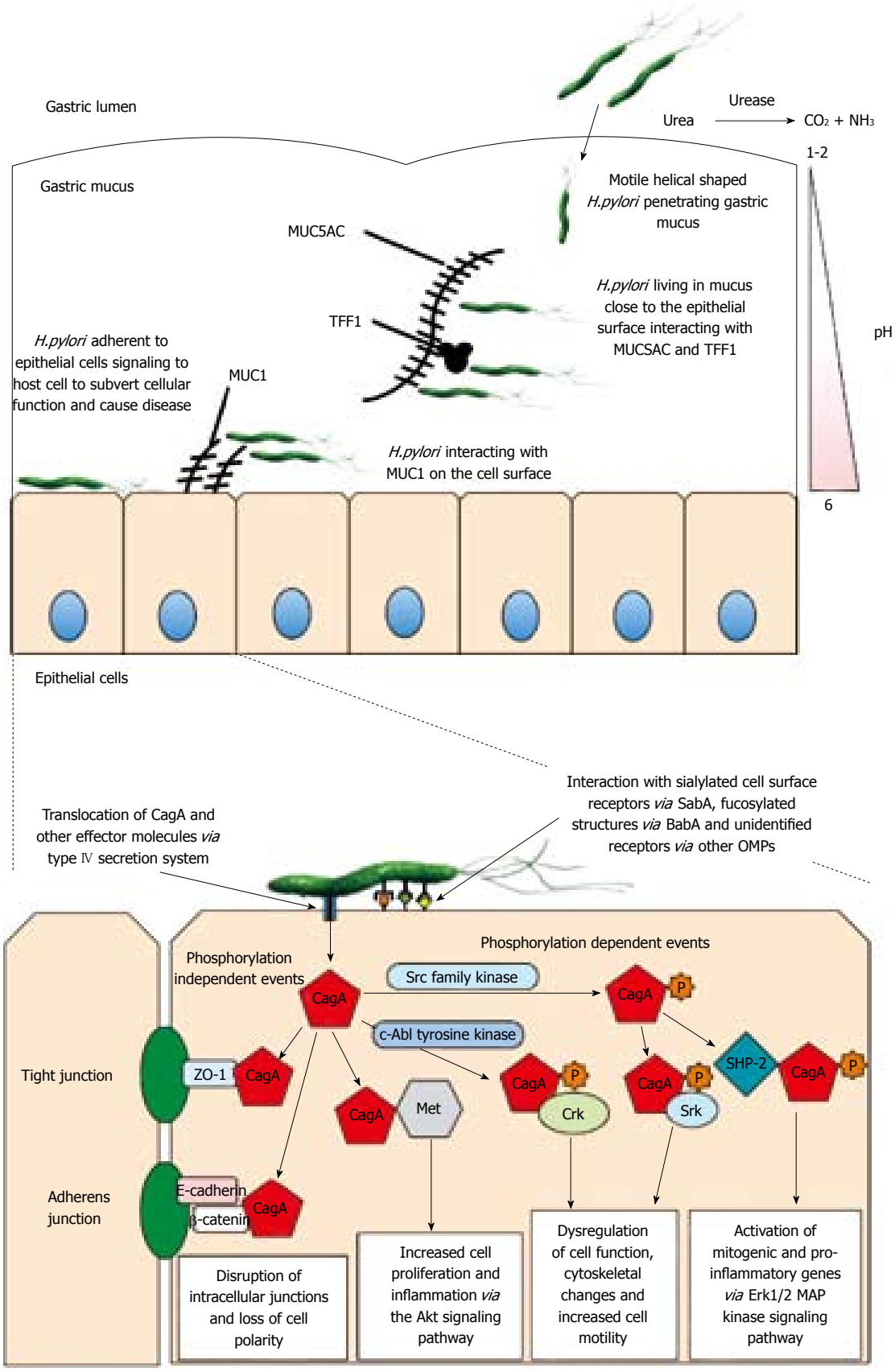

Figure 1 Colonization and infection of the gastric mucosa by *Helicobacter pylori*. The majority of bacteria live in gastric mucus close to the epithelial surface. Motile, helical shaped bacteria can penetrate gastric mucus and escape the acidic conditions of the gastric lumen. Urease acts to generate ammonia in the presence of urea thus raising the pH and protecting the transiting bacteria from the effects of gastric acid. A subset of bacteria interact with gastric epithelial cells. Translocation of cytotoxin-associated gene A (CagA) from the bacteria into the host cell cytosol results in CagA phosphorylation dependent and CagA phosphorylation independent events occurring which subvert epithelial cell function.

Polymorphisms in genes involved in the innate and adaptive immune response play a role in host susceptibility to infection and disease progression. IL-2 plays an important role in mediating the T lymphocyte response including the Th1 phenotype which is known to dominate the immune response to *H. pylori* infection. An increase in IL-2 production as a result of a gene polymorphism, T330G, has been shown to be negatively associated with *H. pylori* infection in adults[130]. Autophagy plays a critical role in the modulation of host immunity/inflammatory responses and is thought to serve as an innate defence mechanism against infection[131,132]. A single nucleotide polymorphism in the autophagy gene, *ATg16L1* (T300A), has been identified as a causal risk variant for Crohn's disease. The functional relevance of this polymorphism is not known but it is thought to result in production of an unstable ATg16L1 protein leading to impaired cytokine responses and antimicrobial autophagy. Induction of autophagy in response to the *H. pylori* VacA cytotoxin is significantly reduced in cells harbouring the T300A polymorphism and a survey of Scottish and German individuals showed that the T300A polymorphism is associated with a higher odds ratio of *H. pylori* infection[133], suggesting that the T300A polymorphism in *ATg16L1* confers a modest but significant host genetic risk of *H. pylori* infection.

There is an association between polymorphisms in the gene encoding the membrane-associated mucin MUC1 which result in shorter MUC1 alleles and *H. pylori* related gastritis[134]. Short *MUC1* alleles have also been linked to the development of gastric adenocarcinoma[135] and intestinal metaplasia in patients with chronic gastritis[136]. In support of the hypothesis that a link exists between short *MUC1* alleles and *H. pylori* related pathologies it has been shown that *Muc1*$^{-/-}$ mice display increased colonization by *H. pylori*, compared to wildtype controls, and also display increased severity of *H. pylori* induced gastritis. Shorter *MUC1* alleles encode proteins with a much smaller extracellular domain, thereby reducing the ability of MUC1 on the surface of cells to maintain a distance between the microbe and the cell surface and thus protect the cell from microbial adhesion and the subsequent development of disease[67].

H. pylori is rarely found in the mucus covering the gastric gland[59] where mucins are capped with a terminal α-1,4-linked *N*-acetylglucosamine[137]. Glycans possessing this residue suppress *H. pylori* growth *in vitro*[61]. The transfer of α-1,4-linked *N*-acetylglucosamine is mediated by a transferase encoded by the gene *a4GnT*[137]. Polymorphisms in this gene may lead to altered expression or function of the encoded transferase. An analysis of single nucleotide polymorphisms in *a4GnT* found that changes in *H. pylori* seropositivity were associated with changes in *a4GnT* and may be related to an increased risk of *H. pylori* infection[138].

The secretor status of an individual is also thought to influence their susceptibility to infection. A Fucα1,2-glycan is common to all three ABH blood group antigens. These carbohydrate structures are expressed along the gastro-intestinal tract of individuals with a positive secretor status[139,140]. Secretor individuals express the secretor-(fucosyl)transferase, an enzyme involved in the synthesis of Fucα1,2-glycan. Non-secretors lack expression of this fucosyl-transferase[141] while weak secretors express a mutated secretor-(fucosyl)transferase with reduced enzymatic activity. Studies involving infected secretor and non-secretor mice have revealed that the non-secretor mice display reduced adhesion of *H. pylori* to gastric tissue[142]. In addition experimental infection of Rhesus monkeys has revealed that the weak secretor phenotype displays reduced *H. pylori* density and reduced inflammation[143]. Non-secretor phenotypes have been shown to express higher inflammatory reactivity and expression of sialylated antigens in response to *H. pylori* infection, and this may explain the higher incidence of peptic ulcer disease previously reported in these individuals[144]. Bacterial factors alone cannot explain why some individuals develop disease upon *H. pylori* infection whereas others remain asymptomatic. The discovery of host factors such as the polymorphisms described above go some way to explaining the different outcomes of infection in different individuals.

CONCLUSION

H. pylori has evolved with humans over thousands of years and is a paradigm of chronic infection. Both bacterial and host factors play a role in mediating bacterial virulence and susceptibility to infection and can explain somewhat the outcomes of infection in different individuals. However, we suggest that *H. pylori* colonization of gastric mucus is key to the ability of the organism to cause chronic infection in humans. The establishment of a reservoir of bacteria in gastric mucus close to the epithelial surface would allow *H. pylori* to avoid removal by mucus flow and killing by gastric acid. The interaction of *H. pylori* with gastric mucus could limit the number of bacteria that can interact with gastric epithelial cells and thus also limit the inflammatory response and promote chronic infection. Modulation of the interaction of *H. pylori* with gastric mucus may be a viable alternative or even adjuvant therapy to antimicrobials for prevention of colonization and the eradication of the organism. Given that the majority of infections in humans and animals occur through mucosal surfaces combined with the results of recent studies which show that alterations in the composition of the gut microflora, sometimes referred to as dysbiosis, are associated with a number of chronic diseases including obesity[145], diabetes[146] and inflammatory disease[147] there is now intense interest in how bacteria living in mucus cause disease. Studies on *H. pylori* are likely to serve as a valuable reference system for how other organisms colonise and infect mucosal surfaces.

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TOPIC HIGHLIGHT

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Blood pressure and stature in *Helicobacter pylori* positive and negative persons

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Abstract

To evaluate vital signs and body indices in *Helicobacter pylori* (*H. pylori*) positive and negative persons. A total of 22 centres entered the study. They were spread over the whole country, corresponding well to the geographical distribution of the Czech population. A total of 1818 subjects (aged 5-98 years) took part in the study, randomly selected out of 38147 subjects. *H. pylori* infection was investigated by means of a 13C-urea breath test. Data on height, weight, systolic and diastolic blood pressure and heart rate were collected at the clinics of general practitioners. The overall prevalence of *H. pylori* infection was 30.4% (402/1321) in adults (≥ 18 year-old) and 5.2% (26/497) in children and adolescents (≤ 17 year-old). Once adjusted for age and gender, only a difference in body mass index remained statistically significant with *H. pylori* positive adults showing an increase of 0.6 kg/m^2 in body mass index. Once adjusted for age and gender, we found a difference in height between *H. pylori* positive and *H. pylori* negative children and adolescents. On further adjustment for place of residence, this difference became statistically significant, with *H. pylori* positive children and adolescents being on average 3.5 cm shorter. *H. pylori* positive adults were significantly older compared to *H. pylori* negative subjects. Once adjusted for age and gender, *H. pylori* infection had no impact on body weight, body mass index and vital signs either in adults or children and adolescents. Chronic *H. pylori* infection appeared to be associated with short stature in children. *H. pylori* infection did not influence blood pressure, body weight and body mass index either in adults or children and adolescents.

Key words: Epidemiology; *Helicobacter pylori*; Czech Republic; 13C-urea breath test; Blood pressure; Heart rate; Weight; Stature; Body mass index

Core tip: Our group studied body indices and basic vital signs in *Helicobacter pylori* (*H. pylori*) positive and negative persons in 2001. The prevalence of *H. pylori* infection decreased significantly in the Czech Republic from 41.7% (2001) to 23.5% (2011). The aim of this multi-centre prospective study was to evaluate body indices and vital signs using comparable methods in the general population from identical geographical areas 10 years later. According to our current results, chronic *H. pylori* infection was associated with short stature

in children. *H. pylori* infection did not influence blood pressure, body weight and body mass index either in adults or children and adolescents.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is the most common chronic bacterial infection in humans. *H. pylori* has been demonstrated worldwide and in individuals of all ages. Infection is acquired at an earlier age and is more frequent in developing countries compared with industrialised ones. *H. pylori* is a cause of chronic gastritis. About one third of all gastric cancers in developed and developing countries, respectively, are solely attributable to *H. pylori*. *H. pylori* infection is a major aetiological agent in peptic ulcer disease[1].

It has been hypothesised that chronic *H. pylori* infection may be associated with and/or contribute towards several extra-gastric diseases, including ischemic heart disease[2-12], arterial hypertension[2,13-20], cerebral non-cardioembolic ischemic stroke[21,22], peripheral arterial disease[23,24], obesity[13,25-27], metabolic syndrome[28,29] and short stature[30-36]. Our group studied body indices and basic vital signs in *H. pylori* positive and negative persons in a large prospective multi-centre study in 2001[37,38]. In the meantime, prevalence of *H. pylori* infection decreased significantly in the Czech Republic from 41.7% to 23.5% within a 10-year period[39,40]. The aim of our current multi-centre prospective study was to evaluate blood pressure and stature in *H. pylori* positive and negative persons in the Czech Republic using comparable methods in a representative sample of general unselected population from identical geographical areas 10 years after the initial study.

SEARCH

Ethics

The study was approved by the University Ethics Committee. All participants received detailed written information about the Project in advance and signed written consent (parents on behalf of their children). For all data obtained, all personal identification information was deleted in compliance with the laws for the protection of confidentiality of the Czech Republic.

Study population

A total of 22 centres entered the study. They included 15 centres of general practitioners for adults and 7 for children and adolescents. These centres covered cities and towns with more than 20000 inhabitants (10 centres), smaller towns (≤ 20000 inhabitants) with surrounding villages (9) and rural areas (3 centres), and were spread over the whole country, corresponding well to the geographical distribution of the Czech population. A total of 1818 subjects (aged 5-98 years) took part in the study, randomly selected out of 38147 registered males and females in this age range.

Urea breath test

Urea breath tests were performed in the morning after overnight fasting by means of 13C-urea breath test[41]. Citric acid solution (3 g dissolved in 150 mL of still water) was given initially as a test drink. Five minutes later two baseline exhaled breath samples were collected into 20-mL vacutainers using a straw. After this, all of the subjects ingested 75 mg 13C-urea (Helicobacter Test INFAI, INFAI GmbH, Köln, Germany) dissolved in 50 mL of still water with 1 g citric acid (at time 0). Breath samples were collected in duplicates using a straw in 20-mL vacutainers after 30 min. Tubes with breath samples were sent to a single analytical centre by post and measured within a one-week period. Breath samples in duplicates were analysed using isotope ratio mass spectrometry (AP 2003, Analytical Precision Products, Cambridge, United Kingdom). The cut-off point was 3.5.

Body indices and basic vital signs

Data on height and weight were collected at the clinics of general practitioners (measured in underwear by nurses). Body mass index was calculated as weight/height^2 (in kg/m^2). Systolic and diastolic blood pressure measurements were performed by a trained nurse in a standard manner[42] at the study clinics. Manual resting pulse measurement at the wrist (radial artery) was performed in sitting position by a trained nurse for 60 s. The blood pressure and heart rate were measured in the morning (from 8 to 12 a.m.).

Questionnaires

Data were collected by self-completed questionnaires distributed to adults and parents of children aged 5-15. The questionnaire included information on the place of residence in childhood, mother's and father's education, access to running warm water in childhood, crowding in childhood and number of siblings. Information on the study subjects' current place of residence, education, marital status, self-reported socio-economic group and smoking habits was also collected in the questionnaire and was used in the analysis of determinants of *H. pylori* positivity in subjects aged 15+ years of age. We combined the mother's and father's education and generated a variable indicating highest education achieved by any of the two parents (or the mother if single). This variable was used in analyses of blood pressure and stature in children and adolescents.

Table 1 Body indices, basic vital signs and socio-demographic characteristics in *Helicobacter pylori* positive and negative children and adolescents (≤ 17 years old)

Parameter	*Helicobacter pylori* negative	*Helicobacter pylori* positive	Statistical significance
	n = 471	*n* = 26	
	(mean ± SD)	(mean ± SD)	
Gender (% males)	47.1	46.1	NS
Age (yr)	11 ± 3	12 ± 3	NS
Weight (kg)	42.3 ± 17.5	40.3 ± 16.9	NS
Height (cm)	145.4 ± 19.6	143.6 ± 19.7	NS
BMI (kg/m^2)	19.1 ± 3.9	18.6 ± 3.7	NS
Systolic blood pressure (mmHg)	107.6 ± 12.6	110.3 ± 10.2	NS
Diastolic blood pressure (mmHg)	65.5 ± 8.6	65.2 ± 8.2	NS
Heart rate (beats per minute)	77.7 ± 7.7	80.9 ± 8.4	0.040
Residence[1]			NS
Larger town	32.0%	32.0%	
Smaller town	24.7%	32.0%	
Village	43.3%	36.0%	
Parental education			0.006
Secondary or higher	66.0%	57.7%	
Vocational	29.3%	23.1%	
Elementary	4.7%	19.2%	

[1]Based on *n* = 491. NS: Not significant; BMI: Body mass index.

Table 2 Body indices, basic vital signs and socio-demographic characteristics in *Helicobacter pylori* positive and negative adults (≥ 18 years old)

Parameter	*Helicobacter pylori* negative	*Helicobacter pylori* positive	Statistical significance
	n = 919	*n* = 402	
	(mean ± SD)	(mean ± SD)	
Gender (% males)	46.3	48.5	NS
Age (yr)	48 ± 20	57 ± 17	*P* < 0.001
Weight (kg)	76.3 ± 16.2	79.1 ± 16.4	*P* = 0.004
Height (cm)	171.6 ± 9.9	170.2 ± 9.9	*P* = 0.020
BMI (kg/m^2)	25.9 ± 4.7	27.2 ± 4.8	*P* < 0.001
Systolic blood pressure (mm Hg)	126.7 ± 16.6	129.9 ± 17.9	*P* = 0.001
Diastolic blood pressure (mm Hg)	77.0 ± 9.2	78.5 ± 9.8	*P* = 0.009
Heart rate (beats per minute)	71.1 ± 7.8	71.0 ± 8.0	NS
Residence[1]:			*P* = 0.023
Larger town	59.0%	52.2%	
Smaller town	16.3%	22.1%	
Village	24.6%	25.6%	
Education[2]:			*P* < 0.001
University	28.9%	22.2%	
Secondary	39.5%	34.5%	
Vocational	20.1%	30.5%	
Elementary	4.2%	10.8%	
Studying	7.2%	2.0%	

[1]Based on *n* = 1320; [2]Based on *n* = 1313. NS: Not significant; BMI: Body mass index.

Statistical analysis

The data was analysed using STATA statistical software (StataCorp. 2011. Stata Statistical Software: Release 12, College Station, TX, United States)[43]. Descriptive statistics, non-paired *t* test and Mann-Whitney test were used. Associations of *H. pylori* positivity with body indices and vital signs were analysed by univariable and multivariable linear regression. Crude and adjusted differences between groups of *H. pylori* positive and *H. pylori* negative study subjects are presented as beta coefficients with 95%CI.

RESULTS

The overall prevalence of *H. pylori* infection was 30.4% (402/1321) in adults (≥ 18 year-old) and 5.2% (26/497) in children and adolescents (≤ 17 year-old). There was no statistically significant difference in prevalence between males and females (Tables 1 and 2). *H. pylori* infection was strongly associated with higher age.

There were no significant differences in weight, height, body mass index or blood pressure among *H. pylori* positive and *H. pylori* negative children and adolescents, while heart rate was statistically significantly higher among those who were *H. pylori* positive (Table 1). There was a statistically significant difference in blood pressure, weight, height, and body mass index among *H. pylori* positive and *H. pylori* negative adults. *H. pylori* positive subjects were significantly older (Table 2).

Once adjusted for age and gender, we found a difference in height between *H. pylori* positive and *H. pylori* negative children and adolescents that was of borderline statistical significance. On further adjustment for place of residence, this difference became statistically significant, with *H. pylori* positive children and adolescents being on average 3.5 (95%CI: 0.2-6.7) cm shorter. This association was affected relatively little by further adjustment for parental education and remained of borderline statistical significance.

A higher average heart rate among *H. pylori* positive children and adolescents was consistently seen in all univariable and adjusted analyses with a difference of 3.4 (95%CI: 0.7-6.2) beats per minute in fully adjusted analysis (Table 3).

Although our crude analyses indicated differences in several body indices and vital signs between groups of *H. pylori* positive *vs* *H. pylori* negative study subjects aged 18+, these findings were mainly driven by substantial differences in age distribution among the *H. pylori* positive compared to *H. pylori* negative groups. Once adjusted for age and gender, only a difference in body mass index remained statistically significant with *H. pylori* positive adults showing an increase of 0.6 kg/m^2 in body mass index. This association weakened and became statistically non-significant on adjustment for place of residence and education (Table 4).

DISCUSSION

The prevalence of *H. pylori* in the Czech Republic in

Table 3 Association of *Helicobacter pylori* positivity with body size and blood pressure in 491 children and adolescents (≤ 17 years old) with no missing data on any variables used in the models; univariable and adjusted analysis

Parameter	β-coefficient (95%CI)			
	Crude	Adjusted for age and gender	+ place of residence	+ parental education
Weight (kg)	-1.1 (-8.2-5.9)	-3.4 (-7.4-0.6)	-3.4 (-7.4-0.5)	-3.4 (-7.4-0.7)
Height (cm)	-0.4 (-8.3-7.4)	-3.2 (-6.5-0.0)	-3.5 [-6.7-(-0.2)][1]	-3.2 (-6.4-0.0)
Body mass index (kg/m^2)	-0.4 (-2.0-1.1)	-0.8 (-2.1-0.5)	-0.7 (-2.0-0.6)	-0.7 (-2.0-0.6)
Systolic blood pressure (mmHg)	3.5 (-1.5-8.5)	2.4 (-1.8-6.7)	2.3 (-1.9-6.6)	2.3 (-2.0-6.5)
Diastolic blood pressure (mmHg)	0.3 (-3.1-3.8)	-0.4 (-3.3-2.5)	-0.4 (-3.3-2.5)	-0.7 (-3.6-2.3)
Heart rate (beats per minute)	3.4 (0.2-6.5)[1]	3.5 (0.3-6.6)[1]	3.7 (0.9-6.5)[1]	3.4 (0.7-6.2)[1]

[1]Statistical significance at 0.05 level.

Table 4 Association of *Helicobacter pylori* positivity with body size and blood pressure in 1312 adults (≥ 18 years old) with no missing data on any variables used in the models; univariable and adjusted analysis

Parameter	β-coefficient (95%CI)			
	Crude	Adjusted for age and gender	+ place of residence	+ own education
Weight (kg)	2.8 (0.9-4.7)[1]	1.3 (-0.5-3.0)	1.1 (-0.6-2.8)	1.1 (-0.6-2.8)
Height (cm)	- 1.4 [-2.6-(0.2)][1]	-0.5 (-1.4-0.3)	-0.5 (-1.3-0.4)	-0.2 (-1.1-0.6)
Body mass index (kg/m^2)	1.4 (0.8-2.0)[1]	0.6 (0.0-1.1)[1]	0.5 (0.0-1.1)	0.4 (-0.1-1.0)
Systolic blood pressure (mmHg)	3.3 (1.3-5.3)[1]	-0.7 (-2.5-1.1)	-1.0 (-2.8-0.8)	-1.1 (-2.9-0.6)
Diastolic blood pressure (mmHg)	1.5 (0.4-2.6)[1]	0.0 (-1.0-1.1)	-0.1 (-1.2-0.9)	-0.2 (-1.3-0.8)
Heart rate (beats per minute)	-0.1 (-1.0-0.8)	-0.2 (-1.1-0.8)	-0.2 (-1.1-0.7)	-0.2 (-1.1-0.8)

[1]Statistical significance at 0.05 level.

2011 was significantly lower compared to the prevalence reported from identical geographical areas in 2001[40]. In our previous project (run in 2001), we evaluated body indices and basic vital signs in *H. pylori* positive and negative persons in a large prospective study[37]. The aim of current research was to assess same parameters in 2011 in a prospective setting, using the same methods in identical geographical areas of the Czech Republic.

In 2001, there was a negative effect of *H. pylori* infection on systolic and diastolic blood pressure in subjects below the age of 25 and a relatively strong positive effect on blood pressure in subjects over 65[37]. We were not able to prove such an association in our current project.

Blood pressure, weight and body mass index were significantly higher in *H. pylori* positive adults compared to *H. pylori* negative ones in our current study. However, *H. pylori* positive adults were significantly older compared to *H. pylori* negative subjects. Once adjusted for age and gender, *H. pylori* infection did not influence body weight, body mass index and basic vital signs either in adults or children and adolescents.

Controversial data have been published in literature on the association of *H. pylori* infection and hypertension with both positive[2,13,17,18] and negative results[14,15]. In a large community-based cross sectional study (1634 *H. pylori* positive and 3267 *H. pylori* negative persons out of 10537 subjects enrolled), *H. pylori* infection had little effect on blood pressure in the general population, mean systolic blood pressure was higher in *H. pylori* infected individuals than in those who were not infected and, although this was significant statistically, the authors concluded that it was unlikely to be clinically important and might be explained by unknown residual confounding factors[15]. Migneco *et al*[17] demonstrated a significant decrease in blood pressure values (in particular in the diastolic one) after successful *H. pylori* eradication. There are several methodological difficulties in carrying out studies to determine a possible relationship between *H. pylori* infection and raised blood pressure, there are several other factors that must be considered (weight gain, salt intake, aging, co-morbidity, antihypertensive therapy, and compliance of patients *etc.*)[44,45].

Several epidemiological studies showed association between *H. pylori* infection and short growth in children[46-49]. However, contradictory data are available on this topic, as other authors did not find any relationship[31,32,50,51]. Results and conclusions of all published papers (including our findings) must be assessed with caution. It is necessary to distinguish intrinsic shortness and delayed or attenuated growth. Several factors can influence growth and stature, including chronic inflammation, nutrition and several gastrointestinal diseases[52].

Family social conditions are possibly a common determinant of *H. pylori* infection and growth. However, quite a large difference in children's height remains after adjustment for parental education, indicating that there may be other mechanisms in place. Possibly, this also might be a causal effect of *H. pylori* infection on children's growth. The adults' socioeconomic conditions and life style may be a common cause of higher body mass index and *H. py-*

lori infection. Although our earlier paper[39] indicated that most subjects become infected in childhood and childhood social conditions may thus be relevant for many different aspects of adult health, including *H. pylori* infection and higher body mass index (or overweight/obesity).

It is necessary to mention another interesting phenomenon: the decreased prevalence of *H. pylori* represents a prominent decline of CagA positive *H. pylori* strains[53,54]. CagA positive *H. pylori* strains are more susceptible to eradication treatment than CagA negative strains. This might partly explain their more pronounced decline[55]. It is necessary to admit that the reasons for decline of *H. pylori* infection have not been fully clarified yet. It is necessary to also consider that the fundamental environmental changes could cause gradual disappearance of *H. pylori* from the human microbiome[56-58].

In conclusion, chronic *H. pylori* infection appeared to be associated with short stature in children. *H. pylori* infection did not influence blood pressure, body weight and body mass index either in adults or children and adolescents.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Biofilm and *Helicobacter pylori*: From environment to human host

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Abstract

Helicobacter pylori (*H. pylori*) is a Gram negative pathogen that selectively colonizes the human gastric epithelium. Over 50% of the world population is infected with *H. pylori* reaching up to 90% of infected individuals in developing countries. Nonetheless the increased impact upon public health care, its reservoir and the transmission pathway of the species has not been clearly established yet. Molecular studies allowed the detection of *H. pylori* in various aquatic environments, even forming biofilm in tap water distribution systems in several countries, suggesting a role of water as a possible reservoir of the pathogen. The persistence of human infection with *H. pylori* and the resistance of clinical isolates to commonly used antibiotics in eradication therapy have been related to the genetic variability of the species and its ability to develop biofilm, demonstrated both *in vivo* and *in vitro* experiments. Thus, during the last years, experimental work with this pathogen has been focused in the search for biofilm inhibitors and biofilm destabilizing agents. However, only two anti- *H. pylori* biofilm disrupting agents have been successfully used: Curcumin - a natural dye - and N-acetyl cysteine - a mucolytic agent used in respiratory diseases. The main goal of this review was to discuss the evidences available in the literature supporting the ability of *H. pylori* to form biofilm upon various surfaces in aquatic environments, both *in vivo* and *in vitro*. The results published and our own observations suggest that the ability of *H. pylori* to form biofilm may be important for surviving under stress conditions or in the spread of the infection among humans, mainly through natural water sources and water distribution systems.

Key words: *Helicobacter pylori*; Biofilm, Water; Infection

Core tip: This review deals with the ability of *Helicobacter pylori* (*H. pylori*) to form biofilm, and the role of the biofilm as reservoir for *H. pylori* infection. The ability of *H. pylori* to grow and form biofilm *in vitro* and *in vivo* could be advantageous for the species to successfully avoid injuries due to chemical stressors - such as antimicrobial therapy *in vivo* - or stress induced by nutrient deprivation. Therefore, the ability of *H. pylori* to form biofilm should be kept in mind when epidemiological strategies are planned to prevent the spread of this ubiquitous pathogen and for treatment of human infection.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram negative pathogen

that selectively colonizes the human gastric epithelium. This species is defined as a urease, catalase and oxidase positive spiraled microaerophilic bacterium, mobile by means of 3-5 polar flagella. Although labile at pH 3.0, *H. pylori* is able to survive for decades in the highly acidic gastric pH by metabolizing urea though its urease enzyme, providing a protective neutral pH in the surrounding of the bacterial cell[1,2].

Over 50% of the world population is infected with *H. pylori* showing a prevalence of 90% in some developing countries where individuals are mainly asymptomatic. In these countries, the pathogen is acquired in the early infancy and its prevalence is higher among individuals belonging to low socioeconomic status groups[2]. The persistent infection with this pathogen is associated to the majority of the gastric pathologies, such as chronic gastritis, MALT Lymphoma, duodenal ulcer, gastric ulcer, and it shows a direct relationship with gastric adenocarcinoma development[3,4]. Nonetheless the wide variety of associated pathologies, only a reduced group of infected individuals develops a clinical severe disease which could be the consequence of environmental factors together with bacterial virulence factors and genetic susceptibility of the host.

H. pylori infected individuals remain colonized by this species during life due to the increased flexibility of the pathogen[5]. The human persistent infection with *H. pylori* and the antibiotic resistance showed by clinical isolates have been related to genetic variability of the species but also to its ability for developing biofilm as it has been demonstrated *in vivo*[6-8] and *in vitro*[9-13]. Thus, various studies have been done searching for biofilm inhibitors and biofilm destabilizing agents[14,15], to decrease recurrent *H. pylori* infections.

PATHOGENESIS AND EPIDEMIOLOGY OF *H. PYLORI* INFECTION

Gastric colonization by *H. pylori* induces superficial chronic gastritis in all infected individuals but few of them develop clinical symptoms[3]. Among the pathologies associated to the persistent infection with the pathogen, gastric adenocarcinoma was a matter of controversy for several years. However, large population studies clearly showed that infection with *H. pylori* significantly increases the risk for developing gastric cancer[16].

H. pylori needs at least four main cellular components to successfully colonize and establish an infection in the gastric mucosa: Urease enzyme, flagella, spiral morphology and adhesins. Additionally, although it is known that the species is able to grow under anaerobic condition[17], it is not clear whether its microaerophilic nature is also a requirement for mucosal colonization. Gastric acid has a pH < 4, which is deleterious for the majority of the bacterial species reaching the stomach by means of drinking water or foods. Thus, successful gastric mucosal colonization by *H. pylori* relies in its ability to convert urea into ammonium, by urease activity, rendering the pH surrounding the bacterial cell neutral, and the 3 to 5 polar flagella together with its spiral form that allows *H. pylori* to leave from the mucosal layer reaching the neutral environment of the gastric epithelial cells[17,18]. While reaching the target epithelial cells, *H. pylori* cells become bound to them through specific adhesion molecules.

Early infection by *H. pylori* and the persistence of this infection are related to an increased risk for developing peptic ulcer disease and gastric cancer[2]. In developing countries, a large number of adult asymptomatic population is usually persistently infected with the pathogen. This increased prevalence has been correlated with gastric cancer development[19]. This disease is considered the second cause of death among individuals died of cancer in the world.

WATER AS *H. PYLORI* RESERVOIR

In spite of the large number of researchs dealing with public health impact, the pathway of transmission and the reservoirs of this species have not been yet elucidated. *H. pylori* has been isolated from the gastrointestinal tract, including saliva and stools, suggesting that oral-oral and fecal-oral routes are the main transmission pathway[20]. However, molecular analyses show that *H. pylori* is also present in various aquatic environments suggesting that human fecal contaminated water sources could be a plausible reservoir of the pathogen[20]. In addition, zoonotic transmission by dogs, cats, sheep and flies as well as iatrogenic transmission by endoscopic procedures[20] have been proposed.

The species has been detected in several water sources, including lakes, rivers, tap water, well water, irrigation water and sea water (Table 1), but also has been detected in water distribution systems. Thus, drinking water could be the pathwayfor returning to humans, even though food and occasionally recreational waters may also participate in the *H. pylori* transmission cycle[21].

H. pylori remains viable after seven days in seawater, 16 d in saline and 11-14 d in distilled water, but this survival property is strongly affected by temperature[22,23]. Nonetheless, one of the main problems to accept that water could be a possible reservoir of *H. pylori* is the inability to routinely isolate the species from water samples in conventional microbiological culture techniques[24-29]. Nowadays, molecular methods, like polymerase chain reaction (PCR), are the most popular to search for *H. pylori* in water, although an intrinsic limitation of these techniques is to accurately differentiate live and dead cells[24,28-37]. However, new analytical methods such as *in situ* fluorescent hybridization (FISH) made it possible to successfully detect *H. pylori* in water distribution systems and various water sources[27,35,38,39] because the techniques detect rRNA, which is indicative not only of bacterial presence but also of viability due to the increased cellular rRNA content[40].

One of the difficulties for isolating *H. pylori* in culture is the ability of the species to change from a spiral

Table 1 Detection of *Helicobacter pylori* in acquatic environments

Methods	Water source	Ref.
Immunofluorescence	Surface and ground water	[60]
Bacteriological culture	Sea water and plankton	[28]
	Wastewater	[24,27]
	Tap water	[26,29]
	Contaminated wells water	[25]
Polymerase chain reaction	Tap water	[29-31]
	Sea water and plankton	[28,32,33]
	River and lake water	[34-36]
	Wastewater	[24,36,37]
Fluorescent hybridization	Tap water and ground water	[38]
	Wastewater	[27,35]
	Sea water	[39]
	Irrigation water	[39]
	River water	[35]

cultivable form to coccoid cells, which is described as a viable but non cultivable state (VBNC). This change is triggered by stressing conditions, like hyperosmolarity or nutritional deprivation[41], suggesting an adaptive mechanism of the species for remaining viable and infective for long periods[42,43]. This state also allows the cells to evade the chloride treatment of water used for human consumption, remaining infective but undetectable by conventional microbiological culture methods[44].

During the last decade, biofilm formation in water environments has been proposed as a strategy of *H. pylori* for surviving in the environment, and has called the attention of water as a reservoir of the pathogen. The first experimental evidence showing the ability of *H. pylori* to grow in biofilm arose in 1999, from the work of Mackay *et al*[45]. The authors found the species in the surface of tap water distribution systems. In 2001, Park *et al*[30] also detected *H. pylori* in the distribution system of tap water in Sweden by molecular analysis, even though the amplified DNA fragments were not sequenced to confirm the detection of the pathogen in the samples. A year later, another study informed the detection of 16S rRNA genes from *H. pylori* in biofilm samples obtained from water distribution system at western Africa[46]. In 2004, the species was detected by Watson *et al*[31] in biofilm and water samples from 11 England residential houses (kitchen, toilet and shower), seven educational establishments and hydrants. Among 151 samples analyzed, the genus was detected by DNA in 26% of the samples (39/151 samples) and in 15% of the samples (23/151 samples) the microorganism was identified at species level. Its presence was found mainly in biofilm samples from showers, suggesting a role of the tap water distribution systems as reservoir of *H. pylori*. Three years later, Braganra *et al*[38] detected, by FISH, spiral *H. pylori* cells (infectious form) in biofilm from water distribution systems in the United Kingdom suggesting that *H. pylori* would be resistant to chloride treatment.

Recently, the coexistence of *H. pylori* or *Legionella pneumophila* in biofilm together with other bacterial species including *V. paradoxus*, *M. chelonae*, *Acidovorax* sp., *Sphingomonas* sp. and *Brevundimonas* sp. has been analyzed[47]. *H. pylori* can be recovered in conventional microbiological cultures before 24 h if the biofilm is formed by only one species (*V. paradoxus*, *Acidovorax* sp. or *Brevundimonas* sp.), but remains cultivable at least 24 h if the biofilm is made by *M. chelonae*, a pathogen commonly found in tap water. This observation suggests that *M. chelonae* may play a role in supporting the incorporation of *H. pylori* to biofilm formed in tap water distribution systems.

BIOFILM: AN OVERVIEW

Bacterial species in natural environments usually live in communities of microorganisms on the surface of different materials, embedded by a self-produced matrix, usually of polysaccharide nature, that allows bacteria to adapt and survive under stress conditions. These structures are called biofilm[48]. The biofilm develops in several steps: (1) conditioning; (2) adhesion; (3) extracellular matrix synthesis; (4) maturation; and (5) dispersion. The processes render a uniform structure of cells deposit surrounded by a matrix that leaves open channels where water can diffuse freely. The matrix is composed by a polymeric substance, including polysaccharide molecules, DNA, proteins and lipids, which modify the bacterial surface and promote first adhesion of cells to surfaces[49].

It is currently accepted that biofilms are implicated in over 80% of the chronic infections caused by bacteria, including middle ear otitis, endocarditis, urinary tract infections and lung infections of patients with cystic fibrosis[50]. The importance of biofilm in medicine is due to its role in persistence of the infection because biofilm is not removed and bacteria in biofilms are 1000 more resistant to antibiotics and host defenses as compared to those free living bacteria[51]. The mechanisms involved in antibiotic resistance are: (1) delay in antibiotic diffusion, allowing the expression of resistance genes; (2) chemical charge of molecules like aminoglycoside antibiotics which show an altered diffusion throughout the negatively charged exopolysaccharide matrix; (3) presence of antibiotic hydrolyzing enzymes (such as β-lactamases); (4) lost of effectiveness of some antibiotics that need active growing cells to exert their function, because bacterial growth ratio is decreased in biofilm[51,52]; and (5) presence of reactive oxygen species as consequence of the oxidative burst by phagocytic cells that increase bacterial antibiotic resistance throughout the increase of mutagenicity in this microorganism by inducing its DNA-break repair systems[52]. In addition, recent experimental evidences suggest that biofilm is a virulence factor in a bacterial community, because the bacterial cells residing in the biofilm may acquire new virulence attributes that free living bacteria do not possess[53].

H. pylori biofilm formation in vitro

Like many of the bacteria investigated, both *in vitro* and *in vivo* experiments have shown that *H. pylori* may have living periods of biofilm forming cells. Roughly, 15 years

after *H. pylori* was successfully grown in culture[54], the first evidence of biofilm formation by this species arose. The biofilm obtained by growing the strain *H. pylori* ATCC 43504 in a chemically defined medium (Brucella broth supplemented with 0.1% β-cyclodextrin) was insoluble in water, and adhered to the glass in the interface glass-water[11]. Five years later, Cole *et al*[10] studied the ability of 19 *H. pylori* clinical isolates and reference strains in Brain Heart Infusion broth supplemented with 0.1% β-cyclodextrin. Both strains were able to form biofilm and the biofilm produced had a similar progression when compared to biofilm formed by other bacterial species[55]. The steps observed were initial binding, expansion to form microcolonies and growth in three dimensions with the presence of water channels for nutrients distribution.

On the other hand, Cole *et al*[10] also analyzed the effect of specific mutations of genes *luxS* and the type Ⅳ secretion gen *cagE* upon biofilm formation by *H. pylori*, detecting that both mutants were surprisingly twice more efficient in biofilm formation than the isogenic wild type parental strain.

The adherence of *H. pylori* to gastric mucosa is a key step in establishing positive interaction with host gastric epithelial cells. This adherence is mediated by BabA adhesin, which binds to Lewis b antigen and facilitates *H. pylori* colonization of human gastric epithelium[56]. On the other hand, gastric epithelial cells are protected by a mucous layer composed mainly by MUC5AC which contains a domain rich in glycans (including the Lewis b antigen), the target for *H. pylori* BabA adhesin. In opposite to MUC5AC, the MUC6 mucin synthesized in the deep mucosa and secreted by glandular mucosal cells works as a natural antibiotic by inhibiting *H. pylori* growth, avoiding the colonization of the deep gastric mucosa layer by the pathogen[56].

Because several papers indicate that mucin avoids adhesion of *H. pylori* to gastric epithelial cells[57], in order to understand what happens *in vivo*, Cole *et al*[10] studied the effect of mucin on the formation of biofilm by *H. pylori*,. It was observed that increasing concentrations of mucin favors significantly the planctonic growth of *H. pylori* over biofilm formation suggesting that this species lives primarily in biofilm but rapidly proliferates as free living bacteria after been in contact with the mucin in the human stomach. Nevertheless, it has been shown that biofilm formation *in vivo* also occurs[6-8].

A study carried out in 2009 with reference strains and clinical isolates showed that all the strains were able to form biofilm in the interface air-water of a cover slip[12]. The increased ability to form biofilm observed in one strain -*H. pylori* TK1402 - isolated from a Japanese patient with gastric and duodenal peptic ulcer disease called the attention of specialists. Scanning electron microscopy analysis showed the presence of outer membrane vesicles produced by this strain only in biofilm, suggesting that these vesicles might play a role in biofilm formation. One year later, the authors[13] search for virulence factors in this strain and showed that none of the traditional virulence factors described in the species were related to the ability of *H. pylori* TK1402 to form biofilm. Thus, new evidences are needed to better understand why this strain shows an increased ability to grow forming biofilm.

In vivo evidences of H. pylori biofilm formation

The first evidence of *in vivo* biofilm formation arose in United States during 2006[6]. The authors compared, by scanning electron microscopy, gastric biopsies from urease positive (presence of *H. pylori*) and urease negative (absence of *H. pylori*) patients, detecting the presence of dense mature biofilm in the pathogen positive biopsies while biofilm was absent in the urease negative biopsies, which is indicative that *H. pylori* is able to form biofilm in the human gastric mucosa.

In 2008, in Italy, a study was conducted to understand if the gastric diseases caused by this species are a consequence of its ability to form biofilm. The study was done with gastric biopsies obtained from patients receiving anti-*H. pylori* therapy three months prior to the analysis. The authors search for presence of the bacteria by culture and detection by reverse transcription polymerase chain reaction (RT-PCR) of the genes *glmM* and *luxS*. Only 30% of the samples were positive for *H. pylori* by culture whilst 90% of positivity was detected by RT-PCR suggesting that *H. pylori* could be present in these patients as VBNC coccid form. The analysis of the samples by scanning electron microscopy showed an S shape or spiral forms of the bacterial cells in all the samples that were also positive by conventional microbiological culture, with several coccid cells embedded in an extracellular matrix. On the other hand, the same study done with those positive samples only by RT-PCR showed predominance of coccid cells. In addition, positive samples for gene *glmM* were also positives for the Quorum Sensing related gene *luxS*, supporting its detection as a confidence marker of biofilm formation in the species. Interestingly, antibiotic susceptibility analysis done to the clinical isolates strains in this study showed that only one strain was resistant to Clarithromycin and none to Amoxicillin (the antibiotics used for eradication therapy) which was suggestive of a biofilm role in the eradication failure.

Anti-biofilm agents

Because antibacterial susceptibility of a particular strain is favored when the biofilm is destabilized[58], it is believed that a combination of antimicrobial agents and anti-biofilm molecules should be synergistic[59]. However, only two anti- biofilm compounds have been assayed against *H. pylori*: Curcumin, a natural dye extracted from *Curcuma longa*, and N-acetyl-cysteine, a mucolytic agent with anti-biofilm proved activity against other pathogens[60]. Curcumin acts upon biofilm formation in a doses dependent way when assayed at sub-inhibitory concentrations, suggesting its utility as coadjuvant to

standard first choice anti-*H. pylori* eradication therapy, especially in recurrent infections associated to biofilm formation.

One year later, N-acetyl-cysteine effect upon the formation of biofilm was analyzed both *in vitro* and *in vivo*[14]. N-acetyl-cysteine was able to avoid biofilm formation and to destabilize the already formed biofilm at concentrations over 10 mg/mL, similar to results observed with other pathogens[60]. On the other hand, *in vivo* studies with two groups of 20 individuals each, one group (treated) received N-acetyl-cysteine for one week before the anti-*H. pylori* first choice treatment while the other group (untreated controls) did not received the destabilizing agent, showed eradication of the infection in 65% of the cases for the N-acetyl-cysteine treated group as detected by the urea breath test. The control group showed only 20% of successful eradication, suggesting that N-acetyl-cysteine act as a biofilm destabilizing agent that favor *in vivo* the activity of antibiotic substances.

PERSPECTIVES

H. pylori biofilm formation and the role of coccid cells found in the environment or among clinical samples extend our frontiers in the understanding of the epidemiological cycle of this pathogen, but new challenges arises dealing with the identification of the molecular mechanisms allowing *H. pylori* to reactivate its metabolism acquiring the active divisionary form immediately after reaching the human stomach mucosa. The role of particular proteins in the regulation of the conversion from coccid to bacillary cells needs to be elucidated. Candidates of rod shape-promoting proteins - *i.e.*, the serendipity finding of YeaZ in *Escherichia coli*[61] - could give new insight evidences into the mechanism of infection of the species.

CONCLUSION

Our understanding of the role of water as reservoir of *H. pylori* infection and the pathway of transmission of the pathogen is still controversial although a fecal-oral route is accepted worldwide. New experimental evidence is needed to improve our knowledge on the survival strategies of *H. pylori* in the environment and how this reservoir contributes to the distribution of the infection among humans.

It has been suggested that biofilm formation is a critical step in bacterial survival in water and other environments. Thus, the ability of *H. pylori* to grow and form biofilm *in vitro* and *in vivo* could be an advantage for the species to successfully avoid injuries due to chemical stressors - such as antimicrobial therapy *in vivo* - or stress induced by nutrient deprivation in the environment. Under these stressing conditions, biofilm formation seems to play a key role for *H. pylori* survival, especially in water - included tap water and distribution systems- because the species may stay as spiral rod (active form) or acquire U form or coccid form (viable but non cultivable form). Thus, the ability of *H. pylori* to form biofilm should be kept in mind when epidemiological strategies are planned to prevent the spread of this ubiquitous human pathogen and treatment in human infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Role of dental plaque, saliva and periodontal disease in *Helicobacter pylori* infection

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Abstract

Helicobacter pylori (*H. pylori*) infection is one of the most common bacterial infections in humans. Although *H. pylori* may be detected in the stomach of approximately half of the world's population, the mechanisms of transmission of the microorganism from individual to individual are not yet clear. Transmission of *H. pylori* could occur through iatrogenic, fecal-oral, and oral-oral routes, and through food and water. The microorganism may be transmitted orally and has been detected in dental plaque and saliva. However, the role of the oral cavity in the transmission and recurrence of *H. pylori* infection has been the subject of debate. A large number of studies investigating the role of oral hygiene and periodontal disease in *H. pylori* infection have varied significantly in terms of their methodology and sample population, resulting in a wide variation in the reported results. Nevertheless, recent studies have not only shown that the microorganism can be detected fairly consistently from the oral cavity but also demonstrated that the chances of recurrence of *H. pylori* infection is more likely among patients who harbor the organism in the oral cavity. Furthermore, initial results from clinical trials have shown that *H. pylori*-positive dyspeptic patients may benefit from periodontal therapy. This paper attempts to review the current body of evidence regarding the role of dental plaque, saliva, and periodontal disease in *H. pylori* infection.

Key words: *Helicobacter pylori*; Dental plaque; Saliva; Oral cavity; Periodontitis; Periodontal therapy

Core tip: *Helicobacter pylori* (*H. pylori*) infection is one of the most common bacterial infections in humans. The mode of transmission of this bacterium has long puzzled researchers. Numerous studies have shown that this microorganism can be detected in dental plaque and saliva of human subjects, suggesting that the oral cavity may be an extra-gastric reservoir of *H. pylori* and play an important role in both transmission and recurrence. Recent data support this hypothesis and indicate that periodontal therapy may play a role in the management of *H. pylori*-associated gastric disease.

Anand PS, Kamath KP, Anil S. Role of dental plaque, saliva and periodontal disease in *Helicobacter pylori* infection. *World J Gastroenterol* 2014; 20(19): 5639-5653 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5639.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5639

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most common bacterial infections in humans[1]. It is a gram negative, microaerophilic, rod-shaped bacterium that colonizes the gastric mucosa. Although its presence in the human stomach has been reported from all parts of the world, the prevalence of *H. pylori* infection is higher in developing countries than developed countries[2,3]. First reported in 1983[4], *H. pylori* (initially termed *Campylobacter pyloridis*) is an important human pathogen associated with the etiology of chronic gastritis, peptic ulcer, gastric cancer, and mucosa-associated lymphoid tissue lymphoma[2,5,6] and has been designated as a Group 1 Carcinogen by the International Agency for Research on Cancer of the World Health Organization (WHO)[6,7]. In addition to gastrointestinal diseases, recent data seem to suggest a possible association of this microorganism with other conditions, such as recurrent aphthous stomatitis[8], anemia[9], altered serum levels of lipoproteins[10], and coronary atherosclerosis[11].

A limited number of anti-microbial agents are effective against *H. pylori* and therapeutic regimens to eradicate the microorganism usually consist of a combination of antibiotics, proton pump inhibitors, and gastroprotective drugs[12,13]. These therapeutic regimens, particularly the combination of two antibiotics and a proton pump inhibitor, can successfully eradicate the microorganism resulting in significant clinical improvement[14]. However, recurrence rates, particularly in developing countries, are high[15-17]. This high rate of recurrence led investigators to study the various possible routes of transmission of the microorganism. Although *H. pylori* may be detected in the stomach of approximately half of the world's population, the mechanisms of transmission of the microorganism from individual to individual are not yet clear. The possible routes of transmission of *H. pylori* include iatrogenic, fecal-oral, oral-oral, and through food and water[2,18-21]. *H. pylori* exists in two different morphological forms, spiral and coccoid. The coccoid form is considered a degenerative or dead form of *H. pylori*, and its role in transmission of disease is negligible[22,23]. There is ongoing debate about its virulence and transformation[24]. Although the coccoid form of *H. pylori* is metabolically active, it cannot be cultured *in vitro*[2]. The organism has been reported to be present in soil samples in public playgrounds[25]. However, no extra-gastric reservoirs of *H. pylori* have been clearly demonstrated, and although organisms resembling *H. pylori* may be detected in other animals, none except non-human primates[26]and cats[27] harbor *H. pylori*. Infections by *Helicobacter* species (*H. heilmannii* and *H. felis*) have been reported in dogs[28,29] and cats[29].

Regarding the various possible routes of transmission of *H. pylori,* the microorganism may be transmitted orally and has been detected in dental plaque and saliva[30-32]. However, the question still persists as to whether the oral cavity is a major extra-gastric reservoir for *H. pylori* or harbors the organism only transiently. If the oral cavity, particularly the dental plaque, serves as an extra-gastric reservoir of *H. pylori*, it may have potentially serious implications regarding the treatment of *H. pylori* infection. This is on account of the fact that treatment of *H. pylori* infection usually involves administration of systemic antibiotics in combination with other drugs, and dental plaque, being a microbial biofilm, provides protection for the resident microorganisms from systemically administered antimicrobial agents. Despite the current treatment regimens that lead to successful management of *H. pylori*-positive chronic gastritis, the re-infection rate is relatively high[14,33]. One of the suggested mechanisms of re-infection is the possible re-colonization from dental plaque[34]. A few studies have also suggested that periodontal disease may also favor colonization of dental plaque by *H. pylori*[35]. This paper attempts to review the role of dental plaque, saliva, and periodontal disease in *H. pylori* infection.

PRESENCE OF *H. PYLORI* IN DENTAL PLAQUE

The prevalence of *H. pylori* in dental plaque has been studied by several investigators. A summary of studies reporting the presence of *H. pylori* in dental plaque of participants is shown in Table 1. The prevalence of reported presence of *H. pylori* in dental plaque in these various studies ranged from 0%-100%. This wide variation in results may be explained by several factors, such as characteristics of the sample population, differing sampling procedures, and differing methodologies used to detect the microorganism in dental plaque.

The diagnostic tests employed by different investigators to detect the presence of the microorganism in dental plaque include urease tests, polymerase chain reaction (PCR) techniques, immunoassays, cytology, and culture. Generally, the prevalence rates reported in studies employing urease tests were higher than studies employing other techniques. Lowest rates of detection have been reported when microbial culture was used to detect the presence of *H. pylori* in dental plaque. The use of urease tests for the detection of *H. pylori* in dental plaque has been subject to controversy. Although urease tests are reasonably specific for detection of the microorganism in gastric biopsy specimens, investigators have doubted its reliability for detecting *H. pylori* in oral specimens[36,37]. This controversy results from the fact that although *H. pylori* is the only urease-positive microorganism known to reside in the stomach, many urease-positive bacterial species, such as *Streptococcus* species, *Haemophilus* species, and *Actinomyces* species, may be detected as part of the normal oral flora. However, it has been reported that only *H. pylori* produces large amounts of urease, such that a positive urease test can occur within 20 min, while other urease-producing microorganisms are not positive within one hour[38]. Moreover, Gürbüz *et al*[39] reported that the rapid urease test for detection of *H. pylori* in dental plaque has a sensitivity of 89.7% and diagnostic accuracy of 86.7%.

Almost all of the studies utilizing urease tests for detection of *H. pylori* in dental plaque were conducted

Table 1 Studies evaluating the presence of *Helicobacter pylori* in dental plaque

Ref.	Method of *H. pylori* detection in dental plaque	Sample population and sample size	Prevalence of *H. pylori* in dental plaque
Agarwal and Jithendra[84], 2012	PCR	India; 30 *H. pylori*-positive and 20 *H. pylori*-negative patients	Overall-42% (n = 21); in *H. pylori*-positive group-60% (18/30); in *H. pylori*-negative group-15% (3/20)
Momtaz *et al*[85], 2012	PCR	Iran; 300 patients with gastro-duodenal diseases	0
Wichelhaus *et al*[86], 2011	PCR	Germany; 11 orthodontic patients	36% (n = 4)
Gao *et al*[46], 2011	PCR	China; 96 patients with *H. pylori* infection	82.30%
Chaudhry *et al*[45], 2011	PCR	Pakistan; 89 dyspeptic patients reporting for endoscopy	51.6% (n = 46) for both genes; 62.9% (n = 56) for 16srRNA; 61.7% (n = 55) for 860-bp DNA region; 73% (n = 65) if either of the 2 regions were considered
Bago *et al*[81], 2011	PCR	Croatia; 56 patients with chronic periodontitis and gastric *H. pylori*-positive	37.5% (n = 21)
Silva *et al*[71], 2010	PCR	Brazil; 115 patients-36 with dyspepsia and periodontal disease, 31 with dyspepsia but no periodontal disease, 22 with neither dyspepsia nor periodontal disease, 26 with periodontal disease and without dyspepsia	11.3% (n = 13)
Silva *et al*[87], 2010	PCR	Brazil; 30 dyspeptic patients	20% (n = 6) by 16S rDNA and 6.7% (n = 2) by vacA
Eskandari *et al*[88], 2010	PCR	Iran; 67 patients with chronic periodontitis-23 with *H. pylori*-positive gastritis	5.97% (n = 4/67)
Assumpção *et al*[89], 2010	PCR	Brazil; 99 adult patients who underwent upper gastro-intestinal endoscopy	72% (71) samples were positive for *H. pylori*. Overall, 63 (89%) of 71 positive dental plaque samples were positive for vacA and cagA. 58/71 (82%) were positive for cagA, while vacA genotypes had a prevalence ranging from 13%-59%
Medina *et al*[90], 2010	PCR	Argentina; 98 patients-43 dyspeptic patients and 55 asymptomatic controls	10.2% (n = 10)
Liu *et al*[70], 2009	PCR	China; 443 dyspeptic patients	59.4% (n = 263)
Gonçalves *et al*[69], 2009	PCR	Brazil; 23 HIV seropositive individuals (13 who had chronic periodontitis and 10 who were periodontally healthy) and 31 HIV seronegative individuals (17 who had chronic periodontitis and 14 who were periodontally healthy)	Not specified; frequency of detection was significantly higher in chronic periodontitis groups compared with periodontally healthy groups
Silva *et al*[91], 2009	PCR	Brazil; 30 individuals who were *H. pylori*-positive with gastric disease (cases) and 32 individuals who were *H. pylori*-positive with no gastric disease (controls)	Overall-17.7% (n = 11). Among cases, *H. pylori* DNA detected in 36.6% (11/30); control group-0%
Morales-Espinosa *et al*[62], 2009	PCR	Mexico; 66 hospitalized patients and 65 dental patients	Overall-19.9% (n = 26); 24% (n = 16) among hospitalized patients and 15% (n = 10) among dental patients
Souto and Colombo[68], 2008	PCR	Brazil; 225 patients-56 periodontally healthy and 169 chronic periodontitis patients	50% in patients with chronic periodontitis and 11.4% in periodontally healthy individuals
Liu *et al*[92], 2008	PCR	China; 214 children	58.9% (n = 126)
Bürgers *et al*[93], 2008	PCR	Germany; 94 patients who underwent upper gastro-intestinal endoscopy	5.4% (n = 5/92 dentate patients)
Teoman *et al*[94], 2007	PCR	Turkey; 67 dyspeptic patients	28.3% (n = 19)
Olivier *et al*[95], 2006	PCR	South Africa; 74 healthy members of a rural community	0
Kignel *et al*[96], 2005	PCR	Brazil; 49 dyspeptic patients	2% (n = 1)
Gebara *et al*[53], 2004	PCR	Brazil; 15 gingivitis and 15 periodontitis patients-all were *H. pylori*-positive in antral mucosa	20% (n = 6) in supra-gingival plaque and 26.6% (n = 8) in sub-gingival plaque
Fritscher *et al*[97], 2004	PCR	Brazil; 53 patients with recurrent aphthous stomatitis (cases) and 52 patients without recurrent aphthous stomatitis (controls)	Overall-3.8%; 5.7% (n = 3) in cases and 1.9% (n = 1) among controls
Umeda *et al*[35], 2003	PCR	Japan; 56 dental patients	25% (n = 14)
Suk *et al*[98], 2002	PCR	Taiwan; 65 patients with dyspeptic symptoms	43.1% (n = 28)
Berroteran *et al*[99], 2002	PCR	Venezuela; 32 dyspeptic patients and 20 asymptomatic controls	Overall-28.9%; 37.5% (n = 12) among dyspeptic patients and 15% (n = 3) among controls
Goosen *et al*[100], 2002	PCR	South Africa; 58 clinically healthy volunteers	1.7% (n = 1)

Song *et al*[63], 2000	PCR	Germany; 15 dyspeptic patients and 6 orthodontic patients	100% (*n* = 21)
Song *et al*[101], 2000	PCR	Germany; 20 dyspeptic patients	In dental plaque-not specified; 100% in oral samples (plaque and saliva)
Song *et al*[58], 2000	PCR	Germany; 42 patients who underwent upper gastro-intestinal endoscopy	97% (*n* = 41)-82% in molar region, 64% in premolar region and 59% in incisor region
Miyabayashi *et al*[79], 2000	PCR	Japan; 47 patients with chronic gastritis or peptic ulcer	38.3% (*n* = 18)
Agarwal and Jithendra[84], 2012	Culture	India; 30 *H. pylori*-positive and 20 *H. pylori*-negative patients	Overall-18% (*n* = 9); in *H. pylori*-positive group-30% (9/30); in *H. pylori*-negative group-0
Loster *et al*[49], 2009	Culture	Poland; 46 dentists without known co-morbidities	48%
Sudhakar *et al*[102], 2008	Culture	India; 50 patients with duodenal and gastric ulcer (study group) and 25 students (control group)	Overall 6.7% (*n* = 5); in study group-10% (*n* = 5), in control group-0%
Teoman *et al*[94], 2007	Culture	Turkey; 67 dyspeptic patients	0
Czesnikiewicz-Guzik *et al*[47], 2005	Culture	Poland; 100 female patients	48.3%
Czesnikiewicz-Guzik *et al*[48], 2004	Culture	Poland; 100 female patients	48.3%
Umeda *et al*[35], 2003	Culture	Japan; 18 dental patients	5.6% (*n* = 1)
Goosen *et al*[100], 2002	Culture	South Africa; 58 clinically healthy volunteers	13.8% (*n* = 8)
Checchi *et al*[103], 2000	Culture	Italy; 35 patients from a Periodontology clinic	8.6% (*n* = 3)
Sambashivaiah *et al*[73], 2011	RUT/CLO test	India; 36 patients in 3 groups-group Ⅰ, healthy subjects, group Ⅱ, chronic periodontitis patients, group Ⅲ, chronic periodontitis patients with type Ⅱ diabetes mellitus	Overall-66.7% (*n* = 24); among group Ⅰ-41.7% (*n* = 5), group Ⅱ-75% (*n* = 9), group Ⅲ-83.3% (*n* = 10)
Bali *et al*[65], 2010	RUT/CLO test	India; 124 dyspeptic patients of which 60 were *H. pylori*-positive (cases) and 64 were *H. pylori*-negative (controls)	Overall-51.6% (*n* = 64); among cases-86.7% (*n* = 52)
Assumpção *et al*[89], 2010	RUT/CLO test	Brazil; 99 adult patients who underwent upper gastro-intestinal endoscopy	52% (*n* = 48/93)
Al Asqah *et al*[64], 2009	RUT/CLO test	Saudi Arabia; 62 dyspeptic patients with periodontitis and 39 dyspeptic patients without periodontitis	Overall-65%; 79% in periodontitis group and 43% in non-periodontitis group
Sudhakar *et al*[102], 2008	RUT/CLO test	India; 50 patients with duodenal and gastric ulcer (study group) and 25 students (control group)	Overall 49.3% (*n* = 37); in study group-70% (*n* = 35), in control group-8% (*n* = 2)
Chitsazi *et al*[40], 2006	RUT/CLO test	Iran; 88 dyspeptic patients-44 with *H. pylori* infection and 44 without *H. pylori* infection	Overall 18.2% (16/88); 36.4% (16/44) in *H. pylori*-positive group
Anand *et al*[104], 2006	RUT/CLO test	India; 65 dyspeptic patients with *H. pylori* infection (cases) and 69 dyspeptic patients without *H. pylori* infection (controls)	Overall-79.9% (*n* = 107/134); 89.2% (*n* = 58) among cases and 71% (*n* = 49) among controls
Gürbüz *et al*[39], 2003	RUT/CLO test	Turkey; 75 dyspeptic patients	91.7% (*n* = 68)
Choudhury *et al*[67], 2003	RUT/CLO test	India; 124 patients with dyspepsia	43% (*n* = 54)
Al-Refai *et al*[105], 2002	RUT/CLO test	Saudi Arabia; 75 dyspeptic patients and 60 healthy controls	Overall-88.1% (*n* = 119); among dyspeptic patients-89.3% (*n* = 67); among controls-86.7% (*n* = 52)
Butt *et al*[74], 2002	RUT/CLO test	Pakistan; 78 dyspeptic patients	100%
Suk *et al*[98], 2002	RUT/CLO test	Taiwan; 65 patients with dyspeptic symptoms	100%
Ozdemir *et al*[106], 2001	RUT/CLO test	Turkey; 81 dyspeptic patients	79% (*n* = 64)
Avcu *et al*[75], 2001	RUT/CLO test	Turkey; 241 *H. pylori*-positive patients with gastric histologic changes	44.8% (*n* = 108)
Namiot *et al*[107], 2010	EIA	Poland; 155 patients	65.6% (*n* = 101)
Leszczyńska *et al*[108], 2009	EIA	Poland; 164 dyspeptic patients referred for endoscopy-95 *H. pylori* infected and 69 non-infected	82.1% in *H. pylori*-positive subjects and 17.7% in *H. pylori*-negative subjects
Checchi *et al*[103], 2000	EIA	Italy; 35 patients from a Periodontology clinic	11% (*n* = 4)
Butt *et al*[74], 2002	Cytology	Pakistan; 78 dyspeptic patients	88%
Butt *et al*[82], 2001	Cytology	Pakistan; 135 dyspeptic patients	81.5% (*n* = 110)
Rasmussen *et al*[109], 2010	Southern blot	Brazil; 78 dyspeptic patients	47.4% (*n* = 37)

HIV: Human immunodeficiency virus; PCR: Polymerase chain reaction; RUT: Rapid urease test; CLO: Campylobacter-like organism; EIA: Enzyme immunoassay; *H. pylori*: *Helicobacter pylori*.

among Asian populations. In studies utilizing urease tests, the reported prevalence of the microorganism in dental plaque generally ranged from 80%-100%, with only one study reporting a prevalence below 40%[40].

The PCR technique for detection of *H. pylori* provides the advantage of detecting the target DNA regardless of the viability of the bacteria and detecting even small numbers of the target species. PCR also provides the advantage of identifying specific genotypes of the micro-organism. The results of studies utilizing PCR techniques

have been very variable, with the reported prevalence ranging from 0%-100%. Generally, the initial studies[41-44] utilizing PCR reported very low prevalence rates, while the later studies reported higher prevalence rates[45,46].

Microbial culture of *H. pylori* permits anti-microbial susceptibility testing as well as detailed study of the isolates[2]. However, the reported rates of prevalence in studies in which microbial culture was employed to detect *H. pylori* in dental plaque was generally low, with only three studies reporting prevalence rates above 20%[47-49]. Moreover, in two of these three studies that reported higher prevalence rates[47,48], each of which had a reported sample size of 100, the prevalence was reported to be 48.3%, which could not be interpreted accurately because the percentage data in a sample size of 100 should have a whole number value rather than a fraction. Low rates of prevalence of *H. pylori* in dental plaque reported in studies which have used culture methods have been attributed to the existence of *H. pylori* in the metabolically active but unculturable coccoid form in the dental plaque. *H. pylori* in the dental plaque, being outside its normal habitat in the stomach, may respond to the altered environment by altering its morphology, metabolism, and growth behavior resulting in the formation of a viable but non-culturable coccoid form[50,51]. Other factors which account for the low rates of prevalence reported with culture methods include the fastidious nature of the microorganism, complex nature of the oral microflora, and inhibition of *H. pylori* by other oral microorganisms[37,52].

Although different rates of detection of *H. pylori* in the dental plaque have been reported by various investigators, data generated from these studies show that the microorganism can be reliably detected in plaque samples, especially when PCR techniques are employed. While some investigators have suggested that the occurrence of *H. pylori* in dental plaque is significant in terms of management of *H. pylori*-associated gastric disease[53], others have suggested that the microorganism is present only transiently in the oral cavity[54].

Dental plaque is a microbial biofilm that adheres tenaciously to teeth and other hard surfaces in the oral cavity, such as restorations. In this biofilm, microbial communities are embedded in an extracellular matrix composed of organic and inorganic materials of both host and microbial origin[55]. The microbial flora of dental plaque is very complex; more than 500 different species of bacteria have been detected in plaque samples[56]. These bacterial species inhabit the biofilm not at random as passive neighbors but interact with other bacterial species through specific interactions. These interactions, along with the biofilm structure, confer a large number of advantages to the resident bacterial species. One major advantage that biofilm bacteria enjoy is an increased resistance to host defense mechanisms and anti-microbial agents[55,57]. Thus, *H. pylori* present in the dental plaque, being biofilm-associated, are protected from systemic antibiotics administered for the management of gastric *H. pylori* infection. As a result, the microorganism may persist in the oral cavity even after successful eradication from the stomach and hence, the possibility exists that dental plaque-associated *H. pylori* may serve as a possible source of re-infection and re-colonization of the stomach.

PREVALENCE OF *H. PYLORI* IN SALIVA

Compared with studies on dental plaque, there are fewer reports on the prevalence of *H. pylori* detection in saliva. Table 2 shows a summary of studies reporting on detecting *H. pylori* in saliva. The majority of these studies utilized either PCR or culture methods. The detection rates in saliva were generally less than in dental plaque, with only few studies reporting detection rates of 50%[46,58-60]. The prevalence rates were even lower in studies in which culture was used for detecting *H. pylori* compared with studies using PCR techniques[30,44,61].

As mentioned previously, detection rates of *H. pylori* from saliva were less than that from dental plaque. This may be due to the fact that, while dental plaque, being a biofilm, allows the bacteria to adhere to solid surfaces, the constant flow of saliva may contribute to a reduction in bacterial load, making detection difficult[62]. As with dental plaque, investigators have differed in their opinions regarding the significance of detection of *H. pylori* in the saliva. The detection of *H. pylori* in saliva and dental plaque may precede or be independent of gastric infection[59,63]. It is not yet clear whether the presence of the microorganism in the oral cavity represents long-term colonization or whether its presence is transient due to either gastric reflux or because it is in route to the stomach. While some authors maintain that *H. pylori* may be a normal commensal organism in the oral cavity with no relation to gastric infection[58,61], others, based on detection of *H. pylori* from dental plaque and saliva of patients with and without *H. pylori* infection, have suggested that the oral cavity may be a permanent reservoir of the organism, acting both as source of re-infection and a route of transmission[59,62].

ASSOCIATION OF ORAL HYGIENE/ PERIODONTAL STATUS WITH *H. PYLORI* INFECTION

Studies that evaluated the association between oral hygiene status and periodontal status with *H. pylori* infection are shown in Table 3. Considerable variability was observed in the methods used in these studies to evaluate oral hygiene status and periodontal status. While some of these studies evaluated the association of oral hygiene and periodontal status with gastric *H. pylori* infection, others evaluated the association of these oral health variables with the oral carriage of *H. pylori*. Few studies considered the presence of *H. pylori* in both the stomach and oral cavity. While the majority of the studies did not show an association between periodontal status and gastric *H. pylori* infection, a few studies showed an association between

Table 2 Studies evaluating the presence of *Helicobacter pylori* in saliva

Ref.	Method of detection of *H. pylori* in saliva	Sample population and sample size	Prevalence of *H. pylori* in saliva
Momtaz *et al*[85], 2012	PCR	Iran; 300 patients with gastro-duodenal diseases	8.3% (*n* = 25)
Gao *et al*[46], 2011	PCR	China; 96 patients with *H. pylori* infection	51.10%
Momtaz *et al*[110], 2010	PCR	Iran; 250 dyspeptic patients	14.4% (*n* = 36)
Silva *et al*[87], 2010	PCR	Brazil; 30 dyspeptic patients	30% (*n* = 9) by 16S rDNA and 6.7% (*n* = 2) by vacA
Medina *et al*[90], 2010	PCR	Argentina; 98 patients-43 dyspeptic patients and 55 asymptomatic controls	9.2% (*n* = 9)
Silva *et al*[91], 2009	PCR	Brazil; 30 individuals who were *H. pylori* positive with gastric disease (cases) and 32 individuals who were *H. pylori* positive with no gastric disease (controls)	Overall-25.8% (*n* = 16). Among cases, *H. pylori* DNA detected in 53.3% (16/30); in control group-0%
Morales-Espinosa *et al*[62], 2009	PCR	Mexico; 66 hospitalized patients and 65 dental patients	Overall-35.9% (*n* = 47); 52% (*n* = 34) among hospitalized patients and 20% (*n* = 13) among dental patients
Suzuki *et al*[111], 2008	PCR	Japan; 326 non-dyspeptic subjects	6.4% (*n* = 26)
Bürgers *et al*[93], 2008	PCR	Germany; 94 patients who underwent upper gastro-intestinal endoscopy	7.4% (*n* = 7)
Kignel *et al*[96], 2005	PCR	Brazil; 49 dyspeptic patients	0
Gebara *et al*[53], 2004	PCR	Brazil; 15 gingivitis and 15 periodontitis patients-all were *H. pylori*-positive in antral mucosa	10% (*n* = 3)
Goosen *et al*[100], 2002	PCR	South Africa; 58 clinically healthy volunteers	3.4% (*n* = 2)
Song *et al*[101], 2000	PCR	Germany; 20 dyspeptic patients	In saliva-not specified; 100% in oral samples (plaque and saliva)
Song *et al*[58], 2000	PCR	Germany; 42 patients who underwent upper gastro-intestinal endoscopy	55% (*n* = 23)
Miyabayashi *et al*[79], 2000	PCR	Japan; 47 dyspeptic patients and 10 healthy controls	34% (*n* = 16) among dyspeptic patients
Umeda *et al*[35], 2003	PCR and culture	Japan; 15 dyspeptic patients	26.7% (*n* = 4)
Czesnikiewicz-Guzik *et al*[47], 2005	Culture	Poland; 100 female patients	54.10%
Cześnikiewicz-Guzik *et al*[48], 2004	Culture	Poland; 100 female patients	54%
Rasmussen *et al*[109], 2010	Southern blot	Brazil; 78 dyspeptic patients	42.3% (*n* = 33)

PCR: Polymerase chain reaction; *H. pylori*: *Helicobacter pylori*.

periodontal disease and gastric *H. pylori* infection[39,64-66]. An epidemiological study conducted in the USA based on the National Health and Nutritional Examination Survey Ⅲ data showed that periodontal disease may be associated with *H. pylori* infection, as determined by serological tests[66]. A positive association between periodontal disease and oral carriage of *H. pylori* has been reported by a few investigators[35,39,64,67-73]. Regarding oral hygiene status, while the majority of the studies did not show an association between oral hygiene status and gastric or oral carriage of *H. pylori*, Gürbüz *et al*[39] reported a positive association between plaque scores and both gastric and oral *H. pylori*. Similar to these findings, Butt *et al*[74] reported a positive association between the amount of plaque and detection of *H. pylori* in the oral cavity, while Bali *et al*[65] have reported that poor oral hygiene was significantly associated with gastric *H. pylori* infection.

As discussed in the previous sections, it is clear that *H. pylori* may be detected in dental plaque. Although the organism is microaerophilic, it has been reported that in the oral cavity, it prefers the supragingival plaque[35,41,75]. However, a few investigators have reported an equal presence of *H. pylori* in supra-gingival and sub-gingival plaque samples[53,76]. Supra- and sub-gingival plaque represent two different microenvironments that differ in their pH, nutrient supply, oxygen availability, and host defense mechanisms[77]. Subgingival plaque is generally present in periodontal disease where tissue destruction results in progressive deepening of the periodontal pockets. The transformation from supra-gingival to sub-gingival environment and from health to disease is associated with a change in the resident microbial flora[57]. Because dental plaque is a biofilm in which several different bacterial species co-exist through specific interactions between different species, survival of *H. pylori* in dental plaque depends on its ability to interact with other bacterial species. Studies have shown that *H. pylori* can selectively adhere to certain bacteria, such as *Fusobacterium* species (*Fusobacterium nucleatum*)[52,78], *Porphyromonas gingivalis*[52], and *Bacteroides forsythus*[35]. Because the numbers of these bacterial species are increased in periodontitis patients, it is more likely that dental plaque in periodontitis patients may harbor *H. pylori* by interacting with these bacterial species.

EFFECTS OF ANTI-*H. PYLORI* THERAPY ON *H. PYLORI* IN DENTAL PLAQUE

Table 4 shows the studies that evaluated the effects of

Table 3 Studies evaluating the association of oral hygiene status and gingival/periodontal status with *Helicobacter pylori* infection

Ref.	Oral health status evaluated	Definition of oral hygiene/periodontal status	Sample population and sample size	Association with oral *H. pylori*	Association with gastric *H. pylori*
Sambashivaiah *et al*[73], 2011	Periodontal status	Mean probing depth > 5 mm	India; 36 patients in 3 groups-group Ⅰ, healthy subjects, group Ⅱ, chronic periodontitis patients, group Ⅲ, chronic periodontitis patients with type Ⅱ diabetes mellitus	Significant	Not evaluated
Silva *et al*[71], 2010	Periodontal status	At least 4 teeth with PD ≥ 5 mm and CAL > 3 mm	Brazil; 115 dyspeptic patients	Significant	Not evaluated
Namiot *et al*[107], 2010	Oral hygiene/ periodontal status	Oral Hygiene index/ Russell's periodontal index	Poland; 155 dyspeptic patients	Non-significant	Not evaluated
Bali *et al*[65], 2010	Oral hygiene status/ periodontal status	Oral hygiene index-simplified/probing pocket depth	India; 124 dyspeptic patients of which 60 were *H. pylori* positive (cases) and 64 were *H. pylori* negative (controls)	Not evaluated	Significant
Gonçalves *et al*[69], 2009	Periodontal status	At least 3 sites with PD ≥ 5 mm and/or CAL ≥ 4 mm and BOP	Brazil; 23 HIV seropositive patients of whom 13 had periodontitis and 10 were periodontally healthy; 31 HIV seronegative patients of whom 17 had periodontitis and 14 were periodontally healthy	Significant	Not evaluated
Al Asqah *et al*[64], 2009	Periodontal status	BOP + PD ≥ 3 mm on at least 4 teeth	Saudi Arabia; Dyspeptic patients-62 patients with periodontitis and 39 without periodontitis	Significant	Significant
Liu *et al*[70], 2009	Gingival status	Gingival index	China; 443 dyspeptic patients	Significant	Not evaluated
Zaric *et al*[72], 2009	Gingival and periodontal status	Mean PD, CAL and gingival index scores	Serbia; 66 dyspeptic patients with *H. pylori* infection of gastric mucosa	Significant for mean PD but not for CAL and gingival index scores	Not evaluated
Bürgers *et al*[93], 2008	Periodontal status	Periodontal Screening Index	Germany; 94 dyspeptic patients	Non-significant	Non-significant
Souto and Colombo[68], 2008	Periodontal status	≥ 10% of teeth with probing depth and/ or clinical attachment loss ≥ 5 mm, or ≥ 15% of teeth with probing depth and/ or clinical attachment loss ≥ 4 mm, and > 10% of sites with bleeding on probing	Brazil, 225 patients-56 periodontally healthy and 169 chronic periodontitis patients	Significant	Not evaluated
Namiot *et al*[112], 2007	Periodontal status	Russell's periodontal index	Poland; 137 *H. pylori*-positive patients with peptic ulcer	Not evaluated	Non-significant
Anand *et al*[104], 2006	Oral hygiene status/ periodontal status	Oral hygiene index-simplified/patients with one or more sites with a PD ≥ 3 mm and CAL ≥ 3 mm at the same site	India; 65 dyspeptic patients with *H. pylori* infection (cases) and 69 dyspeptic patients without *H. pylori* infection (controls)	Not evaluated	Non-significant

Gebara *et al*[53], 2004	Gingival and periodontal status	Gingivitis group-patients with PD ⩽ 3 mm and BOP on at least 4 sites; periodontitis group-BOP + PD ⩾ 5 mm on at least 4 teeth	Brazil; 15 gingivitis and 15 periodontitis patients-all were *H. pylori*-positive in antral mucosa	Non-significant	Not evaluated
Gürbüz *et al*[39], 2003	Oral hygiene/ periodontal status	Plaque index/ Russell's index	Turkey; 75 dyspeptic patients	Significant	Significant
Umeda *et al*[35], 2003	Periodontal status	Presence of periodontal pockets ⩾ 4 mm	Japan; 28 patients who harbored *H. pylori* in stomach/duodenum	Significant	Not evaluated
Choudhury *et al*[67], 2003	Periodontal status	CPI	India; 124 dyspeptic patients	Significant	Not evaluated
Butt *et al*[74], 2002	Oral hygiene status/ periodontal status	Community Periodontal Index of treatment needs	Pakistan; 78 dyspeptic patients	Significant with amount of dental plaque but not with gingival or periodontal inflammation	Not evaluated
Dye *et al*[66], 2002	Periodontal status	Presence of 1 dental site with PD ⩾ 5 mm	United States; data from 4504 participants of National Health and Nutrition Examination Ⅲ Survey	Not evaluated	Significant
Berroteran *et al*[99], 2002	Gingival status	Gingival index-scoring from 0-3	Venezuela; 32 dyspeptic patients and 20 asymptomatic controls	Non-significant	Non-significant
Al-Refai *et al*[105], 2002	Oral hygiene/ gingival/ periodontal status	Plaque index/ gingival index/ Community Periodontal Index of treatment needs	Saudi Arabia; 75 dyspeptic patients and 60 healthy controls	Non-significant	Non-significant

PD: Pocket depth; CAL: Clinical attachment level; BOP: Bleeding on probing; CPI: Community periodontal index; *H. pylori*: *Helicobacter pylori*.

systemic *H. pylori* eradication therapy on oral *H. pylori*. The majority of these studies reported that systemic *H. pylori* eradication therapy alone (*i.e.*, in the absence of any form of periodontal therapy), although successful in managing the gastric infection, had very little effect on oral *H. pylori*[46,79]. Although Gebara *et al*[80] reported an increase in the prevalence of *H. pylori* in dental plaque in their patients after one week of triple therapy, Bago *et al*[81] reported that one week of triple therapy resulted in complete eradication of oral *H. pylori* in all 56 periodontitis patients who had *H. pylori*-associated gastric disease.

The systemic *H. pylori* eradication therapy in these studies usually included two antibiotics and a proton pump inhibitor administered orally. Because microorganisms in the dental plaque are afforded greater protection from systemically administered anti-microbial agents, it may be assumed that systemic *H. pylori* eradication therapy has little impact on oral *H. pylori*, hence the observed failure of these therapeutic regimens to eradicate oral *H. pylori*.

IMPACT OF PERIODONTAL THERAPY ON *H. PYLORI* INFECTION

Studies that evaluated the effect of periodontal therapy on *H. pylori* infection are shown in Table 5. Among these, two studies[73,82] evaluated the effect of periodontal therapy on oral *H. pylori*. While Sambashivaiah *et al*[73] evaluated the effect of non-surgical therapy on oral *H. pylori* in patients with and without type Ⅱ diabetes mellitus, Butt *et al*[82] compared the effects of triple therapy and periodontal therapy alone and in combination in 82 patients who were positive for *H. pylori* in dental plaque. They reported that the greatest reduction in plaque *H. pylori* was in the group of patients who received only periodontal therapy, followed by those who received combination therapy, while *H. pylori* persisted in dental plaque of all of the patients who received only triple therapy. Jia *et al*[83] evaluated the effect of periodontal therapy on prevalence of *H. pylori* in the stomach of dyspeptic patients in whom *H. pylori* was eradicated from the stomach by systemic *H. pylori* eradication therapy prior to periodontal intervention. They reported that 6 mo after periodontal therapy, the prevalence of *H. pylori* in the gastric mucosa was significantly lower among patients who received periodontal therapy compared with controls who did not receive any form of periodontal therapy. However, in this particular study, the authors did not evaluate the presence of *H. pylori* in the dental plaque of the study participants at any time point.

Two of the five studies listed in Table 5 evaluated the effects of periodontal therapy on oral and gastric *H. pylori*[46,72]. Gao *et al*[46] reported that among the 43 *H. pylori*-positive patients who received both anti-*H. pylori* therapy and periodontal therapy, the gastric eradication rates at 4 wk and 1 year after intervention were 81.4% (n = 35/43)

Table 4 Studies evaluating the effects of systemic *Helicobacter pylori* eradication therapy on oral *Helicobacter pylori*

Ref.	Sample population and sample size	Prevalence of *H. pylori* in dental plaque	Type of anti-*H. pylori* therapy	Prevalence of *H. pylori* in dental plaque after anti-*H. pylori* therapy	Effect on *H. pylori* infection
Gao *et al*[46], 2011	China; 80 patients with *H. pylori* infection-37 treated with anti-*H. pylori* therapy (gp A) and 43 treated with anti-*H. pylori* therapy and periodontal therapy (gp B)	82.3% in dental plaque and 51.1% in saliva	Gp A-2 wk proton pump inhibitor or triple therapy; gp B-2 wk triple therapy and initial periodontal therapy (oral hygiene education and scaling)	After 4 wk-29.7% (n = 11) in gp A and 4.7% (n = 2) in gp B; after 1 yr-43.2% (n = 16) in gp A and 18.6% (n = 8) in gp B	Eradication rate of gastric *H. pylori* After 4 wk-73% (27/37) in gp A and 81.4% (35/43) in gp B After 1 yr-32.4% (11/37) in gp A and 62.8% (27/43) in gp B
Bago *et al*[81], 2011	Croatia; 56 patients with chronic periodontitis and gastric *H. pylori*-positive	37.5% (n = 21)	One week therapy consisting of amoxicillin 1 g, clarithromycin 500 mg, and omeprazole 20 mg twice a day	0	Eradication rate in stomach was 76.2% (16/21)
Zaric *et al*[72], 2009	Serbia; 44 patients-21 patients positive for *H. pylori* in subgingival dental plaque and gastric mucosa (G+O+t) and 23 patients who were positive for *H. pylori* only in gastric mucosa (G+O-t)- all 44 received only anti-*H. pylori* (triple) therapy	47.7%	Triple therapy consisting of amoxicillin 2 g/d, clarithromycin 1 g/d, and pantoprazole 80 mg/d for 7 d	In G+O+t-66.7% (14/21)	In the G+O+t group, only 47.6% (10/21) showed eradication of gastric *H. pylori* compared with 87.4% (20/23) in G+O-t
Gebara *et al*[80], 2006	Brazil; 30 dentate patients with gingivitis/ periodontitis and *H. pylori* infection who received anti-*H. pylori* therapy	20% (n = 6) in supra-gingival plaque and 26.6% (n = 8) in sub-gingival plaque	Triple therapy consisting of amoxicillin 1 g, clarithromycin 500 mg, and lansoprazole 30 mg twice a day for 7 d	30% (n = 9) in supra-gingival plaque and 46.7% (n = 14) in sub-gingival plaque	Eradication rate of 90%
Gürbüz *et al*[39], 2003	Turkey; 75 dyspeptic patients of which 61 were *H. pylori*-positive and also had *H. pylori* in dental plaque	90.7% (n = 68); 81.3% (n = 61) had co-infection	Amoxicillin 1 g, clarithromycin 500 mg, and ranitidine bismuth citrate 400 mg twice a day for 7 d	100% in 61 patients	Eradication rate of 83%
Suk *et al*[98], 2002	Taiwan; 65 patients with dyspeptic symptoms	Overall-43.1% (n = 28), 73.7% (28/38) among *H. pylori*-positive patients	Colloidal bismuth subcitrate 1 g, amoxicillin 500 mg, and metronidazole 250 mg four times daily for 2 wk or cimetidine 200 mg, amoxicillin 500 mg, and metronidazole 250 mg 4 times a day for 2 wk	92.9% (26/28)	*H. pylori* eradicated from 84.2% (n = 32/38) *H. pylori* infected individuals
Butt *et al*[82], 2001	Pakistan; 82 patients positive for *H. pylori* in dental plaque-27 received only anti-*H. pylori* therapy (gp 1); 25 received anti-*H. pylori* therapy+periodontal therapy (gp 2); 30 received only periodontal therapy (gp 3)	100%	Gp 1-twice daily omeprazole 20 mg, clarithromycin 500 mg and metronidazole 400 mg; gp 2-triple therapy and dental scaling and chlorhexidine mouthwashes twice daily for 7 d; gp 3-only dental treatment	100% in gp 1; 16% in gp 2 (4/25); 10% in gp 3 (3/30)	Not evaluated

Miyabayashi *et al*[79], 2000	Japan; 47 patients with chronic gastritis or peptic ulcer-48.9% (n = 23) were positive for oral *H. pylori* and 38.3% (n = 18) had *H. pylori* in plaque	48.9% (n = 23) were positive for oral *H. pylori* and 38.3% (n = 18) had *H. pylori* in plaque	Lansoprazole 30 mg/d, metronidazole 750 mg/d, and clarithromycin 400 mg/d for 2 wk	Oral prevalence at 4 wk-34% (16/47)	At 4 wk-91.6% (22/24) of subjects negative for oral *H. pylori* were successfully eradicated of *H. pylori* infection compared to 52.2% (12/23) in oral *H. pylori*-positive patients. At 2 years, 95.8% (23/24) of subjects negative for oral *H. pylori* were successfully eradicated of *H. pylori* infection compared with 69.5% (16/23) in oral *H. pylori*-positive patients

H. pylori: *Helicobacter pylori*.

Table 5 Studies evaluating the effect of periodontal therapy on oral and gastric *Helicobacter pylori*

Ref.	Sample population and sample size	Prevalence of *H. pylori* in dental plaque	Details of periodontal therapy	Prevalence of *H. pylori* in dental plaque after periodontal therapy	Effect on *H. pylori* infection
Gao *et al*[46], 2011	China; 80 patients with *H. pylori* infection-37 treated with anti-*H. pylori* therapy (gp A) and 43 treated with anti-*H. pylori* therapy and periodontal therapy (gp B)	82.3% in dental plaque and 51.1% in saliva	Gp A-2 wk proton pump inhibitor or triple therapy; gp B-2 wk triple therapy and initial periodontal therapy (oral hygiene education and scaling)	After 4 wk, 29.7% (n = 11) in gp A and 4.7% (n = 2) in gp B; after 1 yr, 43.2% (n = 16) in gp A and 18.6% (n = 8) in gp B	Eradication rate of gastric *H. pylori* After 4 wk, 73% (27/37) in gp A and 81.4% (35/43) in gp B, after 1 year, 32.4% (11/37) in gp A and 62.8% (27/43) in gp B
Sambashivaiah *et al*[73], 2011	India; 36 patients in 3 groups-group Ⅰ, healthy subjects, group Ⅱ, chronic periodontitis patients, group Ⅲ, chronic periodontitis patients with type Ⅱ diabetes mellitus	Overall-66.7% (n = 24); among group Ⅰ-41.7% (n = 5), group Ⅱ-75% (n = 9), group Ⅲ-83.3% (n = 10)	Group Ⅱ and Ⅲ patients received full mouth scaling and root planning	Group Ⅱ, 0 and group Ⅲ, 8.3% (n = 1)	Not evaluated
Zaric *et al*[72], 2009	Serbia; 43 patients positive for *H. pylori* in sub gingival dental plaque and gastric mucosa-21 received only anti-*H. pylori* (triple) therapy (G+O+t); 22 received anti-*H. pylori* (triple) therapy)+periodontal therapy (G+O+tp)	100%	Triple therapy consisting of amoxicilin 2 g/d, clarithromycin 1 g/d, and pantoprazole 80 mg/d for 7 d. Periodontal therapy included oral hygiene orientation, plaque and calculus removal with an ultrasonic device, scaling, and root planing, as well as irrigation of periodontal pockets with 0.12% chlorhexidine-gluconate performed during triple therapy, in one sitting	In G+O+t-66.7% (14/21); in G+O+tp-27.3% (6/22)	In the G+O+tp group, 77.3% (17/ 22) showed eradication of gastric *H. pylori* compared with 47.6% (10/21) in G+O+t
Jia *et al*[83], 2009	China; 107 dyspeptic patients in whom *H. pylori* was eradicated from the gastric mucosa-56 received dental plaque control (test) and 51 did not (control)	Not evaluated	Full-mouth scaling, root planning and polishing, and dental plaque control instructions by dentist	Not evaluated	Prevalence of *H. pylori* in gastric mucosa was 19.64% (11/56) in test group and 84.31% (43/51) in control group
Butt *et al*[82], 2001	Pakistan; 82 patients positive for *H. pylori* in dental plaque-27 received only anti-*H. pylori* therapy (gp 1); 25 received anti-*H. pylori* therapy+periodontal therapy (gp 2); 30 received only periodontal therapy (gp 3)	100%	Gp 1-twice daily omeprazole 20 mg, clarithromycin 500 mg and metronidazole 400 mg; gp 2-triple therapy and dental scaling and chlorhexidine mouthwashes twice daily for 7 d; gp 3-only dental treatment	100% in gp 1; 16% in gp 2 (4/25); 10% in gp 3 (3/30)	Not evaluated

H. pylori: *Helicobacter pylori*.

and 62.8% (n = 27/43), respectively, while the eradication rates over the same time periods among the 37 *H. pylori*-positive patients who received only anti-*H. pylori* therapy were 73% (n = 27/37) and 32.4% (n = 11/37), respectively. They also reported that the detection rates of *H. pylori* in dental plaque of patients who received both forms of therapy at 4 wk and 1 year after intervention were 4.7% (n = 2/43) and 18.6% (n = 8/43), respectively, while the corresponding rates for the patients who received only anti-*H. pylori* therapy were 29.7% (n = 11/37) and 43.2% (n = 16/37), respectively. In another study by Zaric *et al*[72], 43 patients positive for *H. pylori* in both subgingival plaque and gastric mucosa were categorized into two groups in which 21 patients received only anti-*H. pylori* therapy and 22 received anti-*H. pylori* therapy along with periodontal therapy. Three months after treatment completion, 77.3% (n = 17/22) of the patients who received both anti-*H. pylori* therapy and periodontal therapy showed gastric eradication compared with only 47.6% (n = 10/21) of the patients who received only anti-*H. pylori* therapy. Among the 22 patients who received both anti-*H. pylori* therapy and periodontal therapy, *H. pylori* was detected in dental plaque of only six (27.3%) patients 3 mo after completion of treatment, whereas the microorganism was detected in 66.7% (n = 14) of the 21 patients who received only anti-*H. pylori* therapy. The authors also reported that eradication in the stomach coincided with eradication in the oral cavity (*i.e.*, all 16 of the individuals who received both forms of therapy and showed eradication of oral *H. pylori* also showed eradication of gastric *H. pylori*). Five of the participants in this group who were positive for oral samples were also positive for gastric *H. pylori*.

The periodontal therapy provided to the patients in these studies consisted of non-surgical periodontal therapy, in which the microbial deposits on the surfaces of the teeth are professionally removed by the dentists, along with other plaque control measures, such as use of mouthwashes and patient education in plaque control. This phase of treatment is referred to as the Etiotropic phase and is considered to be very important because the microbial etiological factors of periodontal disease are removed in this phase. As mentioned previously, because of its biofilm properties, dental plaque provides resistance to the resident microflora from systemically administered antimicrobial agents. Thus, the *H. pylori* present in the dental plaque are seldom affected by systemic *H. pylori* eradication therapy, as shown in previous studies conducted[39,80]. As a result, the removal of *H. pylori* in the dental plaque may necessitate periodontal therapy in which all microbial deposits, along with the resident bacteria, including *H. pylori*, will be eliminated. The initial studies in this regard have shown promising results in the management of both oral and gastric *H. pylori*.

CONCLUSION

Although *H. pylori* has long been known to be detected in the oral cavity, the significance of such findings was controversial. If the oral cavity is an important extra-gastric reservoir of *H. pylori*, then this finding may have major implications because the oral cavity can serve as both a source of re-infection and route of transmission. Because plaque-associated *H. pylori* would be resistant to systemic *H. pylori* eradication therapy, it can affect the success rates of the anti-*H. pylori* therapy. Thus, it is imperative to identify the role of dental plaque, saliva, and periodontal disease in *H. pylori* infection. Once these factors are clearly understood and whether the oral cavity is a major extra-gastric reservoir of *H. pylori* is confirmed, then newer treatment modalities, such as periodontal therapy, may be incorporated in the protocol for the management of *H. pylori* infection. The initial studies on the role of periodontal therapy in the management of *H. pylori* infection have shown promising results, suggesting that oral *H. pylori* may play an important role in re-infection of the gastric mucosa. These observations also create new avenues for both future research and more effective management of *H. pylori* infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Optimal treatment strategy for *Helicobacter pylori*: Era of antibiotic resistance

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Abstract

Standard triple therapy, consisting of a proton pump inhibitor, plus amoxicillin and clarithromycin, has been the most commonly used first-line treatment regimen for *Helicobacter pylori* (*H. pylori*) eradication for many years worldwide. However, as a result of increased resistance to antibiotics, *H. pylori* eradication rates with use of standard triple therapy have been declining and recently reached < 80% in many countries. Several new strategies to enhance the eradication rate of *H. pylori* have been studied. Currently, among the alternative first-line eradication regimens, concomitant and hybrid regimens have shown excellent results and could be the optimal treatment option. Although clinical usefulness of rescue therapy for patients in whom eradication of *H. pylori* with non-bismuth quadruple regimen has failed is unclear, levofloxacin-based quadruple therapy has shown promise as a rescue treatment. The choice of third-line therapy depends on factors such as the local pattern of antibiotic resistance, drug availability, and previous treatment. We hope that a simple method for detection of antibiotic susceptibility using polymerase chain reaction would be a possible alternative to administration of "tailored treatment" in the era of increasing prevalence of antimicrobial resistance.

Key words: *Helicobacter pylori*; Standard therapy; Bacterial eradication; Concomitant therapy; Hybrid therapy

Core tip: Currently, among the alternative first-line eradication regimens, concomitant and hybrid regimens have shown excellent results and could be the optimal treatment option. Levofloxacin-based quadruple therapy has shown promise as a rescue treatment.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is an important factor worldwide influencing progression from chronic gastritis to peptic ulcer and gastric cancer[1]. Nowadays, the incidence of *H. pylori* infection has shown a declining trend. In Korea, in a survey of 5732 asymptomatic patients in 1998, the seropositive rate of *H. pylori* infection was reported as 66.9% in adults[2]. However, it had declined to 59.6% in 2005[3]. The main cause of this difference is treatment for *H. pylori* infection.

Triple therapy, consisting of a proton pump inhibitor (PPI), plus amoxicillin and clarithromycin, was accepted by the international guidelines in 1996 and has been the most commonly used first-line treatment regimen for *H. pylori* eradication in most countries for many years[4,5]. According to Graham *et al*[6], the treatment regimen that meets the category of Grade A [intention-to-treat (ITT)

cure rate is 95%-100%] should be prescribed as a therapeutic regimen for *H. pylori* infection. Regimens scoring as B (ITT cure rate 90%-95%) can be used if Grade A is not obtainable. Unfortunately, *H. pylori* eradication rates with standard triple therapy have been declining and recently reached < 80% in many countries[6].

This phenomenon has been largely related to an increase in bacterial resistance to antibiotics, particularly against clarithromycin[7]. In Japan, resistance to clarithromycin increased from 19% to 28% during 2002-2005[8]. In Korea, a study of 652 isolates from 1994 to 1999 found that resistance to metronidazole and clarithromycin increased from 33% to 48% and 4.8% to 7.7%, respectively[9]. Another recent survey reported that resistance to clarithromycin increased from 16.7% to 38.5% during 2003-2005 and 2007-2009 in Korea[10].

Several strategies to enhance the eradication rate of *H. pylori* have been studied and ongoing results are anticipated. The aim of this review is to suggest timely suitable approaches to management of patients with *H. pylori* infection.

FIRST-LINE THERAPY

Triple therapy with longer duration

There was a simple question regarding whether longer duration of standard triple therapy could result in a higher rate of eradication. In a multicenter study conducted in the US, of 284 patients, the *H. pylori* eradication rate after triple therapy for 14 d was 85% (96/113), which did not differ from 84% (103/123) reported for triple therapy for 10 d[11]. In another study comparing 3-, 7- and 10-d triple therapies with rabeprazole to a 10-d omeprazole control triple therapy, no statistically significant difference was observed between the 7-d rabeprazole-based regimen (77%) and 10-d rabeprazole-based regimen (78%) and omeprazole-based regimen (73%)[12]. In a meta-analysis of the duration of triple therapy, relative risk for eradication was reported as 1.05 (95%CI: 1.01-1.10) for 7-d compared with 10-d triple therapy and 1.07 (95%CI: 1.02-1.12) for 7-d compared with 14-d therapy[13]. Thus, currently, extending the duration of triple therapy beyond 7 d did not show a marked benefit. Other regimens with longer duration have shown promise and are discussed below.

Bismuth-containing quadruple therapy

Bismuth-containing quadruple therapy has been proven as a salvage therapy regimen for failure of standard triple therapy in many countries[14,15]. The regimen consists of PPI, bismuth, tetracycline and metronidazole. However, regarding the first-line therapy, recent results have been disappointing. In a large study, bismuth-containing quadruple therapy [ITT, 82%; per-protocol (PP), 88%] did not show superior eradication rate compared with standard triple therapy (ITT, 78%; PP, 82%)[16]. In a meta-analysis of bismuth-containing quadruple therapy with standard triple therapy [nine randomized controlled trials (RCTs), n = 1679], bismuth quadruple therapy achieved an eradication rate similar to that of primary therapy for *H. pylori* infection compared with standard triple therapy (78.3% *vs* 77.0%, RR = 1.002, 95%CI: 0.936-1.073)[17].

In a recent RCT, the new combination of bismuth containing 2 wk quadruple therapy, including bismuth, PPI, amoxicillin, and clarithromycin, achieved an eradication rate of 90.7% as a first-line treatment[18]. In another RCT, quadruple therapy with moxifloxacin and bismuth showed an eradication rate of 92%[19]. Although conduct of additional studies is needed to validate these results, these studies imply that another bismuth-containing regimen could be an alternative option as a first-line treatment.

Non-bismuth-containing quadruple regimen

In a meta-analysis of the effect of antibiotic resistance status on the efficacy of triple and quadruple first-line therapy for *H. pylori*[20], clarithromycin resistance had a greater effect on treatment efficacy than nitroimidazole resistance. Metronidazole resistance reduced efficacy by 26% in triple therapies containing nitromidazole, tetracycline and bismuth, while efficacy was reduced by only 14% when a gastric acid inhibitor was added to the regimen. Quadruple therapies containing both clarithromycin and metronidazole were the most efficacious; > 80% eradication rate for *H. pylori* infection. Therefore, non-bismuth, three-antibiotic-containing quadruple therapies, consisting of PPI, amoxicillin, clarithromycin and nitroimidazole, could be the key as a new standard first-line regimen.

Sequential therapy: Sequential therapy was introduced as a novel therapeutic approach for *H. pylori* eradication by Zullo *et al*[21,22] in 2000. Ten-day sequential therapy regimen consists of 5 d of dual treatment with a PPI plus amoxicillin, followed by 5 d of triple treatment with a PPI, clarithromycin and nitromidazole. Zullo *et al*[22] reported eradication rates for sequential therapy of 92% by ITT and 95% by PP analysis. Since then, many studies have reported a superior result of sequential therapy compared with standard triple therapy[23,24]. In two meta-analyses of RCTs, sequential therapy showed an association with a higher eradication rate of *H. pylori* at 91%-93.4%, compared with standard triple regimen at 75.7%-76.9%[25,26]. However, these RCTs were conducted before 2008. In recent studies, the success rate of the sequential regimen appeared to be lower, compared to previous trials. In 10 recent RCTs on sequential therapy during 2008-2012, the eradication rate was 80.6% (95%CI: 78.5%-82.7%) for sequential therapy compared to 75.8% (95%CI: 73.5%-78.1%) for standard triple therapy[27]. Theoretically, the objective during the first 5 d of taking amoxicillin is to disrupt the cell wall of *H. pylori* and to prevent activation of efflux channels. Therefore, this damage to the cell wall could help to improve the efficacy of clarithromycin in the sequential phase of treatment[28,29]. However, there is a conflicting opinion against this theory. The main role of sequential therapy

may be related to use of large numbers of antibiotics, including nitroimidazole, not the sequential administration itself[30,31]. In addition, there is a problem with sequential therapy, which is that taking the medication is relatively complex for patients. The patient is required to switch from a dual to a triple regimen[32], which may reduce the compliance of patients and result in a decrease in eradication rates in patients with multiple antibiotic resistance in clinical practice.

In this regard, the triple combination of antibiotics such as clarithromycin plus amoxicillin and a nitroimidazole with PPI has been highlighted or has re-emerged as a non-sequential regimen - concomitant therapy.

Concomitant therapy: In a systemic review reported by Essa *et al*[32], which included nine studies, concomitant therapy achieved an eradication rate of 89.7% in ITT and 92.9% in PP. In a meta-analysis of 15 studies (1723 patients), a significantly higher eradication rate was achieved with concomitant therapy, compared to standard triple therapy (90%, 95%CI: 86%-93%)[33]. A tendency towards better results with longer treatments (7-10 d *vs* 3-5 d) was observed. In our interim analysis of multicenter RCTs (six institutes, 214 patients in ITT analysis, unpublished), the eradication rate of the 10-d concomitant therapy group was significantly higher than that of the 10-d standard triple therapy group (89.3% *vs* 79.3%, $P = 0.049$).

Both sequential therapy and concomitant therapy have shown superiority over legacy triple therapy, especially in cases of clarithromycin resistance. Clarithromycin resistance is the result of different point mutations, which have a different therapeutic impact on current regimens. In a systematic review of sequential therapy, the eradication rate was reported as 75% (41/55, four studies) in clarithromycin-resistant strains[34]. However, the effect of clarithromycin resistance on the efficacy of concomitant regimens was negligible, with 95% efficacy in the clarithromycin-sensitive arm, and 96% in the clarithromycin-resistant arm[20]. In addition, in another study, concomitant therapy was found to be more suitable for patients with dual antibiotic resistances than sequential therapy[35].

Hybrid therapy (sequential-concomitant therapy): Hybrid therapy is a combination of sequential therapy and concomitant therapy. Hybrid therapy regimens consist of PPI and amoxicillin for 10-14 d, with addition of clarithromycin and metronidazole for the final 5-7 d. In a recent study, this regimen showed excellent results, with an eradication rate of 99.1% (95%CI: 97.3%-100%) by PP and 97.4% (95%CI: 94.5%-100%) by ITT analysis[36]. This study is meaningful for achievement of Grade A treatment success for *H. pylori* infection. In another recent RCT ($n = 343$) for comparison with concomitant therapy, the rates of eradication for hybrid and concomitant therapies were 92% (95%CI: 87%-95%) and 96.1% (95%CI: 93%-99%), respectively ($P = 0.07$)[37]. In that study, 23.5% of patients had *H. pylori* strains that were resistant to clarithromycin, and 8.8% were resistant to clarithromycin and metronidazole. This antimicrobial resistance did not impair the efficacy of either of the non-bismuth quadruple regimens. However, larger sample size and conduct of studies in other countries is needed in order to validate the effectiveness of hybrid therapy.

Data from RCTs for the first-line non-bismuth-containing quadruple therapies are provided in Table 1[35,37-42].

SECOND-LINE (RESCUE) THERAPY

After failure of a PPI-clarithromycin-containing regimen, bismuth-containing quadruple therapy is recommended by the Maastricht Ⅳ guidelines[43]. In three studies of bismuth-containing quadruple therapy as second-line therapy, eradication rates of 55%-69.1% have been reported[44-46]. In a recent study conducted in Korea, higher eradication rates were reported for 2-wk bismuth-containing quadruple therapy compared with the same regimen for 1 wk (64.3% *vs* 82.6%, ITT analysis, $P = 0.002$)[47]. However, these data were regarding the rescue therapy after failure of standard triple therapy. Data regarding rescue therapy after failure of non-bismuth-containing quadruple therapy as a first-line treatment are insufficient[48].

The Maastricht Ⅳ guidelines recommend levofloxacin-based triple therapy containing PPI and amoxicillin as a rescue treatment in areas of high clarithromycin resistance[43]. The eradication rate of levofloxacin 500 mg with PPI and amoxicillin was 60%-85% after two eradication failures with key antibiotics such as amoxicillin, clarithromycin, metronidazole and tetracycline[49,50]. However, the recommended regimen had a somewhat disappointing eradication rate as a rescue treatment after failure of sequential or concomitant treatment, below 80%[51,52]. In addition, the rapid acquisition of resistance to levofloxacin may be a problem in future efficacy. In fact, a recent study reported a high prevalence of levofloxacin resistance (29.5%) in *H. pylori* strains isolated from Korean patients[10]. Therefore, a new combination regimen is warranted. In a recent study conducted in Taiwan, 10-d quadruple therapy containing PPI, bismuth, tetracycline, and levofloxacin achieved an excellent result, with an eradication rate of 95.8% after failure of first-line treatment with sequential therapy[53]. This new combination of levofloxacin-based quadruple therapy is expected as the second-line therapy and validation of other combinations with levofloxacin is needed. In addition, high dose of levofloxacin (750 mg), which achieved a better effect against infectious disease[54], should be validated for *H. pylori* eradication. In summary, although further validation is needed, levofloxacin-based quadruple therapy is a promising rescue regimen after failure of first-line non-bismuth-containing quadruple therapy.

THIRD-LINE THERAPY

After failure of second-line treatment, antimicrobial

Table 1 Randomized-controlled trials for the first-line non-bismuth containing quadruple therapies

Author	Country	Publication year		Therapy regimen	Patients (*n*)	Eradication rate (ITT)
Wu *et al*[35]	Taiwan	2010	S	E 40 mg *bd* + AMX 1 g *bd* for 5 d followed by E 40 mg *bd* + CLA 500 mg *bd* + MET 500 mg *bd* for 5 d	115	92.3%
			C	E 40 mg *bd* + AMX 1 g *bd* + CLA 500 mg *bd* + MET 500 mg *bd* for 10 d	117	93.0%
Huang *et al*[38]	Taiwan	2012	S	L 30 mg *bd* + AMX 1 g *bd* for 5 d followed by L 30 mg *bd* + CLA 500 mg *bd* +MET 500 mg *bd* for 5 d	85	80.0%
			C	L 30 mg *bd* + AMX 1 g *bd* + CLA 500 mg *bd* + MET 500 mg *bd* for 10 d	84	88.1%
McNicholl *et al*[39]	Spain	2013	S	O 20 mg *bd* + AMX 1 g *bd* for first 5 d followed by O 20 mg *bd* + CLA 500 mg *bd* + MET 500 mg *bd* for 5 d	170	81%
			C	O 20 mg *bd* + AMX 1 g *bd* + CLA 500 mg *bd* + MET 500 mg *bd* for 10 d	168	87%
Lim *et al*[40]	Korea	2013	S	R 20 mg *bd* + AMX 1 g *bd* for 7 d followed by R 20 mg *bd* + CLA 500 mg *bd* + MET 500 mg *bd* for 7 d	86	75.6%
			C	R 20 mg *bd* + AMX 1 g *bd* + CLA 500 mg *bd* + MET 500 mg *bd* for 14 d	78	80.8%
Zullo *et al*[41]	Italy	2013	S	O 20 mg *qd* + AMX 1 g *qd* 5d followed by O 20 mg *qd* + CLA 500 mg *qd* + TIN 500 mg *qd* for 5 d	90	91.1%
			C	O 20 mg *qd* + AMX 1 g *qd* + CLA 500 mg *qd* + TIN 500 mg *qd* for 5 d	90	85.5%
			H	O 20 mg *qd* + AMX 1 g *qd* for 14 d plus CLA 500 mg *qd* + TIN 500 mg *qd* for last 7 d	90	80.0%
Sardarian *et al*[42]	Iran	2013	S	P 40 mg *bd* + AMX 1 g *bd* for 5 d followed by P 40 mg *bd* + CLA 500 mg *bd* + TIN 500 mg *bd* for 5 d	210	76.7%
			H	P 40 mg *bd* + AMX 1 g *bd* for 14 d plus CLA 500 mg *bd* + TIN 500 mg *bd* for last 7 d	210	89.5%
Molina-Infante *et al*[37]	Spain, Italy	2013	C	O 40 mg *bd* + AMX 1 g *bd* + CLA 500 mg *bd* + NIT 500 mg *bd* for 14 d	172	91.7%
			H	O 40 mg *bd* + AMX 1 g *bd* for 14 d *plus* CLA 500 mg *bd* + TIN 500 mg *bd* for last 7 d	171	90.0%

ITT: Intention-to-treat; S: Sequential therapy; C: Concomitant therapy; H: Hybrid therapy; AMX: Amoxicillin; CLA: Clarithromycin; MET: Metronidazole; TIN: Tinidazole; E: Esomeprazole; L: Lansoprazole; P: Pantoprazole; O: Omeprazole; R: Rabeprazole; *bd*: Two times a day; *qd*: Once a day.

susceptibility testing is needed by obtaining gastric biopsy specimens for culture. In general, the method of agar dilution is needed for *H. pylori* susceptibility testing. However, culture and susceptibility testing require specific equipment, which is not available in most medical centers. In a recent study, *Helicobacter* genotype and the *CYP2C19* polymorphism were examined for detection of antimicrobial resistance[55]. In addition, a new method using polymerase chain reaction (PCR) has been validated for detection of resistance of H. *pylori* to clarithromycin[56]. It is a simple method and requires a shorter time than culture. However, further validation and study for use of a PCR method in real treatment are needed. In the near future, a new tailored regimen for individual patients would be the answer to antibiotic resistance.

CONCLUSION

As a result of increased resistance to antibiotics, conventional triple therapy is no longer standard therapy for naïve *H. pylori* infection. Currently, among the alternative first-line eradication regimens, concomitant and hybrid regimens have shown excellent results and could be the optimal treatment option. After failure of new first-line non-bismuth quadruple treatment, levofloxacin-based quadruple regimens could be an option for rescue treatment. In addition, we hope that development of a simple method for detection of antimicrobial resistance before *H. pylori* eradication and a new tailored treatment for *H. pylori* will be possible in the near future.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori eradication for preventing gastric cancer

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Abstract

Helicobacter pylori (*H. pylori*) infection is a major risk factor for gastric cancer (GC) development, which is one of the most challenging malignant diseases worldwide with limited treatments. In the multistep pathogenesis of GC, *H. pylori* infection slowly induces chronic active gastritis, which progresses through the premalignant stages of atrophic gastritis, intestinal metaplasia, and dysplasia, and then finally to GC. Although eradication of *H. pylori* is a reasonable approach for the prevention of GC, there have been some contradictory reports, with only some long-term follow-up data showing efficacy of this approach. The inconsistencies are likely due to the insufficient number of participants, relatively short follow-up periods, poor quality of study designs, and the degree and extent of preneoplastic changes at the time of *H. pylori* eradication. This review analyzes recent high-quality studies to resolve the discrepancies regarding the eradication of *H. pylori* for GC prevention. The relationship between *H. pylori* eradication and GC/precancerous lesions/metachronous GC is examined, and the cost-effectiveness of this strategy in the prevention of GC is assessed. Although it is assumed that eradication of *H. pylori* has the potential to prevent GC, the feasibility and appropriate timing of this strategy for cancer prevention remain to be determined. As a result, additional well-designed trials with longer follow-up periods are needed to clarify this issue.

Key words: *Helicobacter pylori*; Gastric cancer; Cancer prevention

Core tip: The treatment of gastric cancer (GC) is challenging. Elimination of a major risk factor, *Helicobacter pylori* (*H. pylori*) infection, represents an important approach for the prevention of GC. However, the feasibility and appropriate timing of this strategy remain to be determined. This review highlights the most recent literature and presents a comprehensive evaluation of what is currently known about *H. pylori* infections and GC.

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INTRODUCTION

Gastric cancer (GC) represents one of the most challenging malignant diseases worldwide and is the second leading cause of death with the highest incidence rates observed in Eastern Asia, Japan, Eastern Europe, and the Andean regions of South America[1]. GC develops from the progression of chronic gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and finally invasive carcinoma[2]. Although the development of GC involves a multifactorial pathway, the pathogenesis is believed to begin from a single infectious agent[3,4], *Helicobacter pylori* (*H. pylori*), which is classified as a Group 1 carcinogen

by the World Health Organization (WHO) and International Agency for Research on Cancer (IARC)[5]. *H. pylori* is a leading worldwide infectious agent, accounting for as many as 650000 new cases of non-cardiac GC annually[6], and epidemiological data support a strong causal relationship between *H. pylori* infection and GC[7-11], as well as some animal studies[12-14]. Different countries have different consensus reports about *H. pylori* eradication treatment[15-20]. Among these guidelines, the most consistent recommendation with a high level of evidence is endoscopic resection of early GC[21]. *H. pylori* eradication is recommended to improve gastric atrophy[22]. Although it may seem intuitive that removing the organism would eliminate the risk of GC, only a small proportion of infected individuals develop GC[23]. Furthermore, massive eradication therapy may lead to activation of antibiotic-resistant strains of *H. pylori* in the general population, as well as an over-consumption of medical resources. Therefore, this review integrates information available from recent studies in order to evaluate the benefit of *H. pylori* eradication for GC prevention.

H. PYLORI ERADICATION AND GC

The first well-designed trial to investigate eradication of *H. pylori* for the prevention of GC was performed in 1991 by Correa *et al*[24] and involved Colombian individuals at high risk for GC. Although the cancer incidence was similar in both treated and untreated groups after a 6-year follow-up, this trial showed significant increases in the regression rates of cancer precursor lesions. In 2004, Wong *et al*[25] assigned 1630 patients from the Fujian province in China with *H. pylori* infections to an eradication or a non-eradication group. During the 7.5-year follow-up period, GC was similar in both groups, occurring in 11 out of 813 patients from the non-eradication group and seven of 817 patients from the eradication group. However, the incidence of GC in a subgroup without precancerous lesions receiving *H. pylori* eradication therapy was significantly lower, compared with the non-eradication group; several other trials reported similar results[26-27].

A meta-analysis by Fuccio *et al*[28] examined six randomized trials assessing GC and the progression of preneoplastic lesions during 4-10 years follow-up. Their results indicated that 27 of 3388 patients (1.1%) in the *H. pylori* antibiotic treatment group developed GC, compared to 56 of 3307 (1.7%) not undergoing treatment; the overall relative risk was 0.65. However, this meta-analysis comprised mainly studies performed in Asia, and only two of the studies were of a double-blind design. With a cohort study of 80225 patients, Wu *et al*[29] found that the earlier *H. pylori* is eradicated after peptic ulcer disease, the smaller the risk of GC, with no risk for patients receiving early *H. pylori* eradication as compared to the general population. A later interventional trial in Shandong, China showed that 2 wk antibiotic treatment for *H. pylori* in 3365 patients significantly reduced GC incidence by 39%, during a total follow-up of 14.7 years[30].

Altogether, most of these studies focused on people with gastric precancerous lesions, such as gastric atrophy and intestinal metaplasia (IM), because of the low incidence of GC. However, GC has a long pre-malignancy phase that may mask the ultimate effects of *H. pylori* eradication. Therefore, some results of previous studies are inconclusive, partly due to the insufficient number of participants and the relatively short follow-up[31,32]. Nonetheless, the above studies provide clinical evidence suggesting that successful eradication of this organism is related to a reduction in the risk of GC, although it does not prevent GC completely.

H. PYLORI ERADICATION AND PRECANCEROUS LESIONS

H. pylori infection can cause chronic gastritis. This chronic condition can lead to gastric mucosal atrophy and IM[2], which are considered to be precancerous lesions of GC[2,33-35]. Therefore, improvement or elimination of atrophy and IM with *H. pylori* eradication could potentially inhibit gastric carcinogenesis. Although the effect of *H. pylori* eradication on the incidence of precursor lesions is unknown, many studies have identified alterations in gastric atrophy and IM after *H. pylori* eradication. These reports had contradictory results, with several studies showing improvements in atrophy and IM[36-38], and others showing no improvement in the gastric mucosa after eradication[39-41]. There is also evidence that *H. pylori* eradication can lead to regression of atrophy in other conditions[42]. However, these studies were limited to data from short-term follow-up, small sample sizes, and few points of observation in their design, which may have contributed to the contradictory results.

A study by our team followed chronic atrophic gastritis patients with *H. pylori* infections, with only 92 of 179 patients receiving *H. pylori* eradication. Although the grade of IM increased in the untreated *H. pylori*-infected group after 3 years, the grade of atrophy significantly decreased in the eradication group, suggesting that *H. pylori* eradication may improve gastric atrophy and prevent the progression of IM[43]. However, a more recent meta-analysis that systematically reviewed the long-term effects of *H. pylori* eradication on gastric histology showed that *H. pylori* eradication can improve atrophy but not IM[22]. Recently, a trial from Matsu Island demonstrated that population-based eradication of *H. pylori* infection was associated with a significant reduction in gastric atrophy within the relatively short study period[44]. Evidence for the prevention of GC by reducing the occurrence of precancerous lesions was presented by Kodama *et al*[45], who evaluated the gastric mucosa at five points in the stomach according to the updated Sydney system and showed that atrophy at all sites and IM in the lesser curvature of the corpus were gradually and significantly decreased 10 years after the *H. pylori* eradication.

It is noteworthy to mention that GC can still develop even after successful eradication therapy. One famous

case report describes two patients who were included in one of the first study cohorts that received eradication therapy for peptic ulcer disease, but nevertheless developed GC during long-term follow-up (one at 4 years and the other at 14 years after the *H. pylori* eradication)[46]. Both patients had suffered from IM when the gastric ulcer disease was first discovered. Furthermore, malignant lesions that develop after eradication therapy have a similar characteristic appearance to and therefore may have a common carcinogenesis with cancers that occur in the presence of *H. pylori* infection, although biological features may be changed by the eradication therapy[47,48]. These results suggest that *H. pylori* eradication does not result in the regression of all precancerous lesions, which may depend on the degree and extent of preneoplastic changes at the time of eradication. Moreover, decreased *H. pylori* colonization density may occur in these lesions even without active intervention, with further progression of premalignant lesions less dependent on *H. pylori* infection. Therefore, the key question is whether and when precancerous lesions can be reversed with *H. pylori* eradication. Ongoing clinical studies are focusing on a "point of no return", defined as a situation when certain alterations are no longer reversible by *H. pylori* eradication and GC progression continues.

H. PYLORI ERADICATION AFTER ENDOSCOPIC RESECTION OF EARLY GASTRIC CANCER

Following endoscopic resection of early gastric cancer (EGC), secondary cancers are often found at sites other than the resection site during follow-up, with the rates ranging from 3% to 4% per year[21,49,50], rendering them more likely to be detected in trials compared to the low incidence of GC. Japanese guidelines recommend treatment for *H. pylori* infection in patients following resection of EGC[51,52]. In 1997, Uemura *et al*[53] assigned patients undergoing endoscopic resection for GC to an *H. pylori* eradication group or a non-eradication group. Secondary GC was detected in 10 of 67 patients from the non-eradication group (15%) *vs* none of the 65 patients from the eradication group during about 5 years follow-up, suggesting that *H. pylori* eradication inhibits the development of new carcinomas. Although this was a pioneer study, it was not a randomized controlled trial. Fukase *et al*[21] reported the first multicenter, open-label, randomized study on the incidence of developing metachronous GC following endoscopic resection of EGC. In this study, 544 patients from 51 Japanese institutions were randomly assigned to an *H. pylori* eradication group or a non-eradication group and were followed up over 3 years with annual endoscopy to detect any recurrence of GC. This trial demonstrated a 65% risk reduction for the development of metachronous GC with *H. pylori* eradication. Long-term results of this trail were encouraging[54]. However, two recently published retrospective studies failed to validate these findings, suggesting that *H. pylori* eradication does not significantly prevent metachronous GC[49,55]. Nonetheless, a large retrospective study showed that recurrence rates and recurrence-free survival differed significantly between the non-eradication and eradication groups[56]. As for subtotal gastrectomy, Cho *et al*[57] reported that there was no difference in the development of metachronous GC according to the treatment allocation or final *H. pylori* status, which should be evaluated in further studies because bile reflux was reported to act as a carcinogen for later GC development[58,59].

Unlike gastric resection, endoscopic resection preserves the abnormal mucosa and gastric environment, which may promote the occurrence of secondary cancer in cases with atrophic gastritis or IM caused by *H. pylori* infection. Some available evidence suggests that *H. pylori* eradication reduces the incidence of metachronous GC in patients with a history of gastric adenoma. However, opposing results also indicate that the progression of atrophic gastritis and IM to GC can indeed occur following *H. pylori* eradication. Thus, there must be additional factors, such as genetic and epigenetic alterations, that lead to the progression of these preneoplastic lesions.

COST-EFFECTIVENESS OF *H. PYLORI* ERADICATION FOR GC PREVENTION

Several studies have indicated that the screening and eradication of *H. pylori* is a cost-effective strategy for the prevention of GC in middle-aged adults, even if the treatment prevents only 20%-30% of *H. pylori*-associated cancers[60,61]. Parsonnet *et al*[62] carried out a cost-benefit analysis of *H. pylori* screening and eradication in individuals aged 50 years. With an assumption that *H. pylori* treatment prevents 30% of GC, cost-effectiveness was estimated to be $25000 per year of life saved, and < $50000 per year of life saved for high-risk individuals, such as Japanese-Americans, even at a 5% treatment efficacy. The authors concluded that the screening and eradication of *H. pylori* was therefore a cost-effective strategy for preventing GC, especially in high-risk populations. Another study reported that the screening and eradication of *H. pylori* in young adults has the potential to prevent one in every 4-6 cases of GC in China, and would be considered cost-effective using the GDP per capita threshold[63]. A study from Shin *et al*[64] evaluated the long-term cost-effectiveness of *H. pylori* eradication in a selective population with a high risk of developing GC with estimated model variables based on an extensive review of published reports. Their analysis suggests little difference in *H. pylori* eradication costs ($29780 *vs* $30594 for no eradication) or in saving of lives (mean life expectancy from eradication: 13.60 years *vs* 13.55 years for no eradication). Although screening and eradication appear to be a cost-effective way to prevent GC, shortcomings in the therapeutic armamentarium along with a concern for antibiotic resistance should prevent recommendation of this global screen-and-treat strategy.

A prophylactic *H. pylori* vaccine could be an attractive

alternative strategy for the control of *H. pylori* infections. A 2009 study evaluated the potential socioeconomic benefit of a putative *H. pylori* vaccine in three different simulated scenarios: no intervention, vaccination of infants, and vaccination of school-age children[65]. Results of their direct transmission model indicated that the use of a prophylactic *H. pylori* vaccine was cost-effective in the United States, with vaccination in infancy providing the greatest benefit over at least 40 years, at a cost per quality-adjusted life year of $17684.

CONCLUSION

H. pylori infection induces progressive inflammatory changes in the gastric mucosa that may lead to GC. As the treatment of GC represents a significant medical burden and poor outlook[66], *H. pylori* screening and eradication is likely to be one of the most promising and cost-effective approaches in GC prevention. However, the collective results of previous studies have failed to identify a significant reduction in GC; possibly due to the variable prevalence of *H. pylori* infection between countries and the long course of GC. Nevertheless, younger individuals with no precancerous lesions should consider *H. pylori* eradication for GC prevention, although high-risk groups should combine this therapy with endoscopic surveillance or treatment. Following endoscopic resection of EGC, *H. pylori* eradication should be used to prevent the development of metachronous gastric carcinoma, although study of the benefits in a wider population is needed.

H. pylori, which is often acquired during childhood and associated with low socioeconomic status, is recognized as a necessary but insufficient cause of GC, because the pathogenesis of gastric carcinogenesis is multifactorial. Although the mass eradication of *H. pylori* is potentially feasible, doubts remain about the advisability of such a policy. Differences in the socioeconomic composition of countries and the undesirable side effects of antibiotic use as well as increased incidence of other diseases necessitate further investigation into mass eradication of *H. pylori* as a preventative strategy[67-70]. In addition, the feasibility and appropriate timing of this strategy for cancer prevention remains to be determined. Further systematic data collection comprising large randomized controlled trials designed in multiple geographical areas and with extended follow-up periods is needed to elucidate the role of *H. pylori* eradication for GC prevention in patients with or without precancerous lesions.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Reduced genome size of *Helicobacter pylori* originating from East Asia

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Abstract

Helicobacter pylori (*H. pylori*), a major pathogen colonizing the human stomach, shows great genetic variation. Comparative analysis of strains from different *H. pylori* populations revealed that the genome size of strains from East Asia decreased to 1.60 Mbp, which is significantly smaller than that from Europe or Africa. In parallel with the genome reduction, the number of protein coding genes was decreased, and the guanine-cytosine content was lowered to 38.9%. Elimination of non-essential genes by mutations is likely to be a major cause of the genome reduction. Bacteria with a small genome cost less energy. Thus, *H. pylori* strains from East Asia may have proliferation and growth advantages over those from Western countries. This could result in enhanced capacity of bacterial spreading. Therefore, the reduced genome size potentially contributes to the high prevalence of *H. pylori* in East Asia.

Key words: *Helicobacter pylori*; Genome; Mutation; Epidemiology; Recombination

Core tip: Comparative analysis of strains from different *Helicobacter pylori* (*H. pylori*) populations revealed that the genome size of strains from East Asia was reduced. In parallel with this, the number of protein coding genes and the guanine-cytosine content were decreased. The reduced genome of *H. pylori* from East Asia potentially contributes to the high prevalence of *H. pylori* in East Asia.

Dong QJ, Wang LL, Tian ZB, Yu XJ, Jia SJ, Xuan SY. Reduced genome size of *Helicobacter pylori* originating from East Asia. *World J Gastroenterol* 2014; 20(19): 5666-5671 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5666.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5666

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a major human pathogen causing chronic inflammation of gastric mucosa[1]. Infection by this bacterium is associated with an increased risk for the development of peptic ulcer and gastric cancer[2,3]. There is a dramatic geographical variation in the prevalence of the infection and *H. pylori* associated diseases. In Western countries, the infection rate is approximately 30%, while it is higher than 60% in Eastern countries[4]. The incidence of gastric cancer is much lower as well in Western countries[5].

It is believed that *H. pylori* has established its colonization in human stomachs 100000 years ago[6]. With the human migration out of the Africa, the bacterium carried by its hosts traced the route of migration reaching Asia through India, and then into South East Asia

and Australia[7]. It arrived at Europe through Turkey[7]. The phylogenetic analysis of global strains of *H. pylori* reveals that the bacterium is subdivided into seven populations: hpAfrica1, hpAfrica2, hpEastAsia, hpEurope, hpNEAfrica, hpAsia2 and hpSahul[6-8]. These populations generally correspond to their geographical origins. Up to date, hundreds of strains have been sequenced or being sequenced[9-12]. The purpose of this review is to compare the genomic differences of *H. pylori* from different populations, and discuss their implications in the geographical variation of the prevalence of the infection.

VARIATION OF THE GENOME SIZE AND THE GUANINE-CYTOSINE CONTENT

To explore the genomic differences between *H. pylori* strains, we have determined the genome sequence of *H. pylori* strain D33 isolated from a Chinese patient with gastric cancer. Whole-genome sequencing was performed as described previously[9,10]. Briefly, the raw reads were trimmed and filtered, yielding a total of 2364383 reads with an average length of 260 nucleotides. This corresponded to about 297-fold genome coverage. A total of 74 contigs were assembled. The annotation of the genome was performed using the Glimmer program. This whole genome shotgun project has been deposited in DDBJ/EMBL/GenBank under the accession number ANIO00000000 (The version described here is the first version, ANIO01000000.) The genome of *H. pylori* D33 had a size of about 1555730 bp, with a guanine-cytosine (GC) content of 38.96%. It possessed 1570 protein coding genes. The D33 genome carries a complete *cag* pathogenicity island. It has been found that *H. pylori* from East Asia had a massive decay of molybdenum-related genes[13,14]. There are a total of 12 molybdenum-related genes including three genes encoding molybdenum transport proteins, eight genes involved in molybdenum cofactor synthesis, and only one molybdenum-containing enzyme[15]. Most of them have been fragmented[14]. Consistent with this, seven molybdenum-related genes of strain D33 were fragmented, while intact *modB*, *moaE*, *moeB*, *mogA* and *mobA* genes were present.

H. pylori D33 had a smaller genome compared to strains from Western countries. To identify the genomic size difference between different *H. pylori* populations, a total of 118 sequenced strains with required data available were assigned into different populations based on results of the phylogenetic analysis. Briefly, sequences of house-keeping genes *atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI* and *yphC* were extracted from strain D33 sequenced in this study, 117 sequenced strains available from GenBank (http://www.ncbi.nlm.nih.gov), and 77 strains available from multilocus sequence typing (MLST) database (http://www.mlst.net). The sequences of these genes were concatenated and a neighbor-joining tree was constructed (Figure 1). The strains from the MLST database was used as a standard to assign those sequenced strains into different populations, since the population they belong to is known. These 118 sequenced strains could be assigned into five *H. pylori* populations, including hpEastAsia (30 strains), hpAsia2 (7 strains), hpEurope (39 strains), hpAfrica1 (40 strains) and hpAfrica2 (2 strains). No sequenced strains were assigned into the other two *H. pylori* populations, hpSahul and hpNEAfrica. The average genome size of *H. pylori* was about 1.64 Mbp. However, it is significantly different between the populations. Strains from hpEastAsia had a genome size of 1.60 Mbp on average (Table 1), which is significantly smaller than that from hpEurope ($P < 0.001$) or hpAfrica1 ($P < 0.001$) (Figure 2). The average genome size of strains from hpEastAsia was also smaller than that from hpAsia2 or hpAfrica2, although the difference is not statistically significant probably due to small sample size. These findings suggest that genome reduction occurs in strains of *H. pylori* from East Asia. As shown in Table 1, the genome of *H. pylori* decreased gradually with its spreading. In parallel to the genomic reduction, the number of protein coding genes was decreased ($R = 0.568$) (Figure 3), and the GC content of the genome declined gradually (Table 1).

MECHANISMS OF THE GENOMIC REDUCTION

Bacterial genome size is mainly determined by gains or losses of genes[16]. Bacteria acquired new genes through duplication of genes or horizontal gene transfer[17,18]. Genes may be deleted through mutations or recombination events[19,20]. Genome reduction occurs when gene losses prevail gene gains. The life style of bacteria is a major factor contributing to the genome reduction. Bacterial species have a reduced genome when inhabiting in the host cells or in the extreme conditions[21,22]. It is, however, unusual that a particular bacterium living in similar environments has reduction of genome. Our comparative analyses of genome found that genome reduction occurs in *H. pylori*. This is unexpected as the bacterium only colonizes in the human stomach. It is likely that variation in host genetic backgrounds dramatically influences physiochemical properties, immune and inflammatory responses of the stomach, leading to genomic alterations of *H. pylori*.

Genome reduction may be caused by the deletion of intergenic regions[23]. This leads to an increased density of gene contents and thus a well compacted genome. It is most frequent, however, that genome reduces through removal of non-essential or redundant genes[24]. Our comparative analysis found that the number of protein coding genes of *H. pylori* was closely associated with the genome size. The number decreased with the reduced size of the genome. This demonstrates that the genome reduction in *H. pylori* is caused essentially by deletion of genes. Compared to strains from Western countries, two groups of genes are frequently removed from those from Eastern countries[14]. The first group consists of gene encoding outer membrane proteins, including *oipA-2*, *hopN*,

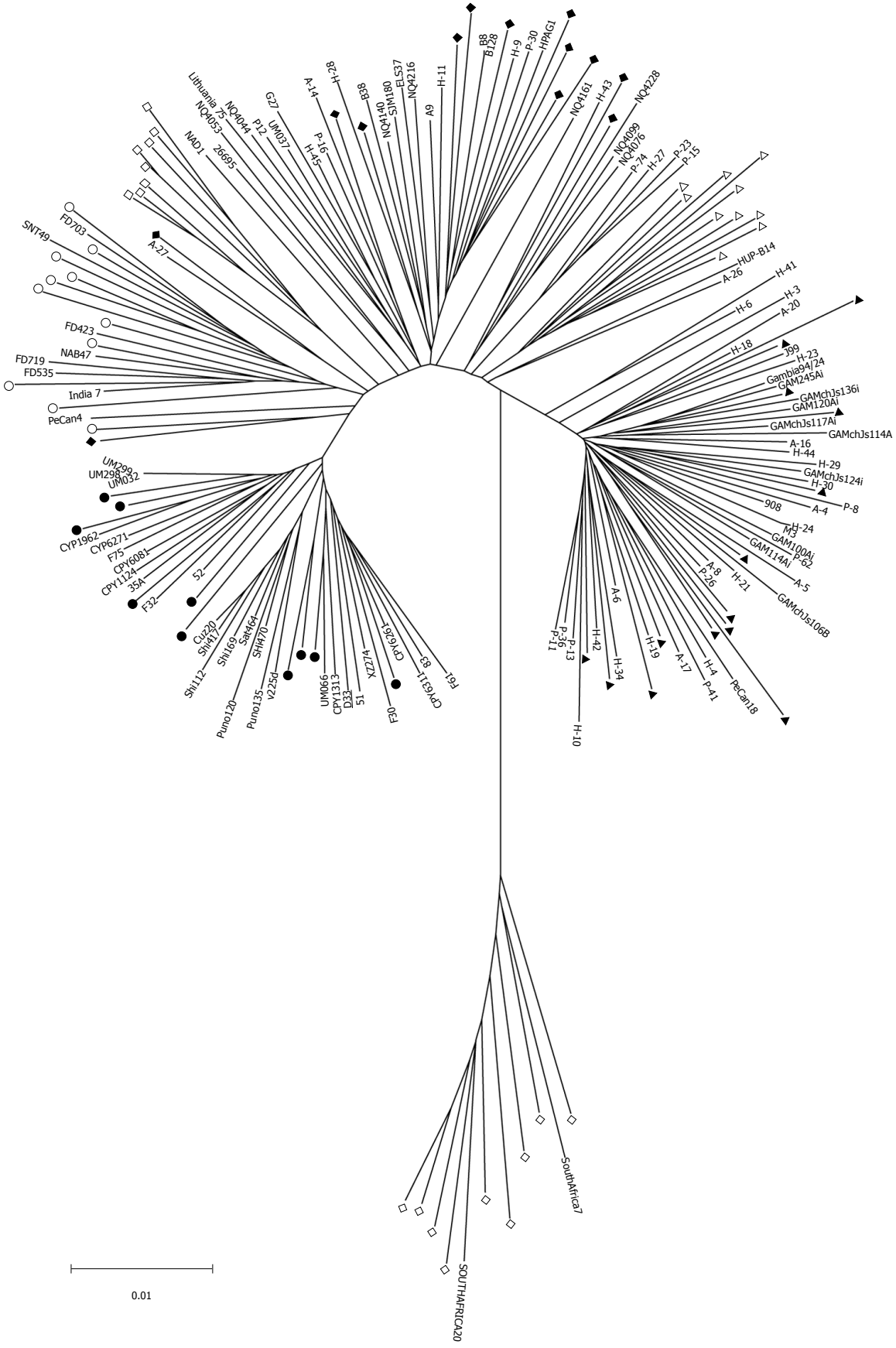

Figure 1 Neighbor-joining tree of 118 sequenced *Helicobacter pylori* strains and 77 strains from multilocus sequence typing database based on the phylogenetic analysis of seven house-keeping genes (*atpA, efp, mutY, ppa, trpC, urel* and *yphC*). The population to which those 77 strains belong is known. The tree was constructed with Mega 5.0 software. Scale bar indicates substitutions per nucleic acid residue (change/nucleotide site). Classification of population/ subpopulation was as previously described[6,7].

Table 1 Genome size, guanine-cytosine content and number of protein coding genes of *Helicobacter pylori* from different populations

Population	*n*	Size (Mbp)	GC content	Protein coding genes
hpEastAsia	30	1.6050 ± 0.0327	38.8167% ± 0.1206%	1548.8000 ± 74.3762
hpAsia2	7[1]	1.6243 ± 0.0282	39.0286% ± 0.0756%	1559.5000 ± 57.2757
hpEurope	39[1]	1.6467 ± 0.0391	38.9231% ± 0.1287%	1627.8684 ± 74.7411
hpAfrica1	40	1.6558 ± 0.0384	39.1800% ± 0.1137%	1660.0250 ± 58.9108
hpAfrica2	2	1.6500 ± 0.0424	38.5000% ± 0.1414%	1637.5000 ± 92.6310
Total	118	1.6379 ± 0.0417	38.9822% ± 0.1986%	1637.5000 ± 71.3128

[1]The number of protein coding gene is only available in 2/7 strains from hpAsia2 and 38/39 strains from hpEurope. GC: Guanine-cytosine.

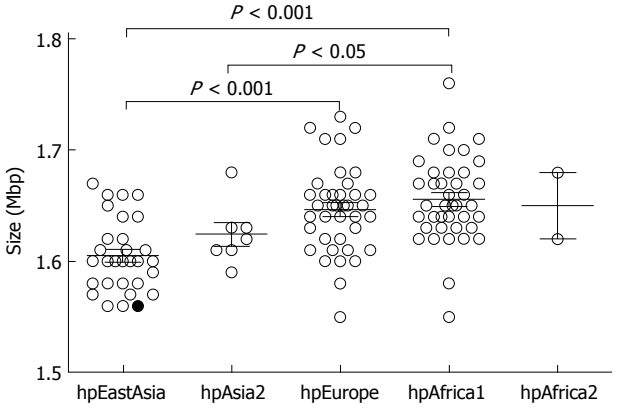

Figure 2 Genome size of strains from different *Helicobacter pylori* populations. Single factor analysis of variance was used for the statistical analysis. The average genome size was significantly different between *Helicobacter pylori* populations.

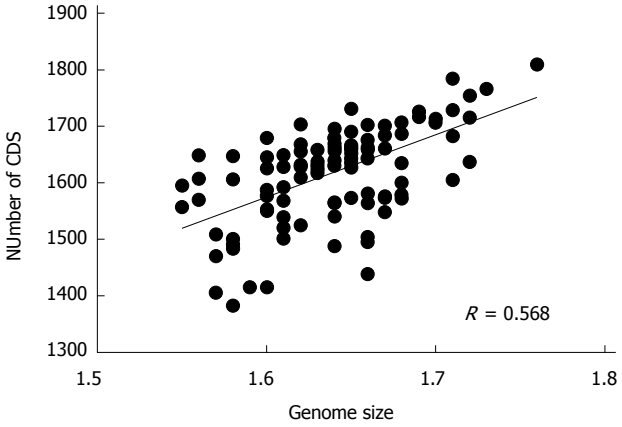

Figure 3 Correlation of the genome size of *Helicobacter pylori* with the number of protein coding genes. CDS: Coding sequence.

babC, *sabB*, *vacA2*, *homB* and *hopQ*. All of these genes have their homologs and are variable in *H. pylori*[25,26]. Therefore, their functions are most likely redundant. The other group of genes deleted in Eastern strains are composed of those involved in central metabolism. These genes include molybdenum-related genes as found in our sequenced strain D33 and other Japanese strains[14], *acoE* encoding acetyl-CoA synthetase[27], *tas* encoding aldoketoreductase involved in carbonyl metabolism and[28] and jhp0585 encoding a putative hydroxyl-isobutyrate dehydrogenase which degrades valine[29]. Functions of these genes could be complemented by their paralogs in the genome. These findings suggest that *H. pylori* strains originating from Eastern countries could eliminate non-essential genes from their genome, resulting in the reduction of genome.

Bacterial genome size is well correlated with the GC content[30]. Events of G-C to adenosine-thymine (C-T) mutations are more common than those of C-T to A-G mutations[31-33]. Thus, GC contents significantly influence the genome size. Our comparative analysis found that the GC content drops with the gradual reduction in the genome size of strains from hpAfrica 1 and 2, to hpEurope and hpAsia2, and finally hpEastAsia. This indicates that mutation is a major factor contributing to the genome reduction in *H. pylori*. Mutations and recombination events frequently occur in this bacterium[34,35]. It has been estimated that the mutation rate is as high as 1.38×10^{5} per site per year. The majority of genes deleted in Eastern strains are possibly caused by mutations. Recombination is also frequent in *H. pylori*[35]. However, recombination usually has no influence on the GC content. Thus, it is unlikely to be a major reason causing the genome reduction.

REDUCED GENOME SIZE AND PREVALENCE OF *H. PYLORI* IN EAST ASIA

The prevalence of *H. pylori* shows considerable geographical variation[36]. In Eastern countries, the infection is much higher compared to Western countries. Family transmission is a major route for the bacterium to spread[37]. Spread of *H. pylori* is influenced by environmental conditions including socio-economic status, inhabiting conditions and hygiene levels[38,39]. These factors may lead to an increased exposure to the bacterium. Spread of the bacterium is also influenced by the individual susceptibility determined by host genetic backgrounds. The single nucleotide polymorphism of the Toll-like receptor gene is closely associated with increased susceptibility to the infection[40]. Bacterial factors related to the spread are, however, unclear.

Bacteria spend the majority of their energy for synthesis of proteins[41]. Genome reduction with reduced number of protein coding genes, thus, requires less cost of energy[42]. Furthermore, genome reduction would

decrease the energy cost for the maintenance of DNA structure and replication[43]. These would promote bacterial growth and proliferation[44]. The lowered energy cost for bacteria means less nutrient needs. Therefore, bacteria with a reduced genome have an increased capacity of adapting to unfavorable environments[16]. Our comparative analysis found that strains form the hpEastAsia population have a smaller genome than those from hpEurope. Thus, *H. pylori* originating from East Asia could have an enhanced capacity of bacterial proliferation and growth, facilitating the spreading of the bacterium. It is conceivable that the reduced genome size contributes to the higher prevalence of *H. pylori* in East Asia.

CONCLUSION

H. pylori shows an unusual variation of the genome size. The reduced genome size of strains from East Asia potentially contributes to the high prevalence of the bacterium in the region. It is worth of further studies to investigate the influence of the genome reduction on the proliferation, growth and spread of *H. pylori*. This will benefit for the understanding of mechanism of the spreading and the prevention of the infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Treatment of early gastric cancer in the Western World

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Abstract

The incidence rate of gastric cancer is much higher in Asia than in the Western industrial nations. According to the different screening programs in Japan and Korea about fifty percent of treated patients had an early tumor stage. In contrast, European and American patients with gastric cancer had an advanced tumor stage. Therefore, the experience for the various therapeutic options for gastric cancer may be different between these regions. In this review we tried to point out the treatment modalities in Western industrial countries for early gastric cancer.

Key words: Gastric cancer; Early cancer; Epidemiology; Diagnosis; Therapy; Western World

Core tip: Early gastric cancer is a challenging disease with varying lymph node infiltration. In the Western industrial countries the frequency of early gastric cancer is much lower than in Asian countries. Especially, carcinomas limited to the mucosa are rare in the West. Therefore the experience of endoscopic therapy or minimal invasive surgery is only in special centers available. Total or subtotal gastrectomy with D2-lymphadenectomy is the standard treatment for early gastric cancer with suspected lymph node metastases. Future studies including prediction of lymph node metastasis are necessary to optimize the therapy according to quality of life.

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INTRODUCTION

Gastric cancer is the second most common cause of death from cancer worldwide[1]. Overall incidence rates for gastric cancer have steadily declined over the past 50 years, particularly in developed countries.

According to the Japanese classification of gastric cancer early gastric cancer (EGC) is defined as gastric cancer in which tumor invasion is confined to the mucosa or submucosa (T1 cancer), irrespective of the presence of lymph node metastasis[2]. Early gastric cancer carries a much more favourable prognosis than advanced cancer, with 5-year survival rates of over 90%[3]. A complete cure can almost always be achieved by conventional gastrectomy with lymph node dissection. Therefore, this treatment has been the gold standard, providing an excellent prognosis in patients with EGC in Eastern as well as in the Western world[3-5]. During the last years limited resection of the primary tumor and minimal invasive surgical approaches have been introduced for early gastric cancer[6]. On the other hand, endoscopic resection (ER) is beneficial, as it is minimally invasive and conserves the whole stomach, and postoperative quality of life is good[7,8].

ER has been accepted as a minimally invasive treatment method for EGC with a negligible risk of lymph node metastasis. But according to the low frequency of EGC in the Western countries the experience with minimal invasive surgical and endoscopic methods are low compared to those in Asian countries.

The aim of this review is to show the actual knowledge about early gastric cancer in Western industrial countries.

DEFINITION

The term "Early Gastric Cancer" is not really well defined. According to the Japanese classification of gastric cancer EGC is defined as gastric cancer in which tumor invasion is confined to the mucosa or submucosa (T1 cancer), irrespective of the presence of lymph node metastasis[2]. Sometimes it is used to contrast it to "Advanced Gastric Cancer". In these cases, EGC includes T2 cancers in addition to T1 cancer. According to the 7th UICC-TNM classification T1 means the tumour has started to grow into the wall of the stomach. T1 is further divided into T1a and T1b. T1a means the tumour is within the inner layers of the stomach (the mucosa). And T1b means the tumour has grown through the mucosa and into a layer of supportive tissue called the submucosa[9]. The N-category describes the number of metastatic lymph nodes (LNM), N0 = no lymph node metastasis, N1 = 1-2 LNM, N2 = 3-6 LNM and N3 more than 6 LNM. The stage-system of the 7th UICC-TNM classification combined the T-, N-, and the M-category. Early gastric cancer is often used in the same manner as stage I tumor. In this stage system T1N0M0 (stage IA), T1N1M0 and T2N0M0 (stage IB) tumors are combined to stage I gastric cancer. T1N2M0 or T1N3M0 are classified to more advanced stages.

EGC AND LYMPH NODE METASTASES

Hölscher *et al*[10] compared the frequency of LNM in patients with pT1 gastric cancer from Asian and European countries. In European countries there were 6.5% for mucosal carcinomas and 23.9% for submucosal carcinomas compared to 2.7% resp. 22.9% in Asian countries. Table 1 shows the results of literature for patients with pT1 gastric cancer in the Western countries[4,5,10,11-15]. One reason for the difference of LNM in pT1a cancer may be the higher frequency of tumor infiltration in the upper layer of the mucosa (m1 and m2) with no lymphatic invasion for Asian patients. The infiltration of the deepest layer (m3) represents an important step for a mucosal carcinoma. In the series from Hölscher *et al*[10] for German patients with mucosal carcinoma the rate of lymph node metastasis springs from 0% in m1 or m2 layers to 12.8% in m3 layer. Another reason for this dissimilarity may be different location of the primary tumor. Patients in Asian have more often tumor in the mid or lower third of the stomach compared to patients in Western countries with higher frequency of tumors in the proximal third[16,17]. Further explanations, like biologic differences or differences in genomic or epigenomic changes, for differences in the frequency of LN metastasis between the two worlds are not really well studied and therefore topics of future studies.

EPIDEMIOLOGY

Nearly for all countries in the world the incidence rate of gastric cancer declined during the last fifty years[1,18]. The main difference between Asian and Western industrial nations is the level of the incidence rate. Whereas in Japan an age standardized incidence rate (ASR) for males of about 80 per 100000 inhabitants were found in European countries the ASR for males is about 16 per 100000 inhabitants.

The frequencies of early gastric cancer in Western countries are not really known. According to the lower incidence rate of gastric cancer there exists no screening programs for this tumor entity. Faria *et al*[19] compared three decades in gastric cancer surgery in a Portuguese hospital and found an increase of EGC from 14.5% in the time span from 1980 to 1989 compared to 20.8% in the time span from 2000 to 2009. Data from Italian Research Group of Gastric Cancer showed a decreasing rate of patients with resected gastric cancer from 1991 to 2005 and during the same time span a decreasing rate of pT1 gastric cancer. But the rate of stage I carcinoma was not different in the three five years sections with 37.8%, 35.4% and 37.4%[20]. In Germany, there are three prospective multicenter observational studies: the German Gastric Cancer Study (GGCS '92) with data from 19 University hospitals in the time span 1986-1989, the quality assurance study of the East German study group for quality assurance and regional development in surgery (EGGCS '02) including the data from 2002 of 80 hospitals and the German multicenter observational studies for gastric cancer for surgical treatment of gastric cancer (QCGC 2007-2009) including the data from 2007-2009 of 141 hospitals[21-23]. Stage I gastric cancer was detected in 27.3% of all cases of GGCS '92, in 34.1% of EGGCS '02 and in 34.2% of cases from QCGC 2007-2009 study. But all these data are from surgical hospitals. During this time span the indication for surgery in advanced cases may be changed. On the other hand, patients with early cancer are more often treated by endoscopic resection. Therefore, it is not clear if the incidence of EGC has changed in the Western.

Based on data from population-based cancer registries the survival rate of Japanese patients with gastric cancer is higher in Japan than in Western countries. One reason may be the high rate of EGC as a consequence of the various screening programs. Other factors of influence like more radical therapy are discussed.

THERAPEUTIC OPTIONS FOR EGC

Treatment options for early gastric cancer range from endoscopic mucosal resection (EMR), endoscopic sub-

Table 1 Survey of the literature on the rate of lymph node metastasis in surgically resected mucosal or submucosal pT1 gastric cancer in Europe

Ref.	Year	Origin	pT1a		pT1b	
			n	% LNM	*n*	% LNM
Folli *et al*[11]	1995	Italy	117	4	106	23
Hayes *et al*[12]	1996	Germany	14	21	14	64
Bösing *et al*[13]	1998	Germany	33	9	24	17
Popiela *et al*[14]	2002	Poland	113	6	125	21
Roviello *et al*[15]	2006	Italy	330	5	322	24
Hölscher *et al*[10]	2009	Germany	47	11	79	25
Nieminen *et al*[4]	2009	Finland	47	2	43	23
Saragoni *et al*[5]	2013	Italy	308	6	221	26
		Europe	1009	5.9	934	24.3

LNM: Lymph node metastasis.

mucosal dissection (ESD), laparoscopic or open distal gastric resection to gastrectomy with radical lymph node dissection.

EMR has become the standard of care for removal of large flat and sessile neoplastic lesions of the gastrointestinal tract in Japan and Korea. The applications of EMR and ESD are expanding and many Western endoscopists are adopting these techniques. But according to the low frequency of early gastric cancer in Western countries and the high frequency of lymph node metastases in those EGC the standard of care is the gastrectomy.

ENDOSCOPIC TREATMENT

Endoscopic treatment (ER): EMR as well as ESD of early carcinomas is only meaningful if lymph node metastasis can be excluded. This is not possible by endoscopic ultrasonography or computed tomography because the size of the lymph nodes (LN) is not a reliable parameter for detection of metastatic infiltration. This has been shown for early as well as advanced gastric cancer[24,25]. Therefore, the indication for endoscopic treatment is an on-going debate between surgeons and gastroenterologists.

EMR: Since endoscopic mucosal resection using the strip biopsy method (two-channel method) was first introduced for endoscopic therapy in 1984[26], various methods of EMR have been developed, including endoscopic resection with a cap-fitted panendoscope (EMRC). They are widely used in Western countries as well as Japan[8,27]. Lesions with an extremely low likelihood of lymph node metastasis have been identified by clinicopathological investigations of multiple surgery cases, and indications for endoscopic therapy have been compiled by the Japan Gastric Cancer Association[2]. In principle, these indications state that the likelihood of lymph node metastasis is extremely unlikely and that the tumor should be of a size and in a site that allows it to undergo en bloc resection. Specifically, the lesion must be (1) a differentiated elevated intramucosal cancer less than 2 cm in size or (2) a differentiated depressed intramucosal cancer less than 1 cm in size without ulcer findings. However, due to the nature of the excision technique, which uses a snare, EMR also has the disadvantage that only a small area can be excised at a time, and so for larger lesions, it has higher rates of piecemeal excision and local recurrence[8]. Manner *et al*[28] summarized the Western results of EMR as follows: Although ER for EGC in Western countries is effective; it is associated with a relevant risk of complications. In view of the possibility of recurrent or metachronous neoplasia, a strict FU protocol is mandatory.

Endoscopic submucosal dissection is a new technique that can remove even large tumours in one piece[29]. In a comparison with endoscopic mucosal resection, resections removing tumours in one piece were more frequent in the endoscopic submucosal dissection group (93% *vs* 56%) and the 3-year recurrence-free rate was higher (98% *vs* 93%), at the expense of a higher rate of perforations (3.6% *vs* 1.2%), which were endoscopically managed in most cases[30]. Several reports from Western referral centers could confirm the high rates of en bloc resection of early gastric neoplasia. However, histology may not always confirm complete resections of horizontal tumor margins[31-33].

Regarding the German S3 guideline for gastric cancer the endoscopic treatment is provided for non ulcerated lesion smaller than 2 cm showing an intestinal type according to the Laurén classification. For bigger lesions and small diffuse carcinomas an endoscopic treatment can be considered[34].

SURGICAL TREATMENT

The surgical resection of the tumor is the only possibility to cure a patient with early gastric cancer and lymph node metastasis. The age-adjusted survival rates for these patients are comparable with the normal population[35]. The presence of lymph node metastasis is one of the most important prognostic factors in patients with gastric carcinoma. Node-negative patients have a better outcome, nevertheless a subgroup of them experience disease recurrence. Baiocchi *et al*[36] analyzed the clinicopathological characteristics of lymph node-negative advanced gastric carcinoma patients submitted to gastrectomy and D2 lymphadenectomy with a retrieved number

of nodes greater than 15, after an actual follow-up of almost 5 years, and evaluate outcome indicators. Data of 301 patients with curative gastrectomy staged as N0 between 1992 and 2002 of 7 centers participating to the Italian Research Group for Gastric Cancer were included. Disease-specific and disease-free survival after 3, 5, and 10 years were 90.4%, 86.1%, 75.9%, and 72.1%, 57.3%, 57.3%, respectively. Mortality was 1.7%. The factors associated with a better disease-free survival at univariate analysis were age < 60, T2 tumors, distal location, intestinal histotype, and number of retrieved nodes > 25; depth of infiltration and histotype were the only 2 independent predictors of 5-year recurrence-free survival at multivariate analysis.

Therefore, the German national guidelines for gastric cancer suggests that only mucosal cancer without LNM can be treated endoscopically if a R0-resection is possible[34].

The guidelines do not dictate the indication for a subtotal gastrectomy, however a D2 lymphadenectomy and a resection margin of 5 cm for intestinal type and 8 cm for diffuse type of gastric cancer is provided. As Hosokawa *et al*[37] show a subtotal gastrectomy to be equal to total gastrectomy concerning the outcome of early remnant gastric cancer patients who underwent endoscopic treatment at first; there is evidence for the efficiency of the limited surgical treatment. But so far, there is a lack of data regarding this approach in early gastric cancer in western countries.

LYMPHADENECTOMY

For many years, clinicians have debated whether an extended lymph-node dissection (D2) for gastric cancer is beneficial. So far, five randomised studies comparing D1 and D2 dissections have been completed. A Cochrane review showed a significantly increased mortality after D2 dissection (RR = 2.23, 95%CI: 1.45-3.45), without a benefit in survival; HR = 0.95, 95%CI: 0.83-1.09[38].

A single-centre randomised trial comparing D1 and D3 dissections was the first to identify a difference (P = 0.041) between overall survival in D1 dissections (53.6%, 95%CI: 44.2-63.0) and D3 dissections (59.5%, 95%CI: 50.3-68.7). No postoperative deaths occurred and morbidity was 12%. Only 13% of patients in this study had pancreatico-splenectomy compared with 23% in the Dutch gastric cancer trial[39]. Analysis of the group that did not undergo a pancreatico-splenectomy in the Dutch trial showed a significant survival advantage for those who had a D2 lymph-node. Thus, a D2 dissection might be beneficial if postoperative mortality can be avoided. Strong *et al*[40] compared pT1 pN0 gastric cancer from United States with those from Korea. They could show that if patients had a D2-lymphadenectomy the prognosis was comparable for both groups of patients. Instead of these results the guidelines for the extension of lymphadenectomy for early cancer is different between Europe and United States. National guidelines from the Netherlands or from Germany recommended a D2-LAD for resectable gastric cancer[34]. The actual version of The NCCN Clinical Practice Guidelines in Oncology for Gastric Cancer recommended for patients with resectable locoregional cancer, a gastrectomy with a D1+ or a modified D2 lymph node dissection (performed by experienced surgeons in high-volume centers). Postoperative chemoradiation is the preferred option after complete gastric resection for patients with node-positive T1-T2 tumors. Postoperative chemotherapy is included as an option after a limited lymph node dissection for this group of patients[41].

PREDICTION OF LYMPH NODE METASTASES

The key issue for the decision of endoscopic or surgical treatment is the existence of lymph node metastasis. For rational LN dissection it is important to know the incidence of metastasis at each LN station. For this purpose a computer program was developed using data from 4302 primary gastric cancers treated at the National Cancer Center Hospital in Tokyo between 1969 and 1989[42]. The accuracy of this program for European patients was evaluated by several authors[43-46]. The Maruyama computer program showed good predictive ability for LN metastases in most of the 16 LN stations. The predictive values could be improved using a neural network for the prediction of LN's[44,45].

The probability of lymph node metastasis is a key criterion for defining subgroups of patients for whom these endoscopic methods are appropriate. The main focus of the current discussion is m3 and sm1. Hölscher *et al*[10] described an algorithm for a group of patients with early cancer in Germany using only variables which are available before therapeutic decision. Using this clinical pathway all carcinomas with lymph node metastasis and 50% of those without LNM were correctly predicted.

A more invasive method is the Sentinel lymph node (SN) biopsy. The technique is based on the concept that the tumor-bearing status of the SN, which is defined as a LN that directly drains a specific cancer, reflects the tumor status of the remaining nodes. In a Japanese multicenter trial the feasibility and accuracy of diagnosis using SN biopsy in T1 gastric cancer was evaluated. The proportion of false negatives was much higher than expected. And the authors conclude that intraoperative histological examination using only one plane is not an appropriate method for clinical application of SN biopsy in gastric cancer surgery[47].

MINIMAL-INVASIVE SURGERY

Since the first report of laparoscopic gastrectomy in 1994 by Kitano *et al*[48] in Japan, much of our knowledge on the feasibility, safety, and benefits of laparoscopy in the treatment of gastric cancer is derived from studies performed

in the East. Data from the West have emerged at a slower pace. This is largely because the disease incidence is much lower and a greater proportion of patients present with locally advanced tumors that render laparoscopic resection less feasible. In addition, up to 50% of gastrectomies for adenocarcinoma in the US are performed at low volume centers where experience with advanced laparoscopic gastric surgery is limited. As a result, conventional open gastrectomy is still performed much more frequently in the US than laparoscopic resection even for patients with early stage disease[6]. Corcione performed during a time span of 12 years laparoscopic gastrectomy with D2-lymphadenectomy in 11 patients with early gastric cancer. The five year survival rate was 100%[49]. In Japan, early stage gastric cancer (T1N0 or T2N0) is regarded as the only indication for laparoscopic gastrectomy. Cancer registry data from United States as well as from the Netherlands showed that more than 90% of the patients with stage I gastric cancer were surgically resected[50].

CONCLUSION

Physicians from Western countries had much learned about treatment of gastric cancer from the great experience of their Eastern colleges. Patients with adequate treated early gastric cancer have the same prognosis as normal population. Therefore the main goal of future research is to reduce the side effects of the therapy and to improve the quality of life after therapy. Actual topic of cancer research is personalized therapy. One example may be the ToGA-trial (Trastuzumab for Gastric Cancer), the first international trial to include patients with human epidermal growth factor 2 (HER-2) positive advanced/metastatic gastric or gastroesophageal junction cancer. For patients with HER-2 positive advanced gastric cancer Trastuzumab, an anti-HER2 monoclonal antibody, survival benefit was shown and becomes the first targeted agent approved in these tumors[51]. But only 20% or less of all patients are HER-2 positive. To date, monoclonal antibody therapy is the only immunotherapy approved by the United States Food and Drug Administration for gastrointestinal cancers. Initial trials validating new immunotherapeutic approaches, including vaccination-based and adoptive cell therapy strategies, for gastrointestinal malignancies have demonstrated safety and the induction of antitumor immune responses. Therefore, immunotherapy is at the forefront of neoadjuvant as well as adjuvant therapies for the treatment and eradication of gastric cancer[52,53].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Pathohistological classification systems in gastric cancer: Diagnostic relevance and prognostic value

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Abstract

Several pathohistological classification systems exist for the diagnosis of gastric cancer. Many studies have investigated the correlation between the pathohistological characteristics in gastric cancer and patient characteristics, disease specific criteria and overall outcome. It is still controversial as to which classification system imparts the most reliable information, and therefore, the choice of system may vary in clinical routine. In addition to the most common classification systems, such as the Laurén and the World Health Organization (WHO) classifications, other authors have tried to characterize and classify gastric cancer based on the microscopic morphology and in reference to the clinical outcome of the patients. In more than 50 years of systematic classification of the pathohistological characteristics of gastric cancer, there is no sole classification system that is consistently used worldwide in diagnostics and research. However, several national guidelines for the treatment of gastric cancer refer to the Laurén or the WHO classifications regarding therapeutic decision-making, which underlines the importance of a reliable classification system for gastric cancer. The latest results from gastric cancer studies indicate that it might be useful to integrate DNA- and RNA-based features of gastric cancer into the classification systems to establish prognostic relevance. This article reviews the diagnostic relevance and the prognostic value of different pathohistological classification systems in gastric cancer.

Key words: Gastric cancer; classification; Laurén; World Health Organization classification; Pathohistology

Core tip: The establishment of a pathohistological classification system for gastric cancer with significant prognostic relevance is highly desirable. Numerous classification systems have been introduced by different authors. Although none of them could reach a consensus, the Laurén classification and the World Health Organization classification are widely used. The characteristics of each classification system as well as the prospect for future developments are presented in this article.

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INTRODUCTION

Gastric cancer is responsible for approximately 10% of cancer-related deaths worldwide; it is the second most common cause of cancer-related deaths and the fourth most commonly diagnosed cancer worldwide[1,2]. Although the incidence is persistently declining due to changes in nutrition and better prevention and treatment, gastric cancer is still associated with a poor prognosis. In the age of microscopic pathology, it has been of greater interest not only to classify the heterogeneous, histological appearance of the tumor cells but also to find a classification scheme with an independent prognostic relevance. To make patient-specific decisions regarding diagnosis and treatment, it is crucial to establish a solid pathohistological classification system. Numerous pathohistological classification systems have been established for gastric cancer thus far, but there is still controversy as to which classifications unify a prognostic correlation with a high validity and practicability in diagnosis and clinical routine.

LAURÉN CLASSIFICATION

Since its establishment in 1965, the Laurén classification of gastric cancer has been the most commonly used and the most studied classification for gastric adenocarcinoma among all of the classification systems. Laurén divided the histology of gastric cancer into two groups, *i.e.*, the intestinal type and the diffuse type (Table 1); later, the indeterminate type was included to describe an uncommon histology[3,4]. Signet ring cell carcinoma is included in the diffuse type. Most studies showed the intestinal type to be the most common, followed by the diffuse and then indeterminate type[5-7]. There is evidence that the intestinal type is associated with intestinal metaplasia of the gastric mucosa and with the presence of *Helicobacter pylori*. In some studies, the incidence of the diffuse type was found to be higher in younger, female patients[8], which may indicate distinct tumor development pathways for intestinal and diffuse adenocarcinoma of the stomach.

The prognostic relevance of Laurén's classification is still controversial. In some studies, Laurén's pathohistological subtypes of gastric cancer did not show a correlation with the patient's outcome[9-11], whereas other studies demonstrated a prognostic significance for the classification system[12]; some investigators even demonstrated that Laurén's classification can be used as an independent prognostic factor[13,14]. In those studies, the presence of a diffuse adenocarcinoma was correlated with a worse outcome. As this correlation has not been verified in other patient cohorts, the prognostic significance of Laurén's classification cannot be viewed as a generally established, but rather as a circumstance of one group's results.

Due to its high clinical relevance, the reliability of the Laurén classification has also been tested. The concordance of intra- and inter-observer agreement was tested using a percentage and kappa statistics that ranged from 77%-95%, indicating a good overall agreement, although a certain rate of mismatches was found. In studies investigating the concordance of biopsy- and specimen-based histological diagnosis in gastric cancer, a mismatch for the Laurén classification was found in 16%-26%. This mismatch was primarily explained as a focal manifestation of diffuse adenocarcinoma in specimens that the biopsy indicated as an intestinal adenocarcinoma[15,16]. With respect to the influence the histology might have in terms of treatment, a control biopsy was recommended in cases of uncertain histology.

Concerning the accuracy of esophagogastroduodenoscopy and endoscopic ultrasound in the diagnosis of gastric cancer, the diffuse type was described as a factor leading to underestimation of tumor infiltration, or T-category[17]. Therefore, in endoscopic treatment of gastric adenocarcinoma, an intestinal type indicated by the Laurén classification is favored in the national guidelines of Japan and Germany and in European Society for Medical Oncology guidelines[18-20]. The German S3 Guidelines also refer to Laurén's classification when recommending a resection margin of 8 cm for the diffuse type and a 5 cm margin for the intestinal type[18]. The rationale for this recommendation is given in several studies by Hornig *et al*[21] who found a sometimes discontinuous proliferation of diffuse gastric cancers[22,23]. These recommendations in national guidelines and the histological findings underline the significance of the Laurén classification and clarify why it is favored over other pathohistological classification systems.

WORLD HEALTH ORGANIZATION CLASSIFICATION

The World Health Organization (WHO) classification issued in 2010 appears to be the most detailed among all pathohistological classification systems. It is remarkable that the WHO classification includes not only adenocarcinoma of the stomach but also all other types of gastric tumors of lower frequency (Table 1)[24]. The gastric adenocarcinoma type is divided into several subgroups including papillary, tubular, mucinous and mixed carcinoma, which can be compared to the indeterminate type in the Laurén classification. The poorly cohesive carcinoma type includes the signet ring cell carcinoma. All other classified gastric adenocarcinomas can be designated as uncommon because of their minor clinical relevance. In the WHO classification, the most common type of gastric cancer is the tubular adenocarcinoma, followed by the papillary and mucinous types. The signet ring cell carcinoma accounts for approximately 10% of gastric cancers and is defined by the presence of signet ring cells in over 50% of the tumor[24-27]. The prognosis of the signet ring cell carcinoma is controversial. Most authors have described a worse prognosis for the signet ring cell carcinoma compared to other subtypes of gastric cancer[28,29]. Recent studies indicate that, on the contrary, signet ring cell carcinoma of the stomach does not differ in prognosis from the other types of gastric

Table 1 Laurén and World Health Organization classification

Laurén classification	World Health Organization classification
Intestinal type	Papillary adenocarcinoma
	Tubular adenocarcinoma
	Mucinous adenocarcinoma
Diffuse type	Signet-ring cell carcinoma and other poorly cohesive carcinomas
Indeterminate type	Mixed carcinoma
	Adenosquamous Carcinoma
	Squamous cell carcinoma
	Hepatoid adenocarcinoma
	Carcinoma with lymphoid stroma
	Choriocarcinoma
	Carcinosarcoma
	Parietal cell carcinoma
	Malignant rhabdoid tumor
	Mucoepidermoid carcinoma
	Paneth cell carcinoma
	Undifferentiated carcinoma
	Mixed adeno-neuroendocrine carcinoma
	Endodermal sinus tumor
	Embryonal carcinoma
	Pure gastric yolk sac tumor
	Oncocytic adenocarcinoma

cancer[30]. Furthermore, signet ring cell carcinoma was shown to have an irregular uptake of ^{18}F-fluorodeoxyglucose during positron emission tomography radionuclide imaging; consequently, this tumor as well as any metastases cannot be detected reliably[31]. Patients with a papillary adenocarcinoma experience a poor prognosis, a tendency for metastatic disease, a higher age at diagnosis and location in the upper third of the stomach[32]. Another study that employed the previous WHO classification found that poorly differentiated and mucinous adenocarcinomas have a worse prognosis than the papillary and tubular subtypes. In the same study, the WHO classification appeared to be an independent prognostic factor[33]. Kawamura *et al*[34] also found a poor prognosis associated with mucinous adenocarcinoma, which suggests a link with advanced stage and metastatic disease. However, unlike most common types of gastric malignancies, the WHO classification is more widely used for studies of infrequent types of gastric cancer. For adenosquamous carcinomas of the stomach, a poor prognosis and a case of simultaneous gastric adenocarcinoma are described[35,36]. In a recent review of the hepatoid adenocarcinoma type, the median survival of 182 patients with a gastric primary lesion was 13 mo, and 63.9% showed lymph node metastasis[37]. Most of the infrequent types of gastric malignancies are described in case reports, so a systematic investigation of their prognoses is not readily available. As the previous WHO classification was renewed in 2010, it is expected that more gastric cancer studies that refer to the most recent WHO classification will be conducted in the near future.

An indication for the significance of the WHO classification can be seen in a similar Japanese classification system. Although the Japanese classification divides the common types of gastric adenocarcinoma into additional subtypes, (*e.g.*, tubular adenocarcinoma is divided into well-differentiated and moderately differentiated adenocarcinoma), a dependence on the WHO classification system is evident[38]. This particular subdivision of tubular adenocarcinoma was based on differences in the submucosal invasion rate, lymph node metastasis and size of the lesions[39].

Table 2 Goseki and Ming classification

Goseki classification
Group Ⅰ
Tubular differentiation-well
Mucus in cytoplasm-poor
Group Ⅱ
Tubular differentiation-well
Mucus in cytoplasm-poor
Group Ⅲ
Tubular differentiation-poor
Mucus in cytoplasm-poor
Group Ⅳ
Tubular differentiation-poor
Mucus in Cytoplasm-rich
Ming classification
Expanding type
Infiltrating type

GOSEKI CLASSIFICATION

In 1992, Goseki *et al*[40] described a new histopathological classification that divides gastric cancer into four groups, as presented in Table 2. In the article, a correlation of the subtypes with the patterns of metastasis and local growth was present in 200 autopsy cases. Other groups showed a correlation of the Goseki classification with the Laurén and the WHO classifications, but there was only a moderate level of inter-observer agreement. A high level of agreement among observers could be achieved concerning the mucus production, and in later studies, the presence of mucus was highly associated with the prognosis[41,42]. An independent prognostic significance of the Goseki classification was subsequently debated. Despite some evidence[43], most studies that focused on this question could not confirm a prognostic independence of the Goseki classification, but did confirm a correlation with the preexisting histopathological characteristics such as those in the union international contre le cancer (UICC) system, grade, and Laurén and WHO classifications[11,44,45].

MING CLASSIFICATION

The Ming classification system is based on the growth pattern of the lesion and recognizes two main growth patterns: the expanding growth pattern and the infiltrating growth pattern (Table 2), which was found to be the less frequent type[46]. In his original work, Ming connected the two growth patterns to specific characteristics, positing that the expanding type originates as an intestinal metaplasia, whereas the infiltrating type emerges from individual cells. The Ming classification system may be

simple and clinically useful, but several subsequent studies could not identify this classification as an independent prognostic factor; a correlation was found with the preexisting classification systems[9,47,48].

OTHER CLASSIFICATIONS

In addition to the aforementioned histopathological classification systems for gastric cancer, some other authors have tried to establish systems based on the preexisting classifications or on histological findings. In 1982, Grundmann *et al*[49] proposed a classification system for gastric cancer with a focus on the depth of invasion. Caneiro *et al*[50] published a classification system for gastric cancer based on morphological appearance and showed an independent prognostic significance for the four subtypes. The Japanese Histological Classification of Gastric Cancer is based on the WHO classification and includes several subtypes in addition to the common histologic types[38]. However, none of these classification systems is used worldwide for research purposes, as no advantages of any one particular classification over the others have been confirmed.

DISCUSSION

There have been many attempts to classify gastric cancer according to its pathohistological characteristics. The very early attempt by Laurén still appears to be the classification system with the highest prevalence in research and clinical practice among all of the classification systems. Many studies illustrate a prognostic independence for the Laurén classification, yet other studies have not validated this relationship. In summary, all of the discussed classifications have demonstrated clinical practicality and relevance as well as providing pertinent data for comparison with Laurén's classification system. Interestingly, all attempts to produce a superior classification system have failed, although all are based on the apparent histopathological characteristics of gastric cancer. The only classification with a comparable significance is the WHO classification, which is the only system that classifies all malignancies with a primary lesion in the stomach irrespective of the cellular origins. The WHO classification system is widely used for the diagnosis and description of infrequent gastric neoplasms and is mentioned in many studies investigating the pathohistological characteristics of gastric cancer. Although the Goseki and Ming classifications can be understood on a cellular level, both classifications have not been proven to be superior to the preexisting systems, with the Laurén classification scheme as the gold standard. That no other attempt has surpassed the classification system of Laurén, established in 1965, is a testament to the complexity of the pathohistological characteristics of gastric cancer. Recent studies recommend that gastric cancer no longer be classified according to histology alone, but with the help of molecular markers or DNA- and RNA-based characteristics[25,51]. Although no reliable concept has been established, with the help of new technologies it is possible to construct a classification of gastric cancer with an independent prognostic relevance. Therefore, it is not necessary to create a completely new classification system; instead, it is easier to complement a preexisting classification with molecular and genetic findings.

Regarding the clinical practice of diagnosis and treatment, the classification systems of Laurén and the WHO predominate in published studies as well as in several national guidelines, such as the German S3 guideline for gastric cancer and the Japanese Classification of Gastric Cancer. As long as there is no widely accepted classification system with prognostic independence, both the Laurén and the WHO classification systems should continue to be used so that data presented in different studies can be compared. Such comparisons are important in order to segregate subgroups of patients with certain clinical features or different outcomes.

This is especially relevant for treatment decisions in clinical practice, where a pathohistological classification system that has an association with the prognosis is highly desirable. Such a classification could lead the way to a more personalized decision-making process for treatment of gastric cancer.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Sentinel lymph node navigation surgery for early stage gastric cancer

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Abstract

We attempted to evaluate the history of sentinel node navigation surgery (SNNS), technical aspects, tracers, and clinical applications of SNNS using Infrared Ray Electronic Endoscopes (IREE) combined with Indocyanine Green (ICG). The sentinel lymph node (SLN) is defined as a first lymph node (LN) which receives cancer cells from a primary tumor. Reports on clinical application of SNNS for gastric cancers started to appear since early 2000s. Two prospective multicenter trials of SNNS for gastric cancer have also been accomplished in Japan. Kitagawa *et al* reported that the endoscopic dual (dye and radioisotope) tracer method for SN biopsy was confirmed acceptable and effective when applied to the early-stage gastric cancer (EGC). We have previously reported the usefulness of SNNS in gastrointestinal cancer using ICG as a tracer, combined with IREE (Olympus Optical, Tokyo, Japan) to detect SLN. LN metastasis rate of EGC is low. Hence, clinical application of SNNS for EGC might lead us to avoid unnecessary LN dissection, which could preserve the patient's quality of life after operation. The most ideal method of SNNS should allow secure and accurate detection of SLN, and real time observation of lymphatic flow during operation.

Key words: Gastric cancer; Sentinel node navigation surgery; Infrared Ray Electronic Endoscopes; Indocyanine Green

Core tip: Two prospective multicenter trials of sentinel node navigation surgery (SNNS) for gastric cancer have been accomplished in Japan. Kitagawa *et al* reported that the endoscopic dual (dye and radioisotope) tracer method for SN biopsy was confirmed acceptable and effective when applied to the early-stage gastric cancer. The ideal method of SNNS should allow secure and accurate detection of sentinel lymph nodes, and real time observation of lymphatic flow during operation. In this review, we attempted to comprehensively evaluate the history, technical aspects, tracers, and clinical applications of SNNS with a special emphasis on the use of Infrared Ray Electronic Endoscopes with Indocyanine Green.

Mitsumori N, Nimura H, Takahashi N, Kawamura M, Aoki H, Shida A, Omura N, Yanaga K. Sentinel lymph node navigation surgery for early stage gastric cancer. *World J Gastroenterol* 2014; 20(19): 5685-5693 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5685.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5685

INTRODUCTION

Complete resection of tumor and dissection of region-

al lymph nodes (LNs) are basic surgical principles for patients with gastric cancer. Gastrectomy with regional LN dissection is the standard procedure for gastric cancer, while less invasive interventions, such as endoscopic submucosal dissection, have emerged as suitable techniques for EGC but not indicated for possible LN metastasis[1].

Laparoscopic gastrectomy was first performed by Kitano *et al*[2] for EGC in 1991. Since then, the usefulness of laparoscopic surgery is recognized, which is widely carried out for EGC in Japan[3]. While early-phase recovery after surgery has been improved by laparoscopic surgery, preservation of late-phase quality of life (QOL) by function-preserving surgery is also important. Therefore, the SN concept has been a much-discussed topic in standard gastric operations such as distal, proximal and total gastrectomy, which are associated with deterioration of QOL by post-gastrectomy syndrome.

The lymph node metastasis rate of EGC is approximately 15%-20%. Therefore, clinical application of sentinel node navigation surgery (SNNS) for EGC might be able to abrogate unnecessary lymph node dissection as well as to reduce the volume of gastric resection. As a result, the post-gastrectomy syndrome may be reduced.

The sentinel lymph node (SLN) is defined as the first possible site to receive cancer cells along the route of lymphatic drainage from the primary tumor. The SLN concept was first advocated by Morton *et al*[4] in patients with melanoma. SNNS has already been validated for breast cancer[5] and malignant melanoma, where a limited or no nodal dissection is performed in case of negative SN.

TNM classification has defined "The SLN is the first lymph node to receive lymphatic drainage from a primary tumor." The absence of metastasis in SLN is believed to correlate with the absence of metastasis in downstream lymph nodes. From the TNM classification Sixth Edition, the presence or absence of metastases in SN is proposed, which is quoted in the 14th Edition of the Rules for gastric cancer in Japan[6].

In this review, we have attempted to evaluate the history of SNNS, technical aspects, tracers for the clinical application of SNNS using Infrared Ray Electronic Endoscopes (IREE) combined with Indocyanine Green (ICG) method for limited lymphadenectomy and modified gastric resection.

CURRENT STATUS OF SNNS FOR GASTRIC CANCER

Reports on clinical application of SNNS for patients with gastric cancers started to be published during early 2000s (Table 1).

Hiratsuka *et al*[7] had reported that SLN biopsy using ICG can be performed with a high success rate, and the SLN status can predict the LN status with a high degree of accuracy, especially in patients with T1 gastric cancer.

Kitagawa *et al*[8] evaluated the technical aspects and clinical application of radio-guided surgery using gamma detection probe technology. Endoscopic injection of technetium-99m-radiolabelled tin colloid was performed before the operation, and radioactive SNs were identified with a gamma probe. The benefit of using moderate magnitude radioisotopes is that the radioisotopes remain for an adequate period in the SN after tracer injection, and thus the identification rate of SLN becomes high even in fatty patients.

Isozaki *et al* reported the results of a regional multicenter clinical trial of SN mapping for gastric cancer using the conventional dye-guided method. Although the patient population enrolled in their study was limited, this first multicenter trial of SN mapping for gastric cancer provided several important messages[9]. This study was designed to investigate the feasibility of SN mapping for gastric cancer using the dye-guided method as a simple method that can be conducted even in community hospitals without special equipment.

Miwa proposed the concept of a "sentinel lymphatic basin," and reported successful mapping of the lymphatic basins in 96.2% of their patients. The dye stained one or more metastatic nodes in 31 patients, but failed to indicate a metastatic node in four patients with a large involved node[9]. This theory can provide an acceptable base for clinical application of SNNS. Their reports have demonstrated that sentinel lymphatic basins contain truly positive nodes, even in cases with a false negative SN biopsy. Therefore, the sentinel lymphatic basins are good targets for focused lymph node dissections in patients with cT1N0 gastric cancer. The distribution of sentinel lymphatic basins and the status of SLN would be useful information to decide the extent of gastric resection.

We have already suggested the clinical usefulness of IREE combined with ICG to detect illuminated SLN in patients with gastric cancer and duodenal tumors as compared with dye alone (Figure 1)[10-15]. The SN identification rate and sensitivity for IREE versus ICG alone were 99.5% *vs* 85.8% and 97.0% *vs* 48.4%, respectively[10].

The lymphatic flow of the stomach is complicated and skip metastases were observed even in EGC. We reported that the group 2 LN metastases were judged as SNs in 32%[11]. The most common locations of the SLN were stations left gastric artery in each of the upper-, middle-, and lower-thirds of the stomach[11]. Our SLN procedure with IREE can detect the SLN and is better than ICG alone. The lymphatic basin dissection (LBD) of the SN basin is required for accurate intra-operative diagnosis of lymph node metastases. LBD dissection based on IREE is an acceptable method of nodal dissection in patients with T1 or limited T2 tumors[12-14].

Ryu *et al*[15] reported a meta-analysis of feasibility studies on SNNS for gastric cancer between 2001 and 2009. The SN identification rate and sensitivity were 97.6% and 87.8%, respectively. Therefore, they advocated that SNB in gastric cancer is probably not clinically applicable for limited lymphadenectomy due to the unsatisfactory sen-

Table 1 Reports on SN mapping for gastric cancer

Ref.	Year	Journal	*n*	Detection rate	Sensitivity
RI method					
Kitagawa *et al*[31]	2000	*Surg Clin N Am*	36	97%	100%
Kitagawa *et al*[8]	2002	*Br J Surg*	145	95%	92%
Gretschel *et al*[32]	2003	*Chirurg*	15	93%	89%
Kim MC *et al*[33]	2004	*Ann Surg*	46	93.50%	84.60%
Uenosono *et al*[34]	2005	*Br J Surg*	104	95.20%	81.80%
Arigami *et al*[30]	2006	*Ann Surg*	61	100%	95.50%
Mochiki *et al*[35]	2006	*Am J Surg*	59	96.60%	83.30%
Yanagita *et al*[36]	2008	*Ann Surg Oncol*	160	98.80%	96.70%
Mean			63.2	96%	90%
Dye method					
Hiratsuka *et al*[7]	2001	*Surgery*	77	99%	90%
Ichikura *et al*[37]	2002	*World J Surg*	62	100%	85%
Carlini *et al*[38]	2002	*J Exp Clin Cancer Res*	40	100%	87%
Miwa *et al*[9]	2003	*Br J Surg*	211	96%	89%
Ryu *et al*[39]	2003	*Eur J Surg Oncol*	71	92%	100%
Song *et al*[40]	2004	*Am J Surg*	27	96%	100%
Osaka *et al*[41]	2004	*Clin Cancer Res*	57	100%	100%
Ishizaki *et al*[42]	2006	*Eur J Surg Oncol*	62	85.70%	96.40%
Park *et al*[43]	2006	*Eur J Surg Oncol*	100	94.60%	78.60%
Rino *et al*[44]	2007	*Hepatogastroenterology*	43	93.00%	81.80%
Mean			70.2	96%	91%
RI + Dye mthod					
Hayashi *et al*[45]	2003	*J Am Coll Surg*	31	100%	100%
Karube *et al*[46]	2004	*J Surg Oncol*	41	100%	92%
Tonouchi *et al*[47]	2005	*World J Surg*	37	94.60%	100%
Gretschel *et al*[48]	2005	*Eur J Surg Oncol*	34	97.10%	95.40%
Ichikura *et al*[49]	2006	*Surgery*	80	100%	92.90%
Saikawa *et al*[50]	2006	*World J Surg*	35	94.30%	50%
Dye + IREE method					
Nimura *et al*[10]	2004	*Br J Surg*	84	99%	100%
Ohdaira *et al*[11]	2007	*Gastric Cancer*	60	100%	100%
Kelder *et al*[12]	2010	*Eur J Surg Oncol*	212	99.50%	97%
Mean				100%	99%
Dye + Fluorcence method					
Miyashiro *et al*[22]	2008	*Ann Surg Oncol*	3	100%	100%
Kusano *et al*[23]	2008	*Dig Surg*	22	90.10%	40.00%
Tajima *et al*[51]	2009	*Ann Surg*	56	96.40%	64.70%
Mean				96%	68%

sitivity and heterogeneity among practicing surgeons, and that more than four SLN should be harvested to improve sensitivity. They also advocated that a tumor-specific SNB method should be developed[16].

Can *et al*[16] investigated a number of reports on SNB for gastric cancer from single institute experiences in order to augment the relevant knowledge base, and demonstrated that the currently established double tracer method (dye and radio-isotope tracers) appeared to be the most efficacious and reliable procedure for identifying true SLN. They commented while conventional dye tracers were still useful, ICG deserves more attention for the current applications. IREE, florescence imaging and near-infrared technology represent the future direction in which the SNNS concept is advancing[17].

Recently, two multicenter prospective studies for SNNS were organized in Japan. The Gastric Cancer Surgical Study Group of the Japan Clinical Oncology Group (JCOG) organized a multicenter prospective study of SN mapping by the dye-guided method using intraoperative subserosal injection of ICG and observation by the naked eye[18]. Another study group was the Japan Society of SNNS, which conducted a multicenter prospective trial of SN mapping by a dual tracer method with blue dye and radioactive colloid[19].

The JCOG study was performed by the dye-guided method using intraoperative subserosal injection of ICG and observed by the naked eye in patients with T1 gastric cancer. Green-stained nodes, representing SLN, were removed first, and then gastrectomy with lymphadenectomy was performed. However, this clinical trial was discontinued because the proportion of false negatives was much higher than expected. Miyashiro *et al*[17] reported that the JCOG0302 trial revealed the unreliability of frozen section examination using just one plane, and highlighted the impact of the learning curve. Nevertheless, we reported that the SN identification rate and sensitivity of ICG alone with the naked eye were 85.8% and 48.4% respectively, which indicates that detection of SN with dye alone is insufficient[10].

Kitagawa *et al*[18] conducted a multicenter, single-arm, phase II study of SN mapping that used a standardized

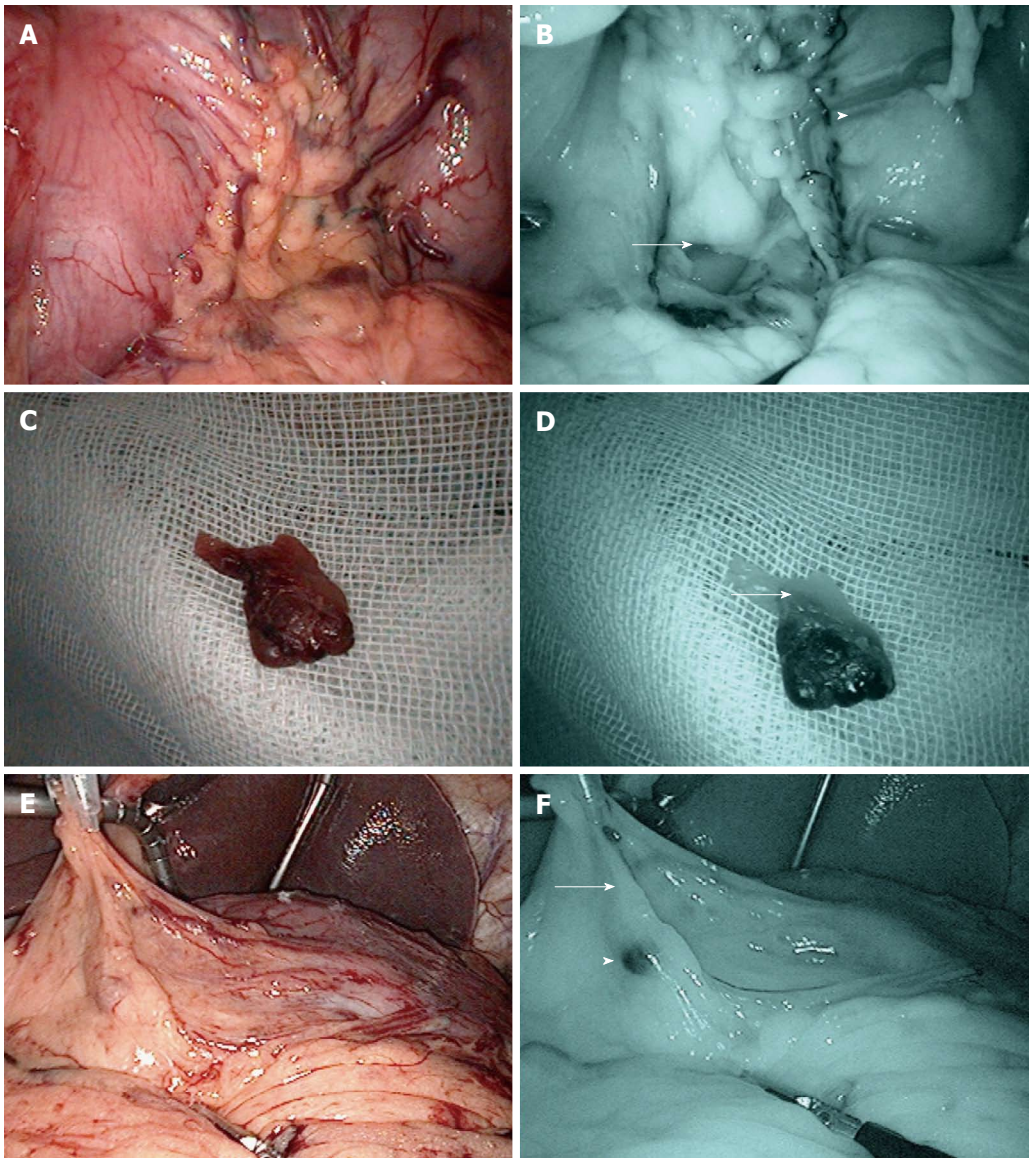

Figure 1 Laparoscopic observation around the left gastric vessels (LN No.3,7). Lymph vessels and LN can be easily detected by IREE with Indocyanine Green (ICG). A: Ordinary light observation of lymph vessels around the left gastric artery; B: Infrared ray observation of lymph vessels around left gastric region (Lymph vessels: arrow; ICG positive node: arrowhead); C: Ordinary light observation of lymph vessels around the right epigastric artery; D: Infrared ray observation around the right epigastric artery shows first drainage lymph vessels, and sentinel lymph node (SLN) (arrow); E: Ordinary light observation of lymph vessels; F: Infrared ray observation of lymph vessels (first drainage lymph vessels: arrow; SLN: arrowhead).

dual tracer endoscopic injection technique. Patients with previously untreated cT1 or cT2 gastric adenocarcinomas < 4 cm in gross diameter were eligible for inclusion in this study. SN mapping was performed by using a standardized dual tracer endoscopic injection technique. The SN detection rate was 97.5%. Of 57 patients with lymph node metastasis by conventional hematoxylin and eosin staining, 93% had positive SNs, and the accuracy of nodal evaluation for metastasis was 99%. Only four false-negative SN biopsies were observed, and pathologic analysis revealed that three of those biopsies were pT2 or tumors > 4 cm. They concluded that the endoscopic dual tracer method for SN biopsy is acceptable and effective when applied to superficial, relatively small gastric adenocarcinomas.

TRACERS AND METHODS OF SNNS

At present, two methods are predominantly used in the detection of the SLN, injection of dye and/or injection of a radio-isotope (RI) with a gamma probe. The SLN is stained blue or green by dye and the hot LNs are identified by the uptake of RI, after injection around the tumor with dye or RI colloid.

Usually, ^{99m}Technetium (^{99m}Tc)-tin colloid was endoscopically injected into the submucosa of the gastric wall around the tumor one day before surgery, and lymphoscintigraphy was performed 2 h later. During surgery, the uptake of the RI in each lymph node was counted using Navigator GPS. The half-life of ^{99m}Tc is short, it produces secure radioactive elements, and the particle size

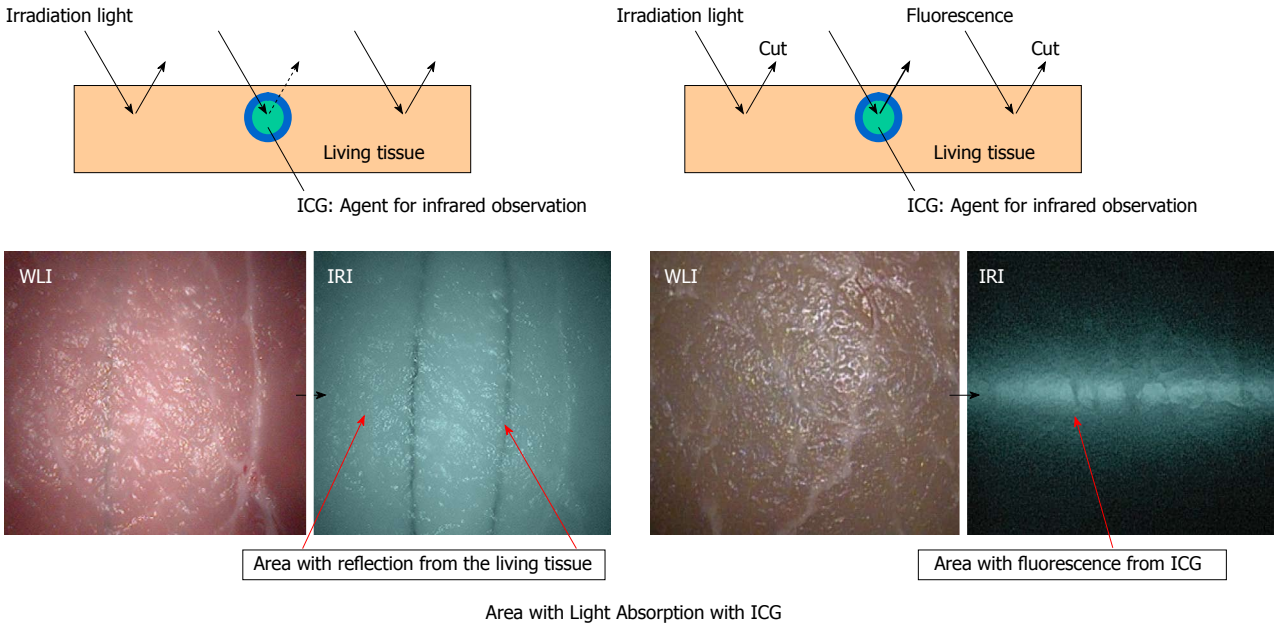

Figure 2 Mechanism of absorption with indocyanine green and fluorescence from indocyanine green. Irradiated with light near the maximum absorption wavelength, the ICG-injected area in the tissue absorbs the light and becomes darker. In the other areas in the background, the light is reflected and those areas become brighter. ICG: Indocyanine Green.

is relatively large. Therefore, ^{99m}Tc is judged to remain in the lymph nodes for approximately 12 h. However, tight regulation and costs of radioactive substances limit the wide-spread use of the probe-guided method in general hospitals.

The dye (Patent blue, lymphazurin, and ICG)-guided method is used in surgery and is safe, convenient, inexpensive and excellent in depiction of not only LNs but also the lymphatic vessels. Particulate-activated carbon and dye have been used to study lymphatic flow previously in Japan[20]. The advantage of these methods is that their transition to the lymphatic system allows intra operative real time evaluation of the lymph flow. Because carbon particles are small and diffuse rapidly to the distal LNs, carbon was judged to be unsuitable for detection of SLN in thick adipose tissue, such as in laparoscopic surgery.

We reported the clinical usefulness of infrared ray IREE combined with ICG to illuminate SLNs in patients with gastric cancer[10-14].

ICG is a tricarbocyanine dye that has been used clinically for over 50 years for hepatic clearance, cardiovascular function testing, and retinal angiography on the basis of its dark green color, which is typically administered at concentrations of 2.5 mg/mL at a typical total dose of 25 mg in adults. ICG conjugates with albumin, which becomes an excellent vascular agent for evaluating both the blood and lymph systems in off-label studies. The incidence of mild adverse reactions of ICG was 0.05% for severe adverse reactions, with no deaths after 1923 procedures[21,22].

It is a known fact that, if combined with plasma protein, ICG has a maximum absorption wavelength of 805 nm. Irradiated with light near the maximum absorption wavelength, the ICG-injected area in the tissue absorbs the light and becomes darker. In the background areas, the light is reflected and those areas become brighter. This is the mechanism of the infrared light absorption observation with ICG (hereinafter referred to as light absorption observation). On the other hand, it is known that ICG which has absorbed the light emits a maximum fluorescence wavelength of 830 nm after it is excited. Compared to the reflected light, the intensity of fluorescence is extremely weak. Thus, infrared fluorescence observation with ICG (herein after referred to as fluorescence observation) is enabled by completely cutting the reflected light and receiving the light near the maximum fluorescence wavelength (Figures 2 and 3).

Utilizing this principle of ICG, the light absorption observation became possible in the conventional infrared endoscope system. The light from the xenon lamp in the light source unit becomes light near the maximum 805 nm of absorption wavelength after passing through the infrared filter which is switchable with the white light filter. Then the light passes through the light guide and the laparoscope to irradiate the subject. The light reflected from the subject is received by an infrared-sensitive charge-coupled device (CCD) incorporated in the camera head, enabling light absorption observation. One of the characteristics of this system is that switching the observation mode between the light absorption observation and the fluorescence observation is made possible.

Recently, ICG with the use of CCD cameras and light-emitting diodes has emerged as an alternative modality in SLN biopsy. The fluorescence method is revolutionary in that it can be observed through a thick adipose tissue, but this needs to be performed in a dark operating room with light off to observe the fluorescence of the

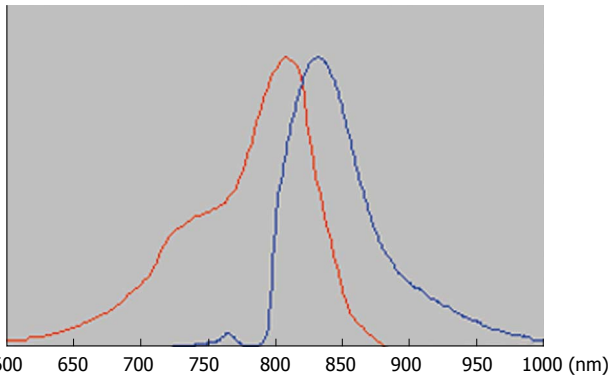

Figure 3 Wavelength of the indocyanine green. Indocyanine green (ICG) has a maximum 805 nm of absorption wavelength. Absorption wavelength band of ICG: Red line; Fluorescence wavelength band of ICG: Blue line.

LNs[23,24].

In order to solve this problem, a system that can describe the ICG near-infrared fluorescence image on the color screen of bright field (hyper eye medical system: HEMS, Mizuho Medical Instruments) has been developed[25], and the usefulness of SNNS of fluorescence observation for breast cancer has been reported[26]. However, these two models do not allow insertion through a trocar, and therefore cannot be used in laparoscopic surgery. In this regard, IREE is especially suitable for use in the SNNS in laparoscopic surgery.

SKIP AND MICRO-METASTASIS OF THE LNS

The precise detection of LN metastasis including micrometastasis is important for SNNS[27]. Kikuchi *et al*[28] analyzed the topographical pattern of lymph node metastasis for pN1 patients with curative resection. Skip metastasis occurred in 5%, and the common stations for such a metastasis were No. 7 and No. 8a. This pattern of metastasis was found in 14% of the patients with single positive nodes. They noted that although perigastric nodes were important first sites of drainage, the distribution of positive nodes depended on the tumor location. Accordingly, in view of both the complexity of the lymphatic flow and skip metastasis, previous studies have recommended the routine use of systemic D2 dissection. However, the feasibility and reliability of SNNS is a prerequisite to less invasive gastric surgery.

Tokunaga *et al*[29] reported that lymphatic flow in LGA and in RGEA are main lymphatic drainage routes of the stomach. Skip metastases were observed in 10%, which were observed in the station 7, 8a, 9, or 11p. The lymphatic stream of the stomach is complicated and multidirectional. Understanding and mapping the complex lymphatic streams of the stomach will allow surgeons to perform more effective lymph node dissection during gastric cancer surgery.

Arigami *et al*[30] reported that the incidence of micrometastasis of SNs detected by RT-PCR was quite high and useful for SNNS. All metastatic lymph nodes were identified within SNs, as judged by RT-PCR. The incidences of metastasis determined by hematoxylin and eosin and IHC were 8.2% and 13.1%, respectively. Micrometastases undetectable by IHC were identified in 23.0% of SNs by RT-PCR.

We have previously reported in patients with lymph node metastasis that positive HE and IHC staining was 12.3% and 23.8%, respectively. Diagnosis of 15 patients without LN metastasis by HE correlated positively with LN metastasis by IHC staining. The 27 positive LNs of these 15 patients consisted of SN which were identified during surgery. Consequently, all these 27 positive LNs judged by IHC staining was micrometastasis or less[13]. Therefore, ICG-positive lymphatic basin dissection by SNNS with infrared ray observation seems to be an adequate method of lymph node dissection for EGC.

When the tumor is located in the lesser curvature, lymphatic fluid is frequently observed to flow from the left gastric artery to the celiac axis area.

OUR METHOD OF SNNS BY IREE

We have reported the clinical usefulness of SNNS for EGC using ICG combined with IREE[10-14].

Briefly, prior to the injection of ICG, the stomach was mobilized by dissecting through the gastrocolic ligament without disrupting the gastro-epiploic vessels. ICG was injected endoscopically in four quadrants of the submucosa surrounding the tumor with an endoscopic puncture needle. Twenty minutes after the injection, SNs stained with ICG were observed with the naked eye as well as IREE. One of the important points regarding ICG injection is that we should not perforate the gastric wall by the needle, for which steady careful injection of ICG into the submucosa is important. It is usually difficult to observe lymphatic flow and the SN if ICG is leaking outside the stomach wall. We usually observe lymphatic flow and SN around the greater and lesser curvature at first, and then the left and right gastric artery and vein by IREE with normal light and the infrared light.

With the rigid endoscope, systems have been developed and we can change normal light and infrared light by a hand switch (Figure 4). Each system has absorption and fluorescence scopes, which can be used in laparoscopic surgery. After careful observation, we make marks around the lymphatic flow area by clips and perform LBD. The portion of the stomach removed depends on the result of intraoperative pathological examination of frozen HE staining and the number of lines of lymphatic flow. If SLN is negative for metastasis and lymphatic flow is single line, we perform a wedge resection. When lymphatic flow is recognized in two directions, segmental resection, pylorus preserving gastrectomy or proximal resection is selected. If SLN is positive for metastasis, a standard gastrectomy with D2 dissection is chosen.

We usually take the extra 60-90 min for SNNS compared to standard operation for EGC. So, the surgeon is

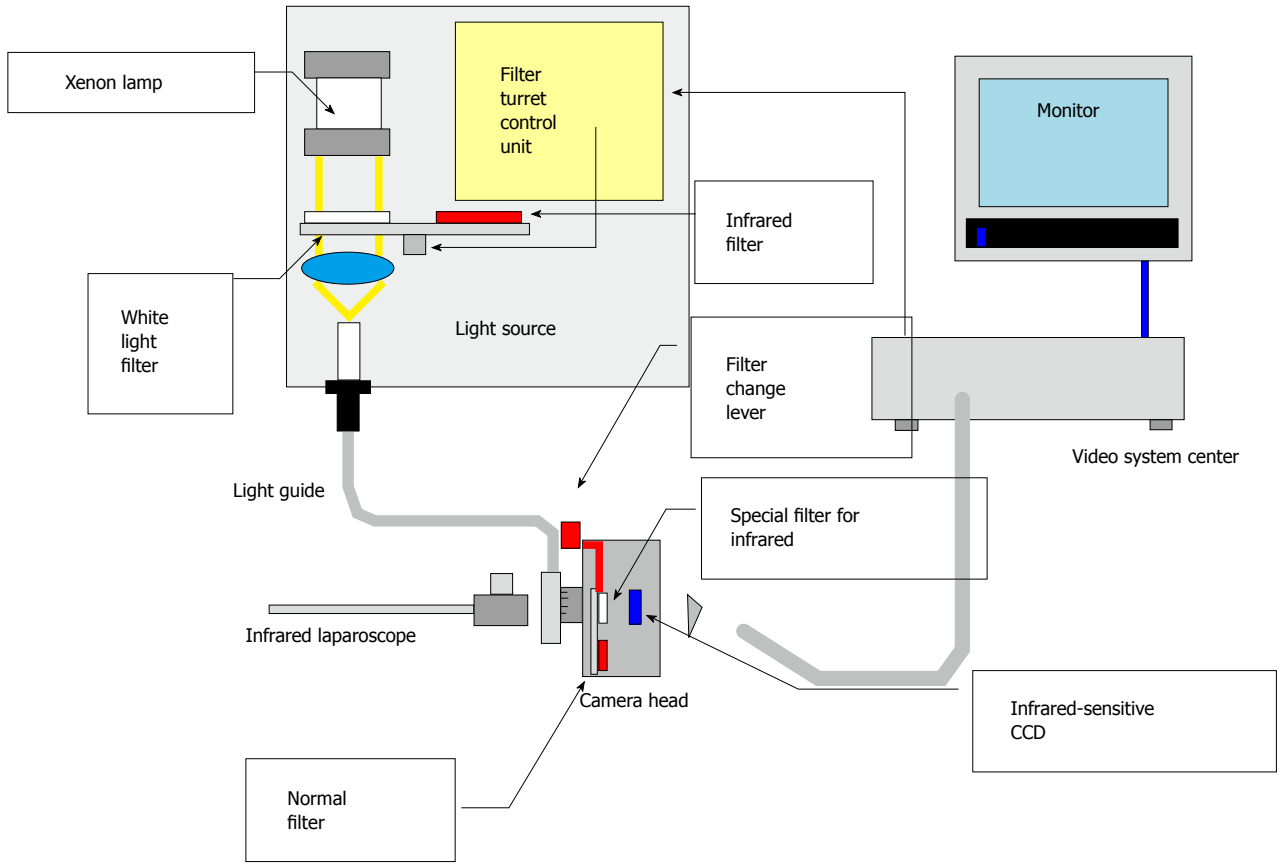

Figure 4 Technological overview of the new infrared observation system ordinary light and infrared ray laparoscopy system can be changed by a handle switch.

required to have a passion for SNNS.

CONCLUSION

Of several methods of SNNS, IREE can detect the SN easily and is superior to ICG alone, and seems to have an equal detection rate as compared to the RI + dye method. LBD of the SN basin is required for accurate intra-operative diagnosis of LN metastases. ICG-positive lymphatic basin dissection by SNNS with IREE seems to be an adequate method of lymph node dissection for EGC.

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TOPIC HIGHLIGHT

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Role of microRNAs in gastric cancer

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Abstract

Although gastric cancer (GC) is one of the leading causes of cancer-related death, major therapeutic advances have not been made, and patients with GC still face poor outcomes. The prognosis of GC also remains poor because the molecular mechanisms of GC progression are incompletely understood. MicroRNAs (miRNAs) are noncoding RNAs that are associated with gastric carcinogenesis. Studies investigating the regulation of gene expression by miRNAs have made considerable progress in recent years, and abnormalities in miRNA expression have been shown to be associated with the occurrence and progression of GC. miRNAs contribute to gastric carcinogenesis by altering the expression of oncogenes and tumor suppressors, affecting cell proliferation, apoptosis, motility, and invasion. Moreover, a number of miRNAs have been shown to be associated with tumor type, tumor stage, and patient survival and therefore may be developed as novel diagnostic or prognostic markers. In this review, we discuss the involvement of miRNAs in GC and the mechanisms through which they regulate gene expression and biological functions. Then, we review recent research on the involvement of miRNAs in GC prognosis, their potential use in chemotherapy, and their effects on *Helicobacter pylori* infections in GC. A greater understanding of the roles of miRNAs in gastric carcinogenesis could provide insights into the mechanisms of tumor development and could help to identify novel therapeutic targets.

Key words: MicroRNA; Gastric cancer; Reverse transcription-polymerase chain reaction; Chemosensitivity; *Helicobacter pylori*; Circulating MicroRNA

Core tip: In this review, we discuss the involvement of miRNAs in gastric cancer (GC) and the mechanisms through which they regulate gene expression and biological functions. Then, we review recent research on the involvement of miRNAs in GC prognosis, their potential use in chemotherapy, and their effects on *Helicobacter pylori* infections in GC. A greater understanding of the roles of miRNAs in gastric carcinogenesis could provide insights into the mechanisms of tumor development and could help to identify novel therapeutic targets.

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INTRODUCTION

Despite the decreasing incidence of gastric cancer (GC) in developed countries, GC remains the second leading cause of cancer-related deaths worldwide[1,2], with 700000 deaths attributed to this malignancy annually[3]. Therefore, the development of novel therapies to improve the

Table 1 Aberrant expression of miRNAs in gastric cancer

Up-regulated miRNAs
let-7a, miR-9, -10a, -10b, -17, -17-5p, -18a, -18b, -19a, -19b, -20a, -20b, -21, -23a, -23b
miR-25, -26b, -27, -29b-1, -30b, -31, -34a, -34b, -34c, -92, -98, -99a, -100, -103, -106a
miR-106b, -107, -125b, -126, -128a, -130b, -138, -142-3p, -146a, -147, -150, -151-5p
miR-155, 181a, -181a-2, -181b, -181c, -185, -191, -192, -194, -196a, -196b, -199a
miR-199a-3p, -200b, -210, -214, -215, -221, -222, -223, -296-5p
miR-301a, -302f, -337-3p, -340, -370, -421, -520c-3p, -575, -601, -616, -658, -1259
Down-regulated miRNAs
let-7a, -7f, miR-7, -9, -22, -29c, -30a-5p, -31, -34a, -34b, -34c
miR-101, -126, -128b, -129, -129-2, -129-3p, -130b, -133b, -135a, -137, -141, -145
miR-146a, -148, -148b, -149, -152, -155, -181b, -181c, -182, -193b, -195, -195-5p, -197
miR-200, -204, -206, -210, -212, -218, -219-2-3p, -302b, -331-3p, -375
miR-378, -408-3p, -429, -433, -486, -495, -551a, -574-3p, -610, -622, -638, -663, -874

prognosis of patients with GC is critical.

MicroRNAs (miRNAs) are a subset of small non-coding RNA molecules, typically 21-23 nucleotides in length, that are believed to regulate the expression of several genes[4]. Mature miRNAs are cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors[4]. The precursor is cleaved by cytoplasmic RNase Ⅲ Dicer into a miRNA duplex[5-7]. One strand of the short-lived duplex is degraded, whereas the other strand serves as the mature miRNA[8]. Mature miRNAs associate with a cellular complex that is similar to the RNA-induced silencing complex that participates in RNA interference[9,10]. Recent studies have reported miRNA-mediated regulation of cell growth and apoptosis[11,12]. Moreover, the measurement of miRNA expression has shown that certain miRNAs are specifically involved in cancer[12-15]. In the context of GC, the number of publications investigating the relationship between miRNAs and GC has been increasing each year. miRNAs have the unique ability to negatively regulate gene expression, thereby resulting in changes in cell development, proliferation, and apoptosis[16]. These biological properties of miRNAs may provide the ability to regulate a variety of human diseases, including cancer[17]. According to recent findings, miRNAs may play important roles in human cancer by acting as potential oncogenes or tumor-suppressor genes[18-20].

In this review, we introduce and discuss the newest knowledge on the relationship between GC and miRNAs.

Aberrant expression of miRNAs in GC

Although more than 1000 miRNAs are thought to exist, no comprehensive analysis of miRNA expression in GC has been performed to date. Each miRNA modulates the expression of hundreds of genes, and we speculate that miRNAs act in a network-type fashion to mediate the expression of genes.

While several reports have published comprehensive expression analysis of miRNAs[21,22], more data, including expression profile analysis by high-throughput real-time reverse transcription-polymerase chain reaction (RT-PCR) or miRNA microarrays, are required. A summary of reported miRNA expression abnormalities in GC is presented in Table 1.

Upregulated miRNAs (oncomirs): Aberrant expression of miRNAs has been observed in many cancers[19,23]. Oncomirs are oncogenic miRNAs that are up-regulated in cancer cells and have been shown to act as oncogenes in GC. One such oncomir is *miR-21*, which has been widely reported as an oncomir in GC[24-26].

Overexpression of *miR-21* has been reported in various cancers, such as esophageal cancer[27], breast cancer[28], and glioblastoma[29]. In GC, the expression of *miR-21* is upregulated compared with normal tissues[22,30,31]. Motoyama *et al*[31] and Cao *et al*[32] reported an inverse correlation between *miR-21* and *PDCD4* expression in GC. *PDCD4*, a direct target gene of *miR-21*, encodes a protein that inhibits cell growth and invasion[33,34]. Moreover, Zhang *et al*[26] reported that *PTEN*, a well-known tumor-suppressor gene, is a target of *miR-21*. Thus, these data support the idea that *miR-21* acts as a key oncomir in GC by inhibiting the tumor-suppressor genes *PDCD4* and *PTEN*.

In addition to *miR-21*, *miR-106a* expression is also upregulated in GC (Table 1), as well as in several other human tumors, compared with adjacent normal tissues[35]. *miR-106a* mimics the function of positive regulators of the G_1-to-S transition[35]. In several human hematopoietic cell lines, *miR-106a* has been shown to target interleukin (IL) 10[36], downregulating the expression of this critical cytokine by binding to the 3' untranslated region[36]. As a further regulatory element, SP1 and EGR1 indirectly downregulate IL10 expression by inducing *miR-106a* expression[36].

Downregulated miRNAs (tumor-suppressor miRNAs): Down-regulated miRNAs in cancer tissue are referred to as tumor-suppressor miRNAs[37-39]. The important target gene of a tumor-suppressor miRNA is usually an oncogene. Therefore, decreased expression of the tumor-suppressive miRNA induces the expression of the oncogene. The genomic loss of *miR-101* in cancer leads to overexpression of EZH2 and concomitant dysregulation of epigenetic pathways, resulting in cancer progression[40,41]. Because *miR-101* targets cyclooxygenase (COX) 2 in GC, downregulation of *miR-101* induces COX2 expression[42]. COX2 activates the arachidonic acid/prostaglandin E2 pathway following cell proliferation[42]. Oncogenic targets of *miR-101* induce cell proliferation in GC. Therefore, *miR-101* may be useful for gene therapy in GC.

Another miRNA that has been suggested to have a role in cancer is *let-7*. Expression of *let-7* reduces the expression of 3 human *RAS* genes, *HRAS*, *KRAS*, and *NRAS*. Moreover, *let-7* expression is lower in lung tumors than in normal lung tissue, whereas expression of

Table 2 miRNAs associated with prognosis in gastric cancer patients

let-7a ,-7i
miR-10b, -20a, -20b, -21, -22, -25, -27a, -30a-5p, -34a, -93
miR-103, -106a, -106b, -107, -125a-5p, -126, -130, -142-5p, -144, -146a, -150
miR-155, -181c, -195, -196a, -199a-3p, -200c, -206, -221, -222, -223
miR-335, -338, -372, -375, -451

the RAS proteins is significantly higher in lung tumors, suggesting a possible role of *let-7* in cancer[43]. The expression of *let-7* miRNA is also reduced in human lung cancer[44], breast cancer[45], and hepatocellular carcinomas[46]. In addition, overexpression of *let-7* inhibits the growth of lung cancer cells *in vitro*[44]. In GC, RAB40C, a target of *let-7a*, has been reported to play an essential role in gastric tumorigenesis[47].

MiR-148a has been shown to act as a tumor suppressor in prostate cancer, and its expression is lower in prostate cancer cells compared with normal prostate epithelial cells[48]. In GC, *miR-148a* is inactivated by hypermethylation of the promoter region[49]. This may result in the upregulation of DNA methyltransferase, which is a target of *miR-148a*[49]. Moreover, *miR-148a* suppresses tumor cell invasion by downregulating ROCK1[50]. In a report from our laboratory, we found that *miR-148a* expression is downregulated in undifferentiated GC[51].

Roles of miRNAs as prognostic factors

Because many factors affect the prognosis of cancer patients, it is difficult to clarify how miRNAs are involved in the prognosis of patients with GC. However, advances in research on the potential role of miRNAs in patient prognosis may lead to the use of miRNAs as tools in medical treatment or diagnosis in the future. The detection of miRNAs involved in the prognosis of GC patients will not only be useful for predicting prognosis but helpful for developing therapeutic targets in the future. In Table 2, we have summarized the current knowledge on the relationship between miRNAs and prognosis. In particular, *miR-21*, *-93*, and *-125* have been well studied in this context. While we mentioned the usefulness of *miR-21* previously, overexpression of *miR-93* in GC cells has been shown to reduce the cellular response to transforming growth factor (TGF)-β (*TGFB1*) by interfering with the synthesis of p21 (*CDKN1A*) and BIM (*BCL2L11*), the 2 most important downstream effectors of TGF-β-dependent cell cycle arrest and apoptosis, respectively[52]. High expression of *miR-98* was found to predict poor survival[53,54]. Interestingly, low expression of *miR-125* in GC has been shown to be an independent prognostic factor for survival[55,56]. One target gene of *miR-125a-5p* is ERBB2 (*HER2*), which is an important molecular target in chemotherapy[56].

Notably, many genes act as prognostic factors for patients with GC; miRNAs may regulate these genes, thereby affecting prognosis. Thus, further analysis of candidate miRNAs is necessary.

Table 3 MiRNAs involved in chemosensitivity

5FU sensitivity
let-7g
miR-10b, -22, -30c, -31, -32,
miR-133b, -143, -144, -145, -181b, -190, -197, -200c, -204, -210
miR-335, -501, -501-5p, -532, -615, -615-5p, -766, -877
miR-1224-3p, -1229, -3131, -3149, -3162-3p, -4763-3p
CPT sensitivity
let-7g
miR-7, -31, -98, -126, -196a, -200, -338
CDDP, CF sensitivity
let-7g
miR-1, -16, -21, -34, -181, -181b, -342, -497

MiRNAs involved in chemosensitivity

Chemotherapy is an important tool for the treatment of GC. However, with currently available tools, it is impossible to predict whether GC patients will respond to chemotherapeutic approaches. The ability to predict the effects of chemotherapy may help reduce the unnecessary use of chemotherapeutics in GC. Current chemotherapeutic agents used in the treatment of GC include 5FU, CDDP, taxan, and irinotecan. Many studies have reported that miRNAs may affect the efficacy of chemotherapy. Wang *et al*[57] identified 9 upregulated miRNAs and 18 downregulated miRNAs involved in 5FU sensitivity by microarray and RT-PCR (Table 3). Moreover, *miR-143*, *miR-145*, and *miR-144* have been reported to be involved in 5FU sensitivity[58,59]. In Table 3, we summarize the involvement of miRNAs in CPT, CDDP, and CF sensitivity. In the near future, we will be able to use miRNA expression as a predictor of chemotherapeutic efficacy. Additionally, gene therapy with miRNAs may be able to induce chemosensitivity in patients with GC.

MiRNAs involved in Helicobacter pylori infection

Helicobacter pylori (*H. pylori*) selectively colonize the gastric epithelium and typically persist for the lifetime of the host. Among colonized individuals, however, only a fraction develop gastric adenocarcinoma, emphasizing the importance of understanding the pathogenic mechanisms through which *H. pylori* promote chronic inflammation and the progression to GC[60]. miRNAs involved in *H. pylori* infections have been reported in several papers. *Let-7* expression has been shown to be downregulated by Cag A after *H. pylori* infection, and Ras, a target of *let-7*, is overexpressed in GC[61]. Additionally, *miR-17/92*, the *miR-106b-93-25* cluster, *miR-21*, *miR-194*, *miR-196*, *miR155*, *miR-222*, and *miR-223* are upregulated in gastric mucosa infected by *H. pylori*[62-64]. Among these miRNAs, Li *et al*[64] revealed that *miR-222* targets RECK, which inhibits the tumorigenicity of GC[64-66]. Further analysis of miRNAs and target genes may clarify the complicated mechanism of GC that occurs in the context of *H. pylori* infections.

Circulating miRNAs as biomarkers

Presently, circulating miRNAs found in the blood of pa-

Table 4 Circulating miRNAs as biomarkers

Up-regulated miRNAs
miR-1, -17, -17-5p, -20a, -21, -27a, -31, -34, -103, -106a, -106b, -107,-194, -200c
miR-210, -221, -223, -370, -376a, -378, 421, 423-5p, 451, -486, 744
Down-regulated miRNAs
miR-218, -375

tients constitute the most promising type of miRNA for clinical use, because these are concise for blood collecting. We summarized the circulating miRNAs in the blood of GC patients in Table 4.

There are a number of reports describing the use of miR-106[67-69], miR-17[67,70], miR-21[67,71], and miR-221 as potential biomarkers[72]. The detection of miRNA in peripheral blood may be a novel tool for monitoring circulating tumor cells in patients with gastric cancers. Moreover, circulating miRNA may be a promising, non-invasive molecular marker for tracking pathological progression, predicting prognosis and monitoring chemotherapeutic effects in gastric cancer.

Finally, the precise mechanism of each miRNA is not well known. Further reports about miRNA are expected to help us better understand cancer mechanisms. This research will be useful for clinical diagnosis or treatment for GC patients.

CONCLUSION

In this review, we presented and discussed the newest knowledge on miRNAs in gastric cancer and their potential usefulness as future medical treatments and diagnostic tools. Although the molecular biology of GC has been well characterized, research on miRNAs in GC is still in its infancy. Thus, in the near future, we anticipate that advances in miRNA research in GC may help to develop novel medical treatments or diagnostic tools, thereby improving the prognosis of GC patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

miRNA polymorphisms and risk of gastric cancer in Asian population

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Abstract

miRNAs are endogenous 19- to 25-nt noncoding RNAs that can negatively regulate gene expression by directly cleaving target mRNA or by inhibiting its translation. Recent studies have revealed that miRNA plays a significant role in gastric cancer development either as a tumor suppressor gene or oncogene. miRNA-single-nucleotide polymorphisms (SNPs), as a novel class of functional SNPs/polymorphisms, have been identified as candidate biomarkers for gastric cancer susceptibility. On the basis of recent data, the present review summarizes current knowledge of the functional effects of miRNA-SNPs and their importance as candidate gastric cancer biomarkers. Additionally, this review also includes a meta-analysis of the most frequently studied miRNA-SNPs in gastric cancer.

Key words: miRNA; Polymorphism; Gastric cancer; Risk

Core tip: Host genetic susceptibility plays an important role in gastric carcinogenesis. In this review, we summarize current knowledge about the functional effects of miRNA-single-nucleotide polymorphisms (SNPs) and their importance in gastric cancer susceptibility. Furthermore, we also conduct a simple meta-analysis about most frequently studied miRNA-SNPs (rs11614913 in miR-196a-2, rs895819 in miR-27a and rs2910164 in miR-146a) in gastric cancer.

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INTRODUCTION

miRNAs are small endogenous noncoding RNAs of about 22 nucleotides that negatively regulate post-transcriptional gene expression by pairing with complementary binding sites located in the 3'-untranslated region (UTR) of mRNA[1]. In 1993, Wightman *et al*[2] and Lee *et al*[3] found the first miRNA, lin-4, which controls developmental timing and cell fate specification in *Caenorhabditis elegans*. To date, thousands of miRNA have been characterized in animal and plant genomes[4]. In human malignancies, miRNA have shown important roles

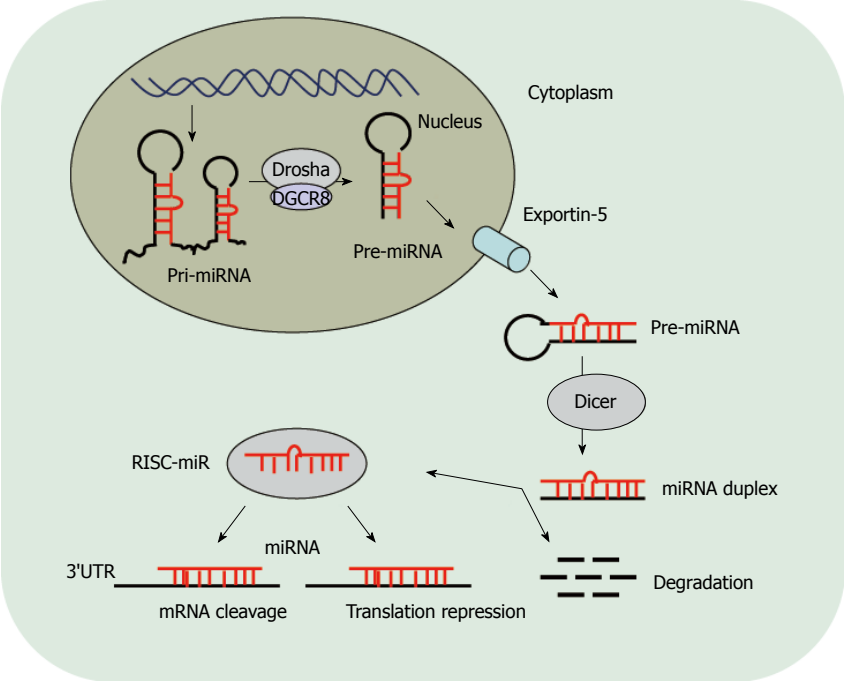

Figure 1 miRNA biogenesis and processing.

in a variety of cellular processes including apoptosis, differentiation, angiogenesis and proliferation[5]. It has been suggested that miRNA regulates almost one-third of the human genes although it represents only a small part of the genome[6]. Moreover, recent evidence shows that more than half of the known human miRNAs are located within cancer-associated genomic regions or fragile sites[7]. Therefore, miRNA can be considered as a potential and ideal biomarker for cancer.

Gastric cancer remains one of the commonest malignant tumors worldwide. In 2013, it was estimated that about 21600 patients were diagnosed with gastric cancer and 10990 died of the disease in the United States[8]. In East Asia, especially in China, gastric cancer has the highest rates of incidence and mortality[9]. Epidemiological studies have identified environmental and lifestyle risk factors that contribute to the development of gastric cancer, such as *Helicobacter pylori* (*H. pylori*) infection or high salt intake[10]. However, only a minority of patients exposed to risk factors such as *H. pylori* infection ultimately develop gastric cancer, which indicates that host genetic susceptibility also plays an important role in gastric carcinogenesis[11-13]. In recent decades, many correlations between single nucleotide polymorphisms (SNPs) in the genome and the risk of various diseases, including gastric cancer, were reported[14,15]. Recently, a class of novel functional polymorphisms in miRNA or its binding sites is the most interesting. Our previous epidemiological studies also provided evidence that the risk of gastric cancer is associated with miRNA-SNPs[16,17]. Therefore, miRNA polymorphisms can be used as specific markers of predisposition for gastric cancer prevention.

In this review, we provide a comprehensive list of potentially functional miRNA-SNPs and have a brief description of miRNA biogenesis, biology and the underlying mechanism of miRNA-SNPs in gastric cancer susceptibility.

miRNA BIOSYNTHESIS AND FUNCTION

miRNA biogenesis is a multistage process[18,19]. Most human miRNA are first transcribed by RNA polymerase II and are encoded by introns[20]. The primary miRNA (pri-miRNA) is excised from the primary transcript of the intron[21]. Then, the pri-miRNA is processed by the RNase Drosha-DGCR8 complex into 70-nucleotide-long precursor hairpin structures (pre-miRNA)[22]. The pre-miRNA is subsequently exported to the cytoplasm by the nuclear membrane protein exportin-5 and cleaved by the RNase Ⅲ enzyme Dicer to produce a transient miRNA duplex composed of a mature miRNA sequence (about 22 nucleotides in length) and its complementary sequence miRNA*[23]. The miRNA duplex is unwound by an RNA helicase and the mature miRNA is incorporated into the targeting miRNA containing RNA induced silencing complex (RISC) together with the argonate (Ago) protein[24,25]. The other strand (or miRNA*) is often degraded. Ago is considered as the heart of the miRNA-induced silencing complex (RISC-miR), which induce the mature miRNA to bind the complementary elements of target mRNA 3' UTR. Lastly, the RISC-miR exerting negatively regulates gene expression function by either mRNA cleavage or inhibiting translation[26] (Figure 1).

As described above, miRNA exert the functionality by sequence-dependent regulation of post-transcriptional gene expression by targeting mRNA for cleavage or translational repression. Target selection is dependent on the extent of Watson-Crick base pairing between the

miRNA and mRNA. Nucleotides 2-8 (from the 5' end of miRNA), also referred to as the "seed sequence", are a major determinant of mRNA target selection[27]. Mutation in either the seed or seed-complementary site could inhibit miRNA activity, which highlights the importance of seed sequence complementarity[28]. Additionally, Grimson *et al*[29] also reported that more than four contiguous Watson-Crick base pairs between nucleotides 12-17 at the 3' end of the miRNA could enhance target recognition. Therefore, miRNA biogenesis and post-transcriptional regulation is highly sequence dependent, and sequence variants (such as SNPs) in either the miRNA sequence or miRNA-target site can have a significant effect on miRNA function. So far, > 1000 miRNAs have been found in humans, which are indicated to regulate up to 30% of all protein-coding genes in the human genome[30]. Moreover, Calin *et al*[7] have suggested that more than half of the human miRNAs are located in cancer-associated fragile regions. This shows that miRNA regulation plays a key regulatory role during development and in various cellular processes such as differentiation, growth and death. These processes are frequently dysregulated in carcinogenesis, implicating miRNA function as oncogenes or tumor suppressors.

miRNA EXPRESSION IN GASTRIC CANCER

Accumulating evidence has strongly indicated that aberrant miRNA expression is an important feature of gastric cancer[31]. In 2012, Wang *et al*[32] summarized 13 miRNA expression profiling studies and suggested that there were 139 miRNAs differentially expressed in gastric cancer tissues compared with neighboring non-cancerous or normal gastric tissues. They also found that the expression levels of miR-21, miR-18a, miR-17 and miR-20a in gastric cancer were significantly upregulated in most studies. Conversely, miR-375 and miR-378 expression levels were downregulated in gastric cancer tissues. Also, expression levels of 29 miRNAs were reported to be inconsistent. In addition, recent evidence using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) also confirmed that miR-17-5p/20a, miR-181a-5p, miR-214 and miR-23a expression levels were upregulated in human gastric cancer tissues, and overexpression of miR-17-5p/20a could promote gastric cancer cell progression and inhibit apoptosis[31,33-35]. Recent studies have indicated that gastric cancer tissue has significantly lower expression of miR-148a and miR-22 compared to non-tumor tissue, and miR-148a and miR-22 inhibit gastric cancer cell migration and invasion. Moreover, low miR-148a levels are associated with lymph node metastasis, N stage, and blood vessel invasion in gastric cancer patients[36,37]. These miRNA expression profiles analyzed by microarray or qRT-PCR could help us understand better the molecular mechanisms of tumorigenesis and contribute to the development of diagnosis and therapy for gastric cancer.

SNPs IN miRNA

SNPs in the miRNA regulatory pathway, as a novel class of functional polymorphisms in the human genome, have been widely implicated in cancer development[38]. In 2005, Calin *et al*[39] reported the first evidence that mutations in miRNA genes are common and might have functional importance in chronic lymphocytic leukemia (CLL). They identified a germ-line mutation in the miR-16-1-miR-15a primary precursor, which causes low levels of miRNA expression and is associated with prognostic factors and disease progression in CLL[39]. Since then, a series of studies has used systematic sequencing or *in silico* approaches to identify more SNPs in miRNA. In our present study, we focused on the miRNA SNPs in gastric cancer susceptibility. Therefore, we searched PubMed to August 2013 to identify all relevant papers, using the key words miRNA, miRNA polymorphism or variant in combination with gastric cancer or gastric tumor. There were 35 epidemiological studies with a focus on the importance of miRNA-related SNPs in gastric cancer susceptibility. We scanned the titles and abstracts and excluded the studies that were clearly irrelevant to the current topic. The remaining articles were read to determine whether they contained information of interest. In addition, to be eligible, studies had to fulfill the following criteria: (1) a case-control study design or prospective study design; (2) number of subjects with each allele or genotype in cases and controls reported; (3) risk estimates OR and 95%CI, or raw data that allowed us to calculate them; and (4) risk estimate was only with miRNA polymorphism, and not with miRNA binding site SNPs. All searches were performed independently by two investigators. Thirteen epidemiological studies were included in our study. As summarized in Table 1, Peng *et al*[40] suggested a significantly increased risk of gastric cancer in subjects homozygous for the variant C of miR-196a-2 (rs11614913) compared with wild-type homozygote TT and heterozygote CT carriers in a Chinese population (adjusted OR = 1.57, 95%CI: 1.03-2.39). Stratified analyses showed that the subjects homozygous for the variant C genotype of miR-196a-2 had a strong association with lymph node metastasis of gastric cancer (adjusted OR = 2.25, 95%CI: 1.21-4.18). Subsequently, Okubo *et al*[41] evaluated the associations of three SNPs (rs11614913, rs2910164 and rs3746444) in pre-miRNA (miR-196a2, miR-146a and miR-499) with the risk of gastric cancer in the Japanese population. The strongest association was observed only in the miR-146a rs2910164 (G > C) SNP. Our research also indicated that miR-27a A/G (rs895819) and miR-146a C/G (rs2910164) polymorphisms had important significance in gastric cancer susceptibility[16,17]. In addition, an increased risk of gastric cancer was obtained with AA genotype in pre-miR-30c compared with GG genotype in the Chinese population (adjusted OR = 1.83, 95%CI: 1.07-3.15).

Table 1 List miRNA-single-nucleotide polymorphisms (SNPs) evaluated in gastric cancer

First author year	Population	miRNA	SNP ID	Allele	Case/control	Risk[1] (OR and 95%CI)
Peng *et al*[40] 2010	Chinese	miR-196a-2	rs11614913	T/C	213/213	1.57 (1.03-2.39)
Okubo *et al*[41] 2010	Japanese	miR-146a	rs2910164	G/C	552/697	1.30 (1.02-1.66)
		miR-196a-2	rs11614913	T/C	552/697	NS
		miR-499	rs3746444	A/G	552/697	NS
Sun *et al*[16] 2010	Chinese	miR-27a	rs895819	A/G	304/304	1.48 (1.06-2.05)
Zeng *et al*[17] 2010	Chinese	miR-146a	rs2910164	C/G	304/304	1.58 (1.11-2.20)
Hishida *et al*[56] 2011	Japanese	miR-146a	rs2910164	C/G	583/540	NS
Mu *et al*[42] 2012	Chinese	miR-30c	rs928508	A/G	240/240	1.83 (1.07-3.15)
Zhang *et al*[43] 2012	Chinese	miR-149	rs2292832	T/C	762/757	NS
		miR-605	rs2043556	A/G	762/757	NS
Arisawa *et al*[44] 2012	Japanese	miR-938	rs2505901	T/C	333/574	0.73 (0.55-0.99)
Ahn *et al*[50] 2012	South Korean	miR-146a	rs2910164	C/G	461/447	NS
		miR-149	rs2292832	T/C	461/447	NS
		miR-196a-2	rs11614913	T/C	461/447	NS
		miR-499	rs3746444	A/G	461/447	NS
Zhou *et al*[53] 2012	Chinese	miR-27a	rs895819	A/G	295/413	0.28 (0.12-0.66)
Yang *et al*[54] 2012	Chinese	miR-27a	rs11671784	G/A	892/978	0.76 (0.63–0.92)
		miR-27a	rs895819	A/G	892/978	NS
Zhou *et al*[57] 2012	Chinese	miR-146a	rs2910164	C/G	1686/1895	1.26 (1.01-1.56)
Wang *et al*[51] 2013	Chinese	miR-196a-2	rs11614913	T/C	1689/1946	0.71 (0.60-0.83)

[1]Risk estimate by authors supplied main results in full text.

Moreover, the gastric cancer risk was especially elevated in older individuals (aged > 60 years), men, non-smokers, and *H. pylori*-infected individuals[42]. More interestingly, Zhang *et al*[43] found that lifestyle-related factors, including tea consumption and smoking, might have the potential to modify the associations between miR-149 and miR-605 polymorphisms and gastric cancer risk. One study also suggested that miR-938 polymorphism (rs2505901) C locus carried a decreased risk overall for gastric cancer (OR = 0.73, 95%CI: 0.56-0.99)[44]. Additionally, many epidemiological studies also reported that miRNA binding site polymorphisms were associated with the risk of gastric cancer[45-47]. As shown in Table 1, the most frequently studied SNPs are rs11614913 in miR-196a-2, rs895819 in miR-27a, and rs2910164 in miR-146a. Therefore, we discuss and perform a meta-analysis to evaluate the association between the three polymorphisms and gastric cancer susceptibility based on all eligible case-control published data. We calculated the pooled OR and 95%CI based on the most frequently adopted genetic model in original literature. The *Z* test was used to determine the statistical significance of the pooled OR, and $P < 0.05$ was considered statistically significant. Additionally, Q and I^2 statistics were used to examine possible heterogeneity of study results[48]. If significant heterogeneity existed ($P < 0.10$ was considered representative of statistically significant heterogeneity), the pooled OR estimate of each study was calculated by the random effects model, otherwise the fixed-effects model was used.

SNP rs11614913 in miR-196a-2

The SNP (rs11614913) in the pre-miRNA region of miR-196a-2 was first identified in a case-control study of 1009 breast cancer cases and 1093 cancer-free controls in a population of Chinese women[49]. In gastric cancer, Peng *et al*[40] suggested a significantly increased risk of gastric cancer in subjects homozygous for the variant C of miR-196a-2 (rs11614913) compared with TT + CT carriers. However, one study conducted in a Japanese population only found the rs11614913 SNP in the miR-196a2 was associated with the degree of *H. pylori*-induced mononuclear cell infiltration, and not gastric cancer risk[41]. To understand this effect better, the SNP was genotyped again in a study conducted on 461 Korean patients with gastric cancer and 447 matched controls. They revealed that miR-196a-2 CC genotype was associated with elevated gastric cancer risk among women (adjusted OR = 1.86, 95%CI: 1.09-3.19)[50]. On the contrary, Wang *et al*[51] recently found that the CC genotype was significantly associated with a reduced risk of gastric cancer compared with the CT + TT genotypes in larger samples of the Chinese population (adjusted OR = 0.71, 95%CI: 0.60-0.83). Therefore, to determine further whether there is an association between rs11614913 and risk of gastric cancer, we summarized the published data from these studies using a recessive model, and showed no significant association between SNP rs11614913 in miR-196a-2 and gastric cancer risk (OR = 1.06, 95%CI: 0.74-1.50, $Z = 0.30$, $P = 0.77$; $Q = 18.85$, $P < 0.01$, Table 2).

SNP rs895819 in miR-27a

A recent study has suggested that miR-27a expression is significantly upregulated in gastric cancer tissues, and high expression levels of miR-27a are associated with poor tumor histological grade[52]. These findings indicate the important role of miR-27a in the development and progression of gastric cancer. Our recent research including 304 gastric cancer cases and 304 cancer-free controls indicated that miR-27a A/G polymorphism

Table 2 miR-196a-2 single-nucleotide polymorphism (rs11614913) in gastric cancer

First author year	Study design	Case/control	Case			Control			Risk[1] (OR and 95%CI)
			TT	CT	CC	TT	CT	CC	
Peng *et al*[40] 2010	HB	213/213	43	94	76	50	107	56	1.56 (1.03-2.35)
Okubo *et al*[41] 2010	HB	552/697	166	281	105	223	350	124	1.09 (0.81-1.45)
Ahn *et al*[50] 2012	HB	461/447	119	242	100	128	232	87	1.15 (0.83-1.58)
Wang *et al*[51] 2013	HB	1689/1946	519	851	319	524	940	482	0.71 (0.60-0.83)
Overall		2915/3303	847	1468	600	925	1629	749	1.06 (0.74-1.50)

[1]OR and 95%CI were calculated under a recessive genetic model (CC *vs* CT + TT). HB: Hospital based.

Table 3 MiR-27a single-nucleotide polymorphism (rs895819) in gastric cancer

First author year	Study design	Case/control	Case			Control			Risk[1] (OR and 95%CI)
			AA	AG	GG	AA	AG	GG	
Sun *et al*[16] 2010	HB	304/304	115	135	54	145	119	40	1.50 (1.08-2.07)
Zhou *et al*[53] 2012	HB	295/413	166	122	7	214	167	32	0.84 (0.62-1.12)
Yang *et al*[54] 2012	HB	892/978	349	437	106	367	472	139	0.93 (0.76-1.13)
Overall		1491/1695	630	694	167	726	758	211	1.04 (0.76-1.42)

[1]OR and 95%CI were calculated under a dominant genetic model (AG + GG *vs* AA). HB: Hospital based.

(rs895819) polymorphism was associated with an elevated risk of gastric cancer. We observed that the patients with variant genotypes (AG + GG) of miR-27a showed a significantly increased risk of gastric cancer relative to AA carriers (adjusted OR = 1.48, 95%CI: 1.06-2.05). The elevated risk was especially evident in older subjects (age > 58 years), men, non-smokers and rural residents. Moreover, we also found that polymorphism of miR-27a might be responsible for elevated miR-27a levels and reduced target gene zinc finger and BTB domain containing 10 (ZBTB10) mRNA[16]. However, another study showed that the SNP rs895819 in miR-27a with the minor allele C (C corresponds to G in the opposite strand) presented a significantly reduced risk of gastric cancer (adjusted OR = 0.77, 95%CI: 0.60-0.99)[53]. Similarly, in the Chinese population, Yang *et al*[54] found that the G/A polymorphism in miR-27a (rs11671784) reduced gastric cancer risk, but not the association with rs895819. Therefore, we extracted the genotypes from the above studies, and calculated the overall OR based on the dominant model (Table 3). There was no significant risk association in the overall pooled analysis (OR = 1.04, 95%CI: 0.76-1.42, Z = 0.25, P = 0.81; Q = 7.82, P = 0.02).

SNP rs2910164 in miR-146a

A G to C polymorphism (rs2910164) located within the sequence of an miR-146a precursor was identified by Shen *et al*[55]. They revealed that the variant predisposed to an earlier age of onset of familial breast and ovarian cancers. In a Japanese population study, Okubo *et al*[41] revealed that rs2910164 CC genotype held a significantly higher risk of gastric cancer when compared to non-cancer subjects (adjusted OR = 1.30, 95%CI: 1.02-1.66). However, we showed that subjects with the variant genotypes (GC + GG) in miR-146a had a significantly higher risk of gastric cancer when compared with CC genotype carriers in the Chinese population (adjusted OR = 1.58, 95%CI: 1.11-2.20)[17]. Additionally, in other stratified analyses for gastric cancer risk, Ahn *et al*[50] found that subjects with miR-146a GG and (CG + GG) genotypes were associated with increased risk of gastric cancer among the non-smokers in a Korean population. In contrast, the study conducted by Hishida *et al*[56] on 583 gastric cancer cases and 540 gastric atrophy cases suggested a lack of association between SNP rs2910164 and gastric cancer risk. These observations were validated by Zhou *et al*[57] in a large sample of the Chinese population (1686 gastric cancer patients and 1895 cancer-free subjects). Their logistic regression suggested a significant association between rs2910164 polymorphism and increased gastric cancer risk (OR = 1.26, 95%CI = 1.01-1.56; GG *vs* CC + CG]. Apparently, these conclusions of the relevant studies are inconsistent, in part because of the small sample size, and the different genetic model and ethnicity of the patients. So, we performed a comprehensive analysis based on published data using a recessive model (Table 4). The combined results indicated that subjects homozygous for the variant C genotype in miR-146a had no statistically significant risk of gastric cancer when compared with (CC + CG) genotypes carrier (OR = 1.08, 95%CI: 0.87-1.33, Z = 0.68, P = 0.50; Q = 10.13, P = 0.04).

CONCLUSION

This review includes a summary and discussion of the current findings evaluating the role of SNPs of miRNA in gastric cancer occurrence and development. Furthermore, we performed a meta-analysis of the most frequently studied miRNA-SNPs (rs11614913 in miR-196a-2, rs895819 in miR-27a and rs2910164 in miR-146a) in gastric cancer.

Table 4 MiR-146a single-nucleotide polymorphism (rs2910164) in gastric cancer

First author year	Study design	Case/control	Case			Control			Risk[1] (OR and 95%CI)
			CC	CG	GG	CC	CG	GG	
Okubo *et al*[41] 2010	HB	552/697	236	243	73	254	322	121	0.73 (0.53-0.99)
Zeng *et al*[17] 2010	HB	304/304	89	153	62	119	132	53	1.21 (0.81-1.82)
Hishida *et al*[56] 2011	HB	583/540	230	271	82	215	254	71	1.08 (0.77-1.52)
Ahn *et al*[50] 2012	HB	461/447	159	231	71	164	221	62	1.13 (0.78-1.63)
Zhou *et al*[57] 2012	HB	1686/1895	286	822	578	393	951	551	1.27 (1.10-1.47)
Overall		3586/3913	1000	1720	866	1145	1880	858	1.08 (0.87-1.33)

[1]OR and 95%CI were calculated under a recessive genetic model (GG *vs* CC + CG). HB: Hospital based.

Based on current data, there was no significant association between these miRNA-SNPs and gastric cancer risk in our meta-analysis. Future studies based on larger samples and independent cohorts are still needed to confirm the predictive signature of miRNA-SNPs in gastric cancer, for a better understanding of gastric cancer carcinogenesis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Molecular diagnosis and treatment of drug-resistant hepatitis B virus

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Abstract

Oral antiviral agents have been developed in the last two decades for the treatment of chronic hepatitis B (CHB). However, antiviral resistance remains an important challenge for long-term CHB therapy. All of the clinically available oral antiviral agents are nucleoside or nucleotide analogues that target the activity of viral reverse transcriptase (RT), and all are reported to have resistant mutations. Since the hepatitis B virus (HBV) RT, like other viral polymerases, lacks proofreading activity, the emergence of drug-resistance occurs readily under selective pressure from the administration of antiviral agents. The molecular diagnosis of drug-resistant HBV is based on sequence variations, and current diagnostic methods include sequencing, restriction fragment polymorphism analysis, and hybridization. Here, we will discuss the currently available molecular diagnosis tools, *in vitro* phenotypic assays for validation of drug-resistant HBV, and treatment options for drug-resistant HBV.

Key words: Hepatitis B virus; Drug-resistance; Molecular diagnosis; Antiviral treatment; Chronic hepatitis B

Core tip: Although several antiviral agents have been developed in the last two decades for the treatment of chronic hepatitis B (CHB), antiviral resistance remains an important challenge for long-term CHB therapy. In this review, we discussed the currently available molecular diagnosis tools, *in vitro* phenotypic assays for validation of drug-resistant hepatitis B virus (HBV), and treatment options for drug-resistant HBV.

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INTRODUCTION

Chronic hepatitis B (CHB) affects 240 million people worldwide and is a leading cause of liver-related morbidity and mortality[1-3]. The last two decades have seen the introduction of oral antiviral agents for the treatment of hepatitis B virus (HBV) infection[4-8]. Long-term antiviral therapy is needed in the majority of patients, and incomplete viral suppression and emergence of drug resistance is a major concern[9]. All of the clinically available HBV drugs are nucleoside or nucleotide analogues that inhibit the activity of viral reverse transcriptase (RT), and all drugs approved as anti-HBV agents are reported to have viral resistance due to specific mutations in the

RT domain[10]. In treatment-naïve patients, the prevalence of such mutations is low and routine mutation analysis is not recommended[11]. The emergence of resistant strains is due to the selective pressure of the therapeutic regimen, although other factors, such as host immune response and therapy adherence, also play a role[12]. The development of antiviral resistance is one of the most important factors predicting the success or failure of CHB treatment[13]. The emergence of antiviral resistance results in the resumption of active viral replication that has been previously suppressed after the initiation of antiviral therapy, and can impair biochemical or histologic improvement[14]. Furthermore, increasing use of antiviral agents for CHB has led to a greater likelihood of antiviral resistance[12]. A recent prospective cohort study showed that antiviral drug resistance increased the risk of hepatocellular carcinoma in decompensated HBV-related cirrhotic patients, especially in those failed rescue therapy[15]. To avoid development of drug resistance, the current guidelines recommend tenofovir (TDF) and entecavir (ETV) as first-line antiviral agents[13,16-18].

In this review, we will discuss methods of diagnosis, phenotypic assays for drug resistance, and treatments for drug-resistant HBV strains.

MOLECULAR MECHANISM OF ANTIVIRAL RESISTANCE

The HBV RT, like other viral polymerases, lacks proofreading activity on the newly synthesized viral genome, resulting in the introduction of random mutations into progeny HBV DNA. The error rate of HBV polymerase is approximately 1 per 10^5 to 10^7 base syntheses due to the highly error-prone nature of the HBV RT[19]. Under selective pressure from the administration of antiviral agents, HBV quasispecies converge on a dominant HBV mutant that can escape selection pressure, producing a drug-resistant HBV strain.

Since the molecular mechanisms and clinical significance of resistance to HBV nucleoside/nucleotide analogue drugs have been extensively reviewed in literature[10,20-22], detailed explanations of the documented resistant mutants will not be reviewed here.

Lamivudine (LMV), a synthetic nucleoside analogue with activity against HBV and HIV, is sequentially phosphorylated to LMV triphosphate by cellular kinases and incorporated into the growing HBV DNA at the 3'-end by HBV polymerase, which induces premature chain termination. The primary resistance mutation to LMV is the rtM204I/V in the YMDD motif. This mutation is usually accompanied by a compensatory mutation including rtL180M, L80I/V, and V173L, which enhances the viral replication of replication-defective rtM204I/V mutants (Figure 1). LMV resistance does not confer cross-resistance to adefovir (ADV) or TDF[20,21].

ADV dipivoxil, an analogue of adenosine monophosphate, can be easily phosphorylated by cellular kinases to the active metabolite ADV diphosphate, which inhibits HBV DNA polymerase by competing with the natural substrate deoxyadenosine triphosphate. The incorporation of ADV diphosphate into the growing viral DNA causes premature DNA chain termination similar to LMV. Genotypic analysis has revealed that ADV resistance is conferred by the rtN236T and/or rtA181T/V mutations. *In vitro* drug susceptibility assays showed that the rtN236T mutation does not affect sensitivity to LMV, telbivudine (LdT), or ETV; however, the rtA181T mutation was shown to decrease susceptibility to LMV (< 10-fold), ADV (2- to 8-fold), and TDF (2- to 3-fold)[32].

ETV, a guanosine nucleoside, is efficiently phosphorylated to the active triphosphate form. By competing with the natural substrate deoxyguanosine triphosphate, ETV triphosphate functionally inhibits the activities of HBV polymerase. ETV is the most potent among the currently available anti-HBV agents. The mutations associated with primary resistance to ETV are the most complex and have not been fully established in patients. Mutations associated with the emergence of ETV resistance have been mapped to the B domain (rtI169T, rtL180M, and rtS184S/A/I/L/G/C/M), C domain (rtM204I/V and rtS202G/I), and E domain (rtM250I/V). ETV resistance does not confer cross-resistance to ADV or TDF[22].

LdT, a synthetic thymidine nucleoside, is the unmodified L-isomer of the naturally occurring nucleoside, thymidine; therefore, phosphorylation to the active LdT triphosphate form by cellular kinases is easily accomplished. The LdT 5'-triphosphate eventually inhibits HBV DNA polymerase by competing with the natural substrate, thymidine 5'-triphosphate. The rtM204I substitution confers primary resistance to LdT treatment and frequently co-occurs with the rtL80I/V and rtL180M substitutions. An *in vitro* study revealed that LdT resistance does not confer cross-resistance to ADV, TDF, or ETV[33].

Clevudine (CLV), a pyrimidine analogue, inhibits HBV polymerase by competing with the natural substrate, thymidine. CLV inhibits the DNA-dependent DNA activity of HBV polymerase, as well as reverse transcription and priming. Since CLV is a fluorinated LdT, it has a similar resistance profile to LMV and LdT. Kwon *et al*[23] identified the rtM204I substitution as the most common mutation during viral breakthrough in four CLV-failure patients, whereas rtL229V was shown to be a compensatory mutation for the impaired replication of the rtM204I mutant. A quadruple mutant (rtL129M+rtV173L+rtM204I+rtH337N) conferred greater replicative ability and strong resistance to both CLV and LMV[23].

TDF disoproxil fumarate is a methyl derivative of ADV with activity against retroviruses, including HIV-1/2 and HBV. TDF demonstrates a mechanism of action and antiviral resistance pattern very similar to ADV. Like ADV, TDF is rapidly metabolized by cellular kinases into the active metabolite, TDF diphosphate. In turn, TDF diphosphate inhibits HBV DNA RT by competing with the natural substrate, deoxyadenosine triphosphate, causing the termination of the growing HBV DNA. *In vitro*

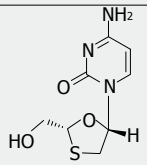
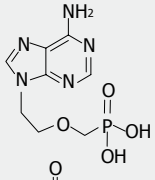
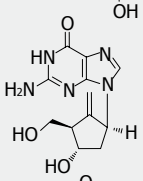
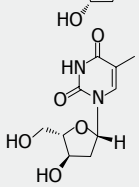
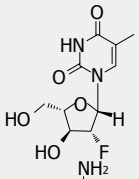
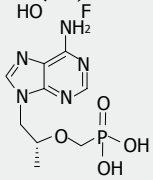
Molecule	Structure	Brand name	Company	Year of FDA approval	*In vitro* IC_{50} (μmol/L)[23]	Resistant mutations	5 yr cumulative resistance rate[25-31]
Lamivudine		Zeffix Heptovir Epivir Epivir-HBV	GlaxoSmithKline	1998 (for adults) and 2000 (for children) in United States	3.3[23]	rtL80V/I rtV173L rtL180M rtM204I/V	70%
Adefovir		Preveon Hepsera	Gilead	2002 (United States)	6.3[23]	rtA181T/V rtN236T	29%-65%[57]
Entecavir		Baraclude	Bristol Meyers Squibb	2005 (United States)	0.01[23]	rtI169T rtL180M rtT184S/A/I/L rtS202G/I rtM204I/V rtM250I/V	1.2%-1.5%
Telbivudine		Sebivo (Europe) Tyzeka (United States)	Idenix, Novartis	2006 (United States)	0.19[24]	rtA181T/V rtM204I	34% (3 yr)
Clevudine		Levovir Revovir	Bukwang Pharm	2006 (South Korea)	0.9[23]	rtL180M rtM204I/V	30% (3 yr)
Tenofovir		Viread	Gilead	2008 (United States)	2.5[23]	rtA181T/V rtA194T rtN236T	0%

Figure 1: Approved anti-hepatitis B virus drugs and their resistant mutations. IC_{50} values are dependent on the duration of drug exposure to cells, the cells used, and the protocol used.

drug susceptibility assay demonstrated that the rtA194T, rtA181T/V, and/or rtN236T mutations are associated with TDF resistance[22]. Resistance to TDF has been proven so far in *in vitro* studies.

MOLECULAR DIAGNOSIS OF DRUG-RESISTANT HBV

The molecular diagnosis of drug-resistant HBV is performed by genotypic assay, which determines the resistance-related mutations in the viral RT gene by comparing the patient-derived viral strain with wild-type. To date, several methods have been developed and clinically used to determine genotypic resistance. These methods include sequencing (PCR-based direct sequencing, cloning and sequencing, and ultra-deep pyrosequencing), restriction fragment length or mass polymorphism (RFLP or RFMP), and hybridization (DNA microarray and line-probe assay). Since the conventional methods for HBV genotyping have been extensively reviewed in literature[20,22], only those technologies that have received lesser attention elsewhere, such as ultra-deep pyrosequencing and RFMP, will be reviewed here in detail.

PCR-based direct or cloning sequencing

Currently, genotypic analysis is largely performed by sequencing-based assays. Only sequencing can provide all of the information on the mutations present in a viral genome. Therefore, this assay is useful to identify novel resistant mutations responsible for the insufficiency of new antiviral drugs[23]. However, limited by sensitivity, this assay can only detect the majority species present in the total viral population and is generally capable of detecting quasispecies comprising more than 20 percent of a viral population. This limitation can be overcome by multiple rounds of cloning followed by sequencing. This method is impractical for use in large cohort studies or clinical laboratories since the process is hard to standardize, labor-intensive, and time-consuming. However, cloning-based sequencing is the only method available to analyze the colocalization of mutations within the same HBV genome.

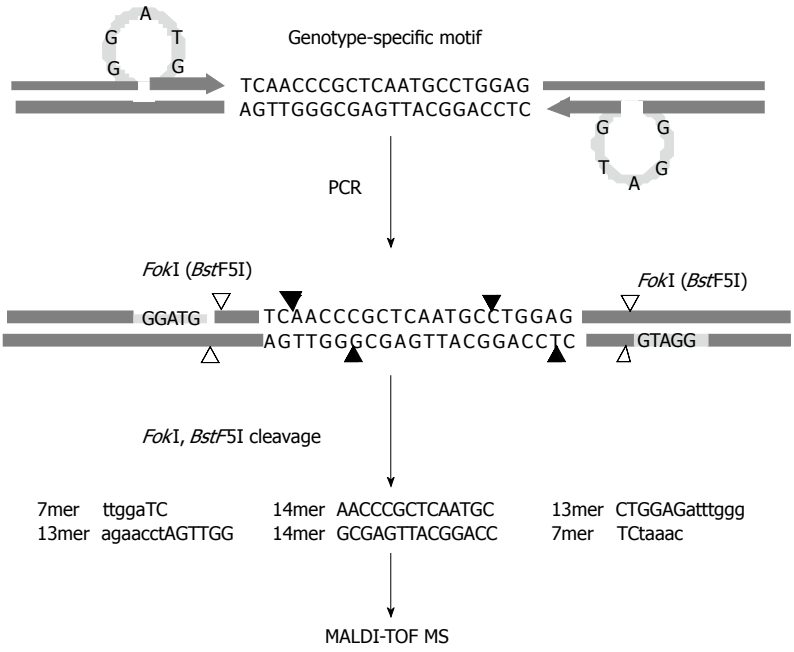


Figure 2 Schematic diagram of the restriction fragment mass polymorphism genotyping strategy. Polymerase chain reaction is performed with primers designed to introduce a type ⅡS restriction endonuclease recognition sequence (*Fok*I) ahead of the genotype-specific motifs upon amplification. The enzymatic cleavage of the products leads to excision of multiple oligonucleotide fragments representing the motifs shown in capital letters, and then the masses of the resulting oligonucleotide fragments are examined by MALDI-TOF MS. Cleavage sites of *Fok*I and *BstF*5I, an isoschizomer of *Fok*I, are indicated by filled and blank arrows, respectively, and recognition sites for both restriction endonucleases are specified by the shaded bars.

Ultra-deep pyrosequencing

Recently, ultra-deep pyrosequencing, a next-generation sequencing technology, was successfully applied to detect HBV quasispecies and also to identify drug-resistant mutants that present at very low concentrations in patient sera[34-37]. This is one of the most sensitive techniques capable of detecting minority virus populations that are less than 0.1% of the total, and that are typically missed by direct or cloning-based sequencing. However, application of this technology in determining drug resistance is limited by its high cost. The one advantage of this next-generation sequencing technique is the production of vast quantities of sequence data without prior information on the sequence of interest. Very recently, Rodriguez *et al*[37] generated approximately 480000 sequences (4010 ± 843 sequences per sample) from ADV-resistant patient sera by ultra-deep pyrosequencing. They found that the dynamics of ADV-resistant viral populations are very complex and more heterogeneous than expected. More importantly, the identified ADV-resistant variants (including rtA181 and rtN236) were already present as minor populations at baseline in most of the treatment-naïve patients who subsequently developed viral DNA breakthrough to ADV therapy.

Restriction fragment mass polymorphism

RFMP technology is based on detection of the mass difference in DNA fragments resulting from drug-resistant mutations in the RT gene[38]. Mass spectrometry generates precise information of the molecular mass of the analytes and enables quantitation of both strands of DNA in parallel using a fully automated procedure. Mass spectrometry directly assesses the nature of the PCR products, whereas other technologies indirectly measure the PCR products either through hybridization or by sequencing reactions.

The assay is based on the amplification and mass detection of oligonucleotides excised using a type ⅡS enzyme digestion and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) as depicted in Figure 2. PCR is performed with primers designed to introduce a type ⅡS restriction endonuclease recognition sequence ahead of the polymorphism site. The use of a type ⅡS restriction enzyme means that this assay does not depend on the fortuitous occurrence of restriction sites, because these enzymes typically have cleavage sites distal to their recognition sites. The enzymatic cleavage of the reaction products leads to excision of multiple oligonucleotide fragments containing the mutated motifs, after which the masses of the resulting oligonucleotide fragments are examined by MALDI-TOF MS. Differences are observed as the presence, absence, or mass change of peaks corresponding to fragments affected by the existence of polymorphisms, including substitutions, deletions, and insertions in the RT gene[38]. In addition to its speed and high-throughput capacity, the RFMP assay is very sensitive and can detect drug-resistant mutants that constitute less than 1% of a total virus population, enabling distinctions between mixed-genotype samples[39]. A clear correlation has been observed between estimated peak heights and real proportions in mixed-genotype pools, indicating the RFMP assay enables better quantitative detection of mixed populations without the need for population-based cloning

and subsequent sequencing. By combining the merits of unique assay chemistry and the established techniques of MALDI-TOF MS, the RFMP assay is able to screen for viral mutants in a robust, high-throughput manner and is known to be capable of simultaneously analyzing 384 samples in 3 h, which is almost 10 times faster than existing methods[40]. The improved sensitivity of the RFMP assay has allowed for its application in monitoring early intervention and prevention in antiviral therapy for HBV. An RFMP analysis of YMDD motifs within 740 consecutive samples collected from 116 hepatitis B patients demonstrated that YMDD mutants occur throughout the course of LMV therapy irrespective of the occurrence of viral DNA breakthrough. This indicates that the mere detection of YMDD mutants is not sufficient to predict viral DNA breakthrough, although the presence of YMDD mutants has been associated with a high incidence of viral DNA breakthrough, and a five-fold predominance of YMDD mutant-to-wild-type virus was significantly associated with viral DNA breakthrough. Periodic testing by RFMP assay was shown to be useful in detecting the predominance of YMDD mutants for monitoring drug resistance, enabling early intervention and prevention of viral breakthrough[40].

Multiplex RFMP methods were developed to have practical advantages over existing methods, enabling the simultaneous detection of several resistant mutations within a large number of samples, and to address the dynamics and evolution of resistance and the relationship between viral genotypes and clinical outcomes[41,42]. This assay has proven to be reliable for clinical virus genotyping in concert with regular measurements of HBV viral load, for the early detection of LMV, ADV, ETV, and MDR mutations, and for the timely introduction of new salvage agents[43-45]. However, RFMP technology cannot provide information on the colocalization of mutations within the same HBV genome and requires access to a mass spectrometer.

Hybridization-based assays

Hybridization-based assays rely on affinity differences between amplified nucleotides harboring mutations and wild-type viral sequences. These techniques include the line-probe (LiPA) assay[46] and DNA microarray[47]. The commercially available LiPA assay (Innogenetics, Belgium) can detect single nucleotide mismatches and discriminate HBV resistance mutations within minor fractions constituting 5% or more of the total viral population[39,46]. DNA chip microarrays can simultaneously detect multiple resistant mutations with relatively low labor and cost[47].

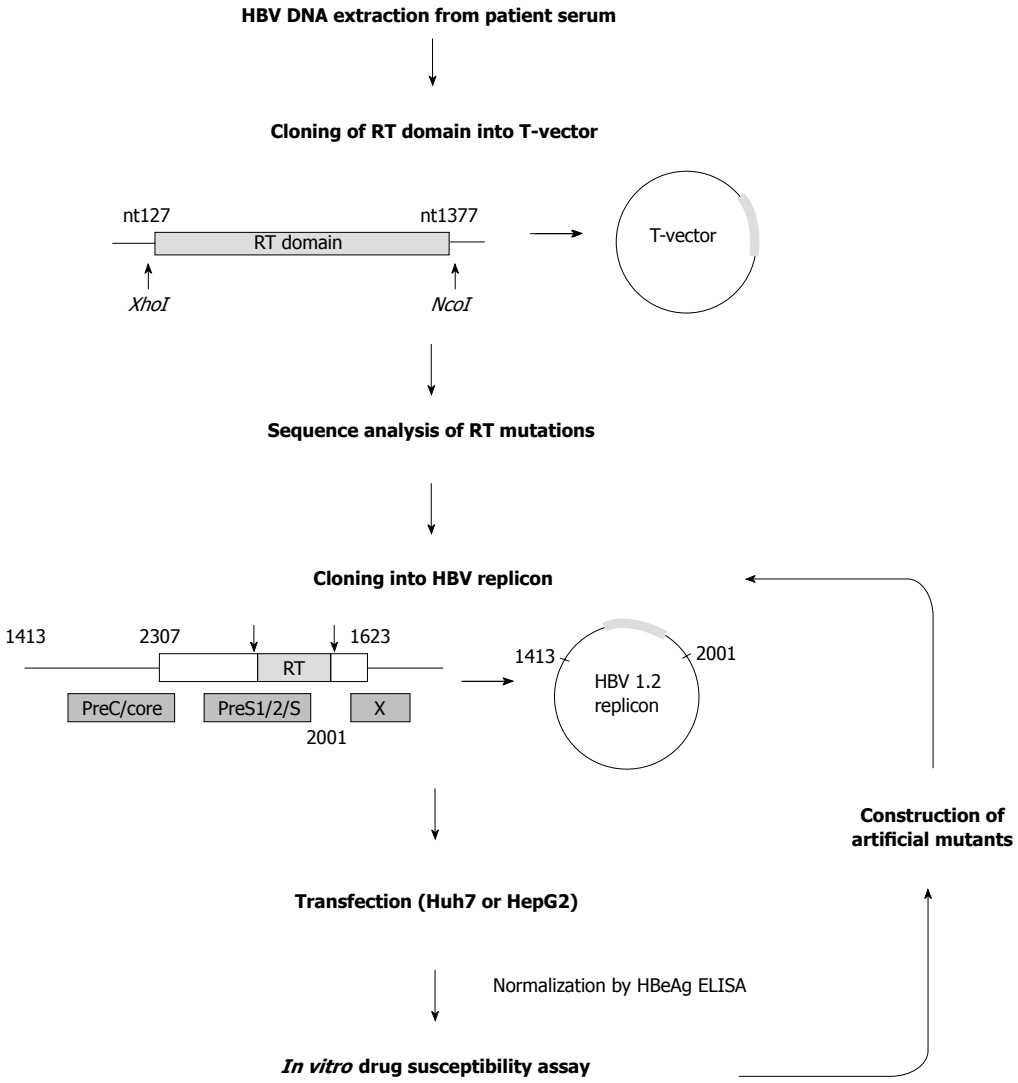
Although hybridization techniques are generally reliable, the main limitations of all hybridization-based assays arise largely from their relatively low specificity and from the fact that they are qualitative rather than quantitative. Moreover, the design and optimization of new sets of specific probes are required for every mutant in order to detect a single nucleotide change because mutations in neighboring regions affect the sensitivity of the target sequence.

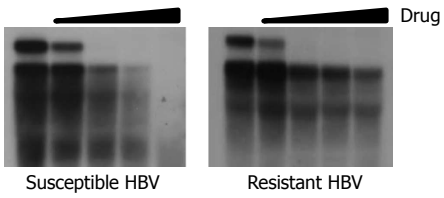
IN VITRO PHENOTYPIC ASSAY FOR VALIDATION OF DRUG-RESISTANT HBV

Only an *in vitro* phenotypic assay can confirm genotypic antiviral resistance. However, current methodology is labor-intensive and time consuming due to the need for construction of a replication-competent HBV replicon and the use of Southern blot analysis. Although the detailed methods for the validation of drug-resistant HBV may vary slightly based on approach, the basic concept is identical[48-50]. A typical approach to analyze phenotypic resistance is presented in Figure 3. The HBV genome is isolated from patient sera, and the RT mutation sequence is analyzed. To assess whether the mutations are colocalized within the same HBV genome and to select a specific RT mutant more easily, the PCR products are subcloned into a T-vector. After sequence analysis, the selected RT mutants are once again cloned into an HBV replicon such as an HBV 1.1mer, 1.2mer, 1.5mer, or dimer. For this procedure, the *Xho*I and *Nco*I restriction sites are preferentially used for insertion into both the T-vector and the replicon. The replicons are then transfected into Huh7 or HepG2 hepatocyte cell lines. Secreted HBeAg is considered to normalize transfection efficiency. Secreted HBsAg cannot be used as a control since the RT and surface genes overlap, and, thus, mutations in the RT gene can affect the antigenicity of HBsAg. Four to five days after treatment with antiviral drugs, cell lysates are subject to Southern blot analysis, which gives the most accurate result. Since drug potency varies, the preferred drug concentrations are indicated in Figure 3. After quantification of replication ability using a PhosphorImager imaging system, the IC_{50} (μmol/L) for each drug is calculated by interpolation. Finally, the fold resistance (R factor) is determined. By doing this, it is possible to determine the presence of drug-resistant mutations in the quasispecies contained in clinical isolates. To identify the sequence elements responsible for drug-resistance, artificial replicons that harbor conserved mutations in clinical isolates need to be constructed and tested for drug susceptibility. An example of the overall characterization of drug-resistant HBV was recently reported, including detailed methods[23]. Alternatively, real-time PCR can be used to measure replication ability, an approach that is suitable for automated and large-scale testing in a hospital setting[51].

TREATMENT

The development of drug resistance is associated with virological breakthrough, biochemical breakthrough, and, sometimes, hepatic decompensation[52]. Furthermore, resistance may also reverse histological improvement and oppose the reduction in disease progression among patients with advanced fibrosis and early cirrhosis[53].





Drug	Preferred drug concentrations (μmol/L)			
LMV	1	10	20	100
ADV	1	10	20	100
CLV	1	10	20	100
TDF	1	10	20	100
ETV	0.05	0.5	1	5

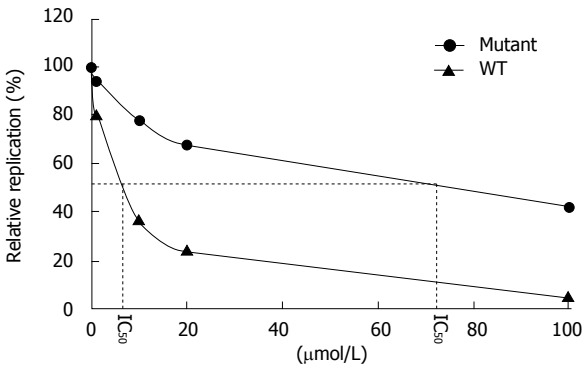


Figure 3 Scheme for *in vitro* phenotypic validation of drug-resistant hepatitis B virus. Hepatitis B virus (HBV) DNA is purified from patient serum, and the sequence of RT mutations is analyzed. After cloning into replication-competent HBV replicons, each mutant is transfected into hepatoma cell lines followed by Southern blot (or real time polymerase chain reaction) analysis. The IC_{50} (μmol/L) value is obtained by quantification of replication ability and curve-fitting. To characterize the specific mutation(s) conferring resistance to antiviral drugs, each artificial mutant must be constructed and individually tested.

LMV is not recommended as a first-line treatment due to high resistance development. Currently, the newer and potent drugs, TDF and ETV are recommended[13,16-18]. Several practice guidelines suggest strategies for treatment of CHB patients with resistance. The American Association for the Study of Liver Diseases guidelines were published in 2009[18]. The updated guidelines from the European Association for the Study of the Liver,

Table 1 Guidelines for treatment of resistance

Resistant drug	AASLD 2009[18]	EASL 2012[16]	APASL 2012[17]	KASL 2012[13]
LMV	Add ADV or TDF Stop LMV, switch to Truvada (TDF + FTC)	Switch to TDF Add ADV (If TDF not available)	Add ADV Switch to TDF Switch to IFN-based therapy	Add ADV or TDF Stop LMV, switch to ADV or TDF + other nucleoside analogues Switch to TDF Stop LMV, consider to switch with Peg IFN
ADV	Add LMV Stop ADV, switch to Truvada Stop ADV, switch to or add ETV	NA-naïve; Switch to ETV or TDF Prior LMV-resistance; Switch to TDF + a nucleoside analogue	Add LMV, LdT, or ETV Switch to TDF Switch to IFN-based therapy	With prior LMV resistance, -Stop ADV, switch to TDF + other nucleoside analogues -Add ETV 1mg ADV as a first-line therapy -Stop ADV, switch to TDF + other nucleoside analogues -Add other nucleoside analogue, if rtA181T, add ETV
ETV	Switch to TDF or Truvada	Switch to TDF Add TDF Add ADV (If TDF not available)	Add TDF or ADV Switch to IFN-based therapy	Add nucleotide analogue
LdT	Same treatment as LMV	Switch to TDF Add TDF Add ADV (If TDF not available)	Same treatment as LMV	Same treatment as LMV
CLV				Same treatment as LMV
TDF		Add ETV, LdT, LMV or FTC (LMV-naïve; Switch to ETV/ prior LMV-resistance; Add ETV)		
Multidrug-resistance		Combination of a nucleoside and a nucleotide (preferably TDF)	ETV + TDF Switch to IFN-based therapy	TDF + ETV 1 mg ADV + ETV 1 mg
Comment	Update is required especially for multidrug-resistance	Recommendation about IFN-based therapy is limited	Recommendation about TDF resistance is limited	Recommendation about TDF resistance and IFN-based therapy is limited

KASL: Korean Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; APASL: Asian Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver; LMV: Lamivudine; ADV: Adefovir; LdT: Telbivudine; CLV: Clevudine; TDF: Tenofovir; FTC: Emtricitabine; ETV: Entecavir; IFN: Interferon.

the Asian Pacific Association for the Study of the Liver, and the Korean Association for the Study of the Liver were published in 2012[13,16,17]. The main principle of these guidelines is to choose antiviral agents without cross-resistance and to begin rescue therapy as soon as possible[13,16,17]. These guidelines are compared and summarized in Table 1.

LMV, LdT, and CLV resistance

LMV, LdT, and CLV are L-nucleoside analogues, and mutations at rtM204 are considered the primary cause of resistance to these agents[20,54,55]. rtM204I or rtM204V, with or without rtL180M mutations, are sensitive to ADV and TDF, but exhibit cross-resistance to ETV and show an eight-fold decrease in sensitivity. The rtA181T mutation has been detected in 5% of LMV-resistant patients. These mutants exhibit cross-resistance to ADV but remain sensitive to ETV[56].

Of the various forms of antiviral drug resistance, treatment for LMV resistance has been the most widely studied. The incidence of the rtM204I/V substitution has reportedly increased from 24% in 1 year to 70% in 5 years[25,54]. ADV monotherapy is not recommended due to the increased risk of ADV resistance, which has been shown to manifest in 18% of patients at 1 year, 25% at 2 years, and up to 65% after 5 years[57-59]. ADV add-on therapy is accepted as a first-line rescue therapy[60]. Many studies have shown that LMV-ADV combination therapy is superior to ADV monotherapy[61-63]. However, the LMV-resistant strain rtA181T has been reported to be continuously detected even after combination therapy with LMV-ADV, so caution is necessary to avoid the possibility of multidrug-resistant HBV[32,64,65]. LdT and ADV combination therapy, or a combination of ETV and ADV, are possible treatment options[66-68].

TDF has shown potent antiviral activity against LMV-resistant HBV and is reportedly superior to ADV monotherapy[69-71]. Therefore, treatment strategies that include TDF seem to be more effective than those involving ADV[13]. One study showed that switching to TDF is as effective as adding TDF to LMV[71]. However, there is one report of TDF resistance in an LMV-resistant CHB patient with HIV co-infection who received TDF monotherapy, so the efficacy of TDF monotherapy requires further evaluation[72]. One recent study also found that the reduction in HBV DNA levels was greater in a TDF-LMV combination therapy group than in TDF monotherapy, ADV monotherapy, or LMV-ADV combination groups[73].

ETV exhibits some cross-resistance with LMV, which has prompted the administration of 1.0 mg doses to LMV-resistant CHB patients[74]. However, ETV resis-

tance was reportedly more frequent than in treatment-naïve patients. A 5-year cumulative genotypic resistance rate of 51% was reported, along with an accompanying virologic breakthrough of 43%[27]. A recent study found that LMV-ADV combination therapy showed superior antiviral efficacy over ETV 1.0 mg monotherapy in LMV-resistant CHB patients[75]. For these reasons, ETV 1.0 mg monotherapy is not recommended.

Peginterferon (PegIFN) is another option for the treatment of LMV-resistant CHB patients. A randomized control trial showed that 48 wk of PegIFN alfa-2a treatment for LMV-resistant CHB patients achieved undetectable HBV DNA levels in 10.6% of patients[76]. Another study showed similar efficacy of PegIFN alfa-2a in a comparison between treatment-naïve patients and LMV-resistant patients with HBeAg-positive CHB[77].

Few data related to LdT and CLV resistance are available. The 2-year risk of LdT resistance was shown to be 25.1% in HBeAg-positive patients and 10.8% in HBeAg-negative patients[78]. In the subgroup that had no genotypic resistance at year 2 and received LdT until year 3, an incremental 1.0% of HBeAg-positive, and 2.1% of HBeAg-negative patients developed genotypic resistance to LdT[79]. The cumulative resistance rate of LdT is 34% after 3 years[29]. In the case of LdT resistance, a switch to, or addition of TDF may be the preferred option[20]. The CLV resistance rate is approximately 20% and 30% in the 2nd and 3rd years of treatment, respectively[30,80]. According to clinical experience with LMV-resistant CHB patients, the general principles for treating LdT or CLV resistance are similar to those of LMV-resistance[13,17].

ADV and TDF resistance

ADV and TDF are nucleotide analogues. rtN236T and rtA181T/V are the primary mutations giving rise to ADV resistance[26,81]. The cumulative incidence of genotypic resistance to ADV is reported to be 0%, 3%, 11%, 18%, and 29% at the end of each successive year of therapy in HBeAg-negative patients[26]. As previously mentioned, ADV monotherapy in LMV-resistant patients leads to a 5-year cumulative ADV resistance of 65.6%[57]. No TDF resistance has been reported during treatment periods up to 3 years in length[82]. However, rtA181T/V and rtN236T mutations confer intermediate resistance to TDF[9]. Among patients who have the rtA181T/V and/or rtN236T substitutions, viral suppression by TDF is reduced[71,83]. rtA194T can decrease susceptibility to TDF by 10-fold in the presence of rtL180M+rtM204V mutations, according to a case study of a patient with HBV and HIV co-infection[72].

TDF significantly suppresses HBV replication in patients exhibiting LMV resistance who have failed to respond adequately to ADV, and in patients who are resistant to both LMV and ADV[83]. TDF alone or TDF plus emtricitabine (FTC) are similarly effective in ADV-treated CHB patients[84,85]. However, reduced sensitivity to TDF has been demonstrated in ADV-resistant HBV infections, indicating potential cross-resistance[71]. The addition of FTC led to a further decrease in serum HBV DNA levels in patients exhibiting ADV resistance and a suboptimal response to TDF therapy[86]. When LMV-TDF combination therapy was given to CHB patients who had previously failed to respond to LMV and subsequent ADV therapy, 64% achieved an undetectable level of HBV DNA after 96 wk of treatment[83].

ETV does not share cross resistance with ADV[56]. ETV has been shown to be effective against both rtA181T/V and rtN236T mutant HBV strains[32,43,87,88]. Switching to ETV monotherapy (1 mg daily) is initially effective in LMV-resistant patients (rtM204I/V), but the subsequent risk of ETV resistance is high[27]. Another study also showed increased ETV resistance risk with ETV monotherapy switching for ADV resistance[89]. In contrast, ADV-ETV combination therapy has been shown to be a better option[13,90]. The ETV-TDF combination can also be considered for multidrug-resistant HBV infections that include ADV resistance[13].

Although rtN236T mutants remain sensitive to LMV, the rtA181T/V mutant exhibits reduced susceptibility to LMV[56] Therefore, LMV-ADV combination therapy is recommended. For similar reasons, LdT or CLV monotherapy is not recommended for the rtA181T/V mutant in order to avoid cross-resistance[13,56], and add-on therapy is preferred[13,17]. One study showed that the LdT and ADV combination can be a good option for ADV resistance[91].

To date, there are no reports of resistance to TDF among patients with CHB monoinfection[31,92-94]. An *in vitro* study found that replication of the rtA194T mutant was suppressed effectively by ETV and intermediately by LdT[95]. The clinical impact of the rtA194T mutation is still unknown[92,96]. One recent report suggested that rtP177G and rtF249A mutants also reduce susceptibility to TDF[97]. In cases of confirmed TDF resistance, an add-on combination with a nucleoside analogue is preferred, while a switch to ETV may be sufficient if the patient had no prior LMV resistance[16].

ETV resistance

ETV is cyclopentene, a type of nucleoside analogue. ETV resistance develops *via* a two-hit mechanism, as previously described. Since this presents a high genetic barrier to ETV resistance, the resistance rate in treatment-naïve subjects is very low. In studies of the long-term follow-up of ETV treatment in CHB patients, the cumulative probability of ETV resistance was reportedly 1.2%-1.5% after 5 years of ETV treatment. However, a resistance rate as high as 51% has been reported after 5 years of treatment in LMV-refractory subjects[27,28,88].

ETV-resistant HBV maintains susceptibility to ADV, which could be considered an initial treatment option, and a clinical case has indicated that ADV can be effective in suppressing the ETV-resistant mutant[98,99]. Adding ADV to ETV would be more reasonable for reducing ADV resistance and improving antiviral efficacy[13,90,100]. Combination therapy of ADV plus LMV could be con-

sidered as another option, since a small study showed that the short-term efficacy of this combination was similar to that of combination therapy of ADV plus ETV[13]. Although TDF has not been fully evaluated in the treatment of ETV resistance, it is expected to be very effective since TDF does not show cross-resistance to ETV *in vitro* and has excellent potency[71]. Therefore, switching to or adding TDF may be preferred for addressing ETV resistance[20].

Multidrug resistance

Multidrug resistance (MDR) is defined as resistance to two or more classes of antiviral drugs[13]. The emergence of MDR is increasing and has raised serious concerns regarding antiviral therapy because it limits the selection of appropriate therapy[45]. Sequential monotherapy is associated with the development of MDR[22,98,101,102]. In these situations, pre-existing antiviral resistant mutations may reappear and become co-localized with newly developed resistant mutations in the same viral genome[98] The development of a triple-drug resistant (LMV, ADV, and ETV) HBV strain has been reported in LMV- and ADV-resistant patients after sequential ETV administration[103].

In patients with MDR, genotypic resistance testing is very useful, and a combination of a nucleoside and a nucleotide should be employed[16]. TDF-ETV combination therapy can be considered in case of resistance to both LMV and ADV[17,20]. A recent study showed that rescue therapy with TDF plus ETV achieved undetectable HBV DNA after a median of 6 mo in 51 (89.5%) of 57 patients, in whom nucleoside/nucleotide analogue therapy (LMV+ADV, ETV+ADV, or TDF+LMV) had failed and who had multidrug-resistant rtA181T/V or other MDR mutations[104].

If TDF is not available, combination therapy with ADV plus ETV is another option[13,90,100,105]. A recently published study showed that the ADV plus ETV combination is superior to the LMV-ADV combination or to ETV monotherapy for multidrug-resistant CHB patients[106]. On the other hand, the LMV plus ADV combination is usually insufficient for treatment due to low antiviral potency[45].

If resistant mutations to LMV, ETV, and ADV are detected in the same patient, combination therapy with TDF plus ETV may be the best option[13].

CONCLUSION AND PERSPECTIVES

Although ETV and TDF are powerful antiviral drugs with a high barrier to resistance, treatment failure due to resistance remains possible, especially among treatment-experienced CHB patients. The mechanisms of drug resistance have been evaluated, and many relevant diagnostic tools have been developed. Current CHB treatment guidelines suggest practical and effective strategies for resistance. However, there are still several treatment-related issues that need further evaluation. First, new diagnostic tools and *in vitro* phenotypic validation methods for drug-resistant HBV need to be developed that can identify mutations more efficiently, with greater accuracy, and with reduced cost. Second, the role of IFN must be evaluated further. A few studies suggest the possibility of PegIFN as a treatment option for LMV-resistant CHB patients. The efficacy of PegIFN in patients with resistance to other agents such as LdT, CLV, ADV, ETV, TDF, and MDR is not yet known. New IFN agents are also under development for hepatitis C virus treatment. These must also be studied for use in drug-resistant HBV infection. Third, the efficacy of new HBV agents in resistance treatment should be evaluated. For example, LB80830 is a new acyclic nucleotide phosphonate with chemistry similar to ADV and TDF. In a phase Ⅱ, open-label, multicenter study among 65 LMV-resistant patients, a dose-dependent reduction in HBV DNA of up to -3.92 log copies/mL was observed at week 12 at the optimal dose of 150 mg daily[107]. These findings clearly need further evaluation. Finally, treatment strategies for MDR should be established. Most guidelines suggest combination therapies of nucleoside and nucleotide analogues, and the ETV plus TDF combination is preferred. However, clinical data on long-term efficacy is still lacking. Furthermore, there are patients who have experienced treatment failure without a known or confirmed genotypic resistance. No treatment guidelines have been suggested for these patients. If more sensitive diagnostic tools are developed, the novel combinations of drug-resistant mutations may be diagnosed in these patients, which can give more treatment options for these patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Hepatitis B virus infection and intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (ICC) is a devastating malignant tumor arising from the peripheral intrahepatic bile duct epithelium. The incidence and mortality of ICC is markedly increasing over the past two decades worldwide, though the cause for this rise in incidence is unclear, thus intensifying the search for alternative etiological agents and pathogenetic mechanisms. Hepatolithiasis, primary sclerosing cholangitis, parasitic infection (*Opisthorchis viverrini* or *Clonorchis sinensis*), fibropolycystic liver disease, and chemical carcinogen exposure are thought to be the risk factors for ICC. Nevertheless, the majority of ICC patients do not have any of these risk factors, and none of the established risk factors can explain the recent increasing trend of ICC. Therefore, identifying other risk factors may lead to the prevention and early detection of ICC. Chronic hepatitis B virus (HBV) infection is the predominant cause of hepatocellular carcinoma in HBV-endemic areas. This review discusses the evidence implicating chronic HBV infection as a likely etiology of ICC and the pathogenetic mechanisms that might be involved.

Key words: Hepatitis B virus; Intrahepatic cholangiocarcinoma; Epidemiology; Etiopathogenesis

Core tip: Intrahepatic cholangiocarcinoma (ICC) is a devastating malignant tumor. Its incidence and mortality is increasing drastically over the past two decades worldwide, though the cause for this rise in incidence is unclear. The etiology and carcinogenesis of ICC remain inconclusive. Recent studies suggest that hepatitis B virus (HBV) infection plays an important etiological role in ICC development. HBV-associated ICC holds many clinicopathological similarities with HBV-associated hepatocellular carcinoma (HCC), and HBV-associated ICC patients may have a better prognosis than ICC patients without HBV infection. HBV-associated ICC and HBV-associated HCC may share a common disease process for carcinogenesis, through a similar long-term inflammatory carcinogenic process, and both possibly arise from hepatic progenitor cells.

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC), a bile duct carcinoma arising from either the second-order or more peripheral branches of the intrahepatic bile duct[1], occurs much less common than hepatocellular carcinoma (HCC). Although the incidence of ICC is very low, it is

the second most common type of primary liver cancer behind HCC, accounting for 10%-15% primary liver cancers and its incidence and mortality are increasing worldwide[2-7]. However, the etiology of ICC is largely unknown. Although several potential risk factors have been established, including chronic biliary tract diseases (*i.e.*, primary sclerosing cholangitis, hepatolithiasis, and Caroli's disease), parasitic infestation of the biliary tract by endemic *Opisthorchis viverrini* and *Clonorchis senensis*[8-10], and nonbiliary diseases such as heavy alcohol use, obesity, nonalcoholic fatty liver disease, chronic hepatitis C, and cirrhosis[11-15], ICC occurs mostly in the absence of these established etiological factors, and none of these risk factors can explain the recent increasing trend of ICC. Therefore, identifying other risk factors may lead to the prevention and early detection of ICC.

HBV is the prototype member of a family of small, enveloped DNA viruses called hepadnaviruses. Chronic HBV infection is the most common cause of HCC worldwide: more than 50% of global HCC cases and 70%-80% of HCC cases in highly HBV endemic regions, such as eastern Asia and sub-Saharan Africa, are attributable to HBV[16-18]. The etiopathogenesis of ICC was once considered to be independent of the presence of chronic HBV infection or HBV-associated cirrhosis, but some recent epidemiological data indicate a causal role for chronic HBV infection or HBV-associated cirrhosis in the development of ICC, particularly in HBV-endemic areas[19-25]. Nonetheless, the role of HBV and its carcinogenesis in the etiology of ICC remain unclear. This review focuses on the evidence and possible pathogenic mechanisms in support of a role for HBV in the etiology of ICC.

EPIDEMIOLOGICAL STUDIES OF ICC-ASSOCIATED HBV

Over the past two decades, there have been 15 epidemiological studies (13 case-control and two cohort studies) examining the relationship between HBV and ICC (Table 1). Of these, four were from China[20-22,26], four from the United States[11-13,27], two from China Taiwan[28,29], two from Japan[15,23], two from South Korea[19,30], and one from Italy[24].

Of the 13 case-control studies, two were population based, all from the United States; the eleven remaining studies were hospital based. Seven found a statistically significant positive association between serum hepatitis B surface antigen (HBsAg) and ICC (range of individual ORs, 2.3-9.67)[13,19-22,26,28], whereas the remaining studies did not[11,12,15,24,27,30]. Six of the seven studies reporting a positive correlation were performed in both regions of high prevalence of hepatobiliary cancers (*i.e.*, China, Japan, and South Korea), with one study in a region of lower prevalence (the United States). One study revealed a positive and significant association for patients with anti-HBc (hepatitis B core antibody), though the results did not reach significance for those with serum HBsAg alone[12]. Age and gender adjustments were reported in all the 13 case-control studies.

Two cohort studies have been conducted. The first retrospective cohort study from the province of Osaka in Japan included 154814 apparently healthy individual blood donors, aged 40-64 years at the time of blood donation in the period 1991-1993[23]. The average observation period was 7.6 years. Incident ICC cases were identified by linking the blood-donor database to the records in the population-based cancer registry for the province. There were 11 incident ICC cases during follow-up, with an incidence rate of 0.88 per 100000 person-years. Compared to those who tested negative for both HBsAg and hepatitis C antibody (anti-HCV), those who tested HBsAg-seropositive had a significantly higher risk for ICC (HR = 8.56; 95%CI: 1.33-55.20). These results suggest that HBV infection is independently associated with ICC development.

The second cohort study was from Taiwan[29] and included 1782401 pregnant Taiwanese women whose HBV serostatus was obtained from the National Hepatitis B Vaccination Registry. Newly diagnosed ICCs were ascertained through data linkage with the National Cancer Registry. Eighteen cases of ICC were recorded during a mean follow-up period of 6.9 years. The incidence rates of ICC were 0.09 and 0.43 per 100000 person-years among women who were HBsAg-seronegative and HBsAg-seropositive, respectively, showing an age-adjusted HR (HRadj) of 4.80 (95%CI: 1.88-12.20). The study also suggests that chronic HBV infection is associated with an increased risk of ICC.

META-ANALYSES OF ICC-ASSOCIATED HBV

To better elucidate a possible association between HBV and ICC, three recent meta-analyses have been conducted, with two focusing on risk factors for ICC alone. All of three studies were published in 2012; of these, two were from China[31,32] and one from The Mayo Clinic, United States[33].

The first meta-analysis by Li *et al*[31] included a total of 18 studies, 16 case-control studies and 2 cohort studies. The pooled risk estimate of all of the studies showed a statistically significant increased risk of cholangiocarcinoma among individuals with HBV infection [RR = 2.66; 95%CI: 1.97-3.60]; compared to those without HBV infection, persons with HBV infection had an increased risk of ICC (RR = 3.42; 95%CI: 2.46-43.74). In a subgroup analysis of HBV infection and risk of ICC, the pooled risk estimate of studies in Asians (RR = 3.63; 95%CI: 2.56-5.13) was higher than that in non-Asians (RR = 1.93; 95%CI: 0.78-4.76). A Begg funnel plot and Egger test revealed no evidence for publication bias.

The second meta-analysis was conducted by Palmer *et al*[33] from the United States. Nine case-control studies investigating hepatitis B as a risk factor from regions of both high and low prevalence of hepatobiliary cancers were selected. All of the studies, except those evaluat-

Table 1 Characteristic of case-control studies of hepatitis B virus infection and intrahepatic cholangiocarcinoma risk

Study	Study design	Dates	Cases		Controls		Source	OR (95%CI) for HBV infection (serum HBsAg, unless stated otherwise)
			Total	With exposure	Total	With exposure		
Donato *et al*[24], 2001	Case-control	1995-2000	26	13%	824	5.5%	Hospital	2.7 (0.4-18.5)
Yamamoto *et al*[15], 2004	Case-control	1991-2002	50	4%	205	2%	Hospital	1.84 (0.34-10.11)
Shaib *et al*[11], 2005	Case-control	1993-1999	625	0.2%	90834	0.2%	Population	0.8 (0.1-5.9)
Choi *et al*[30], 2006	Case-control	2003-2004	51	7.8%	51	9.8%	Hospital	0.8 (0.2-3.02)
Shaib *et al*[12], 2007	Case-control	1992-2002	83	1.2%	236	0.4%	Hospital	2.9 (0.1-236.8) anti-HBc 28.6 (3.9-1268.1)
Lee *et al*[19],2008	Case-control	2000-2004	622	13.5%	2488	5.0%	Hospital	2.3 (1.6-3.3)
Zhou *et al*[22], 2008	Case-control	2004-2006	312	48.4%	438	9.6%	Hospital	8.9 (5.97-13.19)
Tao *et al*[20], 2009	Case-control	1998-2008	61	27.9%	380	5.0%	Hospital	7.3 (3.1-17.2)
Lee *et al*[28],2009	Case-control	1991-2005	160	37.5%	160	13.8%	Hospital	4.99 (2.78-8.95)
Tanaka *et al*[23], 2010	Cohort	1991-1993		9.08%[1]		0.66%[2]	7.6 yr of follow-up	8.56 (1.33-55.20)
Zhou *et al*[21], 2010	Case-control	2003-2006	317	48.6%	634	6.6%	Hospital	9.67 (6.33-14.77)
Peng *et al*[26], 2011	Case-control	2002-2009	98	31.6%	196	12.8%	Hospital	2.75 (1.27-5.95)
Fwu *et al*[29], 2011	Cohort	1983-2000		0.43%[3]		0.09%[4]	6.91 yr of follow-up	4.80 (1.88-12.20)
Welzel *et al*[13], 2011 United States	Case-control	1993-2005	743	1.5%	195953	0.2%	Population	3.1 (1.43-6.58)
Chaiteerakij *et al*[27], 2013	Case-control	2000-2010	612	3.0%	594	3.0%	Hospital	

[1]Incidence rate of intrahepatic cholangiocarcinoma (ICC) per 100000 hepatitis B surface antigen (HBsAg)-seropositive person-years; [2]Incidence rate of ICC per 100000 HBsAg and anti-hepatitis C virus-seronegative person-years; [3]Incidence rate of ICC per 100000 HBsAg-seropositive woman-years; [4]Incidence rate of ICC per 100000 HBsAg-seronegative woman-years. HBV: Hepatitis B virus; Anti-HBc: Hepatitis B core antibody.

ing cirrhosis, diabetes, and obesity, exhibited significant heterogeneity. Of these, the authors excluded one study in which HBV infection was identified solely on the basis of anti-HBc positivity. In the other studies, HBV infection was defined by the presence of HBsAg (6 studies), HBV DNA in serum (1 study), or by ICD9 codes (1 study). The study data collection ranged from 1991 through 2008, encompassing a total study population of 294828 patients. The meta-analysis indicated that the presence of HBV was associated with a combined OR of 5.54, with a 95%CI of 3.19-9.63 for ICC. Five of the analyzed studies were performed in high-prevalence regions in Eastern nations, such as Japan, South Korea, and China, whereas three studies were from Western nations in low-to-intermediate prevalence regions, such as the United States and Italy. A separate analysis of these two groups did not reveal any significant difference between the two regions.

The final meta-analysis by Zhou *et al*[32] included a total of 16 studies: 3 cohort and 13 case-control studies. The combined risk estimate of all the studies showed a statistically significant increased risk of ICC incidence with HBV infection (OR = 3.17; 95%CI: 1.88-5.34). With regard to the case-control studies alone, the combined OR of HBV infection was 2.86 (95%CI: 1.60-5.11), whereas the OR of HBV was 5.39 (95%CI: 2.34-12.44) for the cohort studies. This meta-analysis further suggested that HBV infection is associated with an increased risk of ICC.

In conclusion, there is a large amount of epidemiological data in recent strongly supporting an association between HBV infection and ICC, particularly in HBV-endemic regions.

PATHOLOGICAL STUDIES OF ICC-ASSOCIATED HBV

To date, only four studies have been conducted to investigate the presence of HBV DNA, genes, and proteins in ICC pathology specimens; three of the four studies were from China and one from the United States. The earliest study was from China[34], published in 1998, and analyzed 30 ICC samples and the surrounding hepatic tissue. HBV antigen expression, including HBsAg, hepatitis B core antigen (HBcAg), hepatitis B X antigen (HBxAg), pre-S1, and pre-S2, was found in 12 of the 30 ICC cancerous tissues. Eight cases showed HBxAg positivity, five were positive for pre-S1, and five were positive for pre-S2; in contrast, no case was positive for HBsAg or HBcAg. Five types of probes were applied to detect S gene, pre S gene, C gene, X gene and HBV DNA in the cancerous tissues: HBV DNA was detectable in 23 of the 30 ICC cancerous tissues, and the HBV X (HBx) gene, pre S gene, S gene, and C gene were identified in 20, 13, 12, and 9 cases, respectively.

A second study evaluated 11 ICC samples, with surrounding tissue present for 10 cases[35]. Serum HBsAg was detected in 2 cases, and the 9 remaining cases included no data for serum HBsAg. Although no HBV DNA was detected in the 13 control cases that were seronegative for HBsAg, HBV DNA was detected in 2 (18.2%) of the 11 ICC livers. In 1 case, HBV DNA was detected in both the normal and tumor tissues, whereas it was detectable only in the normal tissue in the other case. In 1 case, the surface, core, and X genes and cccDNA were detected; only the surface and core genes and cccDNA were detectable in the other case. In both

cases, the copy numbers of HBV DNA were low, with less than 200 genome copies per microgram of total liver DNA. Nucleotide sequencing of the surface gene amplicon (672 bp) revealed infection with HBV genotype A in both cases, also revealing marked differences between the 2 cases of HBV genotype A.

The third study included 23 ICC cases between January 2002 and December 2008[36]. HBsAg seropositivity was found in 52.2% (12/23), HBV DNA (X region) in the liver was detectable in 34.8% (8/23), and HBV antigens in liver tissues were detected by immunohistochemistry in 30.4% (7/23). Only HBsAg was detectable, though one case was positive for HBcAg; both HBsAg and HBcAg were detectable in 5 cases. All cases with detected viral protein were also positive for HBV DNA.

The final study[37] included 45 ICC cases, all coexisting with HBV infection. The data collection ranged from December 2008 to April 2009. HBV infection was confirmed by the serological detection of HBsAg, and HBx protein expression was found in 70.4% (38/54) of the paraffin-embedded ICC tissue specimens.

The finding of HBV DNA, genes, HBV antigens, and HBx protein in ICC tissue specimens further supports a potential role for HBV infection in the development of ICC. Moreover, the finding of high rates of HBx protein suggests that HBx may play an important role in the pathogenesis of ICC, though it should be noted that all studies involved relatively small cohorts.

HBV AND CLINICOPATHOLOGICAL FEATURES OF ICC

For further elucidating the role of HBV in the development of ICC, some recent studies have investigated the impact of HBV infection on the clinicopathological features of ICC. A study from Taiwan showed that the mean age of hepatitis B-associated ICC patients (56.4 ± 11.1 years) was 9 years younger than that of hepatitis C-associated ICC patients (65.6 ± 9.17 years), similar to that observed in HCC. Moreover, the profiles of the age distribution between ICC and HCC patients with hepatitis B were essentially consistent[28]. The data from our previous study also demonstrated that the age distribution profile was nearly identical between seropositive-HBsAg ICC patients and HBV-associated HCC patients. We also found that, compared to seronegative-HBsAg ICC patients, seropositive-HBsAg ICC patients were younger, more frequently male (similar to HBV-associated HCC), had a higher incidence of abnormal aminotransferase and serum alpha-fetoprotein (AFP) levels, histological inflammation and cirrhosis, right lobe focus, poor tumor differentiation, tumor encapsulation and microvascular invasion, and had a lower incidence of abnormal serum carbohydrate antigen 19-9 (CA19-9) levels and lymphatic metastasis. These results suggest that HBV-associated ICC shares many clinicopathological similarities with HBV-associated HCC and were further supported by other studies[26,38].

On the basis of gross morphological features, ICC can be classified into three subtypes: mass forming, periductal infiltrating, and intraductal[39]. These different growth patterns suggest that ICCs are heterogeneous tumors having different cells of origin and different pathogeneses. It is likely that periductal and intraductal tumors arise from the malignant transformation of epithelial cells lining the larger bile ducts, whereas the mass-forming type arises from smaller bile ducts or bipotential hepatic stem cells within portal areas. Therefore, etiologies involving distinct molecular pathways may be associated with the subtypes of ICC. Indeed, ICCs associated with hepatolithiasis and *Clonorchis sinensis* infection are nearly always found to have the intraductal growth pattern[40,41], whereas viral hepatitis-associated ICCs are frequently found to have the mass-forming growth pattern[38,42]. ICC can also be classified into two subtypes on the basis of histological features: the cholangiolar and bile duct subtypes. Yu *et al*[42] found that the cholangiolar subtype was more strongly associated with viral hepatitis (seropositive for HBsAg and/or anti-HCV) than the bile duct subtype (OR = 2.71; $P = 0.008$), further supporting aforementioned theory of different etiologies involving distinct molecular pathways in ICC development.

N-cadherin (also referred to as CDH2) is a calcium-binding, single-pass transmembrane cell adhesion molecule and a recently identified marker of hepatobiliary tumors[43]. N-cadherin is expressed in a membranous pattern in hepatocytes, interlobular bile ducts, and ductular reactions but not in extrahepatic and large intrahepatic bile ducts. N-cadherin is more frequently expressed in peripheral than in hilar cholangiocarcinomas[43], which suggests that N-cadherin is more likely to be expressed in tumors differentiating toward small bile duct morphology. A subsequent study found that N-cadherin is an immunohistochemical marker strongly associated with hepatitis virus infection (seropositive for HBsAg and/or anti-HCV) (OR = 5.06; $P = 0.0002$); the prevalence of viral hepatitis in ICC patients with N-cadherin-positive intrahepatic cholangiocarcinoma was 75%, whereas that in N-cadherin-negative ICC patients was only 37%[42].

CK19, a member of the type 1 group of cytokeratins with a molecular weight ranging from 40 to 56 kDa[44], is normally expressed in ductal epithelium (bile ducts, pancreas, and renal collecting tubules) and in the mucosa of the gastrointestinal tract (GIT)[45]. The use of CK19 immunohistochemistry in diagnostic pathology has mainly been implemented to confirm epithelial immunophenotypes in potentially undifferentiated tumors or to establish a biliary/pancreatic/renal ductular origin, usually as part of a larger panel of markers. With regard to tumors, CK19 is expressed in squamous carcinomas of the head and neck, HCCs and more than 50% of renal cell carcinomas[46]. Most GIT adenocarcinomas are CK19 positive, including intrahepatic cholangiocarcinomas[21,47]. Recent studies have reported that CK19 expression occurred more frequently in ICC in the absence of HBV infection compared to HBV-associated ICC (88.96% *vs*

99.38%)[21,47].

HBV AND ICC PROGNOSIS

ICC is notoriously predictive of a poor prognosis, mainly due to poorly encapsulated tumors, periductal invasion, frequent lymphatic involvement, or a greater difficulty of making early diagnoses compared to HCC. Furthermore, these characteristics are more prominent in ICC patients with seronegative-HBsAg than in ICC patients with seropositive-HBsAg[21]. Recent studies also found that high preoperative CA19-9 levels in ICC or CK19 expression in some tumors resulted in a worse prognosis after surgical treatment[47-50]. Compared to HBV-associated ICC, CK19 expression and high CA19-9 levels were found to occur more frequently in ICC in the absence of HBV infection[47,48,50]. Additionally, patients with chronic HBV infection often undergo surveillance with AFP and ultrasound every 3-6 mo for the detection of early HCC, which may lead to the unexpected detection of ICC at a relatively early stage; thus, the proportion of ICC patients who undergo curative resection is significantly higher for HBV-positive than HBV-negative patients[38]. Taken together, relative to ICC without HBV infection, HBV-related ICC shows a trend toward a lower proportion of most of the aforementioned malignant properties and a higher proportion of patients who could be diagnosed early and have undergone curative resection. These results may indicate that ICC patients with HBV infection have a more favorable prognosis compared to ICC patients without HBV infection. Indeed, this hypothesis has been further supported by some recent studies [38,47,51].

POTENTIAL PATHOGENESIS OF HBV-ASSOCIATED ICC

Although epidemiological evidence based on the statistical analyses of patient samples strongly supports the causal role for HBV and HBV-related cirrhosis in the development of ICC, the pathogenesis of HBV-mediated intrahepatic cholangiocarcinogenesis remains largely unknown. To date, only very few studies have been conducted to explore the pathogenic mechanisms of HBV-related ICC.

Cancer stem cells

Recent evidence suggests that some cancers may originate from cancer stem cells, which may derive from the carcinogenesis of normal stem cells[52-55]. A hepatic progenitor cell population, also called oval cell, which gives rise to hepatocytes and cholangiocytes, has been suggested in humans, and the carcinogenesis of such hepatic progenitor cells may cause ICC[54,56]. AFP, with a molecular weight ranging from 68 to 73 kDa in dependence on carbohydrate glycoprotein, is normally produced during fetal development by the fetal hepatocytes, yolk sac cells and gastrointestinal cells[57,58]. The protein levels rapidly decrease after birth, and only trace amounts are detectable in the serum by the second year of life. However, AFP is increased in most of HBV-associated HCC patients. Hepatic progenitor cells were also shown to strongly express AFP mRNA and to produce AFP during differentiation[57,59]. Previous studies reported that HBV-associated ICC patients exhibited a higher incidence of AFP, > 20 μg/L (or > 200 μg/L), than ICC patients without HBV infection[21,26,42,60]. These results may indicate that one mechanism for the development of ICC involves the neoplastic transformation of oval cells and that the oval cell precursors retain the ability to produce AFP through the process of malignant transformation.

Similarities in the demographics of HBV-associated HCC and ICC have been observed, suggesting that common disease processes may be involved. HBV-associated ICC and HCC patients were reported to be younger and more frequently male than ICC patients without HBV infection[21,26,42], and the age and sex distribution profiles were nearly identical between HBV-associated ICC patients and HBV-associated HCC patients[21]. HBV-associated ICC or HCC occurs on average 10 years earlier than HCV-related ICC or HCC[28], and the age profiles of HBV-associated ICC and HCC and HCV-associated ICC and HCC are also similar[28]. The incidence ratio of HCC:ICC: combined hepatocellular cholangiocarcinoma was found to be consistent with the theoretic ratio of hepatocyte number to cholangiocyte number in the liver[28]. Taken together, researchers concluded that HBV-associated ICC and HCC hold common disease processes and that the two types of tumors evolve from hepatic progenitor cells. Using unique mouse models and eloquent hepatocyte fate tracing methods, Fan *et al*[61] and Sekiya and Suzuki[62] have independently demonstrated a compelling alternative to the cellular origin of ICC, namely, through the transdifferentiation and neoplastic conversion of normal hepatocytes into malignant cholangiocytes *via* a mechanism mediated in part by the overexpression of activated Notch. Of further interest, in both studies, ICCs were observed to originate from transdifferentiated hepatocytes in the central areas of the liver lobule and not in the periportal areas, the site of the hepatic stem/progenitor cell niche.

Role of chronic inflammation and cirrhosis

It is well known that most HCC arise in the context of HBV-associated cirrhosis in HBV-endemic areas, clearly suggesting that cirrhosis is the most important risk factor of HCC and is a pre-neoplastic condition per se. The strong association between cirrhosis and HCC suggests a hepatocarcinogenic process that is largely mediated by inflammation, leading to repeated cycles of cell death and regeneration that increase hepatocyte proliferation turnover. Although HBV infection and cirrhosis are generally considered to be unrelated to the mechanism of ICC carcinogenesis[63], a large amount of epidemiological data in recent strongly support that HBV infection

may play an important etiological role in ICC development. Our previous studies have also showed that HBV-associated cirrhosis and histological inflammation are significantly higher in HBV-associated ICC patients than in ICC patients without HBV infection[21,26]. These results indicate that HBV-associated HCC and HBV-associated ICC may share a common disease process for carcinogenesis, through a similar long-term inflammatory carcinogenic process.

Role of HBx protein

The HBx protein (17 kDa) communicates with a variety of host targets and disturbs cellular functions, including cell cycle regulation, apoptosis, signaling, transcriptional regulation, and the expression of cytoskeleton, cell adhesion molecule, tumor suppressor genes, and oncogenes[64-70]. HBx plays a crucial role in HCC development[71-75], up-regulating the expression of such proto-oncogenes as c-myc[76] and c-Jun[77], such transcription factors as nuclear factor kappa B (NF-κB)[78], AP-1[79], AP-2[80], and cyclic adenosine monophosphate response element binding protein[81], and such other viral genes as HBV enhancers in the nucleus[82]. HBx activates mitogen-activated protein kinase (MAPK)/mitogen-activated protein kinase (ERK), stress-activated protein kinase/Jun N-terminal kinase, and protein kinase C signaling pathways to regulate NF-κB and AP-1-dependent transcription[78]. The induction of NF-κB and AP-1 activity leads to the acceleration of cell cycle progression, increased proliferation, and the suppression of apoptosis. HBx can bind to the C-terminus of p53, forming a protein-protein complex and inactivating many functions of p53, including apoptosis. HBx also inhibits the repair of damaged liver cell DNA by interacting with p53 or by binding to the damaged DNA-binding protein, which is implicated in DNA repair and cell cycle regulation, leading to the accumulation of DNA mutations and cancer[83,84]. Similar carcinogenic effects of HBx are expected to occur in ICC. Wang *et al*[34] reported that the *HBx* gene had a high positive rate in cancerous tissues and surrounding liver tissue. Zhou *et al*[37] found that the HBx protein was frequently expressed in the surrounding liver tissues of HBV-associated ICC and that patients with HBx expression had a significantly higher prevalence of elevated serum AFP. Collectively, these results indicate that HBx may also play an important role in the pathogenesis of ICC. Zhou *et al*[37] also found that 33.3% of HBV-relative ICCs exhibited p53 protein expression, though p53 protein expression was not correlated with HBx expression. Given this result, Zhou *et al*[37] concluded that p53 abnormality may not play a significant role in HBx-mediated oncogenicity during ICC carcinogenesis. Performing *in vivo* research in models, Liu *et al*[84] found that the pSmad3L oncogenic pathway was activated in HBx and HCP (hepatitis C virus core)-induced ICC and involved the phosphorylation of p38 by MAPK and p44/42 ERK1/2, indicating the association with the transforming growth factor beta 1 (TGF-β1) signaling pathway in ICC. The knockdown of TGF-β1 by *in vivo* morpholino injections markedly reduced bile duct proliferation, fibrosis, and ICC. These results reveal that TGF-β1 plays an important role in HBx- and HCP-induced ICC development.

CONCLUSION

A large amount of epidemic data strongly support that HBV infection plays an important etiological role in ICC development, particularly in HBV-endemic areas. HBV-associated ICC holds many clinicopathological similarities with HBV-associated HCC, and HBV-associated ICC patients may have a better prognosis than ICC patients without HBV infection. HBV-associated ICC and HBV-associated HCC appear to share a common disease process for carcinogenesis, a similar long-term inflammatory carcinogenic process, and both possibly arise from hepatic progenitor cells.

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TOPIC HIGHLIGHT

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Optimization therapy for the treatment of chronic hepatitis B

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Abstract

Chronic hepatitis B (CHB) is currently medically managed with either interferon-alpha or one of the five nucleos(t)ide analogs. However, there are still a large number of CHB patients whose response to the above therapies remains less than satisfactory, and their incomplete or non-response to antiviral therapies has plagued clinicians worldwide. In recent years, a newly proposed optimization therapy has provided us with a new approach to solve this problem. The key points in this optimization therapy are to initiate antiviral therapy with an appropriate agent at the correct time point, and to adjust treatments in patients with poor early responses by adding a second agent or switching to another more potent agent. In this review, we summarize recent developments in optimization therapy for the treatment of CHB, and provide an outlook for future research in this field.

Key words: Chronic hepatitis B; Nucleos(t)ide analog; Interferon; Suboptimal response; Optimization therapy

Core tip: Optimization therapy is a personalized strategy, and it aims to achieve profound and sustained inhibition of hepatitis B virus replication, and to reduce the likelihood of subsequent disease progression. The key points of optimization therapy are to initiate antiviral therapy with an appropriate agent at the correct time point based on baseline characteristics, and to timely adjust the treatment by dynamic monitoring of the on-treatment response. However, the current understanding of optimization therapy is still very limited, and many issues still need further research.

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INTRODUCTION

An estimated 350 million people are infected with hepatitis B worldwide, and up to 1 million deaths annually can be attributed to hepatitis B virus (HBV)-related complications, including cirrhosis and hepatocellular carcinoma (HCC)[1]. Currently, both interferon-α (IFN-α) and oral nucleos(t)ide analogs (NAs) are used to treat chronic hepatitis B (CHB) patients[2], and a profound and sustained inhibition of viral replication is the most important goal of any CHB treatment, as only such inhibition can reduce the likelihood of subsequent disease progression and viral resistance[3,4].

However, due to the large variation in hosts (gender, age, genetic background, disease duration, extent of liver damage) and viral (genotype and quasispecies complexity) factors[5-8], a considerable number of CHB patients do not have a satisfactory response to therapy. These patients usually have suboptimal response, viral resistance or a lack of sustained curative response. Thus, optimizing existing treatment strategies to maximize efficacy and reduce the emergence of resistance has become a hot research topic over the past few years[9]. In this review, we summarize recent developments in optimization therapy for the treatment of CHB, and provide an outlook on this topic.

IMPORTANCE OF OPTIMIZATION THERAPY FOR CHB

Definition of optimization therapy

The concept of optimization therapy can be traced back to the roadmap approach[10], in which on-treatment adjustment and strategies were proposed for patients with suboptimal responses to antiviral therapy. As there is no uniform definition of suboptimal response, some published reports refer to suboptimal response as poor response or partial response. This definition of optimization therapy has been further broadened to include any therapy with the following two aspects: (1) selecting appropriate drugs for the initial treatment at the correct time point based on baseline characteristics of the patients including serum alanine aminotransferase (ALT) levels, HBV DNA titers, HBV genotypes, and severity of liver damage; and (2) making timely therapeutic changes in CHB patients with poor early response to initial therapy. As an individual measure, more and more clinical trials have demonstrated that the application of optimization therapy can achieve better long-term treatment efficacy[11,12].

For a long time, there was no consensus on the time point for assessing the suboptimal response to NAs. However, recently the European Association for the Study of the Liver (EASL) guideline recommended that suboptimal response should be assessed at the 24th week of treatment for moderately potent agents including agents with a low genetic barrier to resistance [including lamivudine (LAM) and telbivudine (LdT)] and at the 48th week for highly potent agents including agents with a higher genetic barrier to resistance or agents with late emergence of resistance [including entecavir (ETV), adefovir (ADV) and tenofovir disoproxil (TDF)][13].

Why optimization therapy should be considered

TDF and ETV are the currently preferred antiviral agents, given their potent anti-HBV activity and high barrier to resistance. However, these drugs are expensive and their long-term use is often unaffordable for many patients, especially low-income patients from developing countries[14]. Thus, affordable drugs such as LAM, ADV and LdT are still used by a significant number of patients[2]. However, due to the low genetic barrier to resistance and ineffective inhibition of virus replication, these cheaper agents often lead to suboptimal responses and the emergence of viral resistance. An efficacy analysis of different NAs showed that suboptimal virological response (HBV DNA > 300 copies/mL at week 24) was 51%-88% for HBeAg positive patients and 20%-64% for HBeAg negative patients[15], and that the suboptimal virological response to NAs always had a high incidence of resistance and high risk of HCC[16]. In addition, the persistent positive status of HBV DNA caused by the suboptimal virological response also led to extended duration of therapy and increased medical costs, and therefore lowered patient adherence to antiviral therapy[17]. In addition, the baseline HBV DNA levels, ALT levels and histological changes also affected the on-treatment virological response to NAs. Thus, the development of individualized and optimized treatments with NAs is widely accepted by clinicians in clinical practice[6,7,18,19].

Although IFN has both antiviral and immunomodulatory properties against HBV, and a slight serologic advantage over NAs, its strength in suppressing HBV DNA replication is relatively weak. The majority of CHB patients have a low viral response to IFN after completing their full course of treatment, and 65%-80% of them may develop virological rebound in the 6th month after discontinuing therapy, which inevitably diminishes the beneficial effects of the previous therapy. In addition, the antiviral efficacy of IFN is also affected by HBV genotypes[20], and patients with similar clinical characteristics infected with different HBV genotypes may respond differently to the same IFN therapy[21]. Thus, the current NAs and IFN-based antiviral strategies need to be optimized during the course of treatment. Timely and reasonable optimization strategies would help to achieve sustained suppression of HBV replication and remission of liver disease, and to prevent or decrease the occurrence of life-threatening cirrhosis and HCC.

OPTIMIZATION STRATGIES FOR NAS THERAPY

Currently, five NAs are approved for the treatment of CHB, including LAM, ADV, ETV, LdT, and TDF. As NAs only suppress HBV replication at the level of DNA synthesis, most patients require long-term (even lifelong) treatment. In addition, the antiviral strength and genetic barrier to resistance are quite different among these NAs, and non-response, suboptimal response and resistance all limit the use of long-term NAs therapies. Thus, it is necessary to optimize the current antiviral strategies to improve patient responses during and after treatment[22].

Optimization therapy according to baseline characteristics

In the past decade, several factors (including gender, duration of infection, baseline HBeAg level, HBV DNA level, and ALT level) have been used to predict the vi-

rological responses of HBeAg-positive patients to NAs treatments. According to international guidelines, high ALT levels, low HBV DNA levels and high histological activity index prior to NAs treatments can lead to good HBeAg seroconversion[23,24]; and high baseline HBV DNA level was found to be the most important factor associated with virologic breakthrough[7,25-27]. For patients with HBV DNA levels higher than 6.6 log10 copies/mL, virologic breakthrough during LAM monotherapy was as high as 19% at year 1 and 45% at year 2; and was only 6.7% and 18% at years 1 and 2, respectively, for patients with HBV DNA levels lower than 6.6 log10 copies/mL[7], which indicated that LAM may be an effective first-line therapy for HBeAg-positive patients with lower baseline HBV DNA levels. However, further studies are needed to confirm that the baseline HBV DNA level of 6.6 log10 copies/mL is an ideal cutoff value in selecting initial treatment with LAM. The LdT 2-year GLOBE trial results showed that baseline serum ALT and HBV DNA levels could be used to predict the 2-year response in patients treated with LdT. For HBeAg-positive patients with ALT equal to or higher than 2 × ULN and HBV DNA less than 9 log10 copies/mL, more than 47% achieved HBeAg seroconversion at year 2[28]. We previously compared the 2-year efficacy of initial ADV and ETV treatments[29], and found that the efficacy of ADV was inferior to that of ETV in HBeAg-positive patients, however, in HBeAg-negative patients, ADV and ETV achieved similar biochemical and virological responses. Thus, ADV should be considered for HBeAg-negative patients with low baseline HBV DNA.

Considering that high baseline viral load was highly associated with failure to achieve virologic suppression, we recently determined the 96-wk efficacy of highly potent ETV in the treatment of HBeAg-positive patients with baseline HBV DNA higher than 9 log10 copies/mL, and found that the virological response rate was significantly lower in this cohort than in patients with HBV DNA lower than or equal to 9 log10 copies/mL (unpublished data). This suggested that high baseline HBV DNA may be a negative factor influencing the likelihood of virological responses with initial ETV monotherapy. The optimization of treatment for patients with high baseline HBV DNA has become a new challenge, and de novo combination therapies may be good options in solving this problem, however, further research is required.

TDF is a very potent antiviral agent for maintaining long-term HBV DNA suppression, with very low rates of resistance development and a good safety profile. Recently, Gordon *et al*[30] investigated the efficacy of TDF in CHB patients with high baseline HBV DNA (≥ 9 log10 copies/mL), and they found that patients with high baseline HBV DNA could achieve a similar virological response (HBV DNA < 400 copies/mL) to patients with low baseline HBV DNA (< 9 log10 copies/mL), although this tended to take longer in patients with high baseline viral load. Thus, TDF is a good therapeutic option in patients with high baseline HBV DNA.

Recent studies showed that different HBV genotypes also show different sensitivities to NAs therapy. For example, HBV genotype B shows a better virological response to ADV therapy than genotype C[31], and TDF therapy can induce a significant decrease in HBsAg in HBeAg negative patients infected by HBV genotype D. However, the antiviral response of different HBV genotypes to NAs is still unclear[32]. Thus, it remains to be clarified whether optimization strategies of initial NAs therapies should consider HBV genotypes.

Optimization therapy according to on-treatment responses

Suboptimal response is associated with all NAs therapies, and persistent viremia can greatly increase resistance and inevitably lead to aggravation of the disease. Thus, a rapid decrease in HBV DNA to undetectable levels is highly correlated with long-term treatment efficacy[33,34]. In recent years, there has been tremendous progress in the use of on-treatment HBV DNA levels at different time points to predict clinical outcomes in patients[25]. An earlier study of 74 HBeAg-positive patients treated with LAM showed that an HBV DNA level below 4 log10 copies/mL at week 4 can be used to predict the ideal response at year 5 (undetectable HBV DNA level, HBeAg seroconversion, and normal ALT levels)[35]; while patients with HBV DNA levels equal to or higher than 3 log10 copies/mL after 6 mo of LAM therapy had a 63.2% chance of developing resistance[36]. In addition, an early reduction in HBV DNA before week 24 in predicting long-term response was also observed in LAM-treated HBeAg-negative patients[37]. Thus, for LAM-treated patients who fail to achieve this early target, the addition of (such as ADV or TDF or IFN add-on) or a switch to (such as IFN) alternative antiviral agents should be considered. However, data on optimization strategies in LAM suboptimal responders are still limited, especially for IFN add-on or the switch to IFN treatments.

One study from Spain showed that 77% of patients with early virological responses (reduction in HBV DNA ≥ 4 log10 IU/mL at month 6) on ADV therapy achieved undetectable HBV DNA level at month 12 compared to only 5% in those without early virological responses[38]. In China, it was observed that HBV DNA levels at week 24 (HBV DNA < 1000 copies/mL *vs* HBV DNA ≥ 1000 copies/mL) were highly related to the 48-wk virological response, and the rates of 48-wk virological and serological responses were also significantly different between patients with primary non-response and those with virological response at week 12[25]. Currently, the common optimization strategies for suboptimal response to ADV are LAM, LdT and ETV add-on treatments. The IFN add-on or switch to IFN can also be considered in theory. Among 31 HBeAg-positive patients with HBV DNA equal to or higher than 4 log10 copies/mL after 48 wk of ADV monotherapy, the levels of HBV DNA were significantly reduced after 24-wk combination therapy with LAM plus ADV[39]. We further evaluated the combination

strategies of LAM plus ADV or LdT plus ADV for patients with suboptimal responses to ADV, and found that both combination therapies led to a significant decrease in HBV DNA, however, HBeAg serological outcomes were significantly higher in patients treated with LdT plus ADV than those treated with LAM plus ADV[11].

The on-treatment HBV DNA levels for predicting long-term antiviral responses were also determined with LdT treatment. Among HBeAg-positive patients with HBV DNA lower than 300 copies/mL at week 24, the proportions who achieved 2-year undetectable HBV DNA, cumulative HBeAg seroconversion or resistance were 82%, 46% and 9%, respectively[6]; and at year 3, the cumulative HBeAg seroconversion further increased to 54%[40]. A recent study also reported that serum HBV DNA level at week 12 was better than the viral response at week 24 in predicting long-term treatment outcome with LdT[41]. Thus, either ADV or TDF add-on therapy should be used in patients with suboptimal viral response to LdT as early as possible. It is worth mentioning that we recently participated in a large national cohort to evaluate the efficacy and safety of optimization strategy with ADV add-on for suboptimal responders to LdT, and found that change in treatment strategy was essential for suboptimal virological responders at week 24, and ADV add-on optimization therapy increased antiviral potency and lowered resistance without increasing side effects (unpublished data).

Despite the high potency of ETV, some patients treated with ETV only have a suboptimal response (detectable HBV DNA after 12-mo treatment)[42]. The add-ons of ADV, TDF, or IFN, and a switch to TDF or IFN are both considered alternative optimization therapies. One retrospective study from United States compared three different optimization strategies (ADV add-on, switch to TDF, or TDF add-on) in patients with a partial response to ETV, and showed that the TDF add-on therapy and the switch to TDF monotherapy appeared to have similar efficacy in most patients, but the efficacy of ADV add-on therapy was much less than that of either switch to TDF or TDF add-on therapy[43]. However, studies also showed that the vast majority of patients with primary non-response or suboptimal responses to initial ETV treatment (for more than 12 mo) would achieve virological response through prolonged ETV treatment without any other adjustments, and would have only a 1.4% chance of viral resistance[44]. As there are relatively limited data available, more studies are required to determine whether and which optimization therapies are necessary for patients with suboptimal responses to ETV.

There is increasing evidence to show that the change in serum HBsAg titer during antiviral treatment is correlated with changes in covalently closed circular DNA (cccDNA) levels[45]. Monitoring serum HBsAg titer is a reasonable on-treatment indicator of long-term response to pegylated IFN-α therapy. However, the decline in serum HBsAg titer is not significant in patients treated with NAs[46], and its value in predicting responses to NAs therapy is still controversial[47,48]. Thus, further investigation and analysis are needed to determine whether the absolute HBsAg titer or a reduction in HBsAg titer could be used to optimize NAs treatment.

OPTIMIZATION STRATGIES FOR INTERFERON THERAPY

Optimization therapy according to baseline characteristics

IFN therapy results in sustained responses in only a minority of CHB patients, and both host and viral characteristics significantly affect the response to INF. Most recent studies suggest that HBV genotypes, high levels of ALT [$\geqslant 2 \times$ upper limit of normal (ULN)], low levels of HBV-DNA ($< 2.0 \times 10^8$ IU/mL), and female sex predict a long-term viral response with IFN treatment[49]; and patients who can achieve a long-term response to INF are genotype A patients with high ALT and/or low HBV-DNA levels, and genotype B patients with both high ALT and low HBV-DNA levels. However, genotype C and D patients have a low chance of long-term response, regardless of ALT or HBV-DNA levels[21]. In addition, baseline ALT level is also a reliable predictor of HBeAg seroconversion, and the cumulative HBeAg seroconversion rate is significantly high in patients with high ALT levels. For example, the HBeAg seroconversion rate is 22.5% in patients with baseline ALT $> 2 \times$ ULN, and is only 12.5% in patients with baseline ALT $\leqslant 2 \times$ ULN[49].

Thus, determining the correct time and patients for IFN therapy is the core of optimization strategies. A consensus has been reached on the correct time of IFN therapy. The best time to start an appropriate IFN therapy is when patients have changed from the state of immune tolerance to the immune clearance phase, accompanied by increased ALT and decreased HBV DNA[50]. Patients with HBV genotype C and D infection may be considered for NAs treatment, but not IFN treatment[2].

Optimization therapy according to on-treatment responses

Recently, monitoring serum HBV DNA and HBsAg levels has helped to differentiate patients who will quickly respond from those who will require longer treatment and those who are unlikely to respond and therefore need alternative medicines[5]. Thus, monitoring the changes and trends of these indicators during treatment is necessary to develop optimization strategies for IFN therapy.

There is a correlation between HBsAg titer and the levels of cccDNA and total intra-hepatic HBV DNA[45], and the elimination of cccDNA and levels of HBsAg are associated with long-term virological response. Thus, HBsAg and HBV DNA levels may be used to predict long-term responses to IFN therapy. For example, a recent international multicenter trial investigated the role of early on-treatment serum HBsAg levels in predicting long-term response (HBV DNA level < 10000 copies/mL and normal ALT levels at week 72) to IFN in HBeAg-nega-

tive patients[51]. However, other studies showed that a decrease in HBsAg alone was of limited value in predicting long-term responses, but decreases in both HBsAg and HBV DNA (≥ 2 log10 copies/mL) were good indicators of long-term response[51]. Importantly, patients without a change in HBsAg or a significant decline in HBV DNA (< 2 log10 copies/mL) at week 12 are unlikely to have an ideal response[51]. Therefore, a decline in both serum HBV DNA and HBsAg levels at week 12 of IFN therapy have been recommended to determine the choice of subsequent therapies. The absence of a decrease in HBsAg together with a reduction in HBV DNA to below 2 log10 copies/mL at week 12 could serve as the stopping point in HBeAg-negative patients with genotype D HBV infection[52]. Thus, on-treatment HBV DNA and HBsAg kinetics are useful for individual IFN treatment optimization.

A recent report from China showed that extended treatment with pegylated IFNα-2a in combination with LAM or ADV for 96 wk was a promising strategy for achieving high rates of sustainable HBeAg and HBsAg seroconversion and HBV DNA suppression in HBeAg-positive patients[12]. In addition, there is an ongoing multicenter randomized trial in China, which aims to investigate the optimization strategy for pegylated IFNα. In this study, patients with a rapid response (both HBsAg < 1500 IU/mL and HBV DNA < 1.0×10^5 copies/mL at week 24 after treatment) would complete 48 wk of PegIFN monotherapy; while patients with a slow response (both HBsAg ≥ 1500 IU/mL and HBV DNA ≥ 1.0×10^5 copies/mL at week 24) would receive either extended IFN monotherapy to 96 wk or combination therapy of IFN and NAs. The findings of this study will provide further evidence for the combined usage of HBsAg and HBV DNA levels for predicting long-term antiviral efficacy and guiding the choice of optimal treatment strategies (extended treatment *vs* NAs add-on therapy).

CONCLUSION

The primary goal of the above-mentioned optimization therapy is to enable these patients to achieve maximum treatment benefits from current NAs and IFN therapies. At present, optimization therapy is an individualized treatment approach. To establish a rational optimization strategy, patients should be thoroughly assessed for demographic, lifestyle, income and clinical characteristics before starting treatment. Appropriate drugs should be selected according to a patient's baseline host and viral characteristics, followed by timely adjustments of medicines by dynamic monitoring of on-treatment responses. However, research on optimization therapy for CHB is still very limited, and many issues still need to be investigated such as: (1) the concept of poor early response should be strictly defined with unified standards and the difference between various antiviral drugs should be taken into account; (2) besides HBV DNA and quantitative HBsAg, more parameters (including immunology and host genomic-related indicators) should be determined to help develop optimization therapy; (3) broadening drug selection for optimization therapy (not just confined to NAs and IFN-α), and the agents of therapeutic hepatitis B vaccine and immunomodulators should also be considered for use in combination with existing antiviral drugs; and (4) optimization strategies for special populations (including pregnant women, liver transplant patients, viral reactivation during immunosuppression) should also be developed.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Deep vein thrombosis and pulmonary embolism in cirrhotic patients: Systematic review

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Abstract

Patients with liver cirrhosis were traditionally believed to be protected against development of blood clots. Lately, studies have shown that these patients may probably be at an increased risk of venous thrombotic complications. Although the hemostatic changes in the chronic liver disease patients and the factors that may predict bleeding *vs* thrombotic complications remains an area of active research, it is believed that the coagulation cascade is delicately balanced in these patients because of parallel reduced hepatic synthesis of pro and anticoagulant factors. Thrombotic state in cirrhotic patients is responsible for not only portal or non-portal thrombosis [deep vein thrombosis (DVT) and pulmonary embolism (PE)]; it has also been associated with progression of liver fibrosis. The use of anticoagulants in cirrhosis patients is a challenging, and often a scary situation. This review summarizes the current literature on the prevalence of venous thrombosis (DVT and PE), risk factors and safety of prophylactic and therapeutic anticoagulation in patients with chronic liver disease.

Key words: Deep venous thrombosis; Chronic liver disease; Cirrhosis; Thrombosis; Anticoagulation; Pathogenesis; Portal vein thrombosis

Core tip: In this review, the current literature on the risk of venous thromboembolism (VTE) in cirrhosis patients is updated. There is no doubt that these patients are at risk for both venous thrombosis and bleeding, often presenting a challenge to the providers. VTE prophylaxis should be considered in all hospitalized cirrhotic patients, unless absolutely contraindicated. While the risk of bleeding from therapeutic anticoagulation cannot be excluded, a case of careful anticoagulation for treatment of VTE event should be made in the hands of experts.

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INTRODUCTION

Liver cirrhosis is associated with an increased risk of bleeding complications. For decades, it was believed that the increased INR and thrombocytopenia seen in these patients was enough to protect these patients from developing thrombotic complications, and hence the concept of "autoanticoagulation". Over the last few years, grow-

ing body of evidence indicates that liver disease may be associated with an increased risk of thrombotic complications as well[1]. Portal vein thrombosis is common in patients with liver cirrhosis, seen in 10%-25% of the patients, with increased prevalence seen in patients with more severe disease[2-4]. Data is also emerging regarding occurrence of non-splanchnic venous thromboembolic events (VTE) in these patients, mostly lower extremity deep vein thrombosis (DVT) and pulmonary embolism (PE)[5,6].

The economic and health care burden attributable to liver disease is huge, with approximately 1% of the total national health care expenditure spent on care of these patients[7]. Development of VTE is associated with increased length of hospital stay[8-10], hospitalization cost[11] and possibly mortality as well[8]. It has also been proposed that hypercoagulation state may lead to progression of fibrosis, possibly through activation of hepatic stellate cells or as a result of local ischemic changes secondary to hepatic microthrombi[12-15]. A recent study showing decreased risk of decompensation of cirrhosis with prophylactic enoxaparin therapy provides additional support to this burgeoning concept[16]. Understanding of hemostatic pathways in cirrhotics is important not only to predict the bleeding or thrombotic complications in these patients, it also provide us with a therapeutic opportunity to possibly change the natural course of disease[13].

We hereby aim to briefly review the current literature on the changes in the coagulation/hemostatic cascade, prevalence of thrombotic complications and the role of prophylactic and therapeutic anticoagulation in this "high risk" patient population. For the purpose of this review, we will mostly restrict ourselves to the non-portal VTE in cirrhotic patients. The etiology and pathophysiology of hepatic vein thrombosis (Budd-Chiari syndrome) and portal vein thrombosis has recently been reviewed and elucidated and is out of scope of this review[17-19].

MECHANISMS

Over the years, there has been a paradigm shift in our understanding of coagulation abnormalities in cirrhosis. It is now well known that the reduced production of procoagulant factors and platelets is balanced by concomitant decreased levels of anticoagulants (such as protein C and antithrombin), thereby maintaining a delicate hemostatic balance between the two[20]. What tips this balance is not entirely known. It has been proposed that an added insult in the form of sepsis or bacterial infection (possibly through impaired platelet aggregation in the presence of increased endotoxins), renal failure (impaired platelet function) and Vitamin K deficiency (decreased activity of Vitamin K dependent procoagulant factors) may lead to disturbances of the hemostatic balance in favor of bleeding[21].

On the other hand, there are many compensatory hemostatic mechanisms that are seen in cirrhotic patients. It has been shown that these patients have elevated von Willebrand factor (vWF) levels which may contribute to greater platelet adhesion and compensate for defects in platelet number and function[22]. Increased vWF levels may also contribute to increased Factor Ⅷ levels by binding to Factor Ⅷ and thereby preventing its cleavage and clearance[23]. Another possible explanation for increased factor Ⅷ levels seen in cirrhotics is decreased expression of lipoprotein receptor-related protein, responsible for clearance of Factor Ⅷ[20,23]. Low protein C levels and antithrombin levels are secondary to decreased synthetic protein function of diseased cirrhotic liver parenchymal cells[20].

Despite the reduced coagulation factors, *in vitro* studies have shown that the thrombin generation remains preserved in cirrhosis as compared to healthy controls, in the presence of protein C activator like thrombomodulin or snake venom extract[24-27]. At baseline, cirrhosis patients seem to have a procoagulant imbalance which is likely secondary to increased factor Ⅷ levels and decreased protein C levels seen in cirrhotics[28]. The study by Tripodi *et al*[28] showed that in cirrhotic patients the *in vitro* activated protein C (APC) resistance test is impaired. This impaired APC resistance test worsened with progressive deterioration of liver disease from Child Pugh Class A to C. In fact, the hypercoagulability seen in plasma of patients with Child Pugh C cirrhosis has been shown to be similar to that conferred by congenital protein C deficiency or Factor V Leiden mutation[27,28]. A recent *in vitro* study showed that the procoagulant imbalance decreased with addition of exogenous purified protein C to restore the normal levels[29]. Major factors impacting hemostasis in these patients are summarized in Table 1.

PREVALENCE AND RISK FACTORS

A population based nested case control study involving 625 patients with VTE matched with 625 non VTE patients from Olmsted County, Minnesota found that patients with "serious liver disease" had 90% decreased risk of developing VTE. However, out of 1250 patients in the study only 11 patients had severe liver disease (5 with VTE, 6 without VTE)[30]. Patients with acute hepatitis, chronic hepatitis and cirrhosis were all grouped together into the same "serious liver disease" category. As such, this study did not reflect the true magnitude of risk of VTE seen in patients with chronic liver disease, more importantly cirrhotic patients. Since then, evidence has been accumulating with respect to risk of VTE in cirrhotic patients. Till date, there are no prospective randomized trials evaluating the incidence of DVT or PE in this patient population and with the overall low event rate, prospective trials are perhaps impractical.

The incidence of VTE has varied from 0.5% to 8.1% in different series[6,8] (Table 2). In one of the earliest studies, Northup *et al*[6] found that 113 cirrhotic patients out of more than 21000 cirrhotic admissions over an 8 year period developed VTE, giving the incidence of about 0.5%. A retrospective review of 2074 hospitalized cir-

Table 1 Hemostasis changes in chronic liver disease patients

Factors favoring anticoagulant state	Factors favoring prothrombotic state
Thrombocytopenia (decreased thrombopoietin, splenic sequestration)	Decreased liver synthesis of antithrombin III, protein C, protein S
Thrombocytopathy (impaired platelet function)	Elevated von Willebrand factor levels
Vitamin K deficiency	Increased factor Ⅷ levels
Decreased synthesis of coagulation factors including Factor Ⅱ, Ⅶ, Ⅸ, Ⅹ, Ⅴ, fibrinogen	Genetic predispositions like Factor Ⅴ Leiden
Reduced clearance of tissue plasminogen activator	

rhotic patients in Spain showed an incidence of 0.8% (17) for non-portal VTE[31]. Of note, five of the 17 patients who underwent further laboratory testing, all had evidence of antiphospholipid antibodies as well as decreased protein C, protein S and antithrombin Ⅲ.

We had previously shown that hospitalized cirrhotic patients did not have a lower risk of DVT/PE than the matched non-cirrhotic controls without selected co-morbidities including chronic kidney disease, congestive heart failure and solid organ cancers[5]. The incidence of VTE in 963 cirrhotic patients and 12405 non-cirrhotic patients without selected comorbidities including CKD, CHF or cancers who were admitted during the same period was 1.87% and 0.98%, respectively (OR = 1.78, 95%CI: 1.1-2.2, P = 0.007). On multivariable analysis, when adjusted for comorbidities using Charlson Index, presence of cirrhosis was not associated with a higher risk of VTE (OR = 0.87, 95%CI: 0.2-2.6, P = 0.06). The incidence of VTE was significantly lower in cirrhotics (1.87%) as compared to patients with selected comorbidities, including CKD (7%; OR = 0.25, 95%CI: 0.15-0.41), CHF (7.75%; OR = 0.23, 95%CI: 0.14-0.37), and cancer (6.1%; OR = 0.29, 95%CI: 0.17-0.52).

A retrospective cohort study by Dabbagh *et al*[32] showed that a higher INR does not translate to a decreased risk of VTE in cirrhotic patients. The study included 190 patients with chronic liver disease, separated into quartiles using INR values of 1.4, 1.7, and 2.2. Over a 7 year period, 12 patients developed VTE with an incidence of 6.3%. There was no difference in the VTE rates in patients in different INR quartiles. Also, study showed a higher incidence of VTE in patients with Child-Pugh stage C cirrhosis as compared to Child-Pugh stage A cirrhosis (8% *vs* 4.2%, P = 0.6), though not statistically significant. This study again showed that the risk of VTE does not decrease with worsening INR or more decompensated disease (more than 50% of the included patients were Child-Pugh stage C cirrhotics). The risk was still present even with INR > 2.2. Similar results have been seen in other retrospective studies from Indonesia and Saudi Arabia as well[9,33]. In the study by Aldawood *et al*[9], the median length of hospital stay of the patients who developed VTE was significantly longer as compared to the patients without VTE (43 *vs* 8 d, P = 0.004). There was a trend towards higher mean Child Pugh score in patients who developed VTE (10.3 ± 1.97 *vs* 8.25 ± 2.57, P = 0.052). The incidence of VTE was 0.73% (including PVT) and 0.65% in two other case control studies[11,34]. In a study by Walsh *et al*[11] including 27 chronic liver disease patient with VTE and 81 matched CLD patients without VTE (controls), cases had a longer (9 d *vs* 5 d, P = 0.02) and a significantly more expensive ($20137 *vs* $8450, P = 0.03) hospital stay as compared to the controls. One of the reasons for lower incidence of VTE in this study (0.65%) may be greater use of VTE prophylaxis as more than 90% of the patients included in the study received either mechanical or pharmacological prophylaxis.

In a large Danish population based study using data from National Registry of Patients containing records of all hospital discharges, 99444 patients with hospitalization between 1980 and 2005 for index episode of VTE and 496872 population controls (matched by age, gender, and county but not by hospitalization) were included[35]. The study showed that the risk of VTE was higher in patients with liver cirrhosis (OR = 1.74, 95%CI: 1.54-1.95). When the analysis was restricted to unprovoked VTE, risk of VTE was seen to be twice in patients with cirrhosis 2.06 (95%CI: 1.79-2.38) as compared to non-cirrhotic patients. Sub-analysis by stratifying the data into 5 year intervals, authors found that the risk of VTE was decreasing over time, with the highest risk seen in period between 1990 and 1994, perhaps from use of thromboprophylaxis. This study did not have the data regarding severity of liver disease, such as Child Pugh score and could not assess the risk factors in cirrhotics that predict the development of VTE. Also, the cases were compared to population based controls that likely had fewer hospitalizations. The results were similar to another cohort study with nested case-control analysis using General Practitioner Research Database which showed a relative risk of 1.65 (OR = 1.65, 95%CI: 0.97-2.82) for VTE in patients with chronic liver disease[36].

In another population based study by Wu *et al*[8] using US Nationwide Inpatient Sample (1998-2006), prevalence of VTE was assessed in patients with compensated (n = 408253) and decompensated cirrhosis (n = 241626), defined by Baveno Status Classification. Patients with stage I (no ascites or varices) and Ⅱ Baveno (presence of varices without bleeding) were classified as compensated while stage Ⅲ (presence of ascites with or without varices) and Ⅳ (variceal bleeding with or without ascites) as decompensated cirrhosis. The patients were further stratified according to age. The increased risk of VTE was restricted to cirrhotic patients below the age of 45 years, for both compensated (OR = 1.23; 95%CI: 1.04-1.46) and decompensated cirrhosis (1.39; 95%CI: 1.15-1.69). Beyond the age of 45, the risk was modestly lower in compensated cirrhotic patients (OR = 0.90; 95%CI: 0.85-0.95) as compared to controls and similar in decompensated cirrhotics (OR = 0.97; 95%CI: 0.91-1.04).

Table 2 Risk of venous thromboembolism in chronic liver disease patients: major studies

Ref.	Type	*n*	Control	VTE% (*n*) DVT/PE	RR/OR (95%CI)	Comments
Northup *et al*[6], 2006	Case control	21000 cirrhotics	113 (cirrhotic patients without VTE)	0.5 (113) (74 DVT, 22 PE, 17 both)	Risk factor for VTE: low albumin 0.25 (0.10-0.56)	INR, MELD: no correlation
García-Fuster *et al*[31], 2008	Retrospective	2074 cirrhotic patients		0.8 (17) (11 DVT, 7 PE, 1 both)		5 patients had antiphospholipid antibodies
Gulley *et al*[5], 2008	Case control	963 cirrhotics	Control 1:12405 non cirrhotic patients without selected co-morbidities Control 2: non cirrhotic patients with CKD (1692), HF (4489), or solid organ cancer-673	1.87 (18)	Risk factor for VTE: Albumin 0.47 (0.23-0.93) PTT: 0.88 (0.84-0.94)	Cirrhosis not a risk factor on multivariate analysis OR 0.87 (0.2-2.6)
Dabbagh *et al*[32], 2010	Retrospective cohort	190 chronic liver disease patients stratified by INR quartiles		6.3 (12)		Higher INR or higher Child-Pugh stage does not prevent VTE
Lesmana *et al*[33], 2010	Case control	256 cirrhotic patients		4.7 (12)	Risk factor for VTE: Diabetes 4.26 (1.21-15.0)	
Anthony Lizarraga *et al*[34], 2010	Case control	108 CLD patients with VTE (includes 22 patients with PVT)	108 CLD patients without VTE	0.73 (108 out of 14,790 admissions) Includes PVT as well		Cases had lower albumin and hematocrit, higher platelet count, bilirubin and aPTT
Aldawood *et al*[9], 2011	Retrospective cohort	226 cirrhotic patients		2.7 (6)		Significantly longer median LOS in patients with VTE (43 *vs* 8 d, P = 0.004)
Walsh *et al*[11], 2013	Case control	27 CLD patients with VTE	81 CLD patients without VTE	0.65 (17 out of 2606 admissions) (14 DVT, 3 PE)	Risk factor for VTE: low albumin 5.14 (1.05-25.2)	VTE patients had lower transaminases, albumin and hematocrit
Søgaard *et al*[35], 2009	Population based, case control	VTE = 99444	Population controls without VTE 496872		Cirrhosis: RR 1.74 (1.54-1.95)	RR 2.06 (1.79–2.38) for unprovoked VTE
Wu *et al*[8], 2010	Population based, case control	Compensated cirrhotics = 408253 Decompensated = 241626	Non cirrhotic controls = 575057	0.8% for cirrhotics,	Age < 45 yr compensated: 1.23 (1.04-1.46) decompensated: 1.39 (1.15-1.69) Age > 45 yr compensated: 0.90 (0.85-0.95) decompensated: 0.97 (0.91-1.04)	VTE associated with increased mortality and LOS in both compensated and decompensated cirrhotics
Saleh *et al*[37], 2011	Population based	Alcoholic CLD: 4927000 Non-alcoholic CLD: 4565000		0.6% for alcoholicCLD 0.9% for non-alcoholic CLD		
Ali *et al*[10], 2011	Population based cross-sectional	449798 cirrhotic patients		1.80% (1% DVT, 0.9% PE)	Greater morbidity, malnutrition, black race, central venous line associated with higher risk of VTE	DVT associated with longer LOS

DVT: Deep vein thrombosis; PE: Pulmonary embolism; INR: International normalized ratio; MELD: Model for end stage liver disease; CLD: Chronic liver disease; VTE: Venous thromboembolism; CKD: Chronic kidney disease; PVT: Paroxysmalventriculartachycardia; LOS: Length of stay.

VTE was also associated with increased mortality (compensated cirrhotics OR = 2.16; 95%CI: 1.96-2.38, decompensated cirrhosis OR = 1.66, 95%CI: 1.47-1.87) as well as increased length of stay (compensated cirrhotics 1.03 increase, 95%CI: 0.95-1.11, decompensated cirrhotics 86% increase, 95%CI: 78%-94%) in all cirrhotics. The authors concluded that the younger cirrhotic patients may have a higher risk of VTE because of risk conferred by cirrhosis, while in older patients, age related factors may balance or take precedence over cirrhosis related risk factors. This is the largest and the only study to our knowledge that has looked at the differential risk of VTE with respect to the age of the cirrhotics compared to the non cirrhotic patients. In addition, this study also showed an almost two fold increase in mortality and length of hospitalization related to VTE, though the study did not adjust for other possible comorbid conditions like renal failure or respiratory failure. Another population based study included patients with diagnostic codes for chronic alcoholic liver disease and chronic non-alcoholic liver disease who were discharged from short-stay hospitals from 1979 through 2006 using National Hospital Discharge Survey[37]. Study found an overall low rate of VTE, with the prevalence lower in patients with alcoholic as compared to non-alcoholic chronic liver disease (0.6% *vs* 0.9%, $P < 0.0001$). The study did not include data on the severity of the liver disease, reason for hospitalization, proportion of patients hospitalized more than once, and the basis for the diagnosis of liver disease.

Ali *et al*[10] used Nationwide Inpatient Sample Database and included 449798 hospitalizations for cirrhosis in 2005. VTE comprised 1.8% of these hospitalizations though this rate was lower than VTE in overall all hospitalized patients (3.7%, $P < 0.05$). While VTE was not associated with increase in mortality, it was associated with increased LOS by 52% (95%CI: 45%-61% increase LOS) in cirrhotic patients with DVT. In this study, as compared to the study by Wu *et al*[8], cirrhotics with VTE were older as compared to cirrhotics without VTE [36.7% of cirrhotics with VTE were age 65 years and older compared to 29.5% of cirrhotics without VTE ($P < 0.001$)].

The population database based studies have an advantage of large sample size, however, they suffer from limitations including miscoding, missed information as well as lack of laboratory data (thereby MELD score or Child Pugh Score) and clinical details including use of DVT prophylaxis or accurate stratification by severity of liver cirrhosis[8,10,35]. It is obvious that most of the studies above have different study designs, inclusion criteria, period of study, availability of clinical and laboratory data and outcomes. One thing that stands out is that cirrhotic patients have a significant risk of VTE, if not higher than non-cirrhotic patients and this risk cannot be trivialized or ignored.

RISK FACTORS

Studies have attempted to define the risk factors associated with VTE complications in cirrhotics (Table 2). In a case control study by Northup *et al*[6] involving 113 cirrhotic patients with VTE, low serum albumin was an independent risk factor development of VTE (OR = 0.25, 95%CI: 0.10-0.56, $P < 0.001$). Also, INR or platelet counts were not associated with VTE risk. Low albumin was also found to be an independent risk factor in another case control study[5]. This study found that low serum albumin (OR = 0.47, 95%CI: 0.23-0.93, $P = 0.03$) and partial thromboplastin time (PTT) (OR = 0.88: 95%CI: 0.84-0.94, $P = 0.04$) were risk factors of developing DVT/PE in cirrhotics. Diabetes was an independent risk factor for VTE (OR = 4.26; 95%CI: 1.206-15.034; $P = 0.024$) in a retrospective study from Indonesia[33]. Two other retrospective case control series comparing chronic liver disease patients with and without VTE found that VTE cases had significantly lower albumin and hematocrit as compared to non VTE controls, with albumin lower than 1.9 g/dL increasing the risk of VTE more than 5 times compared to patients with albumin greater than 2.7 g/dL (OR = 5.14, 95%CI: 1.05-25.2)[11,34]. It is important to note that both these studies involved patients with portal and nonportal thrombosis. In addition, Anthony Lizarraga *et al*[34] found that chronic liver disease patients with VTE had higher bilirubin (1.71 *vs* 1.11; $P < 0.01$), higher platelet counts (143 *vs* 109; $P = 0.03$), and activated PTT (87 *vs* 60.3 s; $P < 0.01$) as compared with controls. Factors that were associated with higher risk of VTE in cirrhotics included greater comorbidity (as reflected by the Charlson index), black race (OR = 1.25, 95%CI: 1.02-1.55), malnutrition (OR = 1.29, 95%CI: 1.05-1.59) and central venous line placement (OR = 1.7, 95%CI: 1.54-2.04)[10].

Of all the risk factors above, hypoalbuminemia appears to be the most consistent risk factor amongst these studies, with one study finding a five times higher risk in patients with albumin less than 1.9 g/dL. Low albumin may be a reflection of overall decreased liver synthetic function, including a balanced decreased synthesis of the anticoagulant factors like antithrombin Ⅲ, protein C and protein S and the vitamin K dependent coagulant factors Ⅱ, Ⅶ, Ⅸ and Ⅹ. It is interesting to note that elevated INR does not decrease the risk of bleeding and/or thrombosis[6,32].

MANAGEMENT

The current consensus guidelines for VTE prevention do not specifically address the hospitalized cirrhotic patients[38]. Despite the increasing recognition of thrombotic complications in the cirrhotic patients, prophylaxis against VTE in this patient population is frequently avoided. Pharmacological prophylaxis is often not given because of the perceived increased risk of bleeding. Also, mechanical prophylaxis with graduated compression stockings or intermittent pneumatic devices can often be challenging as these can lead to skin breakdown, a condition not so uncommon in cirrhotics with pre-existing

lower extremity swelling and frequently lower extremity cellulitis[39]. In a retrospective study from Saudi Arabia, more than 75% of the hospitalized patients did not receive any mechanical or pharmacological prophylaxis against DVT[9]. Similar low prevalence of DVT prophylaxis was seen in other studies as well[6,32]. In the study by Northup *et al*[6], 21% of the hospitalized cirrhotic patients received prophylaxis, and only 33% of these patients received pharmacological prophylaxis. A greater percentage of chronic liver disease patients (44%) received pharmacological prophylaxis while another 52% received mechanical prophylaxis in a recent study, which may reflect growing awareness about the VTE risk in this patient population[11].

EFFICACY AND SAFETY

To date, there are no randomized studies to evaluate the efficacy and safety of the pharmacological prophylaxis of VTE in hospitalized patients with cirrhosis. A recent meta-analysis of three small retrospective cohort studies with 531 cirrhotic patients (208 with heparin prophylaxis) showed no reduction in the risk of VTE with prophylactic heparin (pooled OR = 1.65 95%CI: 0.36-7.54)[40]. As the authors pointed, the included studies were small in size and had marked clinical heterogeneity with very different inclusion and exclusion criteria[9,41]. Also, the incidence of VTE in the pooled sample was very low (3 events in prophylaxis group and 6 events in no pharmacological group), therefore the sample size was probably insufficient to estimate the real protective effect of the intervention.

The same meta-analysis also found that use of heparin was not associated with higher risk of bleeding in cirrhosis (pooled OR = 0.87 95%CI: 0.34-2.18)[40]. Again, the five studies included in the meta-analysis had very different inclusion criteria (*e.g.*, HCC was an exclusion criteria in one[16] while another study was done in cirrhotic patients with HCC[41]) and involved very different doses of the anticoagulant [prophylactic UFH (unfractionated heparin) or LMWH (low molecular weight heparin)[16,41,42] *vs* therapeutic anticoagulation[43]]. Most of the included studies did not have bleeding risk as the major outcome, therefore the possibility of under reporting or missed bleeding events cannot be excluded. Other than one randomized study, all were retrospective studies and it is possible that patients with perceived higher risk of bleeding did not receive anticoagulation. The largest study evaluating the safety of prophylactic anticoagulation in hospitalized cirrhotic patients found that the prophylaxis was not associated with increased risk of bleeding or death[42]. This retrospective study evaluated 235 patients accounting for 355 discrete hospitalizations to the non ICU bed between 2007 and 2010 who received prophylactic UFH or LMWH. Despite thromboprophylaxis, five patients (1.4%) were diagnosed with VTE (three non-splanchnic DVT, two PE). Nine of 355 (2.5%) had an episode of GI bleeding during hospitalization, five of whom required blood transfusion. Only 3 out of these 9 patients had major bleeding according to standard definition. Heparin induced thrombocytopenia was diagnosed in two patients (0.5%). That no patients died from VTE related complications is of great importance. Prophylactic enoxaparin was also found to be safe in an interesting prospective Italian study[16]. In this randomized study, 34 outpatients with cirrhosis between Child Pugh classes B7-C10 received prophylactic subcutaneous enoxaparin at the dose of 4000 IU/d for 48 wk. Enoxaparin was well tolerated with discontinuation of therapy in only 1 patient at week 36 because of marked thrombocytopenia. Two patients had episodes of bleeding from esophageal varices controlled with conservative endoscopic therapy. Epistaxis was seen in 2 patients. Occurrence of bleeding episodes did not differ between the prophylactic enoxaparin group and control group (P = 0.521). Interestingly, this study showed that prophylactic anticoagulation was associated with significant reduction in risk of development of PVT (HR = 0.098; 95%CI: 0.014-0.697; P = 0.020) occurrence of decompensation (HR = 0.331; 95%CI: 0.148-0.741; P = 0.007), and was associated with survival benefit (HR = 0.366; 95%CI: 0.082-0.795; P = 0.018).

More data is available for safety of anticoagulation in cirrhotic patients with portal vein thrombosis[44-46]. In a study by Delgado *et al*[45] involving 55 cirrhotic patients with portal vein thrombosis, 47 patients received anticoagulation therapy with LMWH with 21 subsequently shifted to vitamin K antagonists (VKA). Remaining 8 patients were initiated and continued on VKA therapy. During the median follow up period of 19 mo, five patients had bleeding that was attributed to the anticoagulation therapy. Platelet count less than 50×10^9/L (P = 0.02) and use of VKA (P = 0.53) were the only factors that were observed more frequently in patients with bleeding secondary to anticoagulation treatment. The anticoagulation treatment with LMWH was also well tolerated in 28 cirrhotic patients with non-neoplastic PVT with no patient requiring interruption of anticoagulation during the treatment duration of more than 6 mo[44].

Although the studies had different designs and used different anticoagulation treatment, the anticoagulation therapy was safely tolerated in all these studies. It is important to realize that in all these studies, patients received primary or secondary prophylaxis for esophageal varices with either endoscopic variceal ligation or use of non-selective beta blockers prior to initiation of anticoagulation. However, anticoagulation treatment was associated with high risk of bleeding complications in a retrospective Spanish study including 17 cirrhotic patients with non-splanchnic VTEs (11 patients with DVT, 7 with PE and 1 with both)[31]. Eleven patients were treated with LMWH while remaining were switched to VKA within a week after initiating LMWH treatment. Majority (83%) of these patients suffered from bleeding complications with six (35%) of them requiring blood transfusions. Only three patients (21%) could continue the anticoagulation treatment beyond six months.

The use of oral VKA has to be considered against the fact that INR is often already elevated in many cirrhotic patients. The target INR is not established as it cannot differentiate between the elevation in INR from underlying cirrhosis *vs* that from VKA[47]. The inter-laboratory variation in the INR in cirrhotic patients is unacceptably high, thereby further complicating the monitoring[48]. It has long been established that INR does not does not predict the bleeding risk in these patients as it fails to capture changes in anticoagulants going on in cirrhotic patients while it measures only the activity of procoagulants[49]. Newer monitoring tests including using a modified INR $_{liver}$[50] rather than INR (using plasma from patients with liver disease rather than plasma from noncirrhotic patients on oral anticoagulants to generate International Sensitivity Index used to calculate INR), thrombin generation assays[27], viscoelastic tests of hemostasis including thromboelastography and thromboelastometry[51] have been proposed but very likely will not offset some of the limitations seen with traditional INR as a marker of coagulation abnormalities seen in these patients.

As discussed above, more data is available with use of LMWH in cirrhotics. While the use of LMWH appears to be safe, its use has its own limitations. In addition to the subcutaneous injection as well as relative contraindication with renal insufficiency, the monitoring of anticoagulant effect in cirrhotics with anti Xa levels is not completely reliable. In a study involving 84 cirrhotic patients requiring prophylactic or therapeutic enoxaparin, Bechmann *et al*[52] found that treatment with standard doses of enoxaparin failed to achieve target anti Xa levels recommended for prophylactic or therapeutic use. Authors also noted negative correlation between the anti Xa levels and the severity of liver disease as assessed by Child Pugh score and the MELD score and concluded that it was likely secondary to decreased synthesis of antithrombin in cirrhotic patients. However, *in vitro* studies evaluating the effect of LMWH on thrombin generation has shown that cirrhotic patients show an increased response to LMWH, in spite of reduced antithrombin and anti-Xa activity levels. The low anti-Xa levels may actually be a laboratory artifact while the efficacy of LMWH is preserved[53,54]. These studies show that increasing the dose of LMWH as a reflex to low anti Xa levels is not necessary and potentially can lead to hemorrhagic complications.

The newer direct thrombin inhibitors like dabigatran and direct factor Xa inhibitors like rivaroxaban and apixaban have advantage of oral intake and are usually given in a fixed dose without requiring any laboratory monitoring in non-cirrhotic patients[55]. However, the data on the use of these agents in cirrhotic patients is limited and the unavailability of any reversible agents in case of active bleeding has limited the clinical applicability of these agents for now.

In summary, we believe that there is enough evidence to make a case for careful anticoagulation in individual cirrhotics-both for prophylaxis as well as treatment for VTE after due consideration for variceal prophylaxis with either endoscopic treatment or non-selective beta blockers or both. In patients with decompensated cirrhosis, use of LMWH may be preferred over oral VKA agents. For reasons stated above, close monitoring of anticoagulation management in the hands of coagulation experts cannot be overemphasized.

CONCLUSION

Chronic liver disease and cirrhosis represent a state of overall decreased liver synthetic function, including a balanced decreased synthesis of the anticoagulant thrombotic factors like antithrombin Ⅲ, protein C and protein S and the vitamin K dependent procoagulant factors Ⅱ, Ⅶ, Ⅸ and Ⅹ on top of thrombocytopenia and/or thrombocytopathia. Though the bleeding risk in advanced liver disease remains the most feared complication of the precariously balanced procoagulant and anticoagulant cascade, VTE complications can certainly not be ignored. These complications are associated with increased hospital length of stay and cost, leading to increased health care burden, in addition to worse patient outcomes. Thromboprophylaxis against VTE should be considered very cautiously in a cross talk between experts in coagulation and hepatology in all these patients. In the absence of absolute contraindications, anticoagulation therapy should be offered of course to all the cirrhotic patients with confirmed VTE.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Pathogenic role of oxidative and nitrosative stress in primary biliary cirrhosis

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Abstract

Primary biliary cirrhosis is a multifactor autoimmune disease characterized by hepatic and systemic manifestations, with immune system dysregulation and abnormalities in the hepatic metabolism of bile salts, lipids, and nutrients, as well as destruction of membrane lipids and mitochondrial dysfunction. Both oxidative and nitrosative stress are associated with ongoing manifestations of the disease. In particular, abnormalities in nitric oxide metabolism and thiol oxidation already occur at early stages, thus leading to the hypothesis that these biochemical events play a pathogenic role in primary biliary cirrhosis. Moreover, the association of these metabolic abnormalities with the progression of the disease may indicate some biochemical parameters as early diagnostic markers of disease evolution, and may open up the potential for pharmacological intervention to inhibit intra- and extra-cellular stress events for resuming hepatocellular functions. The following paragraphs summarize the current knowledge by outlining molecular mechanisms of the disease related to these stress events.

Key words: Aquaporins; Bile salts; Chronic cholestasis; Glutathione; Mitochondria; Nitrosothiols; Nitrotyrosine; Protein sulfhydryls; Thioredoxin

Core tip: Both oxidative and nitrosative stress are associated with ongoing manifestations of chronic cholestasis, and in particular, primary biliary cirrhosis. Abnormalities in nitric oxide metabolism and thiols oxidation already occur at early stages, thus leading to the hypothesis that these biochemical events play a pathogenic role in primary biliary cirrhosis. The association of these metabolic abnormalities with the progression of the disease may indicate some biochemical parameters as early diagnostic markers of disease evolution, and may open up the potential for pharmacological intervention to inhibit intra- and extra-cellular stress events for resuming hepatocellular functions. This article summarizes the current knowledge by outlining molecular mechanisms of the disease related to these stress events.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune liver disease that predominantly affects women and is characterized by chronic and progressive destruction of small intrahepatic bile ducts with portal inflammation and ultimately fibrosis. It is a multifactor condition, mainly due to an immunological disorder of the liver, resulting in a chronic intrahepatic cholestasis that often occurs in asymptomatic subjects, with the diagnosis suggested by the occasional finding of elevated hepatic biochemical markers as early indexes. Appearance of jaundice, dark urine, and pale and fatty stools occur in patients who develop more advanced forms of liver disease.

Little is known about the etiology of PBC. Current theories on its pathogenesis support the hypothesis that the disease develops as a result of an inappropriate immune response following stimulation by an environmental or infectious agent (Figure 1). In particular, liver injury may follow a defect in immunologic tolerance, resulting in the activation and expansion of self-antigen specific T and B lymphocyte clones and the production of circulating autoantibodies, together with a myriad of cytokines and other inflammatory mediators (Figure 2)[1]. The resulting effect of this cascade of events is the activation of pathways leading to oxidative and nitrosative stress (Figures 3 and 4)[2].

Although the disease results from a combination of genetic and environmental risk factors, its serological hallmark is the presence of circulating anti-mitochondrial antibodies. This reflects the presence of autoreactive T and B cells to the culprit antigens, the E2 subunits of mitochondrial pyruvate dehydrogenase. The existence of a genetic predisposition is indicated by the higher familial incidence of the disease, particularly among siblings, and the high concordance rate among monozygotic twins[3]. Environmental xenobiotics/chemical compounds triggering events appear to be crucial in disrupting a pre-existing unstable immune tolerance of genetic origin allowing, after a long latency, the emergence of clinical disease[4]. Finally, up-regulation of WAF1 and p53 related to apoptosis of biliary epithelial cells represents the major pathogenic process leading to bile duct loss in PBC as the expression of genotoxic damage (Figure 1)[5].

It remains unknown why the disease evolution displays a faster pace in some PBC patients compared to others in which it is milder. Therefore, one of the major efforts of the current research is to answer this clinical question and, as a consequence, to identify easily assessable biochemical abnormalities serving as prognostic markers, as well as a target for potential therapeutic intervention. Up to now, however, such a search has not been particularly successful, although a number of biochemical and physiological alterations (toxic bile salt accumulation, oxidative mitochondrial dysfunction, and changes in lipid metabolism)[6] appear to be actively implicated in the onset and evolution of the disease.

PBC is mainly characterized by biliary obstruction and impaired canalicular bile secretion[7], with a progressive retention of hydrophobic bile salts in the liver determining detergent activity and cytotoxic damage[8]. Inflammatory changes[9] and parallel metabolic disorders contribute to the reduced detoxification capacity of the liver[8,10,11], and the enhanced generation of reactive oxygen (ROS) and nitrogen species[12] responsible for oxidative and nitrosative stress[13].

The changes in bile secretion involve complex pathways and result in retention of toxic compounds in the liver. This metabolic and functional impairment is associated with alterations of some physiological mechanisms that regulate bile flow and water transport into the bile canaliculus in response to transient osmotic gradients originating from active solute transport[7,14]. Indeed, at least one third of the bile flow is driven osmotically by the amount of hepatic glutathione (GSH) secreted into canalicular spaces. Beyond the importance of this secretory mechanism for bile formation, the excretion of GSH also represents an important way to discharge toxic anionic compounds deriving from hepatic metabolism of exogenous and endogenous substances. Derangement of hepatic and/or biliary GSH status occurs in several experimental animal models of cholestatic liver injury and in patients with cholestasis[15]. Chronic cholestasis, and in particular PBC, are associated with decreased biliary secretion of GSH, which may affect thiol-dependent bile flow and the hepato-biliary transport of toxic organic compounds[16], and can occur before the reduction in biliary bile salt secretion[17].

On the other hand, the canalicular secretion of water is mediated by aquaporin-8 (AQP8)[18], a water channel protein found at multiple compartments of hepatocytes, including the canalicular plasma membrane and related subapical vesicles[19,20], smooth endoplasmic reticulum, and mitochondria[21,22]. The transport of water from the sinusoidal blood into the hepatocyte is conversely facilitated by AQP9, an aquaporin channel with a broad selectivity[23,24]. AQP9 also represents the major pathway by which glycerol is imported by hepatocytes to supply gluconeogenesis during starvation[25].

During choleresis, hepatocytes rapidly increase their canalicular membrane water permeability by modulating the abundance of AQP8. Studies in several experimental models of cholestasis, such as extrahepatic biliary obstruction, estrogen-induced cholestasis, and sepsis-induced cholestasis, have shown that a decrease in canalicular AQP8 expression contributes to the development of cholestasis. Thus, it has been suggested that the combined alterations in hepatic expression of solute transporters and AQP8 hamper the efficient coupling of osmotic gradients and canalicular water flow, indicating that cholestasis may also result from a mutual occurrence of impaired solute transport and decreased water permeability[26,27]. Since glucagon, a hormone stimulating

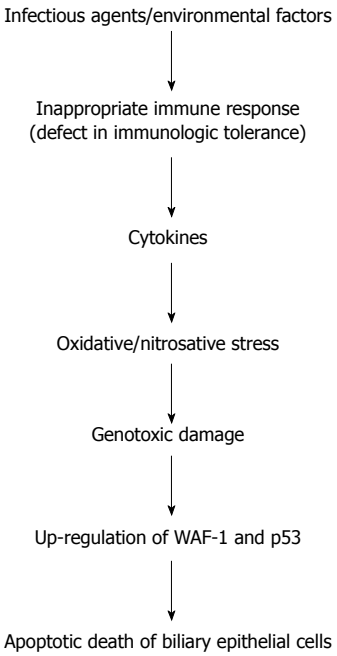

Figure 1 Flow-chart illustrating the cascade of events playing a potential role in the pathogenesis of liver injury in primary biliary cirrhosis. Important steps include the altered immune response linked to increased oxidative/nitrosative stress and genotoxic damage. The final result is the apoptotic death of biliary epithelial cells. WAF1: Cyclin-dependent kinase inhibitor 1; p53: Tumor protein 53.

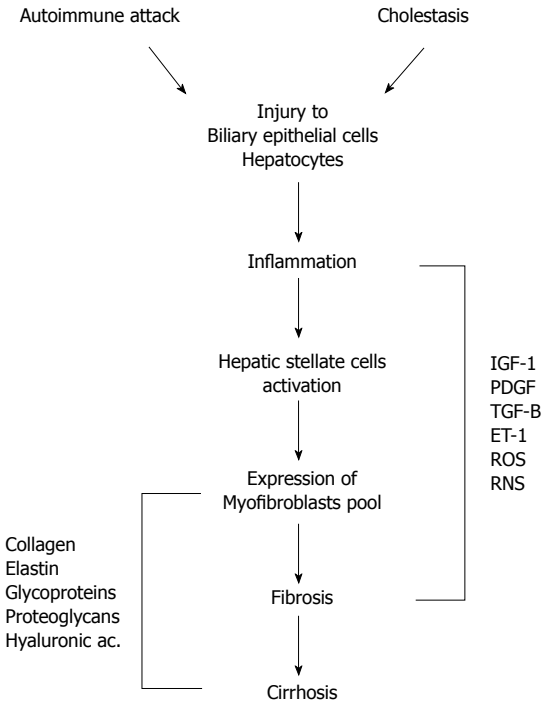

Figure 2 Pathophysiological mechanisms of liver damage in primary biliary cirrhosis. Autoimmune attack and cholestasis are both responsible for the injury to biliary epithelial cells and hepatocytes. Cell damage results in inflammatory processes with activation of hepatic stellate cells and expression of myofibroblasts leading to fibrosis and cirrhosis. A myriad of mediators have a role in these events. IGF-1: Insulin-like growth factor-1; PDGF: Platelet-derived growth factor; TGF-β: Transforming growth factor beta; ET-1: Endothelin-1; ROS: Reactive oxygen species; RNS: Reactive nitrogen species.

hepatocyte bile formation[28], induces the gene expression of AQP8, but not AQP9 water channels in rodent hepatocytes[29], it is reasonable to hypothesize that the most critical step in coupling solute transport and secretion of water takes place on the canalicular membrane of hepatocytes, and that it may be influenced by stress conditions.

Therefore, the associations between inflammation and oxidative and nitrosative stress-induced structural and functional alterations of hepatocytes, including subcellular organelles, render the interpretation of these relationships extremely intriguing, and open to potential therapeutic challenges. In the following paragraphs, the current knowledge on the molecular mechanisms of the disease related to oxidative and nitrosative stress is concisely summarized.

Oxidative stress

Cholestatic events may evolve towards oxidative changes which may directly affect the nature and function of membrane lipids and embedded proteins[30,31], and occur in both the liver and some extrahepatic tissues, albeit recognizing different pathogenic moments.

The transsulfuration pathway is impaired in rats undergoing bile duct ligation (BDL), an experimental model resembling human chronic cholestatic conditions, as well as in humans with cholestatic liver diseases[6,32,33]. This alteration is associated with changes in thiol disposition and redox status. GSH is the main thiol detoxifying molecule playing a role in bile formation and biliary excretion of toxic compounds, as well as in maintaining protein sulfhydryls (PSH) in their reduced form[34,35]. Among these proteins exerting a number of major cell functions are those involved in mitochondrial respiration and membrane transition pore integrity[36,37]. Indeed, most of the redox changes occurring in proteins involve the redox state of cysteine residues, and lead to the formation of intermolecular or mixed disulfides.

In some studies[6,33], more aggressive forms of cholestasis have been associated with primary changes in hepatic and extrahepatic membrane protein redox status and lipid composition. In particular, abnormalities in hepatocyte membranes have been reported in cirrhotic patients[38] that have been associated with an imbalance between the transmethylation and the transsulfuration pathways[39]. Similar to hypercholesterolemic subjects[40], cirrhotic patients show lipid alterations in erythrocyte (RBC) membranes in relation to changes in the circulating lipoproteins[41,42]. Such alterations are particularly important in patients with PBC who accumulate toxic bile salts and show early alterations in hepatic lipid metabolism with increased synthesis of cholesterol[43]. Indeed, both bile salt excess and lipid changes have been implicated in the early modification of membrane fluidity and transport function[44] at both hepatocyte and RBC levels[45,46]. Common biochemical derangements[47], such as the alteration of Na^+/K^+ ATPase activity of hepatocyte

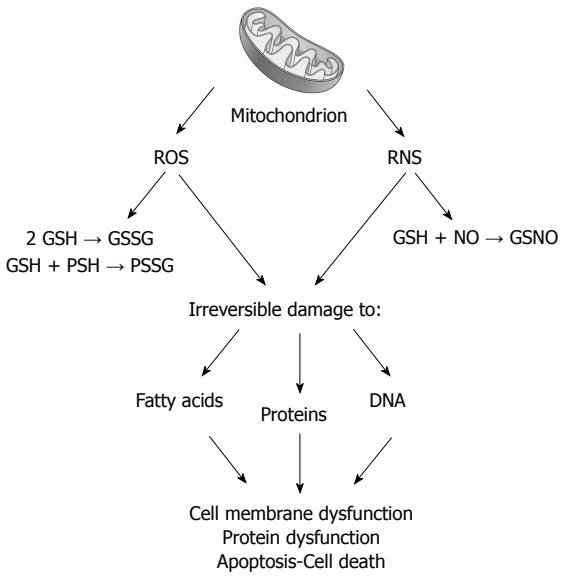

Figure 3 Following chronic cholestasis, liver mitochondrial impairment is associated with increased delivery of reactive oxygen species and reactive nitrogen species. Reactive oxygen species (ROS) promotes the oxidation of glutathione (GSH to GSSG) and of protein sulfhydryls (PSH to PSSG). Reactive nitrogen species (RNS) favors the formation of nitrosothiols (GSNO). Both steps are responsible for irreversible damage to lipids, proteins, and nucleic acids, ultimately leading to membrane and protein alteration.

membranes[48], have also been claimed.

RBC membrane alterations, which parallel those found in hepatocytes[47], include a decreased content of PSH and an increased aliquot of oxidized proteins (carbonyl), especially in patients with stage Ⅲ-Ⅳ PBC[49]. In cell membranes, PSH has structural and functional roles, and confers resistance to damaging insults[50]. Membrane PSH maintenance in the reduced form is therefore a major program for all biological systems to assure functional integrity. Together with changes in ratios of the membrane cholesterol:phospholipids, the described oxidation rate of ghost proteins in RBC likely explains the high susceptibility to *in vitro* hemolysis observed in PBC patients. Furthermore, with the application of logistic regression analysis, it has been observed that advanced forms of PBC could be identified by the simultaneous presence of significant changes of some RBC membrane parameters[50]: PSH lower than 40 nmol/mg protein (r = -0.817), protein carbonyls higher than 2.7 nmol/mg protein (r = 0.653), cholesterol greater than 550 nmol/mg protein (r = 0.744), and phosphatidylethanolamine lower than 25% (r = -0.731).

Taken together, these observations explain, at least in part, the changes to membrane fluidity, transport[41], receptor activities[51-53], and the increased hemolysis observed in patients with PBC[54]. Indeed, the possible link between protein oxidation and altered lipid composition in the RBC ghost membrane of PBC patients may be partially explained by metabolic changes occurring in the liver. In particular, the increased hepatic production of phosphatidylcholine, which is formed through the methylation of phosphatidylethanolamine *via* the transformation of S-adenosyl methionine in S-adenosyl-L-homocysteine, may play a role in these processes[55-57].

Many clinical and experimental observations have found that oxidative stress also participates actively in cholestasis-induced hepatocyte damage, as suggested by the accumulation of lipid and protein oxidative products[9]. However, the molecular regulatory mechanisms by which oxidative stress induces modifications of antioxidant capacities at the same time as maneuvering cellular adaptation by increasing the availability of redox active compounds is still debatable.

Studies performed in BDL rats have shown that both free and protein thiols (GSH and PSH) are lower within the hepatocyte cytosol, and that these alterations occur at early stages. These changes parallel the high concentrations of oxidized glutathione (GSSG) observed with ongoing cholestasis and the increased enzymatic activities of both GSH-peroxidase and GSSG-reductase, thus asserting the participation of oxidative stress in cholestatic hepatic injury[58].

One of the most interesting molecules in this context is thioredoxin, an oxidative stress-induced redox-active protein[59]. Thioredoxin possesses a number of biological activities[60], including the maintenance of protein-SH groups[61], the regulation of redox-sensitive molecules[59], and the protection of membrane permeability[62]. Circulating levels of thioredoxin are increased in patients with a high rate of free radical generation[63], in which it also interferes with the modulation of the immunological response[64]. In PBC patients, circulating thioredoxin is increased in the early disease stage[65] and decreased in patients with a more advanced disease stage, in which it is inversely related with circulating nitrosothiols (r = -0.609), the liver fibrosis marker hyaluronate (r = -0.432), and with histology (r = -0.757)[65]. In RBC, thioredoxin concentrations are higher in PBC stages I to Ⅲ, but lower in stage Ⅳ patients compared to healthy subjects[65].

More detailed changes in thioredoxin disposition have been studied in BDL rats. In these cholestatic animals, serum thioredoxin concentrations were found to be increased at 3 d after surgery and then decreased. Similarly, such changes were observed in the whole liver and in isolated mitochondria, but no variations were documented in RBC. Therefore, by considering the difference in the average time changes from baseline among serum, hepatic, and RBC levels, it emerged that circulating thioredoxin is likely of hepatic origin.

Other important organs having a role in cholestatic liver damage and reparation are oxidatively damaged during chronic cholestasis; this is the case of the intestine. In this regard, significant redox alterations have been early observed in the intestine of BDL rats, where the mucosal concentrations of GSH are low in both the ileum and colon. The hypothesis on oxidative consumption of GSH has been confirmed by early increases of GSSG and protein carbonyls, as well as a decrease of PSH[58]. However, under cholestatic conditions, decreased avail-

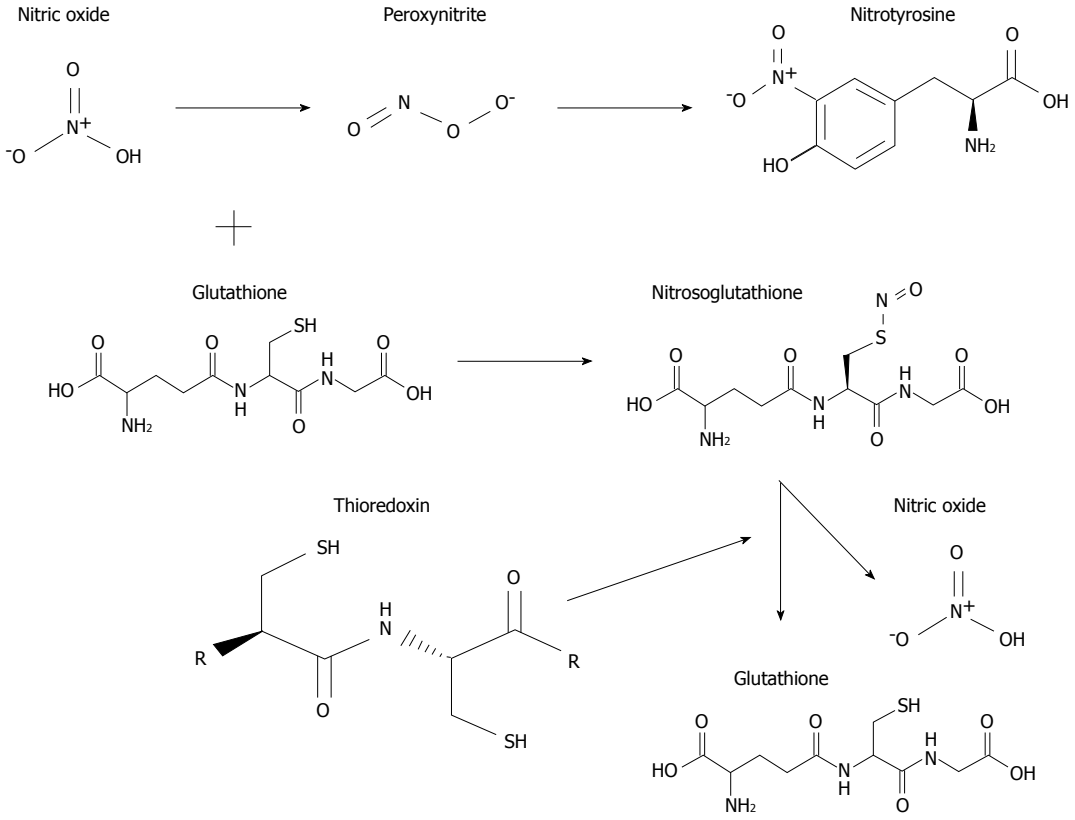

Figure 4 Structural formula and interaction of some important molecules involved in oxidative and nitrosative stress associated with chronic cholestasis.

ability of biliary GSH, which currently represents a considerable source of luminal GSH, may also take part in the depletion of intestinal GSH. At this level, the changes in mucosal proteins are associated with a decreased concentration of total proteins and lower activity of GSH-dependent antioxidant enzymes (peroxidase and reductase). This may depend on the reduced synthesis of proteins linked to a decreased availability of bile salts, which enhance gene expression and regulate the synthesis of proteins at the intestinal level[66,67]. Bile salt deficiency, conversely, promotes mucosal injury[68] and reduces protein concentrations[69], which, at least in part, explains the suppression in protein synthesis in the intestinal mucosa of cholestatic rats. These biochemical changes are associated with profound functional alterations of colonic ionic transport and a significant overall decrease of all electrophysiological parameters.

Considering that the impairment of the GSH-dependent antioxidant system, the changes in mucosal permeability and transport capacity, and the increased susceptibility to toxic injury all occur during the early stage of cholestasis, it is conceivable that these alterations may favor intestinal bacterial translocation and the absorption of toxic molecules[70]. The latter two phenomena are likely to be implicated in the appearance of hepatic injuries, and this would demonstrate the existence of a close interrelation between the liver and the intestine, both in the physiological state and in the case of cholestasis. Finally, cholestatic rats show a progressive reduction in their daily food intake, and as a consequence, show a deficient availability of dietary precursors, which is likely to occur earlier in the intestine and liver than in other organs. This aspect should always be considered when studying cholestatic animals.

Nitrosative stress

It is known that a number of cellular and circulating factors can modulate cell membrane activity and cholestatic disease progression. In cholestatic livers, nitric oxide (NO), a potential free radical intermediate, is mainly released by inducible NO synthase (iNOS)[71,72], which is likely sustained by the enhanced inflow of gut-derived endotoxins[58,72,73]. Indeed, it is ascertained that extrahepatic factors actively participate in the determination of cholestatic liver injury. In this respect, prolonged interruption of enterohepatic circulation of bile salts results in intestinal permeability alteration and favors portal endotoxemia[74] with worsening or even promotion of hepatic damage[75] by enhancing free radicals generation, GSH depletion, and impairing detoxification defense[6,32,33].

The excess generation of NO already present in early disease stages favors the formation of hyperreactive derivatives that ultimately result in nitrotyrosine deposition[76-78]. This compound accumulation reflects the excess formation of the highly reactive molecule peroxynitrite, and supports a major injuring role for NO in chronic cholestasis. This view is also consolidated by

the observation that the serum of PBC patients contains significantly higher levels of nitrotyrosine compared with control subjects. Moreover, the trend in nitrotyrosine levels seems to parallel the disease stage.

Conversely, nitrosothiols are formed by conjugation of some reactive NO forms ($NO^{\bullet}$) with thiols and GSH in particular, and thereby oppose dangerous side reactions such as peroxynitrite formation[60]. Nitrosothiols also act as intracellular messengers, and exert a major regulatory effect on cellular and mitochondrial functions[79] through the nitrosylation of proteins and enzymes[80,81]. Conversely, circulating nitrosothiols mainly act as a reserve for NO, whose release after molecule decomposition serves for vascular tone modulation[81]. Serum nitrosothiols are significantly higher in PBC patients than in healthy controls, with the increase being directly proportional to disease severity and paralleling the serum concentrations of hyaluronate[65] and nitrotyrosine[82]. Indeed, it is known that circulating levels of NO derivatives are particularly high in all cirrhotic conditions[83].

Compared to RBC, in which no variations have been noted, serum and hepatic nitrosothiols significantly and progressively increase with ongoing cholestasis in the BDL model; increasing more than tenfold in value at day 10 after the BDL. Following BDL, the NOx (nitrate plus nitrite) content is significantly increased in the liver.

Overall, the importance of the observations reported for early stages of experimental cholestasis and their presence in patients with milder forms of PBC definitely point to an active participation of these pathophysiological events in the generation of cholestatic liver damage. In fact, the increased hepatic content of NOx and nitrosothiols following BDL point to enhanced hepatocellular NO production, although some studies have shown no increase or even a decreased delivery of hepatic NO by endothelial cells and macrophages in pre-cirrhotic BDL rats[84,85]. On the other hand, others have reported increased hepatic iNOS expression in PBC patients[77] and in the hepatocytes of BDL rats[70]. Indeed, the physiological role of nitrosothiols in biological systems has also not been completely clarified as of yet. Nitrosothiols are unstable thioesters exerting different functions within the cell and in the extracellular compartment[86]. Nitrosothiols are not exclusively formed in cells generating NO, and can be taken up by cells *via* amino acid transport systems[87]. In a concentration dependent manner, intracellular nitrosothiols seems to be actively involved either in redox signaling or as nitrosative stress mediators[88], whereas, by representing a storage pool of thiols and NO, extracellular nitrosothiols have an importance in the regulation of the protein redox status at the cell surface[89]. In tissues, the formation of nitrosothiols adducts occurs under conditions of nitrosative stress and counteracts excess NO radicals[60]. Therefore, increased levels of tissue nitrosothiols may result from increased formation and/or decreased decomposition[86,90], such as in the presence of reduced thioredoxin availability.

Interaction between oxidative and nitrosative stress

The interplay between antioxidant molecules and oxidative and nitrosative stress derivatives renders these interactions particularly fascinating in the field of chronic cholestasis, where adaptive processes, in particular those at the subcellular level, are ultimately responsible for hepatocyte death or survival. Indeed, mutual and interrelated changes between circulating and hepatic oxidative and nitrosative stress markers have been reported, and point to an active and interrelated participation of these biochemical events in both liver injury and extrahepatic changes during cholestasis[6,33,65]. In fact, chronic cholestasis results in early and significant interactive changes between thioredoxin and nitrosothiols both at the circulating level and in the liver. The close relationship between the levels of these molecules with liver histology in patients with PBC may account for their active implication in the progression of the disease itself.

This is an important observation since thioredoxin represents one major system playing a regulatory role for several subcellular activities. Thioredoxin has an active role in oxidative/nitrosative stress interplay; in fact, it is also involved in the regulation of some NO activities through the cleavage of nitrosothiols[90,91] and the suppression of peroxynitrite formation[60]. Serum thioredoxin levels appear to discriminate between aggressive and benign forms of chronic non-cholestatic liver disease conditions[92]. In the context of PBC, the increased circulating levels of thioredoxin in patients with stage Ⅰ-Ⅱ suggest that it is likely induced to counteract increased ongoing oxidative stress or that it is an early adaptive measure to regulate NO metabolism, nitrosothiols decomposition, and to maintain surface PSH in the reduced form. Conversely, the decreased circulating level of thioredoxin in patients with more advanced stages of PBC likely reflects a diminished hepatic synthesis and delivery into the vasculature.

However, with the progression of cholestasis, the decreased production of thioredoxin in the liver, likely due to a down-regulation process associated with excess retention of hydrophobic bile salts and toxic molecules, may indirectly contribute to the progressive appearance of oxidative alterations, and therefore the promotion of fibrosis. In fact, adequate thioredoxin levels effectively protect against stellate cells activation and opposite collagen synthesis and hepatic fibrosis[93]. As a consequence of thioredoxin changes, the serum levels of nitrosothiols and the oxidation of erythrocyte PSH are increased in stage Ⅳ patients, indicating that circulating thioredoxin serves to protect surface PSH from oxidation and regulates nitrosothiols levels.

In this regard, it is known that nitrosothiols can be decomposed either by enzymatic homolytic cleavage in a NADPH dependent reaction involving the thioredoxin system, by a redox-sensitive metalloprotein, or *via* a non-enzymatic one electron reduction[86,91,94]. Notably, one recent study has reported that thioredoxin-deficient cells

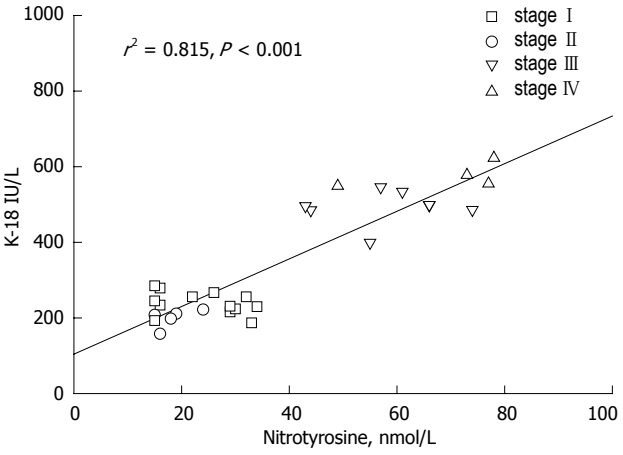

Figure 5 Link between nitric oxide derivatives and inflammatory products is linearly evidenced in this graph reporting the relationship between serum levels of nitrotyrosine and keratin-18. Patients with primary biliary cirrhosis (n = 30) were divided in stages according to liver histology: stage Ⅰ; stage Ⅱ; stage Ⅲ; stage Ⅳ. Data adapted from Grattagliano and Portincasa (unpublished). Nitrotyrosine and keratin-18 (K-18) concentrations measured as previously reported[82].

denitrosate S-nitrosothiols less efficiently[91]. Given the fact that increased nitrosothiols formation is associated with disease progression in PBC patients, they may have a potential use as a prognostic indicator in these patients.

The close link between oxidative and nitrosative stress parameters in PBC is further shown by the inverse relationship existing between serum thioredoxin and nitrotyrosine (r = -0.838), as well as between thioredoxin and cytokeratin 18 (r = -0.838), a major cytoplasmic intermediate filament protein in hepatocytes and cholangiocytes whose serum levels are elevated in chronic liver disease as a consequence of hepatic inflammation, and thus directly reflect the expression of cellular apoptotic processes (Figure 5)[95]. In PBC patients, cytokeratin 18 correlates well with serum nitrotyrosine (r = 0.894)[82].

Hepatic mitochondrial changes

Prolonged cholestasis is known to be associated with changes in mitochondrial morphology (*i.e.*, shortening of cristae, appearance of inclusions, and matrix darkening) and energy metabolism[8,10,96]. In fact, both the number and volume ratio of mitochondria increase and parallel the duration of biliary obstruction in rats[97]. These findings point to adaptive processes in response to mitochondrial dysfunction[98]. Mitochondria are plastic organelles constantly changing their shape to fulfill their various functional activities[99].

In this regard, the permeability transition pore (PTP), which is formed across the mitochondrial membranes, has been hypothesized to have a major part in the rapid movements of water into and out of the mitochondrial matrix underlying the changes in volume that characterize the organelle[100]. The aqueous pore formed by the AQP8 isoform across the inner mitochondrial membrane is found to be more relevant to mitochondrial processes, such as release of H_2O_2[101] and import of ammonia by the organelle (urea cycle)[102-104], than underlying mitochondrial volume homeostasis. It has been observed that the mitochondrial changes accompanying the ongoing cholestasis occur early and parallel the progressive decrease of mitochondrial GSH concentrations and the increase of GSSG levels. In our context, therefore, low levels of mitochondrial GSH are associated with increased susceptibility to oxidative damages[105], resulting in protein oxidation responsible for the activation of cell death pathways[106,107]. The amount of mitochondrial PSH lowers with the cholestasis progression and, similarly to the extra-mitochondrial compartment, both GSH-peroxidase and GSSG-reductase activities increase (Table 1). The decrease in PSH parallels the increased content of protein disulfides (PSSG) and decreased expression of AQP8 (Figures 6 and 7), thus testifying that cholestasis is associated with mitochondrial oxidative changes of proteins and sulfhydryls, as well as functional impairment.

These oxidative changes of mitochondria are functionally important for the energetic activities known to be damaged early during cholestasis, as reported previously[10]. With this in mind, the observation that GSH content declines earlier and at a greater extent in mitochondria than in the cytosolic compartment, as observed in rodent models of cholestasis, points to selective mitochondrial damage. This behavior of mitochondrial GSH might imply two different hypotheses: defective *ex novo* synthesis of cytosolic GSH, which is then imported by mitochondria, or increased mitochondrial oxidative consumption. Both may follow the intrahepatic accumulation of hydrophobic bile salts (*i.e.*, tauro- and glycochenodeoxycholate) and may also coexist. Indeed, it is known that liver mitochondria exposed to hydrophobic bile salts generate a great amount of ROS, and that damaged mitochondria result in a higher release of radicals[108,109]. The former hypothesis is also supported by the increased expression of mRNA for γ-glutamylcysteine synthetase and by the enhanced activity of methionine adenosyltransferase following administration of ursodeoxycholate (UDCA)[110]. UDCA (molecular formula $C_{24}H_{40}O_4$, molecular weight: 392.572) is the 7β-epimer of the primary dihydroxy bile acid chenodeoxycholic acid (representing the hepatic catabolic product of cholesterol)[111]. UDCA is found in mammalian bile, following bacterial reduction in ileum and colon. As a therapeutic agent, UDCA has hydrophilic and less cytotoxic properties. The latter hypothesis is supported by the association of a marked increase of GSSG and PSSG with the increased activity of GSH-related antioxidant enzymes. Furthermore, mitochondria from BDL rats exhibited an altered lipid composition, with a two- to threefold increase in the cholesterol/phospholipid ratio in the mitochondrial inner membrane[97], thus suggesting the possibility of impaired transport of GSH into mitochondria.

Moreover, it is important to note that mitochondrial

Table 1 Time-related changes of glutathione peroxidase and glutathione reductase activities in liver cytosol and mitochondria of sham-operated and bile duct ligated rats

Cytosol	Day 0	Sham Day 3	BDL Day 3	Sham Day 10	BDL Day 10
GSH-Px	351 ± 32	336 ± 35	504 ± 54[1]	335 ± 20	566 ± 32[1,2]
GSSG-Rx	0.31 ± 0.11	0.27 ± 0.10	0.37 ± 0.08[1]	0.28 ± 0.05	0.60 ± 0.10[1,2]
Mitochondria					
GSH-Px	977 ± 27	913 ± 44	1061 ± 95	890 ± 42	1568 ± 105[1,2]
GSSG-Rx	1.12 ± 0.17	0.93 ± 0.09	1.33 ± 0.12[1]	0.89 ± 0.07	1.98 ± 0.12[1,2]

Glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-Rx) are reported as mU/mg protein/minute. Significantly different ($0.01 < P < 0.05$) compared to [1]baseline and [2]sham-operated rats at each time point. Adapted from Grattagliano and Portincasa (unpublished data). Enzyme activities assessed as previously described[58]. BDL: Bile duct ligation.

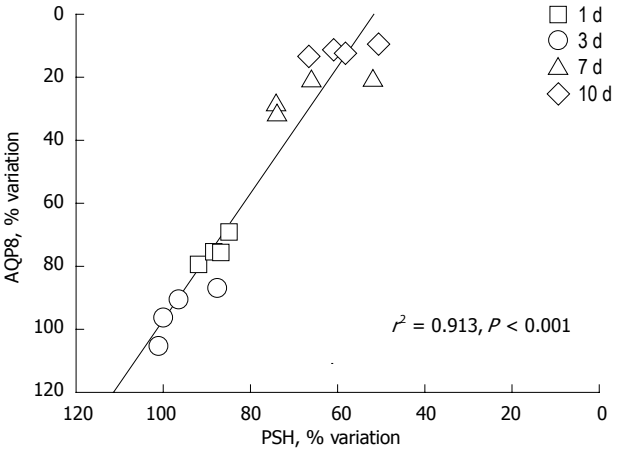

Figure 6 Aquaporin 8 is a specific water channel actively involved in the regulation of mitochondrial volume in the liver. Protein sulfhydryls (PSH) are a diffuse category of proteins playing important roles in mitochondria, including structural proteins and respiratory complexes to channel pores. The graph depicts the correlation between densitometric values of aquaporin 8 (AQP8) immunoreactivity and PSH content in the liver mitochondria of sham-operated and bile duct ligated rats at different time-points. AQP8 expression varies in relation to the changes in PSH content (n = 16, r = +0.96, r^2 = 0.913, P < 0.0001). N = 4 experiments per time point with 1 d, 3 d, 7 d and 10 d; BDL. Data adapted from Grattagliano and Portincasa (unpublished). Measurements performed as previously reported[65].

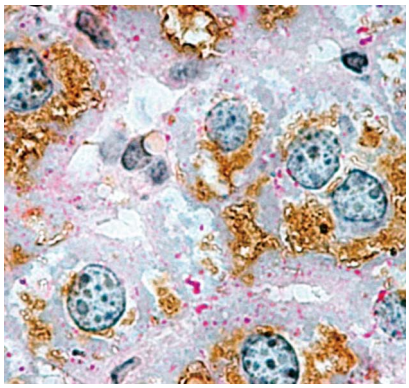

Figure 7 Immunohistochemical localization of aquaporin 8 in mouse hepatocytes. Considerable aquaporin 8 (AQP8) immunoreactivity (brown staining) is seen within the cytoplasmic compartment of most hepatocytes. Periodic acid-Schiff (PAS) reactivity (red staining) is seen over glycogen granules. In rodents, hepatocyte AQP8 is expressed at multiple subcellular levels, including the apical (canalicular) plasma membrane, subapical vesicles, smooth endoplasmic reticulum, and mitochondria. The AQP8 channel features conductance to water, ammonia, and H_2O_2, and is suggested to be involved in primary bile secretion, ammonia detoxification, ureagenesis, and mitochondrial reactive oxygen species generation. Original magnification, × 1000. (micrograph from Ferri *et al*[22], reproduced with permission from Wiley Online Library).

protein content is generally decreased in BDL rats and, together with their increased oxidation rate, deserves some consideration. Firstly, mitochondria exhibiting a decrease of functional proteins are less prone to rapidly changing their shape and, therefore, are less able to adapt their function in response to environmental stress. Secondly, the decreased content of proteins in mitochondria may be the consequence of down-regulation processes to which several proteins undergo during cholestasis when the excretory function is severely impaired[112].

Recent studies have shown that mitochondrial concentrations of nitrosothiols are increased after BDL to an even higher extent than that observed in the extra-mitochondrial compartment[65]. Indeed, NO is known to stimulate hepatocyte GMPc dependent signaling pathways and, in mitochondria, it is an important physiological reactant. NO controls mitochondrial ATP synthase, gene expression, and PTP through protein nitrosylation and nitrosation[88,113]. Mitochondria are known to possess their own NO metabolism[114] and a specific NOS that generates the highest rate of intracellular $NO^{\bullet}$[88,115]. Moreover, NO may provide beneficial effects at this level by supporting cell survival, thus resulting as a determining factor for mitochondriogenesis[116,117].

Among redox active compounds, thioredoxin is a molecule having an important role for the mitochondrial energetic domain. In fact, overexpression of mitochondrial thioredoxin and thioredoxin reductase attenuates damages associated with excess NO[118] by increasing the mitochondrial membrane potential[119]. In particular, mitochondrial thioredoxin is critical for defense against oxidative stress induced cell apoptosis, and thioredoxin reductase is the only known enzyme catalyzing thioredoxin reduction in mitochondria. However, thioredoxin reductase is known to be sensitive to inactivation by electrophiles, such as some lipoperoxidative products. Glutaredoxin, a system helping to keep thioredoxin reduced during stress events, also contributes to anti-apoptotic signaling. This mitochondrial thiol-disulfide oxidoreductase, in fact, is relatively independent from the cytosolic compartment. In particular, thioredoxin and glutaredoxin systems consist of NADPH, thioredoxin reductase[120],

Table 2 Therapeutic doses of ursodeoxycholic acid and its potential effects in the treatment of patients with primary biliary cirrhosis

Effective dose: 13-15 mg/kg per day indefinitely
Mechanisms of action
Promotion of endogenous bile acids secretion
Replacement of hepatotoxic (endogenous) bile acids
Stabilization of biliary epithelial cell membranes
Alteration of HLA Ⅰ-Ⅱ expression on biliary epithelial cell
Inhibition of biliary cell apoptosis
Improvement of hepatocyte redox status
Signaling for glucose and insulin metabolic pathways
Delays disease progression and improves transplant-free survival

and thioredoxin[121], or NADPH, GSH-reductase, GSH, and glutaredoxin[122], respectively. Within mitochondria, thioredoxin and glutaredoxin play critical roles in controlling protein folding and regulating cell growth/apoptosis[123-126]. An experimental-computational study converged on the idea that GSH/thioredoxin scavenging systems in mitochondria are both essential for keeping minimal levels of H_2O_2 emission[127]. The crosstalk between these two systems may support each other's functions, but could not substitute for each other, probably due to their respective functions and different substrates. Thioredoxin is more active in catalyzing the reduction of intra- or inter-chain disulfides of protein substrates, is an electron donor for peroxiredoxins, a major player in the elimination of H_2O_2[128], and regulates the activity/activation of transcription factors or apoptosis signaling factors through modulating the redox-regulatory disulfides of the protein. Mitochondrial thioredoxin in the reduced form is usually an indicator of cell survival, while the predominance of its oxidized form is an indicator of cell death. Glutaredoxin is not inactivated by oxidation[122] and its insensitiveness increases the chance of protecting mitochondrial thioredoxin from oxidation when the reductase is inactivated. It is specifically active in catalyzing the deglutathionylation of proteins at the expense of GSH.

Protein S-glutathionylation is an important reversible post-translational modification of proteins that performs thiol-redox signaling for many cellular events, including apoptosis[129]. Indeed, it is known that nitrosative stress is critically important in promoting *S*-nitrosylation and S-glutathiolation of various mitochondrial proteins, leading to mitochondrial dysfunction, decreased energy supply[130], and increased hepatic injury[130]. With the progression of cholestasis, however, the decreased availability of thioredoxin, together with excess NO and GSH depletion[131], may result in enhanced protein nitrosation[76] and PSH oxidation. These events have obviously negative consequences for hepatocyte survival and bile duct integrity[78,132]. Of course, whether over-expression of mitochondrial glutaredoxin and thioredoxin systems might have therapeutic potential in diseases such as PBC where mitochondrial oxidation plays a dominant role is an intriguing challenge for future investigations.

CONCLUSION

The data discussed here from integrated and translational studies unequivocally suggests that oxidative and nitrosative events determine disease appearance and progression in patients with PBC. Changes in circulating markers, such as thioredoxin and nitrosothiols, may be used to monitor disease progression and to identify patients at risk of disease evolution. Finally, it may be argued that pharmacological interventions directed to sustain hepatic GSH, thioredoxin, and glutaredoxin levels by activating nuclear factors and up-regulating gene transcription[133-135] may favorably contrast NO deranged metabolism in the early phase of chronic cholestatic conditions and may yield hepatocyte protection by favoring mitochondrial proliferation through the maintenance of protein redox status. In fact, the modulation of the protein oxidation/denitrosation process has been suggested as a mechanism of either cytoprotection or cellular damage. In this connection, recent studies have revealed new therapeutic aspects of some compounds. UDCA, initially introduced in therapy to counteract the cholestatic components of PBC, was subsequently shown to have also anti-inflammatory and immunomodulatory properties (Table 2). The use of farnesoid X receptor agonists in animal models of cholestasis has confirmed that bile acids are not only toxicants and inflammagens, but also repressors of innate and adaptive immunity[136]. In fact, other than a well-established role in the digestion and absorption of dietary lipids, bile acids are currently recognized as signaling molecules in a wide range of metabolic processes in which they contribute to improve hyperglycemia and insulin resistance[137].

For this reason, understanding the complexity of the inflammatory mechanisms leading to bile duct epithelial injury represents a crucial step for the future development of therapies aimed at inhibiting ongoing biliary tract destruction in PBC. Both oxidative and nitrosative injurious mechanisms are effectors of damage in the liver, and therefore, interventions aimed at rectifying these processes may have therapeutic effects. Recently, UDCA has shown to protect against oxidative and nitrosative stress, at least in the early stages of PBC[65,138].

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TOPIC HIGHLIGHT

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Antitubercular therapy in patients with cirrhosis: Challenges and options

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Abstract

Tuberculosis (TB) has been a human disease for centuries. Its frequency is increased manyfold in patients with liver cirrhosis. The gold standard of TB management is a 6-mo course of isoniazid, rifampicin, pyrazinamide and ethambutol. Although good results are seen with this treatment in general, the management of patients with underlying cirrhosis is a challenge. The underlying depressed immune response results in alterations in many diagnostic tests. The tests used for latent TB have many flaws in this group of patients. Three of four first-line antitubercular drugs are hepatotoxic and baseline liver function is often disrupted in patients with underlying cirrhosis. Frequency of hepatotoxicity is increased in patients with liver cirrhosis, frequently leading to severe liver failure. There are no established guidelines for the treatment of TB in relation to the severity of liver disease. There is no consensus on the frequency of liver function tests required or the cut-off used to define hepatotoxicity. No specific treatment exists for prevention or treatment of hepatotoxicity, making monitoring even more important. A high risk of multidrug-resistant TB is another major worry due to prolonged and interrupted treatment.

Key words: Antitubercular therapy; Drug hepatotoxicity; Multidrug-resistant tuberculosis; Immune dysfunction

Core tip: Treatment of tuberculosis (TB) in patients with underlying cirrhosis is a challenge because of the compromised liver functions and high risk of hepatotoxicity. There is no consensus regarding the treatment and monitoring of TB in this group of patients. This paper reviews the differences in diagnosis, treatment, monitoring, hepatotoxicity and other issues in treatment of TB in patients with cirrhosis. Suggestions for treatment of TB in patients with different grades of cirrhosis, as well as monitoring guidelines, are provided. Finally, issues such as liver transplantation, multidrug-resistant TB and reactivation of TB by interferon are briefly reviewed.

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INTRODUCTION

Tuberculosis (TB) has afflicted humans for many centuries[1]. About one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). TB is widely prevalent worldwide, especially in the developing countries in Africa and Asia, with an estimated 40%-50% of the adult population being infected[2]. India has the highest TB burden in the world according to World

Health Organization (WHO) statistics for 2011, giving an estimated incidence of 2.2 million cases in India out of a global incidence of 8.7 million cases[3]. Primary infection with *M. tuberculosis* leads to clinical disease in only approximately 10% of individuals and in the rest, latent TB infection develops. In 5%-10% of latently infected persons, the infection reactivates and causes active TB[4]. The progression from latent infection to active disease depends on a number of factors, of which the most important is the presence of an underlying immunodeficient state[5]. Cirrhosis is a widely prevalent disease that leads to immunosuppression and a higher prevalence of TB than in the general population[6]. However, treatment in patients with underlying cirrhosis is complicated by poor tolerance, higher incidence of hepatotoxicity, no consensus regarding monitoring and treatment regimens, and higher chances of multidrug-resistant (MDR) TB. This paper reviews the differences in diagnosis, treatment, monitoring, hepatotoxicity and other issues in treatment of TB in patients with cirrhosis.

CIRRHOSIS AND TB

Prevalence and relationship

Liver cirrhosis is also a relatively common condition with autopsy studies showing a prevalence of 5%-10%[6]. Evidence suggests a higher prevalence of TB in patients with cirrhosis as compared to the general population. The high incidence of TB in patients with cirrhosis has been ascribed mainly to immune dysfunction with associated higher virulence as compared to the general population[7]. In a cohort study of patients with liver cirrhosis from Denmark (1977-1993), the incidence of TB was 168.6 per 100000. It was highest in men aged > 65 years, with an incidence of 246 per 100000[8]. Furthermore, patients with cirrhosis who acquired TB had a poor prognosis in that study. A study conducted in Western India showed that the prevalence rate was 15 times higher than in the general population[9]. Another study from India showed that there is nearly five times higher prevalence of TB in cirrhosis patients (8.1%) compared to the general population (1.6%), with pulmonary TB being the commonest form[10].

Pulmonary TB is generally responsible for 80%-85% of all cases of TB reported[11]. Cirrhosis has been suggested as a risk factor for extrapulmonary TB in a previous study[12]. In a Korean study, 31% patients with cirrhosis had extrapulmonary TB, as compared to 12% in the non-cirrhosis control group with a predominance of peritoneal TB[7]. There are several reports of unusual manifestation of TB in patients with cirrhosis[13]. Little is known about the immunopathogenesis of TB in such clinical conditions. Although most of the host defense systems, especially the clearance capacity of the reticuloendothelial system, are thought to be diminished in patients with cirrhosis, there is no simple explanation as to how this immune dysfunction results in patients being more likely to develop extrapulmonary TB than pulmonary TB.

Cirrhosis-associated immune dysfunction

Cirrhosis-associated immune dysfunction syndrome is a multifactorial process in which the ability to clear cytokines, bacteria and endotoxins from the circulation is decreased[14]. The liver is the major organ of the reticuloendothelial system and contains 90% of the cells of the reticuloendothelial system that are central to clearing bacteria, such as Kupffer cells and sinusoidal endothelial cells[14]. There is reticuloendothelial system dysfunction in patients with cirrhosis, which leads to significantly reduced monocyte spreading, chemotaxis, bacterial phagocytosis, and bacterial killing in cirrhosis compared with controls, and hence compromised innate immunity[14]. These patients also have decreased neutrophil mobilization and phagocytic activity with reduced oxidative bursts; a phenomenon that has been shown to correlate with the severity of liver disease. Hyperammonemia and hyponatremia have been shown to lead to reduced neutrophil function and impaired phagocytosis[15]. Furthermore, specific etiology of liver disease, such as alcohol and hepatitis B and C, have been shown to be associated with additional impairment in immune function and/or increase in proinflammatory cytokines[16].

Toll-like receptors (TLRs) are encoded pattern recognition receptors that play a central role in host cell recognition and responses to microbial pathogens. About 10 functional human TLRs (TLR1-10) have been described; each one being involved in the sensing of distinct microbial products[17]. TLR-2 is capable of recognizing pathogen-associated molecular patterns expressed by *M. tuberculosis*, such as a 19-kDa lipoprotein, lipoarabinomannan, and soluble TB factor[18]. Immune evasion allows *M. tuberculosis* to establish persistent or latent infection in macrophages and results in TLR-2-dependent inhibition of MHC class Ⅱ transactivator expression, MHC class Ⅱ molecule expression, and antigen presentation[19]. TLR-2 genetic polymorphisms have been shown to influence susceptibility to pulmonary TB. TLR-2 variants play a role in the development of TB phenotypes, probably by controlling the expansion of natural killer cells[20]. Patients with stable alcoholic chronic liver disease show an attenuated TLR-2-mediated innate immune response[21]. The extent to which cirrhosis interacts with TLR polymorphism in promoting mycobacterial immune evasion is not known.

Diagnosis of TB in cirrhosis

Diagnosis of latent as well as clinical TB can be challenging in the setting of cirrhosis. There can be overlap between the symptoms of TB and decompensation of cirrhosis leading to delay in diagnosis. These patients demonstrate impaired delayed-type hypersensitivity; hence, there is a higher likelihood of false-negative tuberculin test results[22]. The exact mechanism of anergy to skin testing is not well known. Schirren *et al*[23] have shown that, although in patients with alcoholic liver cirrhosis, T-cell-dependent functions are impaired *in vivo*,

T-cell-activation pathways are not responsible for the observed immune defect. A strong association was observed between increased soluble intercellular adhesion molecule (ICAM)-1 concentrations and impairment of delayed-type hypersensitivity skin tests, suggesting that soluble ICAM-1 may be implicated in the immune depression seen in patients with chronic liver disease[24]. In the same study, serum alkaline phosphatase levels were also correlated with the impaired delayed-type hypersensitivity skin test[24]. The tuberculin skin test (TST) is further confounded by the etiology of cirrhosis. A recent study by Çelikbilek *et al*[25] showed that TST findings were more often falsely positive in the end-stage liver disease caused by viral as compared to nonviral etiology.

Interferon (IFN)-γ release assay(IGRA) is an alternative to purified protein derivative (PPD) testing. The test requires only a single contact with a patient. In addition, unlike the PPD, which is subject to interpretation bias, IFN-γ release assays are machine read and have single cut-offs. Thus, there is little subjectivity to the reading of results. IFN-γ-release assays have been tested and found to perform reasonably well in healthy populations as well as in patients with end-stage liver disease[26]. Several controversies still exist regarding their operational value, such as their discordance with the TST, role in immunocompromised subgroups, role in healthcare workers, role of serial testing, and ability to identify people who are likely to progress to active[27]. In high-burden settings, IFN-γ release assays tend to have decreased sensitivity because of the confounding effects of malnutrition, nontuberculous mycobacterium (NTM) exposure (especially *Mycobacterium kansasii* and *Mycobacterium marinum*), leprosy, and parasitic and other tropical infections that may alter the host T helper 1/T helper 2 cell balance[28].

Great efforts have been made globally to accelerate the development and expansion of new diagnostic technologies. However, pulmonary TB case detection remains dependent upon sputum smear and culture, radiography and clinical symptomatology. The role of sputum smear and radiography in the presence of cirrhosis is similar to that in patients without underlying cirrhosis. The *M. tuberculosis*-specific nucleic acid amplification tests (NAAT) performed on bronchopulmonary specimens is the most frequently used molecular test for laboratory diagnosis of pulmonary TB. NAAT results can be available to the clinician within 1 d after obtaining sputum or bronchoalveolar lavage (BAL) fluid and can have important implications for the management of patients. Unfortunately, NAAT amplification targets are not standardized and the diagnostic accuracy of the tests is highly heterogeneous[28,29]. In individuals with positive acid-fast bacillus (AFB) sputum smears, the sensitivity of NAAT to detect *M. tuberculosis* nucleic acid on these specimens is > 95%[29]. When AFBs are found on sputum or BAL smears, the presumptive diagnosis of TB can thus be rapidly confirmed. Apart from rare exceptions, a negative NAAT result in this situation strongly indicates the presence of an NTM species. Currently available serological tests cannot be recommended for the diagnosis of TB because of poor sensitivity and specificity. Recently, Steingart and colleagues conducted a meta-analysis of the published studies of distinct single antigens and multiple-antigen combinations in terms of their performance in diagnosing pulmonary TB[30]. The authors concluded that none of the antigen sensitivity was high enough to replace sputum smear microscopy. A recent test has been approved by the FDA (MTB/RIF test), which provides sensitive detection of TB and rifampin resistance directly from untreated sputum in < 2 h with minimal hands-on time. The role of this test in cirrhosis needs to be evaluated as the proportion of patients with pulmonary TB is much lower than in the general population[31].

The diagnosis of extrapulmonary TB in cirrhosis is similar to the disease in the general population. TB peritonitis possibly mimics spontaneous bacterial peritonitis. TB peritonitis occurs in less-advanced cirrhosis and ascitic fluid analysis usually shows lower white blood cell counts, higher proportions of mononuclear cells, and higher levels of protein and adenosine deaminase (ADA)[32]. In developed countries where TB peritonitis is uncommon, the diagnosis of TB peritonitis should prompt a workup for cirrhosis. In a study from the United States, > 50% of TB peritonitis cases had underlying cirrhosis, predominantly alcohol-related[33]. Although ADA level is generally helpful in the detection of TB peritonitis, the presence of cirrhosis may reduce its sensitivity to 30%[33-35]. In addition, abdominal TB is a paucibacillary disease and AFB smears are generally negative in such patients. Sometimes the TB manifestation in cirrhosis could be just the worsening of the liver function.

Drugs used in tuberculosis

There is no consensus regarding the use of antitubercular drugs in patients with cirrhosis. The potential hepatotoxicity of antitubercular drugs is a major concern. First, in the setting of pre-existing liver disease, the likelihood of developing drug-induced hepatitis may be higher. Second, the outcome of drug-induced hepatitis in patients with compromised liver function may be poor. Third, monitoring of drug-induced hepatitis may be confounded in the presence of underlying liver disease due to fluctuating liver function tests related to the pre-existing liver disease[36-38].

First-line drugs

Isoniazid: Isoniazid is a synthetic analog of pyridoxine and the most potent tuberculocidal drug[39]. It is an essential component of all regimens. Isoniazid is effective against both intra- and extracellular organisms because it inhibits the synthesis of mycolic acids in the bacterial cell wall[39]. Isoniazid is metabolized in the liver through two main pathways. Acetyl hydrazine, a nontoxic metabolite, is formed when metabolism proceeds along the N-acetyltransferase (NAT) 2 pathway, while hydrazine, the toxic metabolite, is formed when it proceeds along the amidase pathway[40]. Most previous research had identified acetyl

hydrazine as the toxic metabolite of isoniazid[41,42]. Later studies, however, suggested that hydrazine, and not isoniazid or acetyl hydrazine, was most likely to be the cause of isoniazid-induced hepatotoxicity[43].

An asymptomatic, self-limited increase in aminotransferase levels is observed in the majority of patients treated with isoniazid, which does not progress to more serious forms of liver injury[44]. Frequency of liver damage increases with age and in general is < 2%. A meta-analysis of six studies estimated the rate of clinical hepatitis in patients given isoniazid alone to be 0.6%[45]. Hepatotoxicity due to isoniazid therapy seems to be idiosyncratic in most patients and does not recur with rechallenge, hence, it can be reintroduced after complete clinical recovery[46].

Rifampicin: Rifampicin is a bactericidal agent that inhibits mycobacterium DNA-dependent RNA polymerase. It has profound early bactericidal activity against rapidly dividing cells and also against semidormant bacterial populations[47]. Transient elevation of hepatitis enzymes are however routinely observed in these patients. However, they return to normal on continuation of therapy. Yee *et al*[48] reported a rate of 0.05 per 100 person-months for hepatitis caused by rifampicin. Conjugated hyperbilirubinemia probably results from rifampicin inhibiting the major bile salt exporter pump, impeding secretion of conjugated bilirubin at the canalicular level[49]. Rifampicin can cause hepatocellular changes such as centrilobular necrosis, associated with cholestasis[37].

Pyrazinamide: Pyrazinamide is a weak bactericidal drug. Its active form, pyrazinoic acid, disrupts the bacterial membrane and inhibits membrane transport functions. It exerts greatest activity against the population of dormant or semidormant organisms contained within macrophages or the acidic environment of caseous foci[50]. Historically, it was considered the most hepatotoxic antitubercular drug. When the drug was first introduced in the 1950s, a high incidence of hepatotoxicity was reported and the drug was nearly abandoned[51]. This appeared to be related to the high dosage of 40-70 mg/kg used at that time. Toxicity is rare when pyrazinamide is used at a daily dose of < 35 mg/kg[52]. In murine models, pyrazinamide inhibits CYP45058 activity and NAD59 levels are altered in association with free-radical-species-mediated hepatotoxicity[53]. Bridging necrosis, lymphocytic infiltration, focal cholestasis, increased fibrosis, and micronodular cirrhosis have been observed in the liver of a patient who died of rifampicin- and pyrazinamide-induced hepatotoxicity[54]. The rate of hepatotoxicity of pyrazinamide monotherapy in its currently used dose is unknown. However, more data on the safety of pyrazinamide are needed to clarify its use in patients with cirrhosis.

Ethambutol: Ethambutol is a bacteriostatic antibiotic approved for the treatment of TB. It works by preventing the formation of the bacterial cell wall. Hepatotoxic effects of this agent are not clinically significant[55].

Second-line antitubercular drugs

The second-line drugs are considered as the reserved therapy for TB. These drugs are often used in special conditions. When situations like resistance to first-line therapy, extensively drug-resistant tuberculosis (XDR-TB) or MDR-TB arise, the second-line drugs are implemented[56]. These include: (1) aminoglycosides such as amikacin and kanamycin; (2) polypeptides such as capreomycin, viomycin and enviomycin; (3) fluroquinolones such as ciprofloxacin, levofloxacin and moxifloxacin; (4) thioamides such as ethionamide and prothionamide; (5) cycloserine; and (6) terizidone.

Third-line antitubercular drugs

Third-line antitubercular agents include rifabutin, macrolides (clarithromycin), linezolid, thioacetazone, thioridazine, arginine, and vitamin D. These drugs may be considered third-line because they are not very effective (*e.g.*, clarithromycin) or because their efficacy has not been proven (*e.g.*, linezolid)[57].

ANTITUBERCULAR THERAPY IN CIRRHOSIS: THE CHALLENGES

Challenges in the treatment of TB in patients with cirrhosis arise because three of the first-line antitubercular drugs are potentially hepatotoxic. The administration of these drugs can lead to worsening liver function with decompensation of stable cirrhosis and sometimes cause fulminant hepatic failure, with a high mortality. There is no consensus on the drugs to be given for different grades of liver injury, although the WHO guidelines mention that the more unstable or severe the liver disease is, the fewer hepatotoxic drugs should be used[58].

Incidence of antitubercular drug hepatotoxicity

There is a high incidence of hepatotoxicity ranging from 2% to 28%. TB is usually treated with multiple drugs to prevent emergence of MDR strains. This makes the determination of the exact drug responsible for hepatotoxicity difficult. Temporal data are sometimes helpful in providing evidence for hepatotoxicity of particular drugs. Therefore, there are limited data on toxicity rates of individual antitubercular drugs, except for isoniazid, which has been widely used as prophylactic monotherapy for latent TB infection. A meta-analysis of development of toxic hepatitis with isoniazid and rifampicin alone and in combination was done by Steele *et al*[59]; a summary of which is provided in Table 1.

An asymptomatic, self-limited increase in aminotransferase levels was observed in most patients treated with isoniazid. Approximately 0.5% of all patients treated with isoniazid monotherapy for latent TB developed clinically important increases in aminotransferase levels in a large study. The percentage was higher in combination thera-

Table 1 Incidence of hepatotoxicity of isoniazid and rifampicin individually, and in combination[49]

Drugs used	Total no. of patients	Patients with hepatotoxicity	Incidence of hepatotoxicity
INH	38257	210	0.6%
RIF	NA	NA	NA
INH + RIF	6155	168	2.73%
INH + other drugs	2053	33	1.6%
RIF + other drugs	1264	14	1.1%

INH: Isoniazid; RIF: Rifampicin.

py[59]. Isoniazid-induced hepatotoxicity is seen mainly as hepatocellular steatosis and necrosis, and it has been suggested that toxic drug metabolites may bind covalently to cell macromolecules[60].

Hepatotoxicity associated with rifampicin is usually idiosyncratic. Rifampicin may occasionally cause dose-dependent interference with bilirubin uptake due to competition with bilirubin for clearance at the sinusoidal membrane, resulting in mild, asymptomatic unconjugated hyperbilirubinemia or jaundice without hepatocellular damage. Occasionally, rifampicin can cause hepatocellular injury and can potentiate hepatotoxicity of other antitubercular drugs[49].

Hepatotoxicity is a major toxic effect of pyrazinamide. Previously reported studies have shown high rates of hepatotoxicity with high doses of pyrazinamide. Doses used currently (< 35 mg/kg per day) are considered much safer[60].

A study by Park *et al*[38] in patients with chronic liver disease and TB found that the incidence of hepatotoxicity was 17%, with no difference in patients with or without cirrhosis. The incidence of antitubercular-drug-induced hepatotoxicity when used as part of combination regimens in various studies is shown in Table 2[38,61-75].

Treatment of TB in compensated cirrhosis

Due to better functional reserve, patient with compensated cirrhosis have more treatment options and better tolerability. There has been no study to date comparing the full antitubercular therapy course with regimens containing only two potentially hepatotoxic drugs. Some authors do not favor the use of pyrazinamide, but at currently used doses, pyrazinamide has not been shown to be more hepatotoxic as compared to isoniazid or rifampicin[60]. Pyrazinamide is generally substituted with a fluoroquinolone or an aminoglycoside as per the clinician preference. It is prudent to use only two hepatotoxic drugs in treating compensated cirrhosis until a randomized controlled trial (RCT) proves the safety of low-dose pyrazinamide-containing combinations of three potentially hepatotoxic drugs. Proposed regimens are: (1) rifampicin, isoniazid, pyrazinamide and ethambutol for 2 mo followed by 4 mo rifampicin and isoniazid; (2) rifampicin, isoniazid, fluoroquinolone/aminoglycoside and ethambutol for 2 mo followed by 4 mo rifampicin and isoniazid; and (3) rifampicin, isoniazid, and ethambutol for 2 mo followed by 7 mo rifampicin and isoniazid.

Treatment in decompensated cirrhosis

Treatment of TB in decompensated cirrhosis is challenging because treatment is a double-edged sword. Treatment may lead to hepatotoxicity and progressive TB may lead to liver decompensation. Treatment regimens should ideally contain one of either isoniazid or rifampicin because they are the most potent antitubercular drugs. Currently, rifampicin is generally the preferred single hepatotoxic agent due to its potentially lower hepatotoxicity, although this has not been proven in an RCT. The high efficacy of isoniazid against mycobacteria warrants a head to head comparison between isoniazid and rifampicin when only one agent can be used. Other agents that are combined in regimens with single hepatotoxic agents include ethambutol, fluoroquinolone, injectable aminoglycoside, and cycloserine. No data on duration of therapy are available but treatment duration usually exceeds 12 mo, depending upon the site and extent of the disease.

In patients with advanced liver disease with complications of cirrhosis and signs of liver failure, it may not be possible to use even a single hepatotoxic drug. The presence of hepatorenal syndrome or other renal dysfunction further complicates the situation, limiting the use of aminoglycosides. Altered mental status may also hamper administration of oral drugs. The outcome in such group patients is poor, with high mortality due to the underlying poor hepatic function. There are no data to guide the choice of agents or the duration of treatment, or that indicate the effectiveness of such a regimen. Expert opinion suggests that a regimen of this sort should be given for at least 18-24 mo[58]. The American Thoracic Society (ATS) guidelines advise the use of ethambutol with fluoroquinolone, cycloserine and capreomycin or aminoglycoside for 18-24 mo if the patient has liver cirrhosis with encephalopathy[45]. Proposed regimens are: (1) rifampicin, ethambutol, fluoroquinolone with/without aminoglycoside for 9-12 mo; (2) isoniazid, ethambutol, fluoroquinolone with/without aminoglycoside for 9-12 mo; and (3) ethambutol, fluoroquinolone with/without aminoglycoside for 12-24 mo.

We propose treatment options according to Child's class as shown in Table 3. Studies are needed in this grey zone. It would be interesting to evaluate the safety and efficacy of low-dose isoniazid and rifampicin in advanced decompensated cirrhosis.

There is generally no difference in treatment of pulmonary or extrapulmonary TB but there could be a need for prolongation of antitubercular therapy in cases of central nervous system or skeletal TB. Bone infections have always been difficult to eradicate, which is why prolonged antitubercular therapy (9-18 mo) is routinely prescribed in endemic countries such as India[76]. No consensus or data on the duration of antitubercular therapy in these conditions with concomitant cirrhosis is available.

Monitoring for development of hepatotoxicity

Drug induced liver injury usually occurs in the first 2

Table 2 Studies on hepatotoxicity of antitubercular drugs in combination therapy

Ref.	Definition of hepatotoxicity	Incidence	Risk factors
Døssing *et al*[61] 1996	AST > 6 × ULN and confirmation by re-challenge	2.0	Female sex, advanced age
Ormerod *et al*[62] 1996	ALT > 5 × pre-treatment level	2.3	Advanced age
Tost *et al*[63] 2005	ALT/AST > 10 × ULN	2.6	Alcoholism, hepatitis B carrier state, other hepatotoxic drugs
Yee *et al*[48] 2003	ALT > 3 × ULN	3.0	Advanced age, female sex, Asian, HIV positive
Van Hest *et al*[64] 2004	ALT > 5 × ULN	3.4	Female gender
Teleman *et al*[65] 2002	ALT/AST > 3 × ULN	5.3	Abnormal baseline values, female sex, advanced age
Fernández-Villar *et al*[66] 2004	ALT/AST > 5 × ULN	8.1	Abnormal baseline liver function, low BMI, hepatitis B/C, other drugs
Pukenyte *et al*[67] 2007	ALT > 5 × ULN	10.7	Baseline CD4 < 100 cells/mL, bilirubin > 13 mmol/L or ALT > 51 U/L
Schaberg *et al*[68] 1996	ALT/AST > 3 × ULN	11.0	Advanced age, past history of hepatitis, female sex
Saigal *et al*[69] 2001	AST/ALT > 5ULN or > 400 IU/mL Bilirubin rise > 2.5 mg/dL	12.9	Advanced child status
Breen *et al*[70] 2006	ALT/AST > 5 × ULN	13.0	HIV infection, Asian
Huang *et al*[71] 2003	ALT > 3 × ULN	15.0	Advanced age, low BMI, slow acetylator status, CYP2E1 c1/c1 genotype
Sharma *et al*[72] 2002	ALT/AST > 5 × ULN, or any increase + symptoms	16.1	Advanced age
Park *et al*[38] 2010	ALT > 3 × ULN	17.0	Female sex, total no. of hepatotoxic drugs administered and baseline ALP levels
Ungo *et al*[73] 1998	ALT/AST > 3 × ULN	19.0	HIV or hepatitis C infection
Sharifzadeh *et al*[74] 2005	ALT > 3 × ULN with or > 5 × ULN without symptoms	27.7	No significant risk factors
Pande *et al*[75] 1996	AST > 3 × ULN	ND	Advanced age, high alcohol intake, slow acetylators

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; ULN: Upper limit of normal; BMI: Body mass index; HIV: Human immunodeficiency virus.

Table 3 Proposed treatment according to stage of liver disease

Child's status	Treatment
A	Two hepatotoxic drugs can be used namely isoniazid and rifampicin with/without pyrazinamide (low dose). Duration 6-9 mo
B	Ideally one hepatotoxic drug is used in combination. Pyrazinamide generally avoided Duration generally 9-12 mo
C	No hepatotoxic drugs to be used. Can use second-line drugs like streptomycin, ethambutol, fluoroquinolones, amikacin, kanamycin for extended duration of 12 mo or more. Role of aminoglycosides may be limited due to reduced renal reserve in these patients

mo of treatment. Clinical, biochemical and histological features of drug hepatotoxicity are hard to distinguish from viral hepatitis[44,77]. The signs and symptoms of liver injury include but are not limited to jaundice, abdominal pain, nausea, vomiting and asthenia[78]. Antitubercular treatment drug hepatotoxicity (ATDH) is usually reversible on withdrawal of the offending drug. Monitoring liver function tests more frequently at the start of therapy is a reasonable way to identify these patients. No recommendation for monitoring interval duration exists but once weekly liver function test for the initial 2 mo followed by once monthly should be reasonable. It should be supplemented by liver function tests in between if clinically warranted (Figure 1).

Diagnosis of hepatotoxicity

The definition of hepatotoxicity in patients with previous liver diseases is controversial, because of difficulty in defining the influence of the natural evolution of the underlying liver disease. There is a need to define better the level of aspartate aminotransferase (AST)/alanine aminotransferase (ALT) and serum bilirubin at which to consider hepatotoxicity to avoid unnecessary treatment withdrawal and to avoid dangerous continuation of antitubercular therapy when hepatotoxicity has set in. The baseline AST/ALT and serum bilirubin are already elevated prior to the institution of antitubercular therapy. Although it is generally recommended that therapy be interrupted when transaminase levels increase to 3-5 times the upper limit of normal, this limit has not been defined in patients with transaminase values already elevated before starting therapy[79]. Schenker *et al*[80] reported that elevations in the ALT and/or AST levels to 50-100 IU/L more than the baseline levels might define toxicity. In a study by Saigal *et al*[69], hepatotoxicity was diagnosed if ALT/AST levels increased to more than fivefold of the baseline level, or to more than 400 IU/L, or if the bilirubin increased by 2.5 mg/dL after exclusion of superimposed acute hepatitis. The role of fibroscan and other

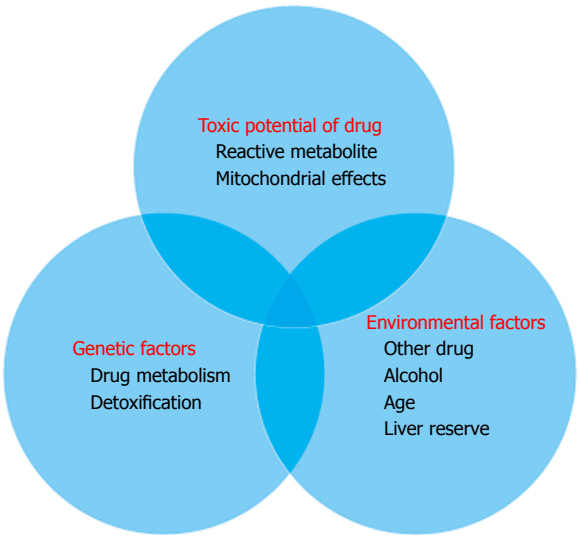

Figure 1 Interaction of factors to produce hepatotoxicity in cirrhosis.

newer blood test needs to be evaluated in early detection of hepatotoxicity and for differentiation of hepatic adaptation from toxicity.

REINSTITUTION OF ANTITUBERCULAR DRUGS

Guidelines for management of ATDH have been published by the ATS, British Thoracic Society (BTS), Task Force of the European Respiratory Society, WHO and International Union against Tuberculosis and Lung Disease[81-83]. No universally accepted consensus on management is available. All confounding factors like superimposed acute viral hepatitis and recidivism towards alcohol should be investigated. Usually, asymptomatic transaminase elevation resolves spontaneously. When the initial antitubercular regimen has been interrupted due to hepatotoxicity, it is reasonable to maintain at least 3 nonhepatotoxic drugs if possible. These generally include ethambutol, a fluoroquinolone and an aminoglycoside.

After TB treatment has been stopped because of hepatotoxicity, both the BTS and ATS advise restarting the antitubercular drugs one at a time. The Task Force advises restarting all the drugs simultaneously; after a second episode of hepatotoxicity the drugs need to be reintroduced consecutively. These recommendations are in general and not specific to groups of patients with underlying cirrhosis. It is more prudent to start one drug at a time after the serum bilirubin and AST/ALT levels have returned to near the baseline. After bilirubin and AST/ALT levels return to baseline, rifampicin may be restarted first at a reduced dose of 150 mg/d and increased every 3 d with simultaneous liver function test monitoring to the full dose. After successful reintroduction of one hepatotoxic drug, the second agent isoniazid may be restarted at a reduced dose of 50 mg/d and increased slowly every 3-4 d like rifampicin. Rifampicin is generally restarted first because it is thought to be less likely to cause hepatotoxicity than isoniazid. There is no data on reintroduction of pyrazinamide after development of hepatotoxicity episode. The rationale for reintroduction is that majority of hepatotoxicity episodes are hepatic adaptation and it is likely that rechallenge in a gradual manner may be easily tolerated without any evidence of hepatotoxicity. If any single drug is implicated as the cause, it is permanently eliminated from the regimen. If a second episode of hepatotoxicity occurs after full institution of antitubercular therapy, all hepatotoxic drugs should be stopped and extended duration antitubercular therapy with no potentially hepatotoxic drugs should be provided (Figure 2).

Liver transplantation

ATDH can worsen the liver function in patients with cirrhosis and lead to drug withdrawal. This makes the situation difficult because ongoing infection is generally considered as a contraindication for liver transplantation. In these cases, the strategy for the treatment of TB is poorly defined. In patients with acute decompensation and/or intolerance of antitubercular drugs, liver transplantation has been performed on an urgent basis[84]. In such cases in a post-transplantation setting, rifampicin should be used carefully because drug interactions may change the drug levels significantly and switching to rifabutin may be beneficial[85]. There is also a risk of graft rejection by rifampicin-induced reduction in the level of immunosuppressant because rifampicin is a strong enzyme inducer.

Special situations

Hepatitis B and/or C infections are common causes of the chronic liver disease that is frequently seen in populations at risk for TB infection, and these patients have increased risk of ATDH. In a study from Korea, amongst 110 inactive hepatitis B surface antigen (HBsAg) carriers and 97 controls without hepatitis B infection, 38 inactive HBsAg carriers (35%) and 19 controls (20%) developed elevated liver enzyme levels during antitubercular therapy ($P = 0.016$). A higher proportion of inactive HBsAg carriers who received antitubercular therapy experienced moderate-to-severe drug-induced hepatotoxicity when compared with the controls (8% *vs* 2%, $P < 0.05$)[86]. Ungo *et al*[73] showed that the relative risk of developing hepatotoxicity if the patient had hepatitis C or was HIV positive was fivefold and fourfold, respectively ($P < 0.05$). If a patient was co-infected with hepatitis C and HIV, the relative risk of developing drug-induced hepatitis was increased by 14.4-fold ($P < 0.002$). Alcoholism is associated with a higher risk of ATDH because of enzyme induction. Patients with ongoing alcohol abuse and concomitant use of other hepatotoxic drugs also have an increased risk of hepatotoxicity. In the USPHS surveillance study[87], alcohol consumption appeared to more than double the rate of probable isoniazid hepatitis, with daily consumption increasing the rate more than four times. It is highly likely that this subgroup of patients may have additional risk for hepatotoxicity as compared to other patient

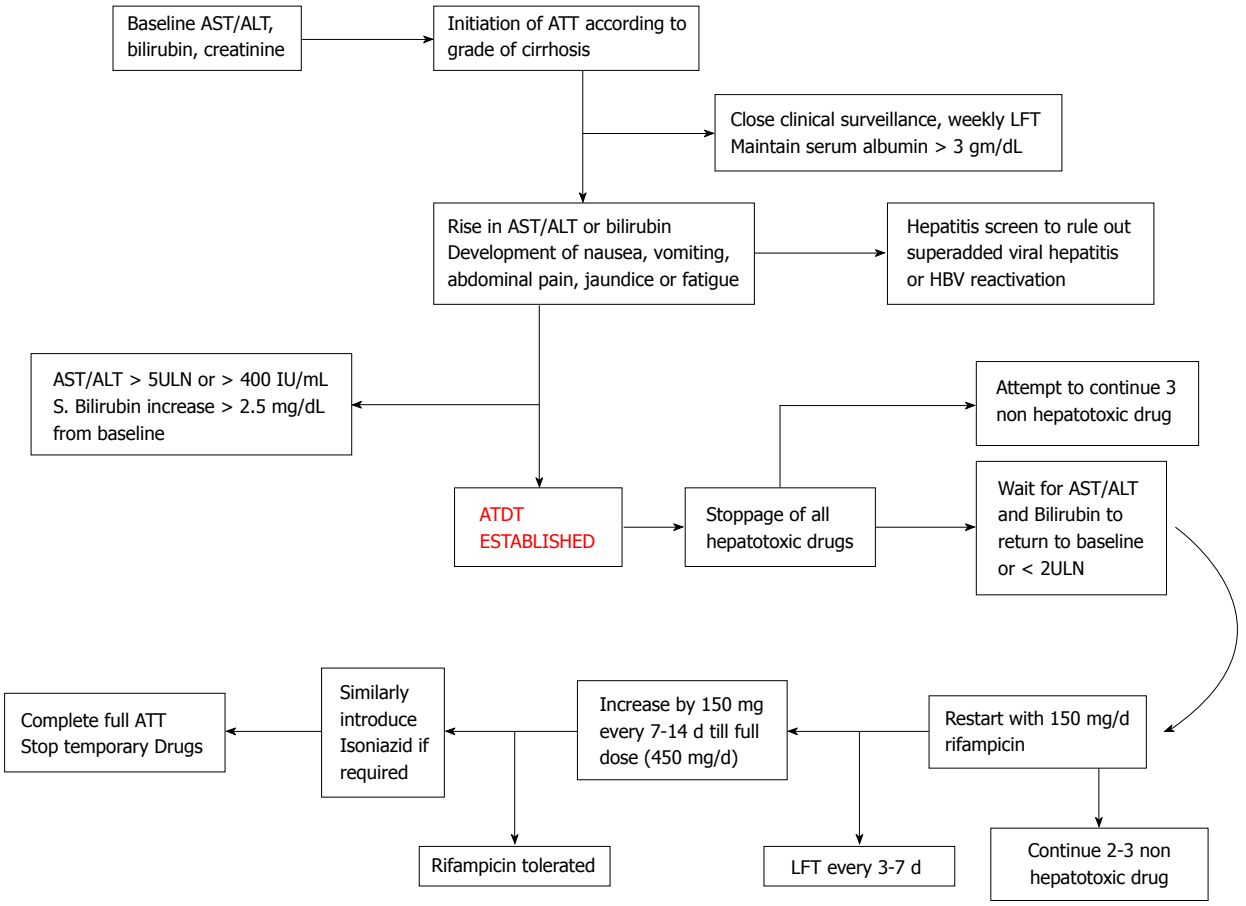

Figure 2 Proposed algorithm for monitoring and management of Antitubercular treatment drug hepatotoxicity. ALT: Alanine aminotransferase; ATDH: Antitubercular treatment drug hepatotoxicity; AST: Aspartate aminotransferase; ULN: Upper limit of normal; ATT: Antitubercular therapy; LFT: Liver function test.

groups with cirrhosis, and warrants close monitoring.

Genetic polymorphisms in drug-metabolizing enzymes affect enzyme activity. This may lead to differences in treatment response or drug toxicity, for example, due to an increased formation of reactive metabolites. Data on genetic risk factors for ATDH are still limited. Human genetic studies have shown that cytochrome P450 2E1 (CYP2E1) is involved in ATDH. Huang *et al*[71] demonstrated that slow acetylators for isoniazid have a more than twofold risk of developing ATDH compared with fast acetylators. Deficiency of glutathione S-transferase (GST) activity, because of homozygous null mutations at GSTM1 and GSTT1 loci, may modulate susceptibility to drug- and xenobiotic-induced hepatotoxicity. Polymorphisms at GSTM1, GSTT1 and CYP2E1 loci have been linked to various forms of liver injury[88].

Prevention of ATDH

There are few effective treatments available for ATDH. This emphasizes the importance of early detection of hepatotoxicity and prompt withdrawal of the offending drug. Polypharmacy should be avoided to prevent inadvertent use of other potentially hepatotoxic drugs. Close clinical and biochemical monitoring are strictly needed for early detection of this potentially reversible liver injury.

Genetic profiling of patients for polymorphisms associated with increased risk of hepatotoxicity will be helpful but is currently not available in the clinical setting. NAT2 genotype could be used to divide patients into low and high isoniazid dose groups. N-Acetyl cysteine (NAC) has been shown in one study to prevent antitubercular-therapy-induced hepatotoxicity[89]. In that RCT, 60 new TB patients aged ⩾ 60 years were randomized into two groups. In Group I (n = 32), the drug regimen included daily doses of isoniazid, rifampicin, pyrazinamide, and ethambutol. Patients in Group Ⅱ (n = 28) were treated with the same regimen and NAC. The mean values of aspartate aminotransferase and alanine aminotransferase were significantly higher in group I than in group Ⅱ (with NAC) after 1 and 2 wk of treatment[89]. This study proved that NAC protects against antitubercular-drug-induced hepatotoxicity. More studies are needed on the potentially protective effect of such compounds in humans and possible interactions with antitubercular drugs. A hepatoprotective effect of silymarin on ATDH has been shown in rats[90]. A study in patients with cirrhosis is warranted to demonstrate that NAC efficacy may strengthen the already depleted armor in the fight against TB.

The herbal formulation of *Curcuma longa* and *Tinospora cordifolia* prevented hepatotoxicity significantly and improved the disease outcome as well as patient compliance, without any toxicity or side effects in a random-

ized study[91]. Caution must be exercised before using any indigenous drug formulation due to unknown drug interactions and side effects. Ultimately, a strategy that incorporates new analytical approaches - addressing both the immune response and pharmacogenetic vulnerability - can be envisioned.

MDR TB

Many studies for risk factors for drug-resistant TB have found that the presence of hepatic cirrhosis is a risk factor for the development of drug-resistant TB[92]. A study for risk factors for drug-resistant TB found that the prevalence of drug-resistant TB was 46% among patients with cirrhosis, although the number of patients with cirrhosis was only 11[93]. Drug resistance may occur from reduced immune response and the inability to use the most potent drugs in many patients due to the risk of hepatotoxicity.

IFN-induced reactivation of TB - a special scenario

The standard of care for patients with chronic hepatitis C is pegylated IFN-α (Peg-IFN) and ribavirin. IFN treatment induces immunomodulation[94]. Theoretically, IFN-induced immunomodulation should increase TB occurrence as well as other bacterial infections but there is a paucity of reported cases. There have been few case reports of patients developing reactivation of TB as a consequence of IFN therapy, but overall, there is a paucity of data about development of TB in patients after IFN treatment[95-98]. Recent unpublished data from India have shown 10 cases of IFN-induced reactivation of TB. There are many cases of TB occurring after completion of treatment[99]. There could well be under-reporting of cases, leading to lower incidence of TB seen with IFN administration, and such under-reporting is normally high in developing countries. Hence, there is a need for close surveillance of TB in patients receiving IFN for hepatitis C.

New drugs

There is an urgent need for development of new drugs with high efficacy and low hepatotoxicity to reduce the incidence of ATDH. A new drug, bedaquiline, has recently been approved for the treatment of MDR TB[100]. Bedaquiline is a member of the diarylquinoline class of drugs and has a unique mechanism of action, targeting ATP synthase of *M. tuberculosis*. ATP synthase is used by the bacterium for generation of its energy supply. Bedaquiline is active against both *M. tuberculosis* and drug-resistant bacteria that cause MDR TB. Laboratory tests and clinical trials have shown it to have strong bactericidal and sterilizing properties[100]. More data on the safety of this drug are required. Moxifloxacin has been shown to be the most efficacious fluoroquinolone *in vitro*. Many studies with this drug in various combinations are ongoing[101]. Many drugs are in various stages of development, namely DprE inhibitors, indazoles, mycobacterial gyrase inhibitors, pyrazinamide analogs, nitroimidazoles and RNA polymerase inhibitors[102,103].

CONCLUSION

Patients with cirrhosis are predisposed to TB, especially extrapulmonary TB. Diagnosis of TB in patients with cirrhosis is challenging, due to hampered immune response and reduced sensitivity of the available diagnostic tests. Successful completion of antitubercular drug therapy remains a challenge in patients with cirrhosis due to reduced hepatic reserve and higher incidence of hepatotoxicity. Close monitoring and early detection are the mainstay to prevent drug-induced liver injury. Successful reintroduction of antitubercular drugs is possible and should be done in stable patients. Liver transplantation is possible in patient's not recovering but post-transplantation antitubercular therapy is difficult with ongoing immunosuppression. Ongoing research for potent non-hepatotoxic antitubercular drugs should be expedited.

FUTURE DIRECTIONS

RCTs are needed to decide the optimal regimen of antitubercular therapy in cirrhosis, depending on Child's score. Better diagnostic methods are needed for detection of latent TB, especially in patients with hepatitis C, prior to starting IFN regimens and as part of pre-transplant evaluation. The efficacy of hepatoprotective agents to reduce drug-induced liver injury when given in combination with antitubercular therapy needs to be studied.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Autophagy in hepatitis C virus-host interactions: Potential roles and therapeutic targets for liver-associated diseases

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Abstract

Autophagy is a lysosome-associated, degradative process that catabolizes cytosolic components to recycle nutrients for further use and maintain cell homeostasis. Hepatitis C virus (HCV) is a major cause of chronic hepatitis, which often leads to end-stage liver-associated diseases and is a significant burden on worldwide public health. Emerging lines of evidence indicate that autophagy plays an important role in promoting the HCV life cycle in host cells. Moreover, the diverse impacts of autophagy on a variety of signaling pathways in HCV-infected cells suggest that the autophagic process is required for balancing HCV-host cell interactions and involved in the pathogenesis of HCV-related liver diseases. However, the detailed molecular mechanism underlying how HCV activates autophagy to benefit viral growth is still enigmatic. Additionally, how the autophagic response contributes to disease progression in HCV-infected cells remains largely unknown. Hence, in this review, we overview the interplay between autophagy and the HCV life cycle and propose possible mechanisms by which autophagy may promote the pathogenesis of HCV-associated chronic liver diseases. Moreover, we outline the related studies on how autophagy interplays with HCV replication and discuss the possible implications of autophagy and viral replication in the progression of HCV-induced liver diseases, *e.g.*, steatosis and hepatocellular carcinoma. Finally, we explore the potential therapeutics that target autophagy to cure HCV infection and its related liver diseases.

Key words: Autophagy; Hepatitis C virus; Steatosis; Cirrhosis; Hepatocellular carcinoma

Core tip: Hepatitis C virus (HCV) is a major cause of chronic liver disease and is associated with over 170 million infected individuals worldwide. However, a successful strategy for completely eradicating HCV infection is still limited. Autophagy is a catabolic process that delivers cytosolic components to lysosomes for breakdown. HCV has been shown to activate autophagy to promote viral growth *in vitro*. In this review, we outline the recent findings on the physiological significance of autophagy in the HCV life cycle and propose a potential role of autophagy in the development of HCV-related liver diseases as well as a perspective on therapeutics targeting autophagy to cure HCV infection.

Ke PY, Chen SSL. Autophagy in hepatitis C virus-host interactions: Potential roles and therapeutic targets for liver-associated diseases. *World J Gastroenterol* 2014; 20(19): 5773-5793 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5773.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5773

INTRODUCTION

Hepatitis C virus (HCV) infection is a global public health burden. Approximately 170 million people are infected with HCV worldwide, and most of these patients become persistently infected. Furthermore, HCV infection in some patients may progress into chronic liver diseases, such as steatosis, cirrhosis, and hepatocellular carcinoma[1,2]. The successful rate of curing HCV infection using the current therapy that combines interferon (IFN) and ribavirin is still limited due to its low efficacy, drug resistance, and severe side effects in a particular population of infected individuals[3]. Additionally, preventive vaccines against HCV are not yet available[4,5]. Hence, a new antiviral drug with high potency and/or a protective vaccination against HCV infection are urgently needed. Autophagy is an evolutionarily conserved, catabolic pathway by which eukaryotic cells degrade unnecessary cytoplasmic compartments to recycle nutrients and maintain cellular homeostasis[6,7]. Recent studies collectively indicate that HCV activates autophagy to promote viral growth through regulating different steps of the viral life cycle by affecting different host cellular signaling processes[8-11]. Because autophagy has widely been shown to contribute to the progression of human diseases[12,13], HCV-activated autophagy could be physiologically significant in the pathogenesis of HCV-associated liver diseases. Most importantly, interference with the autophagic process can suppress HCV replication[8-11], suggesting that inhibition of autophagy can serve as a novel therapeutic strategy against HCV infection. Therefore, in this review, we outline the current findings on the functional roles of autophagy at each stage of the HCV life cycle and the molecular mechanism by which HCV activates the autophagic response. Lastly, we also discuss the possible impacts of the autophagic response on the development of HCV-related liver disorders as well as provide a perspective on the implications of modulating autophagy to control HCV infection.

HEPATITIS C VIRUS INFECTION

An unknown infectious agent that caused non-A, non-B post-transfusion hepatitis was first discovered in the mid-1970s[14]. In 1989, the nucleic acid sequence of this unidentified virus was cloned, reported, and formally named HCV[1]. It is estimated that over 3% of the human population is infected by HCV. Most of the infected individuals become chronically infected, and HCV infection often progresses to severe, liver-associated diseases, such as cirrhosis, steatosis, and hepatocellular carcinoma[2]. To date, due to the low efficacy of the combined therapy of pegylated IFN-α and ribavirin[3], options for complete eradication of HCV infection and a preventive strategy are still absent[4,5]; therefore, HCV infection is a global public health problem.

HCV is a membrane-enveloped, positive-sense, single-stranded RNA virus belonging to the *Hepacivirus* genus and the *Flaviviridae* family[2]. Until now, the known isolates of HCV were classified into seven genotypes, *i.e.*, genotypes 1 through 7, with 20%-30% sequence divergence, and an array of subtypes could be grouped within each genotype[15]. The genetic heterogeneity of these HCV genotypes could result in the variable degree of risk for progressive liver diseases and the different treatment outcomes of IFN-based therapy[16]. For instance, a higher prevalence of progression into hepatosteatosis and cirrhosis occurs in cases infected with HCV genotype 3[17,18]. Regarding the efficacy of standard IFN/ribavirin treatment, the successful rates of patients infected with genotypes 2 and 3 are higher than those of patients infected with genotypes 1 and 4[19-22].

Hepatocytes in the liver are the predominant targets of HCV infection, and the entry of HCV into hepatocytes is a stringently coordinated process that relies on successive and concerted interactions between the envelope glycoproteins E1 and E2 and host cellular factors that are present on the cell surface, *i.e.*, the so called "entry (co)receptors" (Figure 1). The known (co)receptors, including the tetraspanin CD81[23-25], scavenger receptor class B member I (SCRAB-I)[26,27], Claudin 1 (CLDN1)[28], and Occludin (OCLN)[29], have been shown to mediate HCV entry into hepatocytes (Figure 1). In addition, the low-density lipoprotein receptor (LDLR)[30], highly sulfated heparin[31], and the dendritic cell-specific intercellular adhesion molecule three grabbing non antigen[32] were reported to be involved in attachment to and concentration of lipoprotein-associated viral particles on the cell surface of infected cells (Figure 1). After attachment to the cell surface, the virions bind to CD81 and SCRAB-I on the plasma membrane through the interaction between the E2 protein and these two entry (co)factors[33-35]. Subsequently, the association of CD81 or SCARB-I with CLDN1 on the basolateral surfaces of hepatocytes facilitates the formation of entry complexes[36-38] (Figure 1), thus promoting the internalization process of viral particles *via* the clathrin-mediated and pH-dependent endocytosis pathway[39,40]. Following internalization into cells, the envelopes of the virions fuse with the endosomal membrane, allowing uncoating and release of the viral genomes into the cytoplasm, where the translation of viral proteins and replication of viral RNA occur[39,40] (Figure 1). The exact physiological role of OCLN in the entry of the HCV virion is still unclear, although the second extracellular loop of this protein, along with CD81, has been shown to determine the host tropism of HCV infection[29]. In addition to CLDN1 and OCLN, other tight junction proteins such as CLDN6 and CLDN9 have been reported to participate in the entry of HCV into peripheral blood mononuclear cells, which lack CLDN1 expression[41,42]. This represents an alternative route for HCV infection in extrahepatic compartments[42].

In addition to these entry (co)receptors, epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) have recently been identified as additional

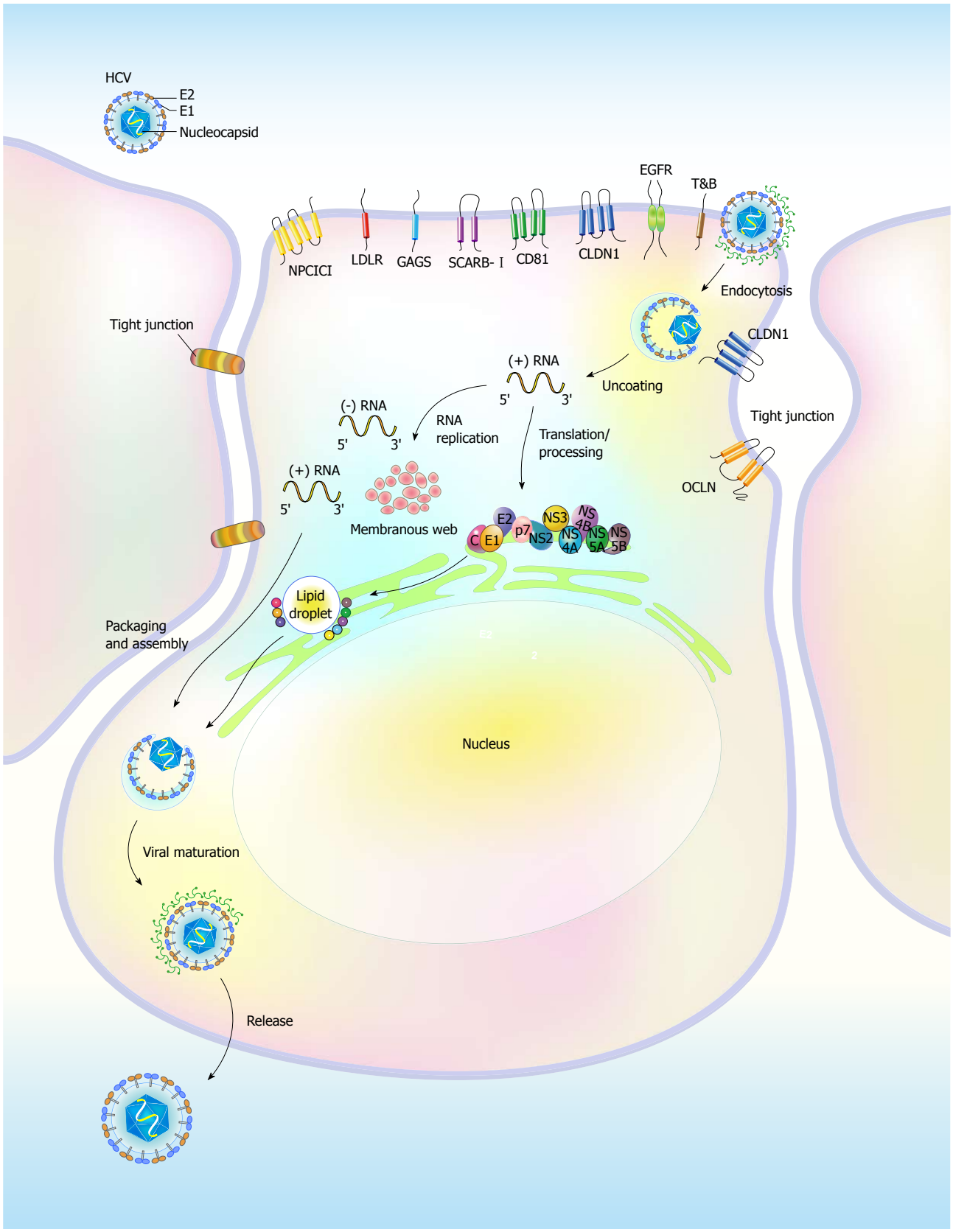

Figure 1 Life cycle of hepatitis C virus. Hepatitis C virus (HCV) replication involves binding of the virion to the entry molecules on the cell surface of host hepatocytes; endocytosis; viral genome uncoating and translation; polyprotein processing; RNA replication on the ER-associated membrane structure, called the "membranous web"; and virion packaging, assembly, maturation, and release. Glycosaminoglycans (GAG), low-density lipoprotein receptor (LDLR), scavenger receptor class B member Ⅰ (SCARB-Ⅰ), two tight junction molecules Claudin 1 (CLDN1) and Occludin (OCLN), epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), Niemann-Pick C1-like L1 (NPC1L1), and transferrin receptor (TfR) constitute the entry receptors. In addition to participating in viral RNA replication, the NS proteins are recruited onto the surface of lipid droplets (LDs) to promote viral RNA replication and the assembly of infectious viral particles, which are composed of the core, E1, and E2 proteins.

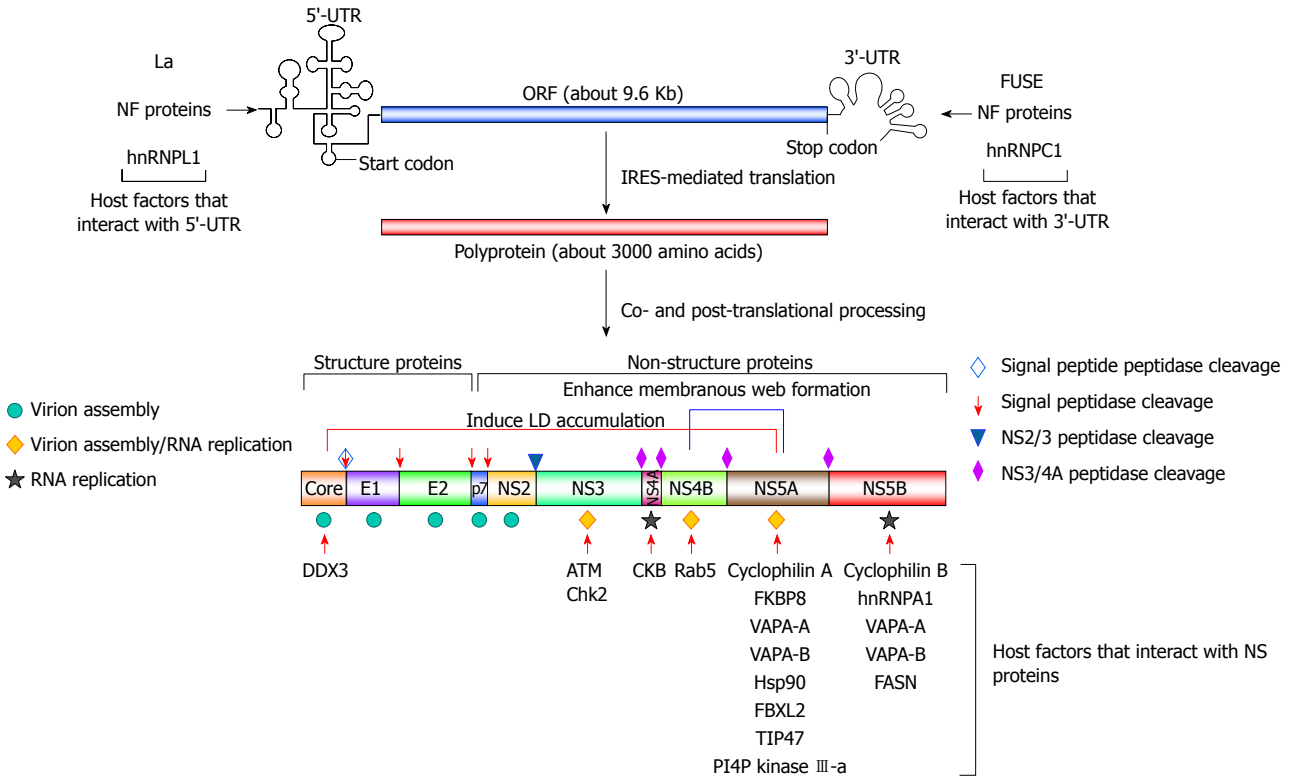

Figure 2 Genome organization of hepatitis C virus and cellular proteins that modulate viral RNA replication. The positive-sense, single-stranded hepatitis C virus (HCV) genome, containing an open reading frame (ORF) of 9.6 Kb that is flanked by the 5'- and 3' untranslated regions (UTR), is translated using an internal ribosome entry site (IRES) to a polyprotein, which is processed by cellular and viral proteases to mature into three structural proteins (core, envelope glycoproteins E1 and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The role of each viral protein in virus assembly, RNA replication, or both is indicated by the symbol shown on the left. The cellular proteins that modulate HCV replication and their interacting viral counterparts are shown. Host factors that interact with the 5'-UTR and 3'-UTR, as indicated, regulate IRES-mediated translation and viral RNA replication, respectively. Membrane-associated proteins, as shown, modulate HCV replication through interactions with NS5A and NS5B. Other cellular factors, such as DDX3, ATM and Chk2, CKB, and Rab5, promote the replication of HCV viral RNA *via* interacting with core, NS3, NS4A, and NS4B, respectively.

(co)factors for HCV entry by facilitating the CD81-CLDN1 interaction[43] (Figure 1). Additionally, the receptor tyrosine kinase activities of these two molecules were shown to enhance the membrane fusogenic activity of HCV envelope glycoproteins[43]. In addition, the entry of HCV virions into host cells can be mediated by an association with cholesterol *via* the Niemann-Pick C1-like L1 (NPC1L1) cholesterol uptake receptor[44]. Likewise, transferrin receptor (TfR), which is an iron absorption receptor, was recently demonstrated to be involved in the internalization of HCV virions into hepatocytes[45] (Figure 1). These studies collectively indicate that infection of target cells by HCV is a highly regulated process.

INTERPLAY OF HCV AND HOST CELLULAR PROTEINS

The viral genome of HCV is a positive-stranded RNA of approximately 9.6 Kb in length[46] that contains two untranslated regions (UTRs) located on the 5' and 3' termini, which flank a major open reading frame (ORF) region[46] (Figure 2). The ORF of the HCV genome can be translated into a polypeptide of approximately 3300 amino acids using an internal ribosome entry site (IRES) that is located within the 5'-UTR. The large polyprotein is then co-translationally processed into structural (core and the envelope glycoproteins E1 and E2) and nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) *via* a combination of viral and cellular proteases[46] (Figure 2). The structural proteins constitute the viral particle[2,46], whereas the NS proteins, in conjunction with cellular proteins, participate in viral RNA replication by organizing the replication complex within a multi-vesiculated, membranous web[47,48] (Figure 2). The NS proteins are also concertedly localized on the surface of lipid droplets (LDs), which is a process that is required for virion assembly[49,50] (Figure 2).

In addition to the involvement of viral proteins that function as cis-acting elements for HCV replication, many host cellular proteins have been known to regulate viral RNA replication through their interaction with viral proteins and RNAs. Playing a pivotal role in the IRES-mediated translation of a precursor polypeptide that is subsequently processed into individual viral proteins, the eukaryotic translational initiation factors and RNA-binding proteins, such as the La autoantigen[51,52], nuclear factors (NF) NF45, NF90, and NF110[53], the far upstream element-binding proteins[54], and heterogeneous nuclear ribonucleoproteins[55-57], have been implicated in viral

RNA translation and replication *via* interactions with the 5'- and 3'-UTRs (Figure 2), thus promoting HCV replication. In addition to these host factors that modulate viral translation, other host factors, such as cyclophilin B and hnRNP A1[57,58], may directly modulate viral RNA replication by forming a protein complex with the RNA-dependent RNA polymerase (RdRp), NS5B, and regulating replicase activity (Figure 2). The other cyclophilin family proteins, including cyclophilin A and FK506-binding protein 8 (FKBP8), have been reported to interact with NS5A, and this association recruits heat shock protein 90 to form a protein complex that promotes the efficiency of viral RNA replication[58-62] (Figure 2). On the other hand, a variety of vesicle-associated membrane proteins (VAPA), such as VAPA-A and VAPA-B, have been reported to positively regulate HCV replication by interacting with NS5A and NS5B[63,64] (Figure 2). Additionally, the geranylgeranylated protein F-box/LRR-repeat protein 2 is a host protein that interacts with NS5A to promote HCV replication[65,66] (Figure 2). In addition, other host factors, such as DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked[67], ataxia telangiectasia mutated (ATM), checkpoint Chk2 kinases[68], creatine kinase B (CKB)[69], and the small Ras GTPase-binding protein 5 (Rab5)[70], may regulate HCV replication *via* binding to core, NS3, NS4A, and NS4B, respectively (Figure 2).

In addition to cellular proteins that interact with viral proteins, several host cellular proteins exert their trans-acting function in the HCV life cycle by altering lipid metabolism. The findings that support this notion originate from observations suggesting that the expression of sterol regulatory element-binding proteins can be enhanced by HCV infection and the ectopic expression of individual viral proteins, *e.g.*, core, NS2, and NS4B[71-73] (Figure 3). The SREBP-mediated transactivation of lipogenic genes enhances cholesterol biogenesis and biosynthesis of fatty acids, which, in turn, promote the storage of neutral lipids within LDs[74]. Therefore, these results indicate that HCV infection may activate the gene expression of lipogenes, thus modulating the metabolic pathways of lipids to support the HCV life cycle. In line with this, the gene expression of fatty acid synthase, which is involved in the synthesis and transport of fatty acids, was reported to be upregulated and required for HCV viral RNA replication[75]. Recently, FASN was demonstrated to interact with NS5B to enhance RdRp replicase activity, thereby promoting HCV replication[76] (Figure 2).

Apart from these proteins that directly function in the modulation of lipid biosynthesis, a new subset of host cellular factors has emerged based on their roles in HCV replication *via* altering the expression and subcellular distribution of phosphatidylinositol-4-phosphate (PI4P). HCV infection was reported to increase the intracellular level of PI4P *via* PI4P kinase (PI4PK) Ⅲα and PI4PKⅢβ[77-83]. Interference with the gene expression of these two PI4P kinases dramatically inhibits HCV replication[77-83]. Furthermore, recent studies indicated that the NS5A protein can recruit (PI4PK) Ⅲα to the membranous web, which is a multi-vesiculated structure that supports efficient replication of HCV viral RNA, thus upregulating the level of PI4P to maintain the membranous web architecture[77,78,81,84,85]. Reciprocally, (PI4PK) Ⅲα can modulate the phosphorylation status of NS5A, thus regulating the morphogenesis of viral replication compartments[86]. On the other hand, annexin A2 and proline-serine-threonine phosphatase-interacting protein 2 (PSTPIP2), two host membrane-associated proteins, were also shown to regulate HCV replication *via* facilitating the formation of the membranous web[87,88]. In addition, the tail interacting protein of 47 kDa (TIP47), which is an LD-associated protein, has recently been shown to positively modulate HCV RNA replication by interacting with NS5A[89,90] (Figure 2). Collectively, these studies indicate that host cellular factors may regulate HCV replication *via* directly facilitating the reconstitution of the membranous web or modulating LDs by interacting with viral NS proteins.

HCV-ASSOCIATED LIVER DISEASES

The disease progression in HCV-infected patients reveals a nonlinear and long-term period mode. At the beginning of infection, most infected patients are non-symptomatic, although hepatitis, jaundice, and fulminant hepatic failure can be detected in some cases of acute infection[16,91] (Figure 3). However, more than 50% of HCV infection cases develop viral persistence, which often leads to liver steatosis, fibrosis, cirrhosis, and ultimately to hepatocellular carcinoma (HCC)[16,91-95] (Figure 3). In the majority of infected individuals, the disease progression to end-stage HCV-related diseases, such as liver cirrhosis and HCC, often occurs 20-30 years after acute infection, and approximately 20%-30% of the infected patients will progress to end-stage liver diseases[16,91-95] (Figure 3). In addition to the end-stage liver diseases, chronic HCV infection in some patients is also highly associated with extrahepatic diseases such as mixed cryoglobulinemia vasculitis, which is an inflammatory symptom of small blood vessels, due to the precipitation of cryoglobulin-containing immune complexes in blood vessels of the skin and other tissues[96,97]. Additionally, chronic HCV infection is associated with metabolic syndromes such as diabetes and insulin resistance[98,99]. The development of these diseases often requires long periods of time, thus affecting the health quality of patients and imposing a heavy burden on medical care for the treatment of HCV-related diseases. Therefore, it is urgent to design a new, efficacious therapeutic strategy and/or to develop prophylactic vaccines.

IMPLICATIONS OF HCV IN LIVER DISEASES

Many HCV proteins are known to participate in the pathogenesis of HCV-related diseases. For instance, the HCV core protein was reported to coat the surface

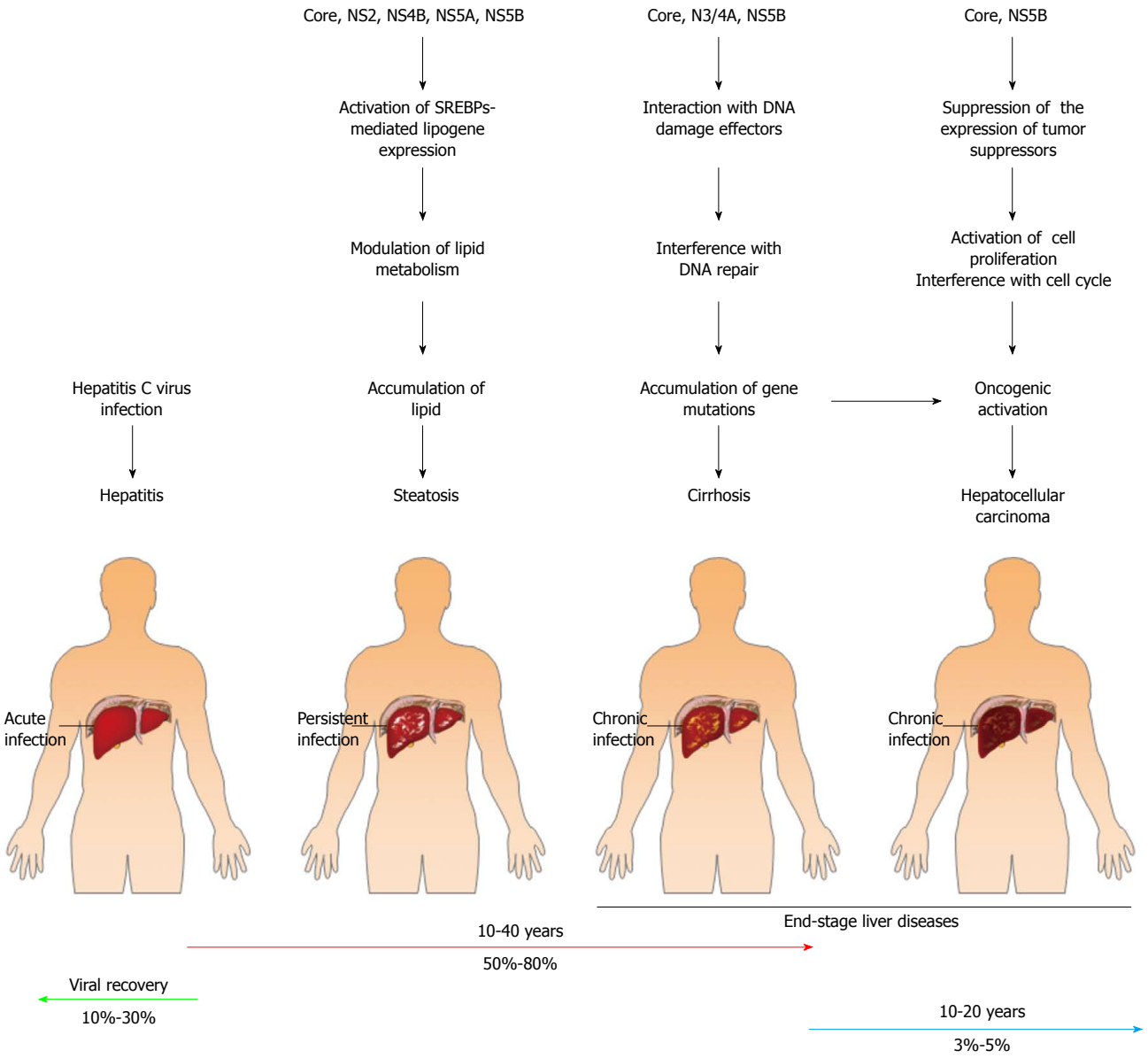

Figure 3 Contribution of viral proteins to the pathogenic changes leading to hepatitis C virus-related liver diseases. After acute hepatitis C virus (HCV) infection, approximately 10%-30% of cases will spontaneously recover, whereas the majority of HCV infection cases (approximately 50%-80% of infected cases) become persistently infected. Chronically HCV-infected hepatocytes in the livers of these patients progress into hepatosteatosis and liver cirrhosis. Ultimately, some (approximately 3%-5%) of the chronically infected individuals develop end-stage liver disease, *i.e.*, hepatocellular carcinoma. The potential contributions of the viral proteins to the development of HCV-associated liver diseases are shown. The HCV core protein and the NS2, NS4, NS5A, and NS5B proteins can promote hepatic steatosis by mediating the clustering of LDs and regulating the SREBP-mediated transactivation of lipogenes, respectively. The binding of core and NS5B to tumor suppressors downregulates the expression of these tumor suppressors, thus activating cell proliferation and interfering with cell cycle progression. Moreover, HCV core, NS3/4A, and NS5B can bind to DNA damage response-associated effectors, thereby inhibiting the DNA repair mechanism and inducing genome instability. Deregulated cell growth, cell cycle progression, and genome instability may, in turn, trigger oncogenic activation in infected cells, which finally leads to the development of hepatocellular carcinoma.

of LDs and induce the emergence of LDs from the endoplasmic reticulum (ER) and the clustering of LDs in a cultured cell model[100-102]. On the other hand, accumulating lines of evidence have shown that the HCV core protein can induce hepatic steatosis in an *in vivo* transgenic model[103-106]. Most importantly, the genetic variation within the core sequences of different HCV genotypes has been reported to critically determine the status of hepatic steatosis. The prevalence and severity of hepatic steatosis are higher in patients infected with HCV genotype 3 than in those infected with other genotypes[107-109]. This greater extent of steatosis in genotype 3-infected individuals may be due to the substitution of phenylalanine for tyrosine at amino acid residue 164 of the core protein in the genome of HCV genotype 3[110]. Despite this amino acid variation, several specific polymorphisms in the core protein of different HCV genotypes have been shown to increase the intracellular lipid levels and, thus, contribute to hepatic steatosis[111,112]. Cumulatively, these studies suggest that HCV infection may lead to hepatic steatosis through core-induced LD accumulation. Apart from the impacts of altering intracellu-

lar lipids, HCV core was shown to suppress the expression of the tumor suppressors p53 and cyclin-dependent kinase (CDK) inhibitor p21, thereby enhancing CDK2 activity and increasing the phosphorylation status of retinoblastoma, RB, in cells[113] (Figure 3). In turn, phosphorylated RB stimulates the DNA binding ability of E2F transcriptional factor 1 and activates the expression of downstream genes, such as S phase kinase-interacting protein 2, which is an initiating signal for cell proliferation[113] (Figure 3). Moreover, HCV NS5B has also been demonstrated to interact with RB and target it for proteolysis, thus activating downstream E2F-responsive promoters and cell proliferation[114] (Figure 3). Due to their suppression of the expression of tumor suppressors and promotion of cell proliferation, the HCV core and NS proteins may contribute to the progression of uncontrolled hepatocyte growth, thereby increasing the occurrence of hepatocellular carcinoma.

The interaction between HCV NS3/4A with ATM kinase has been shown to lead to the cytoplasmic retention and dephosphorylation of ATM, thus interfering with the activation of the DNA repair mechanism and desensitizing Huh7 cells to ionization[115] (Figure 3). In addition, the ATM and Chk2 kinases bind to the HCV NS5B protein to promote HCV viral RNA replication[68] (Figure 3). On the other hand, recent studies indicate that HCV infection may interfere with multiple signaling pathways of DNA repair *via* interactions between HCV core and the Nijmegen breakage syndrome protein 1 (NBS1), which is a downstream effector of the ATM-associated DNA damage response[116] (Figure 3). Taken together, these findings imply that HCV infection may interfere with the host DNA damage/repair response, thus benefiting viral growth. Interference with the integrity of the DNA repair mechanism may introduce error-prone effects on DNA replication, which leads to the accumulation of gene mutations, gene instability, and oncogenic activation in infected cells and, eventually, promotion of the progression of infected cells into hepatocellular carcinoma.

TOOLS USED IN HCV RESEARCH

Innovation of new therapeutic strategies against HCV infection relies on a comprehensive understanding of the entire viral life cycle and HCV-host interactions. Although HCV was identified more than two decades ago, our knowledge of how HCV infection leads to a homeostatic balance with host cells is still limited due to the lack of an *in vitro* cell culture model that can support the complete HCV cycle. The replication assay utilizing a subgenomic replicon that harbors only the HCV nonstructural genome in human hepatoma Huh7 cells was established in the late 1990s. This model allows one to study HCV RNA replication and the biological functions of each of the viral NS proteins[117-120]. The advent of a replicon system also facilitates the identification of adaptive mutations in the HCV viral genome and promotes the discovery of potent anti-HCV agents[118,121]. On the other hand, the establishment of an HCV pseudoparticle system (HCVpp), in which the HCV E1 and E2 glycoproteins are incorporated onto retro- or lentiviral particles, provides an efficient system to study HCV entry, identify entry (co)receptors, and screen for neutralizing antibodies[122-125]. In 2005, the robust production of infectious HCV in a cell culture system (HCVcc) based on the entire genome of the JFH1 strain, which is an HCV genotype 2a virus that was isolated from a fulminant hepatitis patient in Japan, was developed[126-128], thus marking a great achievement in the HCV research field. The generation of infectious HCVcc allows one to investigate each step of the viral life cycle and HCV-host cell interactions *in vitro* and will be useful for the screening and testing of new antiviral drugs. Nevertheless, the availability of an *in vivo* model for HCV research was limited to chimpanzee, which has been used as a model for studying viral replication kinetics, the immune response, and vaccine development[129-135]. The immune-deficient mouse system transplanted with human hepatocytes serves as an additional research tool to analyze the HCV life cycle in a humanized, small animal model[135,136]; however, the study using these two animal models is circumscribed by their high cost and the inconvenience of the experimental manipulations. Recently, a model that allows investigation of the complete HCV life cycle in an immune-competent mouse system was successfully developed by genetically engineering human CD81, SCARB- I , CLDN1 and/or OCLN into mice[137,138]. This system provides a new research platform for studying HCV infection *in vivo* and screening anti-HCV drug and vaccine candidates. Nevertheless, the low level of viral replication and virion production in this HCV-rodent model hampers the use of this *in vivo* model. Thus, further efforts are needed to develop an improved version of this rodent system.

AUTOPHAGY

Autophagy is considered a "self-eating" process in eukaryotic cells that engulfs unwanted cytoplasmic components within double-membranous vacuoles and delivers these cargos to lysosomes for breakdown. The autophagic process promotes the turnover of damaged organelles and aggregated proteins through lysosomal degradation to ensure the recycling of cellular constituents, thus maintaining cellular homeostasis[6,7]. The concept of "self-eating" was originally described in the mid-1950s in Christine de Duve's work on biochemical characterization of the lysosome in liver tissue[139,140]. Soon after this study, she and other researchers independently utilized transmission electron microscopy to show that dense bodies similar in size to mitochondria are present in the cytosol of renal and hepatic cells[141-146]. These observed dense bodies formed unique single- and double-membranous vesicle structures that were associated with lysosomes and contained mitochondria and endoplasmic reticulum (ER)[141,144,145]. Based on these observations, de Duve proposed a new term, "autophagy", to illustrate this de novo

process of sequestrating cytoplasmic organelles within a double membrane-enclosed vesicle termed an "autophagosome". Per Seglen's group then investigated the process prior to autophagosome formation in autophagy and identified the expansion of the "phagophore", which is an initial, membrane-rearranged structure, into the autophagosome[147-149]. Additionally, Mortimore and Schworer showed that amino acid deprivation activates autophagy and proteolysis in rat liver, and they were the first to suggest that energy imbalance and/or an insufficient nutrient supply can stimulate the initiation of the autophagic process[150-153]. In the early 1990s, the detailed molecular basis of autophagy began to be uncovered through Yoshinori Ohsumi's study using the yeast *Saccharomyces cerevisiae*[154,155]. Using the advantage of well-established genetic manipulation techniques and the well-known genomic background in the yeast model system, Ohsumi's and Klionsky's groups began to identify the genes involved in the autophagic process (ATGs)[156-168]. Most homologues of the yeast ATG genes also exist in humans and other eukaryotes, and the human orthologs of the yeast ATG genes can carry out similar functions[161,169]. Finally, a unified nomenclature for all ATGs in the different model systems was denoted[170-172]. These significant breakthroughs enhanced the understanding of the mechanism underlying how autophagy initiates and terminates and provided a crucial foundation for further investigation of autophagy-related processes.

MOLECULAR EVENTS LEADING TO AUTOPHAGY

Autophagy is the concerted action of the core molecular machineries that are involved in the initiation stage of the isolation membrane (IM)/phagophore, the middle step of elongation and enclosure of the autophagosome, and the late stage of autophagosome fusion with a lysosome and the subsequent degradation of the sequestrated components within the autolysosome by acidic proteases[6,7] (Figure 4). The completion of autophagy requires a dynamic membrane rearrangement process and multiple signaling pathways[171-174] (Figure 4). Upon nutrient deprivation, autophagy begins with the suppression of mammalian target of rapamycin (mTOR), which is a serine/threonine protein kinase that is required for cellular metabolism. Inhibition of mTOR leads to translocation of the downstream unc-51 like-kinase (ULK) complex, *i.e.*, ULK1-ATG13-FIP200-ATG101, from the cytoplasm to the unique ER membrane-associated compartments[175,176] (Figure 4). The class Ⅲ phosphatidylinositol-3-OH kinase (class Ⅲ-PI3K) complex, *i.e.*, PI3K-Vps15-Beclin-ATG14, is then recruited to the ER-derived nucleation site, where the P3K complex catalyzes the formation of phosphatidylinositol-3-phosphate (PI3P)[177] (Figure 4). The newly synthesized PI3P, in turn, recruits downstream effectors, including the double-FYVE-containing protein 1 (DFCP1) and the WD-repeat domain PI3P-interacting (WIPI) family proteins, resulting in the formation of an ER-associated Ω-like structure called the "omegasome"[178]. After the nucleation step, elongation and enclosure of the IM/phagophore to form an autophagosome require two ubiquitin-like conjugation complexes, *i.e.*, ATG12-ATG5-ATG16L and ATG4-ATG3-LC3Ⅱ (Figure 4). The ATG12-ATG5 conjugate is first produced through the concerted action of the ATG7 (E1-like) and ATG10 (E2-like) enzymes, and it then binds to ATG16L, which forms the ATG12-ATG5-ATG16L trimeric complex. The conjugation of microtubule-associated protein 1 light chain 3 (LC3), *i.e.*, the ATG8 homologue in mammals, to phosphatidylethanolamine (PE) begins with the proteolytic cleavage of its C-terminus by ATG4. Subsequently, the cleaved form of LC3 is conjugated to PE through the catalytic cascade of ATG7 and ATG3, thus generating the lipidated form of LC3, *i.e.*, LC3-Ⅱ[179]. Finally, the autophagosome fuses with the endosome and lysosome to form a mature autophagolysosome, named an "autolysosome", in which the engulfed materials are broken down and recycled for further use by cells[7]. Despite the emergence of an IM/phagophore from the ER[180,181], a variety of organelles, such as the plasma membrane (PM)[182], mitochondria[183], and Golgi apparatus[184], can also serve as membrane sources for the initiation of autophagy in mammalian and yeast cells (Figure 4). Nonetheless, what and how signaling pathways regulate each step of the membrane rearrangement processes must be further investigated.

AUTOPHAGY AND DISEASES

Apart from nutrient starvation, multiple stresses, including ER stress, accumulation of aggregated proteins and damaged organelles, and pathogen infections, can activate the autophagic response[7,185,186]. Upon infection by bacteria and viruses, the autophagic process is triggered in cells to directly engulf incoming pathogens and deliver them to the lysosome for degradation, *i.e.*, xenophagy[187-189]. In addition, autophagy may induce the innate immune defense to repress microbial infection, such as enhancing Toll-like receptor-mediated innate immune signaling and promoting the presentation of antigens derived from viruses such as vesicular stomatitis virus and Epstein-Barr virus onto a major histocompatibility complex class Ⅱ molecule[6,190-193]. Thus, autophagy can function as a restrictive route to eliminate pathogens. In addition, autophagy was shown to be exploited by many RNA viruses, such as mouse hepatitis virus, poliovirus, and rhinovirus, to promote their life cycle by serving as a membranous compartment for RNA replication[194]. On the other hand, autophagy also plays critical physiological roles in the pathogenesis of various diseases, including neurodegenerative disorders[12,13], inflammatory diseases[195,196], liver-associated diseases[197], and cancers[198]. Because of its impacts on a wide array of physiological and pathological conditions, autophagy has become an attractive field of biomedical research. Nevertheless, further studies are needed to better understand the regulation of autophagy and its exact physiological significance

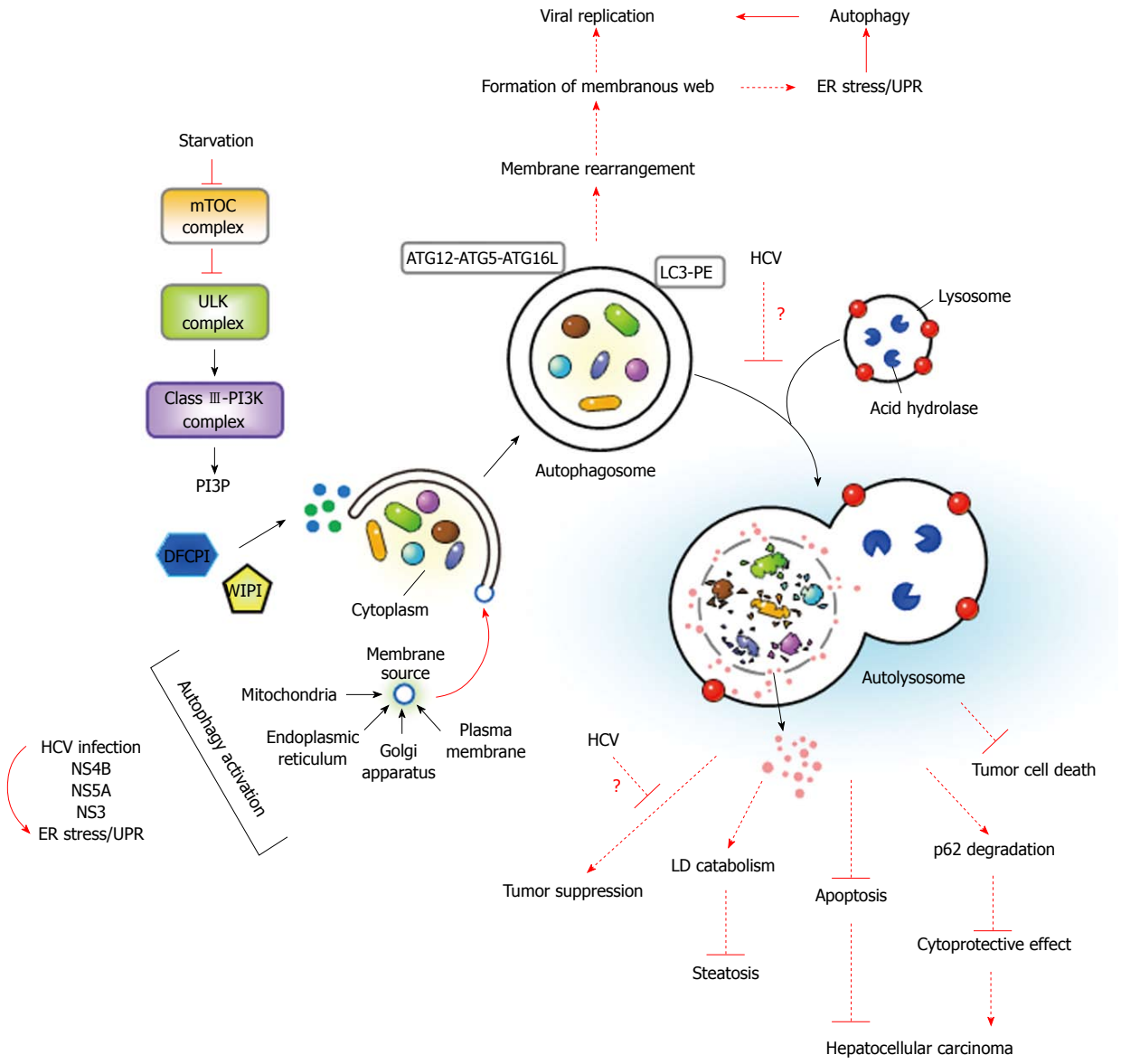

Figure 4 Sequential and coordinated events of autophagy and their impact on viral replication and hepatitis C virus-associated liver diseases. When cells undergo nutrient starvation, the activity of the mammalian target of rapamycin (mTOR) complex is inhibited, resulting in dephosphorylation, activation, and translocation of the unc-51 like-kinase (ULK) complex to the ER, where the ULK complex activates the class Ⅲ phosphatidylinositol-3-OH kinase (class Ⅲ-PI3K) complex to generate PtdIn(3)P (PI3P). PI3P, in turn, recruits double-FYVE-containing protein 1 (DFCP1) and WD-repeat domain PI3P-interacting (WIPI) protein into the isolation membrane, which may originate from the ER, mitochondria, plasma membrane, or Golgi apparatus. Two ubiquitin-like conjugation systems, the ATG12-ATG5-ATG16L and LC3-PE conjugation cascades, coordinate the elongation and enclosure of the autophagosome. Finally, the autophagosome fuses with a lysosome to degrade the sequestrated cytoplasmic components. Hepatitis C virus (HCV) infection, which activates ER stress/the unfolded protein response (UPR), as marked by a curved arrow, and ectopic expression of NS4B, NS5A, and NS3 are known to activate autophagy. The dashed lines indicate the potential implications of autophagy in HCV replication, HCV-related steatosis, and hepatocellular carcinoma. Activated autophagy may contribute to the rearrangement of the membrane as a resource of the membranous web for viral RNA replication. It is surmised that the HCV-induced blockade in autolysosome formation, as shown by a "?" during fusion of the autophagosome with a lysosome, may disrupt the effect of autophagy on LD and p62 degradation, thus contributing to the development of steatosis and hepatocellular carcinoma. On the other hand, interference with the effects of autophagy on tumor suppression by HCV, as indicted by a "?", or the inhibitory effects of autophagy on the apoptotic signaling and killing of tumor cells in HCV-infected patients may also enhance the progression to hepatocellular carcinoma.

in the pathogenicities of various diseases.

HCV AND AUTOPHAGY

Infections of flaviviruses such as HCV, Dengue virus, and Japanese encephalitis virus were shown to activate autophagy *in vitro*[8-11,199-202]. Many studies have reported that HCV induces the autophagic response in HCV viral RNA-expressing and HCVcc-infected cells[8-11]. By analyzing the viral expression of HCV genotype 1a H77 strain in immortalized human hepatocytes (IHH), Ait-Goughoulte's group demonstrated that autophagic vesicles and GFP-LC3-labeled punctate structures accumulate in viral RNA-transfected cells[8] (Table 1). These authors also found that the H77 HCV-induced autophagic response is accompanied by increases in Beclin expression

Table 1 Roles of autophagy in hepatitis C virus-host cell interactions

HCV genotype	Expression	Model	Analysis of autophagy activation	Physiological significance	Ref.
HCV-H77 (1a)	Transfection of viral RNA	IHH cells	1 Detection of GFP-LC3 punctate structure formation 2 Accumulated autophagosome in TEM analysis 3 Upregulation of Beclin expression and ATG5-ATG12 conjugate	Promotion of viral RNA replication	Ait-Goughoulte *et al*[8]
HCV-JFH1 (2a)	Transfection of viral RNA	Huh7.5 cells	1 Upregulated expression of LC3-II 2 No overlapping signal of GFP-LC3 punctate with lysosome 3 Autophagic activation by UPR 4 An incomplete autophagic process lacking enhanced autophagic degradation of long-lived proteins and p62	Promotion of viral RNA replication	Sir *et al*[11]
HCV-JFH1 (2a)	HCVcc infection	Huh7 cells	1 Upregulation of LC3-II 2 Accumulation of GFP-LC3 dot-like vesicles 3 No colocalization of autophagic vacuoles with viral proteins	Enhanced translation of the incoming viral RNA	Dreux *et al*[9]
HCV-JFH1 (2a)	HCVcc infection	Huh7.5-1 cells	1 Increase of GFP-LC3 dot-like structures 2 No colocalization of autophagic vacuoles with viral proteins	Promotion of virion assembly	Tanida *et al*[207]
HCV-JFH1 (2a)	HCVcc infection	Huh7 cells	1 Transient interaction of ATG5 with NS5B and NS4B 2 Association of ATG5 with membranous web	Promotion of viral RNA replication by organizing membranous web	Guévin *et al*[205]
HCV-JFH1 (2a) infection	HCVcc infection	Huh7 cells	1 Detection of early- and late-stage autophagic vacuoles by TEM analysis 2 Colocalization of autophagic vacuoles with lysosome 3 Accumulated LC3B-II expression by interference with autolysosome maturation 4 Complete autophagic process by HCV infection	Promotion of viral RNA replication by suppressing antiviral immunity	Ke *et al*[10]
HCV-H77 (1a); HCV-JFH1 (2a)	HCVcc infection	IHH	1 Activated IFN response in the HCV-infected cells by silencing of Beclin and ATG7 2 Increased caspase-dependent apoptosis by knockdown of Beclin and ATG7 in the HCV-infection cells	Promotion of viral RNA replication by suppressing antiviral immunity	Shrivastava *et al*[203]
HCV-Con1 (1b) and JFH1 (2a)	Replicon viral RNA transfection	Huh7 cells; Huh7.5-1 cells; Liver biopsy	1 An inverse correlation between hepatic steatosis and activation of autophagy in liver biopsy samples of infected patients 2 Colocalization of autophagic vacuoles with LDs	Promotion of catabolism of LDs	Vescovo *et al*[208]
HCV-JFH1 (2a)	Replicon viral RNA transfection	Huh7 cells; HCV-transgenic mice	1 Enhanced ROS level in mitochondria in HCV viral RNA-transfected cells 2 Activated autophagy by expression of HCV NS proteins 3 Alteration of antioxidant response by upregulation of antioxidant enzymes in HCV NS protein-expressing cells	Regulation of oxidative response in mitochondria	Chu *et al*[210]
HCV-JFH1 (2a)	HCVcc infection	Huh7 cells; Huh7.5-1 cells	1 Accumulation of mito autolysosome in HCV-infected cells 2 Stimulation of Parkin and Pink 1 expression in HCV-infected cells 3 Activation of mitophagy *via* a Parkin-dependent pathway	Elimination of damaged mitochondria and promotion of viral RNA replication	Kim *et al*[209]
HCV-JFH1 (2a) HCV-N (1b)	HCVcc infection; Replicon viral RNA transfection	Huh7 cells	1 Activation of autophagy through AKT1-TSC-mTORC1 signaling 2 Activation of autophagy *via* UPR	Promotion of viral RNA replication	Huang *et al*[214]
HCV-JC1 (2a) infection; HCV NS4B	HCVcc infection; Ectopic overexpression	Huh7.5 cells	1 Activation of autophagy by HCV NS4B amino acid 1-190 2 Requirement of Rab5 and PI3K for autophagic activation	Organization of virus replication site	Su *et al*[216]
HCV-JFH1 (2a)	HCVcc infection	IHH cells	1 Transcriptional activation of Beclin gene expression 2 Autophagy activation in a Bcl2-Beclin dissociation- and mTOR inhibition-independent manner	Promotion of viral RNA replication	Shrivastava *et al*[215]
HCV-JFH1 (2a)	HCVcc infection	Huh7.5 cells	1 Activation of autophagy through IRGM 2 Interaction of HCV NS3 with IRGM	Promotion of viral RNA replication; modulation of innate immunity (?)	Grégoire *et al*[237]

HCV: Hepatitis C virus; ROS: Reactive oxygen species; NS: Nonstructural; IRGM: Immunity-associated GTPase family M; UPR: Unfolded protein response; ATG: Autophagy-related gene; GFP-LC3: Green fluorescence protein-conjugated microtubule-associated protein 1 light chain 3; LDS: Lipid droplets.

and the ATG12-ATG5 conjugate[8]. Shortly after their study, Sir *et al*[11] reported that HCV triggers autophagosome formation in full length, JFH1 (genotype 2a) viral RNA genome-transfected Huh7 cells (Table 1). They further showed that HCV triggers incomplete autophagy, *i.e.*, autophagolysosome maturation is interrupted. This conclusion was based on the observation that the degradation of long-lived proteins and p62, which is a substrate of autophagic degradation, was inhibited despite increased numbers of LC3-Ⅱ and GFP-LC3-labeled dot-like vesicles in JFH1 RNA-transfected cells[11] (Table 1). Their study also demonstrated, for the first time, that autophagy positively regulates HCV growth *via* promoting viral replication because knockdown of ATG7 and LC3 dramatically suppressed viral RNA expression in HCV RNA-transfected cells[11] (Table 1). Whether autophagy is analogously activated by HCV in the HCV infection system had not been determined until Druex and her colleagues investigated autophagic activation in Huh7.5 cells infected with the JFH1 HCVcc virus in 2009[9] (Table 1). These authors provided first-line evidence demonstrating that HCV infection can induce the autophagic response to enhance the translation of the incoming viral RNA, rather than regulate viral growth, when virus RNA replication is established[9].

Utilizing the JFH1 infection system, we demonstrated that HCV infection of Huh7 cells enhances the autophagic flux and triggers the complete autophagic process throughout the formation of the mature autophagolysosome[9,10] (Table 1). Several lines of evidence supported this conclusion, including (1) the detection of early and late-stage autophagic vacuoles in the TEM analysis of HCV-infected cells; (2) the accumulation of LC3-Ⅱ expression by blocking the fusion of the autophagosome with a lysosome; (3) the predominant expression of RFP, but not GFP, fluorescence of a mRFP-GFP-LC3 reporter in the infected cells; and (4) the high degree of colocalization of GFP-LC3 puncta with lysosomes in infected cells[9,10] (Table 1). Moreover, we showed that silencing of the ATG genes and treatment with pharmacological inhibitors of autophagolysosome maturation repress HCV viral RNA replication[9,10]. However, interfering with autophagy had no detectable effect on virus entry or the translation of viral RNA[9,10]. Most importantly, inhibition of the HCV-activated complete autophagy drastically upregulated the IFN response that was mediated by the HCV pathogen-associated molecular pattern (PAMP), which is located within the poly-U/UC region of HCV 3'-UTR[9,10]. Consistent with our study, Shrivastava *et al*[203] reported that gene silencing of Beclin or ATG7 inhibits HCV growth and activates IFN and interferon-stimulated gene expression in HCV-infected human IHH cells (Table 1). Together, these two studies imply that autophagy may represent a repressive mode to protect HCV-infected cells against an excessive IFN antiviral response, thereby promoting viral RNA replication.

FUNCTIONS OF AUTOPHAGY IN HCV INFECTION

In addition to its suppressive effect on antiviral immunity, autophagy was reported to promote viral RNA replication through other mechanisms[204,205]. Guevin and colleagues showed the transient interaction of ATG5 with NS4B and NS5B as well as the detection of ATG5 in the HCV-induced membranous web, which suggested a proviral role of the autophagic machinery in the formation of the HCV replication complex[205] (Table 1 and Figure 4). Sir *et al*[204] further showed that NS5A, NS5B, and nascent viral RNA were colocalized with the autophagosome and argued that the HCV-induced autophagic membrane can be used as a membrane-associated compartment for the replication of viral RNA (Table 1). In addition to its pivotal role in viral RNA replication, autophagy was shown to regulate the assembly of infectious virions and protection of infected cells from death[206,207] (Table 1). Tanida and colleagues first reported that knockdown of ATG7 and Beclin gene expression moderately downregulates the extracellular titer of HCV virions without showing an apparent effect on the intracellular level of viral proteins and RNAs[207] (Table 1). This study suggests that HCV-activated autophagy may modulate the egress of HCV virions into infected cells. Additionally, HCV was shown to activate autophagy to protect infected cells from cell death[206] (Table 1). In this study, severe cytoplasmic vacuolation and cell death accumulated in Con1-HCV (genotype 1b)-transfected cells through interference with autophagy *via* ectopic expression of a protease-inactive mutant ATG4B^{C47A}[206], which implied that HCV may exploit autophagy as a cellular surveillance machinery to counteract the overloaded stress that is triggered by viral replication.

The HCV-induced autophagic process was also shown to regulate host cellular metabolism, including eliminating excess lipids and degrading damaged mitochondria[208,209] (Table 1 and Figure 4). Vescovo *et al*[208] studied the correlation of autophagy markers with the clinical parameters of lipid metabolism in liver biopsies of patients chronically infected with HCV and found an inverse relationship between autophagy activation and the extent of steatosis in those patients (Table 1). The authors further showed that autophagy participates in the catabolism of LDs in cells transfected with the HCV subgenomic RNA replicon[208], implying that HCV may utilize autophagic degradation to promote LD breakdown and circumvent virus-triggered lipid accumulation in host cells (Figure 4). In addition to its degradation role in lipid metabolism, a unique form of autophagy, termed "mitophagy", was recently shown to eliminate damaged mitochondria in HCV-infected cells in a Parkin-dependent manner[209,210] (Table 1). Knockdown of Parkin and Pink gene expression suppresses HCV viral RNA replication[209], suggesting a critical role of mitophagy in HCV replication.

INDUCTION OF AUTOPHAGY IN HCV INFECTION

The molecular mechanism for how HCV initiates autophagy is not fully understood, although several studies have shown that ER stress and the unfolded protein response (UPR) can stimulate autophagy activation[211-213] (Table 1 and Figure 4). Remarkably, two independent reports showed that the UPR is required for activation of autophagy by HCV[10,11]. Recently, Huang *et al*[214] showed that HCV can inhibit the protein kinase B (PKB)-tuberous sclerosis (TSC)-mTOR complex 1 (mTORC1) signaling pathway *via* virus-induced ER stress, thus activating autophagy. Nevertheless, Shrivastava *et al*[215] demonstrated that HCV induces autophagy by transcriptionally activating the expression of the Beclin mRNA and triggering mTOR signaling. In addition to virus-triggered ER stress and the UPR, viral protein expression seems to be another signal for HCV-activated autophagy (Figure 4). Su *et al*[216] showed that HCV NS4B can trigger incomplete autophagy *via* an interaction with Rab5 and Vps34 (Table 1). Moreover, HCV NS5A was reported to be sufficient to trigger the autophagic response[215] (Figure 4). On the other hand, Gregoire and colleagues demonstrated that several RNA viruses, including HCV, could modulate autophagy *via* the interaction of the immunity-associated GTPase family M (IRGM) with ATG5 and LC3[217] (Table 1). They also showed that HCV NS3 is sufficient to activate IRGM-mediated autophagy[217]. Collectively, these studies reveal that multiple signaling pathways may be involved in the HCV-activated autophagic response. However, further investigations are necessary to determine how HCV RNA or proteins cooperate with those cellular signaling pathways to modulate autophagy.

POTENTIAL ROLE OF AUTOPHAGY IN INHIBITING HCV-RELATED LIVER DISEASES

Although HCV infection is shown to positively induce the autophagic process in the cultured human hepatocyte system[8-11], the evidence for autophagy activation in an *in vivo* animal model and liver specimens from infected patients is still limited. Autophagy activated by HCV infection has been demonstrated to promote HCV growth in host cells *via* regulating RNA replication, the translation of incoming viral RNA, and the assembly of infectious viral particles[9-11,207]. In addition to its proviral role in the HCV life cycle, upregulation of autophagy functions in suppressing innate immunity[10,203,218], altering the apoptosis pathway[215] (Figure 4), and maintaining the surveillance of infected cells[206]. In addition, recent studies provide a new horizon for autophagy and its role in protection of host cells from excess LDs due to HCV infection and the elimination of damaged mitochondria *via* the degradative process[208-210]. These studies also suggest that the autophagic response is utilized to maintain cell homeostasis *via* promoting the breakdown of excess lipids and damaged organelles that are induced by HCV[208-210] (Figure 4). However, how these cell-signaling pathways, in turn, affect cellular metabolism or alter cell homeostasis, which lead to the development of HCV-associated liver diseases, still remains to be investigated (Figure 4).

The potential role of autophagy in the progression of HCV-induced steatosis and fibrosis emerges from the recent findings of Singh *et al*[219]. This group showed that autophagy regulates lipid metabolism in hepatocytes *via* a selective degradation process, *i.e.*, lipophagy[219] (Figure 4). Lipophagy represents a new mode of autophagy in lipid metabolism that catabolizes LDs in the liver[219]. Moreover, Singh *et al*[220] proposed another function of autophagy in the control of body lipids through regulating the differentiation of adipose tissues. Collectively, their studies imply that modulation of autophagy in the liver may affect the metabolic cycle of lipids. In line with their findings, Vescovo's group reported that HCV might subvert the degradative process of autophagy to promote the catabolism of LDs[208]. Based on the *in vitro* HCV replicon study and *in vivo* investigation of liver biopsies from patients chronically infected with HCV[208], Vescovo *et al*[208] concluded an inverse interrelationship between the extent of autophagy activation and the level of steatosis in HCV patients (Figure 4). This notion was based on their observations that the autophagic process facilitates LD breakdown in HCV replicon cells and that interference with autophagy leads to an elevated cholesterol level in HCV JFH1-infected cells. Although HCV-activated autophagy acts as a counteracting mechanism to prevent excessive accumulation of lipids that are induced by virus infection, it remains to be determined whether virus-induced autophagy affects the cell metabolism balance during the enhanced catabolism of LDs in liver cells, such as by altering the homeostatic levels of related lipids or interfering with the balance between the lipogenesis and lipolysis pathways. Moreover, whether activation of lipophagy by HCV infection affects the regular cellular functions and leads to pathological changes in the infected hepatocytes warrants further investigation. Nevertheless, the autophagy-mediated regulation of lipid metabolism may represent a mechanism of deregulation that interferes with metabolic homeostasis, thus promoting the progression of HCV-associated metabolic syndrome.

POSSIBLE LINK BETWEEN AUTOPHAGY AND THE HCV MEMBRANOUS REPLICATION COMPLEX

A recent study has shown that ectopic expression of HCV NS4B is sufficient to activate incomplete autophagy by interacting with Rab5 and Vps34 in human hepatoma cells[216], which suggests that activation of autophagy by the NS4B protein may be related to membranous web formation (Figure 4). Reciprocally, the HCV NS4B-induced membranous web accumulation could trigger a

stress response, such as ER stress, which was indicated to be an inducer of HCV-triggered autophagy[10,11] (Figure 4). It is still unknown whether HCV NS4B can utilize the autophagy-mediated membrane rearrangement process to generate double-membrane vesicles (DMV) within the membranous web, which is required for HCV replication. Notably, several studies indicated that HCV activates incomplete autophagy[11,216], which may serve as a means of inducing the accumulation of DMV. Moreover, the autophagosomal membrane has recently been demonstrated to be a site for HCV viral RNA replication[204]. In addition to NS4B, HCV NS5A was shown to activate autophagy *via* enhancing phospho-mTOR expression and its downstream target 4EBP1 in IHH cells[215]. HCV NS5A being a critical regulator for modulating the local concentration of PI4P, which is a critical component of the membranous web[77,78,81,84,85], implies again that HCV may exploit autophagy to regulate the formation of the membranous web (Figure 4). These studies collectively imply that the extent of host cellular autophagy may affect the pathogenesis of HCV-associated liver diseases through modulating the status of HCV replication and membranous web formation (Figure 4).

AUTOPHAGY AND HCV-RELATED LIVER CANCER

In addition to the possibility of participating in the development of liver-associated diseases by altering lipid metabolism, HCV-activated autophagy may contribute to the development of hepatocellular carcinoma (Figure 4). The role of autophagy in inhibiting tumor development originated from the investigation of the functional impact of knocking out the ATG genes in mice[221-225]. The heterozygous loss of the Beclin gene with a repressed autophagic process in mice promoted tumorigenesis and increased the occurrence of spontaneous malignancies, which indicated, for the first time, that Beclin may serve as a tumor suppressor in tumor progression[221]. Likewise, mosaic knockout of ATG5 and the conditional depletion of ATG7 in mice also resulted in spontaneous formation of benign liver cancer[223]. Additionally, inhibition of tumor suppressor genes, such as phosphatase and tensin homolog (PTEN) and p53, suppresses the basal autophagic response[223,226,227], suggesting an association of autophagy with tumor formation. On the other hand, recent studies from Komatsu's group showed that the accumulation of p62, which is a substrate of autophagy, by interference with autophagy promotes the formation of hepatocellular carcinoma[224,228] (Figure 4). This process occurs through direct interaction of p62 with Kelch-like ECH-associated protein 1 (Keap1), which is a component of Cullin3-associated ubiquitin E3 ligase, and ablation of the Keap1-mediated degradation of activating nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) and, therefore, leads to persistent activation of the expression of Nrf2 downstream cytoprotective genes[224,228]. These studies collectively unveil a novel role of autophagy in tumor suppression (Figure 4). Along with these findings, it would be interesting to investigate whether HCV-activated autophagy interferes with the suppressive effect of basal autophagy in tumor progression, thus promoting the development of liver cancer. Currently, how HCV-induced autophagy interacts with and/or affects basal autophagy *in vivo* to promote tumor formation is still unclear and needs to be studied.

On the other hand, accumulated evidence has indicated that cancer cells may activate autophagy to alter the tumor microenvironment and promote cell surveillance, which would, therefore, protect tumor cells from cell death[198,229-235] (Figure 4). The tumor microenvironment often faces stringent stress conditions of hypoxia and restricted nutrients; thus, tumor cells activate autophagy to counteract these stress responses[198,232,235]. Additionally, activation of autophagy is exploited by cancer cells to trigger resistance against anti-cancer therapy[229-231]. Hence, suppression of autophagy has been shown to synergistically enhance the efficacy of anti-cancer drugs to kill cancer cells[229,230]. The potential role of the HCV-activated autophagic response in the establishment of the tumor microenvironment and chemo-resistance of hepatocellular carcinoma has not yet been determined, but such a hypothesis is conceivably reasonable. For instance, the autophagy that is triggered by the virus may protect chronically HCV-infected cells from stress-induced cell death, such as through apoptosis, which would promote cell survival and possibly result in the development of tumors (Figure 4). Nevertheless, further investigations on the relationship between autophagy and the pathogenesis of HCV-related liver diseases and tumor progression are urgently needed. Without a convenient small animal model that supports the entire HCV life cycle and allows the monitoring of the HCV-associated disease progression, a large gap must be crossed before investigation on the *in vivo* relevance of autophagy in the development of end-stage HCV-associated liver diseases becomes feasible.

AUTOPHAGY AS AN ANTIVIRAL TARGET

Suppression of autophagy has emerged as a means to inhibit HCV replication[9-11,207]; therefore, the implications of repressed autophagic activity in anti-HCV therapy can be envisioned. Our recent studies indicated that pharmacological inhibitors of autophagy, such as chloroquine (CQ) and bafilomycin A1 (BAF-A1), can specifically inhibit HCV infection through activation of type I IFN antiviral immunity in the *in vitro* HCVcc model[10]. CQ and BAF-A1 were also shown to inhibit HCV entry *via* inhibiting the endocytosis pathway[39], and CQ has been demonstrated to inhibit the development of pancreatic tumor formation in a rodent model[233,234]. Recently, *in vivo* gene transfer of transcriptional factor EB, which is a master gene that regulates autophagy in the livers of mice, can promote clearance of mutant, hepatotoxic alpha-1-anti-trypsin, which is a protein aggregate that commonly causes liver injury[236]. This finding implicates

that modulation of cellular autophagy may provide an innovative and feasible therapeutic strategy for curing liver-associated diseases. Therefore, it is anticipated that these autophagic inhibitors, along with small molecule of inhibiting autophagy, could be therapeutically applied in the treatment of HCV infection and possibly HCV-associated liver diseases. Again, an *in vivo* small animal model for studying HCV infection and the progression of liver-related diseases is required for screening and testing the efficacy and safety of a potential therapeutic strategy.

CONCLUSION

Autophagy has emerged as an important topic in HCV research. However, the detailed mechanistic action of how HCV activates the autophagic process and comprehensive knowledge of the physiological significance of autophagy at each step of the HCV life cycle still remain to be investigated. Moreover, autophagy may contribute to the pathogenesis of HCV-associated liver diseases. In the future, studies on the exploration of the clinical relevance of autophagy in HCV-infected patients and *in vivo* investigations using small animal models that can support the complete HCV replication cycle shall provide mechanistic insights into the functional impacts of autophagy-HCV interactions in the pathogenesis of HCV-derived liver diseases. The results of these studies will benefit the development of new therapeutic strategies that are capable of curing HCV infection and elucidate the pathogenesis of HCV-associated liver diseases. These results will also facilitate the design of an efficacious vaccine that can protect the human population against HCV infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Laparoscopic splenectomy for hypersplenism secondary to liver cirrhosis and portal hypertension

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Abstract

Since the first laparoscopic splenectomy (LS) was reported in 1991, LS has become the gold standard for the removal of normal to moderately enlarged spleens in benign conditions. Compared with open splenectomy, fewer postsurgical complications and better postoperative recovery have been observed, but LS is contraindicated for hypersplenism secondary to liver cirrhosis in many institutions owing to technical difficulties associated with splenomegaly, well-developed collateral circulation, and increased risk of bleeding. With the improvements of laparoscopic technique, the concept is changing. This article aims to give an overview of the latest development in laparoscopic splenectomy for hypersplenism secondary to liver cirrhosis and portal hypertension. Despite a lack of randomized controlled trial, the publications obtained have shown that with meticulous surgical techniques and advanced instruments, LS is a technically feasible, safe, and effective procedure for hypersplenism secondary to cirrhosis and portal hypertension and contributes to decreased blood loss, shorter hospital stay, and less impairment of liver function. It is recommended that the dilated short gastric vessels and other enlarged collateral circulation surrounding the spleen be divided with the LigaSure vessel sealing equipment, and the splenic artery and vein be transected en bloc with the application of the endovascular stapler. To support the clinical evidence, further randomized controlled trials about this topic are necessary.

Key words: Laparoscopy; Splenectomy; Liver cirrhosis; Portal hypertension; Hypersplenism

Core tip: With meticulous surgical techniques and advanced instruments, laparoscopic splenectomy is becoming a technically feasible, safe, and effective procedure for hypersplenism secondary to cirrhosis and portal hypertension, and contributes to decreased blood loss, a shorter hospital stay, and less impairment of liver function. It is recommended that the dilated short gastric vessels and other enlarged collateral circulation surrounding the spleen be divided with LigaSure vessel sealing equipment, and blunt dissection be avoided. Use of the vascular stapler is reported to shorten and facilitate hilar dissection compared with the former techniques of ligation or clipping.

Zhan XL, Ji Y, Wang YD. Laparoscopic splenectomy for hypersplenism secondary to liver cirrhosis and portal hypertension. *World J Gastroenterol* 2014; 20(19): 5794-5800 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5794.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5794

INTRODUCTION

Millions of patients with chronic hepatitis B or C infec-

tion and chronic alcohol consumption may develop liver cirrhosis[1,2], which can lead to portal hypertension and hypersplenism. Portal hypertension increases the risk of variceal bleeding and results in a bleeding tendency due to thrombocytopenia[3]. For patients with cirrhosis, bleeding portal hypertension, and secondary hypersplenism, splenectomy and devascularization or shunt surgery were necessary[2]. Recent advances in interferon therapy have contributed to the elimination of hepatitis C virus both in patients with compensated cirrhosis and in those without cirrhosis[4,5]. Patients who have hypersplenism with splenomegaly, however, cannot receive such treatment due to thrombocytopenia, leukocytopenia, or both[5,6], so they are good candidates for splenectomy.

Open splenectomy (OS) has been performed for hypersplenism since 1950[7], but OS is excessively invasive in terms of blood loss and wound pain. For patients with hypersplenism who have poor liver function, splenectomy is associated with high rates of morbidity and mortality. Catheter-based arterial embolization has gradually become more popular, but some severe complications have been reported, which limit its wide use[2,8].

Laparoscopic splenectomy (LS) was first reported in 1991[9]. Since then, many studies have demonstrated the advantages of the laparoscopic approach over OS including a shorter hospital stay, decreased blood loss, faster recovery, and better quality-of-life outcomes. LS has become the golden standard for removal of normal to moderately enlarged spleens, even in malignant splenic disorders[10-16]. Mastery of the laparoscopic skills and the advances in technology have led to an increasing use of LS for hypersplenism secondary to liver cirrhosis and portal hypertension[2,3,17-24], but the safety of LS for patients with hypersplenism has not yet been established. Portal hypertension from liver cirrhosis is still considered a contraindication to LS in many institutions[25,26]. The aim of this article is to give an overview of the latest development in laparoscopic splenectomy for hypersplenism secondary to liver cirrhosis and portal hypertension, as well as to evaluate the feasibility and safety of laparoscopic splenectomy in portal hypertension.

SURGICAL INDICATIONS

Mastery of laparoscopic skills and the advances in technology have allowed a wide range of minimally invasive procedures to replace their open counterparts and led to better outcomes, allowing surgeons to apply the technique to disease processes that were previously regarded as contraindications to LS[27]. In keeping with the precept that laparoscopic surgery should hold to the same indications as the corresponding open procedure, the indications for LS are the same as those for OS. Splenectomy can be used in the prevention of increased elimination of the corpuscular elements of the blood and relieving symptoms caused by an enlarged spleen, possibly including abdominal distension, pain, and fullness or early satiety, or it may be used for staging purposes in case of malignant diseases[25].

Splenectomy is generally indicated for cirrhotic patients whose platelet count drops below (30-50) $\times 10^9$/L[2,20]. Watanabe *et al*[20] did LS in hepatocellular carcinoma patients with platelet counts $\leqslant 30 \times 10^9$/L, or in patient receiving interferon therapy with a platelet count $\leqslant 50 \times 10^9$/L or with a past history of severe thrombocytopenia caused by interferon therapy. Hirooka *et al*[28] reported that splenectomy was performed according to the following criteria: (1) thrombocytopenia (platelet count $< 8 \times 10^9$/L); (2) leukocytopenia (white blood cell count $< 2 \times 10^8$/L); or (3) in the case of consenting splenectomy. To date, splenectomy has been recommended for those patients with liver cirrhosis who have a bleeding tendency due to thrombocytopenia, endoscopic treatment-resistant esophagogastric varices or difficulty in undergoing surgical treatment or those patients who remain unsuitable for chemotherapy for hepatocellular carcinoma due to thrombocytopenia[5,28,29], and also for cirrhotic patients who require antiviral therapy[5,30-32] or have portal hypertensive gastropathy[33]. For patients with cirrhosis, bleeding portal hypertension and secondary hypersplenism, LS and devascularization were indicated[2,21,22,29,34].

Low-risk patients with stage Child-Pugh A or B liver cirrhosis are preferred to receive the procedure[3,20,21]. The absolute contraindications for laparoscopic surgery in portal hypertension are patients who can not tolerate general anesthesia, have intractable coagulopathy, and/or have any contraindications to laparoscopy[21].

SURGICAL TECHNIQUES

Laparoscopic splenectomy

LS can be performed using a lateral, semilateral, or supine approach depending upon the surgeon preference, spleen size, patient characteristics, and the need of concomitant procedures[25]. The anterior or supine position allows for a good access to the omental pouch and an excellent visualization of the splenic hilum. Some authors stated that this approach may be advantageous in case of very large spleens[14,35]. The splenic artery may be ligated early, thereby diminishing the risk of severe hemorrhage[21,35]. The anterior (or supine) position is indicated in case concurrent procedures need to be performed (*e.g.*, cholecystectomy, or biopsies of other organs)[26,35]. Difficulties arise in exposing and dissecting the ligamental structures as well as the dorsal vessels and the splenic hilum, due to their close relationship to the tail of the pancreas[25,26]. With the fully lateral approach, the patient is positioned at a 90-degree angle to the operating table. The spleen and viscera fall medially due to gravity, facilitating the dissection of the ligaments and hilar structures. Thus, this approach allows for safe vascular control. Visualization of the tail of the pancreas is good, thereby minimizing the risk of pancreatic injury[25,26]. For the majority of indications the hemi- (or semi-) lateral approach is preferred by most of the authors[35]. With this approach, the patient's position can be adjusted to surgical requirements by tilt-

ing the table so that a fully supine or fully lateral position is obtained. Some authors prefer a hemilateral position at the beginning of the procedure for easy access to the lower sac and division of the short gastric vessels[25,27,29]. The table then can be tilted to a more lateral position in which the spleen and other organs (stomach and intestine) fall medially by gravity. This allows for easier access to the posterior face of the spleen and the perisplenic ligaments[25,26]. Thus the dissection and ligation of the vessels at the splenic hilum are facilitated while the tail of the pancreas is spared.

The surgeon operates from the right side of the operating table using a 10-mm 30-degree scope. In general, four operative ports are used for LS[27,35], and the placement of the trocars depends upon the size of the spleen. A 10-mm trocar is placed to the superior right of the umbilicus for the camera. Another 10-mm operating trocar is placed in the left midclavicular line just below the border of the spleen to pass the LigaSure vessel-sealing equipment (LigaSure AtlasTM; Tyco Healthcare, Boulder, CO, United States) or harmonic shears (Harmonic Scalpel; Ethicon EndoSurgery, Cincinnati, OH, United States). A 5-mm trocar is placed in the subxiphoid space for allowing the use of a supplementary retractor or grasper. A 12-mm trocar is placed in the left midaxillary line halfway between the costal margin and the iliac crest or below the border of the spleen for the application of the endoscopic linear vascular stapling device and other supplementary instruments[27].

The procedure begins with the division of the splenocolic attachments and the opening of the gastrocolic ligament to access the lesser sac. Whenever possible, the splenic artery is dissected and tied at the upper border of the pancreas in patients with splenomegaly[27,29]. The splenogastric ligament (including short gastric vessels) and the splenorenal ligament are divided with the LigaSure vessel-sealing equipment or harmonic shears. The splenic hilum is dissected cautiously, and the splenic artery and vein are transected en bloc with the application of a linear laparoscopic vascular stapler (EndoGIA; AutoSuture, Norwalk, CT, United States, or Endolinear Cutter; Ethicon Endosurgery)[21,27]. Use of the vascular stapler can shorten and facilitate hilar dissection compared with the former techniques of ligation or clipping[25], also the clipping technique is not a safe procedure for massive splenomegaly because of the high conversion rate[36]. In addition, should bleeding ensue within the hilum, vascular staplers or suture must be used to control the bleeding. As few clips as possible are used because these may interfere with the use of vascular staplers and sutures. The remaining spleen diaphragmatic attachments are divided using the LigaSure vessel-sealing equipment or harmonic shears, therefore completing the splenectomy. The resected spleen is placed in a large specimen bag, then morcellated and retrieved[27]. Some authors reported that an electromechanical morcellator was used to remove the large spleen without a cumbersome intracorporeal bag or enlarged incision[29,37].

Laparoscopic azygoportal disconnection

Most patients underwent the laparoscopic Hassab's operation. The patient is put in the reverse Trendelenburg position, and another 10-mm operating trocar is placed in the left midclavicular line just below the costal margin. After finishing the splenectomy, dissection begins by approaching the left crus, which can be accomplished with the LigaSure vessel sealing equipment or harmonic shears, followed by opening the gastrohepatic ligament and identifying the right crus. The gastric coronary vein is visualized and its branches toward the esophagus and proximal stomach are divided near the esophagus and stomach walls with the LigaSure. At least 6-10 cm of the distal esophagus is dissected through the hiatus, and the paraesophageal venous collaterals are divided[21,22,34,38].

Five patients underwent the laparoscopic modified Sugiura procedure in one study. After finishing the paraesophagogastric devascularization, the lower esophagus of 2 patients was transected and then reanastomosed with a circular stapler (EEA; Ethicon) through an 8-10-cm accessory incision, and the spleen was extracted through the accessory incision. Gastrotomy in 3 patients was made on the anterior wall of the stomach, and a circular stapler (ILS, ECS25; Ethicon) was then introduced through the gastrotomy. The esophagus was tied around the stapler rod with a 3-0 polypropylene thread, then the stapler was fired and removed. The gastrotomy was closed with a straight stapler. The spleen was put into a big bag and then morcellated and retrieved[21].

The most important intraoperative complication during LS and azygos-portal disconnection is bleeding, which is the main cause of conversion. Capsule or small vessel tears may cause oozing, which contaminates the operating field and make the surgical procedure more difficult. It is very hard to manage massive hemorrhage from the major vessel or capsule fracture by laparoscopy; therefore, the prevention of bleeding during the procedure is fundamental. Generally, the harmonic shears can divide a 3-mm diameter vessel, and LigaSure vessel-sealing equipment can divide a 7-mm diameter vessel safely. It is recommend that the dilated short gastric vessels and other enlarged collateral circulation surrounding the spleen, distal esophagus and proximal stomach be divided with the LigaSure vessel sealing equipment, and blunt dissection be avoided[21,27].

Hand-assisted technique

Hand-assisted laparoscopic surgery is an alternative laparoscopic approach in which a minilaparotomy is planned and performed to enable the surgeon to introduce his or her hand while the pneumoperitoneum is maintained and the dissection maneuvers are performed under videoendoscopic control. It simplifies the performance of difficult procedures for experienced surgeons and can initiate the less experienced surgeons in advanced laparoscopic surgery[39].

Hand-assisted LS is a valid approach. It should be considered to avoid conversion to open surgery for mas-

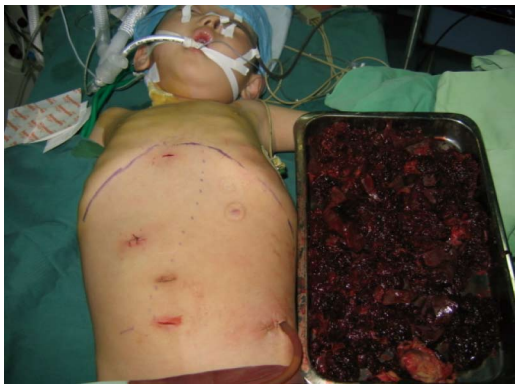

Figure 1 Morcellated and retrieved spleen after laparoscopic resection of massive splenomegaly and hypersplenism secondary to portal hypertension in a child.

sive splenomegaly. The inserted hand allows for tactile feedback and can assist in the surgical process during dissection, retraction, and placement of the enlarged spleen into the retrieval bag. Furthermore, unexpected situations such as hemorrhage or adhesions can be controlled. The spleen then can be removed via the additional incision, often without morcellation. Some studies have shown that hand-assisted LS is a very feasible and appropriate procedure for cirrhotic patients with splenomegaly and hypersplenism, and it significantly facilitates the surgical procedure and reduces the operational risk[40-44]. In cases of splenomegaly, hand-assisted LS results in shorter operating times, lower conversion rates, and fewer perioperative complications compared with the purely laparoscopic approach[25]. The main drawback of hand-assisted laparoscopic surgery is that it requires an additional incision, thus increasing trauma[39].

Laparoscopic splenectomy for portal hypertension in children

LS in the pediatric population is a relatively uncommon procedure, but it has shown the same advantages over OS as for adults, such as similar or less blood loss, a similar or lower complication rate, a shorter hospital stay, and better cosmesis. Less postoperative pain and earlier return to normal activities are especially important for pediatric patients. If splenectomy is indicated for children, the laparoscopic approach should be preferred[25,45]. Only there are few reports about LS with or without devascularization for hypersplenism and portal hypertension[46,47]. One study involves 6 cases of hypersplenism secondary to portal hypertension, and the results have shown that LS for children with portal hypertension and massively enlarged spleens is technically feasible, safe, and effective (Figure 1). Splenomegaly is not a contraindication for LS in children; in fact, significant benefits might be gained with the use of the laparoscopic approach[47]. Another study reports LS and periesophagogastric devascularization for portal hypertension in 6 children, and the conclusion is that laparoscopic massive splenectomy with selective devascularization of the lower esophagus and the upper stomach is a technically feasible, effective, and safe surgical procedure. It has all the benefits of minimally invasive surgery and offers a new alternative modality for children with bleeding portal hypertension and hypersplenism[46].

Historically, splenomegaly was considered a contraindication for LS because the working space is limited, especially in children[47]. Large spleen size can, in fact, interfere with visualization of the spleen and with the identification, isolation, and division of its vessels. Very large spleens can be more fragile and therefore more prone to bleeding from tearing. Moreover, the size can interfere with spleen extraction using either a bag or additional incision. Although massive splenomegaly is not a contraindication to laparoscopic splenectomy, the parents should be informed of the longer duration of surgery and the theoretically higher risk of complications[45].

FEASIBILITY AND SAFETY

In this article, we selected 19 studies to evaluate the feasibility and safety of LS in portal hypertension. If there were multiple reports about this topic in an institution, the earliest study was used for analysis. All studies were observational, including 9 non-randomized comparative studies[2,3,20,22-24,38,44,48] and 10 case series[5,17,19,21,29,37,42,43,46,49]. A total of 302 LS procedures were performed in 9 studies. The rate of conversion varies between studies, from 0% to 9.6%. In total, 15 (4.97%) conversions from laparoscopic to open surgery were necessary mainly because of massive intractable bleeding, 13 patients underwent LS using the hand-assisted technique. Five reports involve the comparison between laparoscopic and open splenectomy for hypersplenism secondary to liver cirrhosis[2,20,23,24,48]. Four studies show that LS required longer operating times than OS in portal hypertension[2,23,24,48], but one does not[20]. The duration of LS reported by different authors varies widely, from 150 to 237.7 min. Compared with patients who underwent OS, patients who underwent LS suffered less intraoperative blood loss and required fewer blood transfusions[2,20,23]. Five cases of post-operative bleeding were reported, and the postoperative hospital stay was also shorter after LS than OS[2,23,24,48]. The outcomes for selected studies of LS are shown in Table 1.

One hundred and thirty-eight patients underwent LS and devascularization in 6 studies, and 11 (7.97%) patients required conversion to open surgery. Two reports involve the comparison between laparoscopic and open surgery for bleeding portal hypertension, and show significantly less blooding duing laparoscopic surgery[22,38]. Laparoscopy resulted in fewer cases of pleural effusion, earlier passage of flatus, and shorter hospital stays. During a postoperative follow-up period of 2 to 50 mo, esophagogastric variceal rebleeding occurred in five patients (6.3%) who underwent laparoscopic surgery and in six (8.2%) patients who underwent open surgery (P = 0.638), and the 4-year mortality rates for these two

Table 1 Outcomes for selected studies of laparoscopic splenectomy in portal hypertension

Ref.	*n*	Operative time (min)	Blood loss (mL)	Conversion	Major complications	Hospital stay (d)	Additional procedures
Hashizume *et al*[29], 2002	73	210.1 ± 101.9	374.7 ± 352.4	7	0	31 ± 25.5	Devascularization (15)
Kercher *et al*[19], 2004	11	189 (79-245)	141 (10-60)	HALS (4)	0	2.6 (1-6)	0
Watanabe *et al*[20], 2007	25	173 ± 53	359 ± 280	HALS (4)	0	NR	0
Hama *et al*[3], 2008	17	171 ± 68	248 ± 312	HALS (3)	Bleeding requiring re-surgery (2)	10.0 ± 4.0	0
Zhu *et al*[2], 2009	81	174 ± 42	150.6 ± 135.4	5	0	8.2 ± 2.0	0
Akahoshi *et al*[5], 2010	21	237.7 ± 43.5	138.2 ± 190.6	HALS (2)	0	12.6 ± 7.3	0
Cai *et al*[23], 2011	24	224 ± 44	162 ± 126	1	Bleeding requiring re-surgery (1)	7.5 ± 1.7	Devascularization (5)
Ando *et al*[48], 2012	10	224 ± 56	342 ± 513	0	0	14.6 ± 3.5	0
Wang *et al*[24], 2013	40	150 ± 30	150 ± 110	2	Postoperative bleeding (2)	6.1 ± 2.2	0

LS: Laparoscopic splenectomy; HALS: Hand-assisted laparoscopic splenectomy; NR: Not reported.

Table 2 Outcomes for selected studies of laparoscopic splenectomy and devascularization

Ref.	*n*	Operative time (min)	Blood loss (mL)	Conversion	Major complications	Hospital stay (d)
Hashizume *et al*[17], 1998	10	287.5 ± 66.0	515.5 ± 507.9	1	0	12.7 ± 4.9
Wang *et al*[21], 2008	25	246 (180-330)	100-400	1	0	9 (6-15)
Li *et al*[46], 2009	6	214 ± 18	135 ± 48	0	0	NR
Zheng *et al*[22], 2009	7	180	100	0	Gastric perforation (1)	12
Jiang *et al*[37], 2013	10	288.0 ± 53.9	240.0 ± 217.1	0	0	11.3 ± 3.2
Cheng *et al*[38], 2013	80	254.4 ± 65.2	191.2 ± 163.2	9 (11.3%)	IH (2), EVR (2)	10.1 ± 2.5

LS: Laparoscopic splenectomy; NR: Not reported; IH: Intra-abdominal hemorrhage; EVR: Esophagogastric variceal rebleeding.

Table 3 Outcomes for selected studies of hand-assisted laparoscopic splenectomy in portal hypertension

Ref.	*n*	Operative time (min)	Blood loss (mL)	Conversion	Major complications or mortality	Hospital stay(d)	Additional procedures
Yamamoto *et al*[42], 2006	7	184.3 ± 54.9	166.4 ± 152.7	0	Mortality (1)	NR	Devascularization (7)
Uehara *et al*[49], 2009	5	237 ± 12	229 ± 100	0	Paralytic ileus (1)	16.7 ± 2.5	0
Wang *et al*[44], 2012	19	124 ± 42	92 ± 65	0	0	7.2 ± 2.8	0
Ando *et al*[48], 2012	6	341 ± 94	531 ± 390	0	Massive ascites (1)	19.8 ± 8.7	Devascularization (6)
Kakinoki *et al*[43], 2013	28	227 ± 100	236 ± 246	1	Bleeding requiring re-operation (1)	NR	Hepatectomy (4), Cholecystectomy (4), RFA (1), Devascularization (5)

HALS: Hand-assisted laparoscopic splenectomy; NR: Not reported; RFA: Radio frequency ablation.

surgical approaches were similar[38]. The outcomes for selected studies of LS and devascularization are shown in Table 2. Sixty-five patients underwent hand-assisted LS in 5 studies. Among them, 13 underwent splenectomy and devascularization using the hand-assisted technique. The outcomes for selected studies of hand-assisted LS are shown in Table 3.

The influence of laparoscopic surgery in patients with deteriorated liver function is of great concern. One study showed that no changes in liver function were noted 2 wk after LS for patients with hypersplenism secondary to liver cirrhosis[20]. Other studies investigated the effect of LS and OS procedures on liver function and found that the increases of aspartate aminotransferase, alanine aminotransferase, total bilirubin and direct bilirubin after surgery were less significant in the LS group, which indicated minor liver function impairment[2,50,51].

The immune responses in the LS group were significantly lower than those in the OS group. The LS group exhibited better preserved cellular immune response and faster recovery than the OS group on post-operative day 7[52].

CONCLUSION

Despite a lack of randomized controlled trial, a common consensus maintains that with meticulous surgical technique and advanced instruments, LS is a technically feasible, safe, and effective procedure for hypersplenism secondary to cirrhosis and portal hypertension, and contributes to decreased blood loss, a shorter hospital stay, and less impairment of the liver function. The results obtained will encourage surgeons to attempt a wider range of minimally invasive procedures as a replacement to their open counterparts. However, further randomized trials comparing open and laparoscopic splenectomy are

mandatory for patients with liver cirrhosis and portal hypertension.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (18): Pancreatitis

Acute pancreatitis: The stress factor

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Abstract

Acute pancreatitis is an inflammatory disorder of the pancreas that may cause life-threatening complications. Etiologies of pancreatitis vary, with gallstones accounting for the majority of all cases, followed by alcohol. Other causes of pancreatitis include trauma, ischemia, mechanical obstruction, infections, autoimmune, hereditary, and drugs. The main events occurring in the pancreatic acinar cell that initiate and propagate acute pancreatitis include inhibition of secretion, intracellular activation of proteases, and generation of inflammatory mediators. Small cytokines known as chemokines are released from damaged pancreatic cells and attract inflammatory cells, whose systemic action ultimately determined the severity of the disease. Indeed, severe forms of pancreatitis may result in systemic inflammatory response syndrome and multiorgan dysfunction syndrome, characterized by a progressive physiologic failure of several interdependent organ systems. Stress occurs when homeostasis is threatened, and stressors can include physical or mental forces, or combinations of both. Depending on the timing and duration, stress can result in beneficial or harmful consequences. While it is well established that a previous acute-short-term stress decreases the severity of experimentally-induced pancreatitis, the worsening effects of chronic stress on the exocrine pancreas have received relatively little attention. This review will focus on the influence of both prior acute-short-term and chronic stress in acute pancreatitis.

Key words: Pancreatitis; Acute stress; Chronic stress; Heat shock proteins; Tumor necrosis factor alpha

Core tip: Depending on the timing and duration, stress can result in beneficial or harmful consequences. Regarding the exocrine pancreas, a previous acute-short-term stress decreases the severity of experimentally-induced pancreatitis. This protection is conferred by distinct heat shock proteins (HSP) including HSP27, HSP60 and HSP70. Conversely, chronic stress increases the susceptibility of the exocrine pancreas, aggravating pancreatitis episodes. These worsening effects are mainly mediated by tumor necrosis factor alpha.

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INTRODUCTION

Acute pancreatitis is an inflammatory disorder of the pancreas with an overall mortality of approximately 5%[1]. Etiologies of pancreatitis vary, with gallstones accounting for the majority of all cases, followed by alcohol. Other causes of pancreatitis include trauma, isch-

emia, mechanical obstruction, infections, autoimmune, hereditary, and drugs[2].

The main events occurring in the pancreatic acinar cell that initiate and propagate acute pancreatitis include inhibition of secretion, intracellular activation of proteases, and generation of inflammatory mediators[3]. These cellular events can be correlated with the acinar morphological changes (retention of enzyme content, formation of large vacuoles containing both digestive enzymes and lysosomal hydrolases, and necrosis), which are observed in the well-established *in vivo* experimental model of supraphysiological cerulein-induced pancreatitis[4], as well as in human acute pancreatitis[5]. Chemokines released from damaged pancreatic cells attract inflammatory cells, whose systemic action ultimately determined the severity of the disease. Indeed, severe forms of pancreatitis may result in systemic inflammatory response syndrome and multiorgan dysfunction syndrome, characterized by a progressive physiologic failure of several interdependent organ systems[6].

Stress can be defined as "threatened homeostasis", and stressors can include physical or mental forces, or combinations of both. The reaction of an individual to a given stressor involves the stimulation of pathways within the brain leading to activation of the hypothalamic-pituitary-adrenal axis and the central sympathetic outflow[7]. This can result in visceral hypersensitivity through the release of different substances, such as substance P and calcitonin gene-related peptide from afferent nerve fibers[8].

The main source of pancreatic innervation comes from both vagus nerves and the celiac ganglion complex. The cephalic segment is innervated by the right celiac complex and the hepatic and mesenteric plexus coming from the right vagus. The splenic segment is innervated by the left celiac nerve and the splanchnic nervous network. Except for the gastro-duodenal branches network, most of the nerves enter the gland by its periphery and concentrate in the cephalic segment, which exhibits an important number of ganglion cells. These characteristics of the macroscopic innervation decrease in a significant and progressive fashion towards the splenic segment[9,10].

While it is well established that a previous acute-short-term stress decreases the severity of experimentally-induced pancreatitis[11-17], the worsening effects of chronic stress on the exocrine pancreas have received relatively little attention[18-20]. This review will focus on the influence of both prior-acute-short-term and chronic stress in acute pancreatitis.

ACUTE STRESS

Preceding acute-short-term stress is a well-known inductor of cellular protection against numerous pathological conditions, including renal ischaemia, heart ischaemia, brain ischaemia, enterocolitis and pancreatitis[11-17,21-25]. Exposure of organisms to an initial sublethal stress leads to the synthesis of heat shock proteins (HSP) and confers protection against further stress[26]. HSP comprise a highly conserved family of proteins with molecular sizes ranging from 10 to 110 kDa. These molecular chaperones are involved in synthesis, folding, transport and degradation of proteins, and can be induced by stressful conditions such as infection, inflammation, hypoxia, starvation, heat shock, water immersion, and oxidative stress[27-29].

The eventual protection conferred by acute stress-induced HSP in pancreatitis, seems to be stressor- and disease-inducer-dependent[30,31]. Water immersion and heat shock induce pancreatic HSP60 and HSP70, respectively, and protect rats from cerulein-induced acute pancreatitis by inhibiting autophagy, which prevents the subcellular redistribution of cathepsin B and the activation of trypsinogen[14,32,33]. Additionally, hyperthermia- or chemical-stimulated HSP70 also decrease the production of inflammatory mediators by downregulation of NF-κB[34,35]. Remarkably, transgenic mice knock-out for HSP70 (*HSP70.1*$^{-/-}$) develop spontaneous activation of pancreatic trypsinogen[36]. However, transgenic knock-in mice over-expressing HSP72 do not exhibit protection for development of cerulein-induced acute pancreatitis, but HSP72 over-expression accelerates tissue injury recovery by lessening NF-κB signaling[37]. Heat shock also induces pancreatic protection against cerulein hyperstimulation by upregulating HSP27[38]. Indeed, over-expression of HSP27 preserves the actin cytoskeleton of pancreatic acinar cells and protect against cerulein-induced pancreatitis in a specific phosphorylation-dependent manner[39]. HSP27 exerts a similar protective effect in coronary arteries[40]. Vessels (endothelial and/or smooth muscle cells) from patients with ischemic heart disease exhibit decreased levels of HSP27 (in particular phospho-HSP27), which correlates with destabilization of the actin cytoskeleton[40]. Regardless of the underlying mechanism, disorganization of the actin cytoskeleton is associated with dysregulation of pancreatic enzyme secretion[41]. Interestingly, HSP27 seems to coordinate activity with other HSP members to provide the full extent of resistance to injury[42]. For instance, , depletion of HSP70 in renal cells does not impede association of HSP27 with actin, but prevents maximal cytoprotective effect against energy depletion[42].

Other pancreatitis-induced models exhibit some differences with the previously mentioned, secretagogue hyperstimulation. Thus, hyperthermia protects against subsequent L-arginine-induced acute pancreatitis in rats by increasing pancreatic expression of HSP70 and HPS27, and phosphorylation of HSP27, but without changing HSP60 levels[15,43]. As observed in the cerulein model, transgenic mice over-expressing HSP72 do not exhibit protection for L-arginine-induced acute pancreatitis[37]. However, HSP72 over-expression does not accelerate tissue injury recovery in L-arginine treated animals[37]. Although both hot and cold water immersion induce pancreatic HSP72 and HSP60, respectively, only cold water

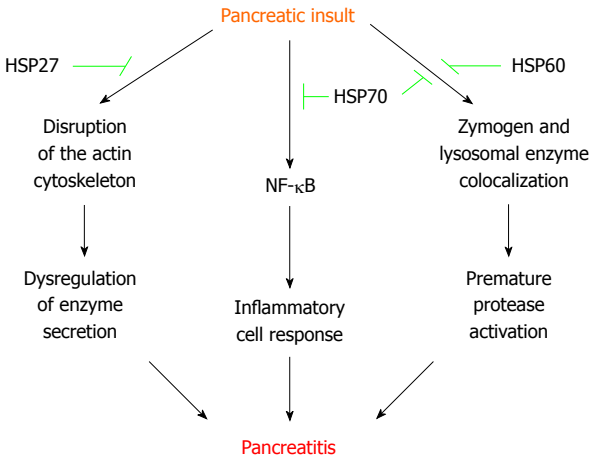

Figure 1 Hypothetical mechanisms underlying prior-acute-short-stress protects against pancreatitis. Pancreatic insults may provoke dysregulation of enzyme secretion, premature protease activation and inflammatory acinar response, which result in the development of pancreatitis. Different stressors such as hyperthermia, hypothermia, hypoxia, energy depletion and chemicals, can induce pancreatic heat shock proteins (HSP) by a prior-acute-short-stress exposition. Distinct HSP avoid the disruption of the actin cytoskeleton, zymogen/lysosomal enzyme colocalization and activation of the pro-inflammatory nuclear factor-kappa beta (NF-κB) caused by the pancreatic insult. These HSP-mediated effects seem responsible for the protection against pancreatitis. The specific pathway inhibited by each HSP is depicted in green.

immersion slightly protect rats from sodium tauracholate-induced acute pancreatitis, pointing the transcendence of the subcellular redistribution of cathepsin B in this necrohemorrhagic pancreatitis model[13].

Nevertheless, prior-acute-short-term stress protects against pancreatitis by distinct HSP, which seem to exert its beneficial effects through different pathways (Figure 1).

CHRONIC STRESS

Chronic stress has been proved to increase the susceptibility of different rat organs, such as the small intestine, colon and brain, to inflammatory diseases[8,20,44-46], as well as to aggravate atherosclerotic lesions in mice[47].

Even though oxidative stress and inflammation each occur in the pancreas during the early stage of supramaximal cerulein-induced acute pancreatitis model, neither oxidative stress nor an inflammatory insult alone cause the characteristic changes of acute pancreatitis[48]. However, chronic stress leaves the exocrine pancreas susceptible to pancreatitis by submaximal cerulein stimulation[20]. Pancreatic tissue from rats chronically exposed to restraint exhibit measurable levels of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) as well as a low but detectable leukocyte infiltrate and myeloperoxidase activity[20], suggesting leukocytes as a feasible source of TNF-α induced by chronic stress. Interestingly, *in vitro* incubation of mice pancreatic acini with phorbol-12-myristate-13-acetate-activated neutrophils or macrophages directly induce intracellular trypsinogen activation and cell death, being protease activation and necrosis mediated by leukocyte-secreted TNF-α in a cathepsin-B and calcium-dependent manner[49].

TNF-α has an important role in various biological functions, including cell proliferation, cell differentiation, survival, apoptosis and necrosis[50], and in stress-related inflammatory disorders[45-47,51]. For a long time, it has been known that TNF-α participates in the inflammatory cascade which propagates pancreatitis[52]. Nevertheless, its relevance in the genesis of this debilitating disease only recently captured the attention of research investigation[20,49].

Secretion of TNF-α by several stress stimuli has been demonstrated *in vitro* in many cell types, including pancreatic acinar cells[53-60], and *in vivo* in different tissues[47,51,61-63]. Our lab has shown that *in vitro* hypoxia-reoxygenation conditions also induce TNF-α secretion by acinar cells[20]. These conditions are concomitant with ischemia-reperfusion processes, which can be the result of microcirculatory disturbances generated by stress[64]. Indeed, local pancreatic blood flow is reduced by stress[65]. Hence, alternate vasoconstriction and vasodilatation leading to tissue ischemia and reperfusion could reflect another putative local origin of chronic stress-derived TNF-α found in the pancreatic tissue. This is supported by the increased levels of the transcription factor hypoxia inducible factor 1 alpha (HIF-1α) observed in experimentally stressed rats[20]. HIF-1α is induced by hypoxic conditions and is involved in different inflammatory processes, such as dermatitis, rheumatoid arthritis[66], and also pancreatitis[67].

Different reports evaluated the response of pancreatic acinar cells to exogenous TNF-α, showing disruption of the typical filamentous actin distribution[20,68]. A similar redistribution of actin from apical to basolateral membranes was observed in pancreatic acini supra-stimulated with CCK[69]. While TNF-α alone does not stimulate amylase secretion in human pancreas[70] or in isolated rat pancreatic acini[20,68], it certainly inhibits submaximal CCK-stimulated amylase secretion[20]. Although necessary, the inhibition of pancreatic enzyme secretion alone is not sufficient to induce pancreatitis[3]. Nonetheless, TNF-α also activates pancreatic acinar nuclear factor-κB (NF-κB), a key transcriptional regulator of the expression of inflammatory molecules[20,68,71,72]. Consistently, rat pancreatic acinar cells treated with high doses of exogenous TNF-α, exhibit a notable increase in the production of cytokines interleukin (IL)-1β, IL-4, IL-6, IL-10, as well as TNF-α[73].

TNF-α has been shown to regulate the activity of distinct protein kinase C (PKC) isoforms in diverse cell types, including the pancreatic acinar cell[72,74,75]. PKC family comprises at least 12 members differing in tissue distribution and activation requirements. There are three subclasses: classical PKC isozymes (-α, -β1, -β2, and -γ), which require calcium and are activated by diacylglycerol and phorbol ester; the novel PKC isozymes (-δ, -ε, -η, and -θ), which are activated by diacylglycerol and phor-

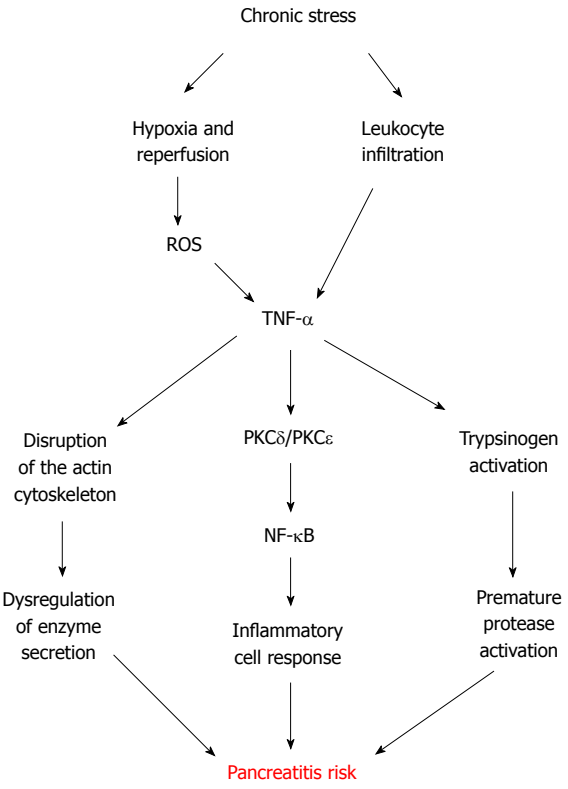

Figure 2 Hypothetical mechanisms involved in chronic stress sensitizes to pancreatitis. Chronic stress compromises the exocrine pancreas by generating ischaemia and reperfusion processes, as well as attracting leukocytes to the pancreatic parenchyma. Ischaemia and reperfusion induce hypoxia and reoxygenation conditions that generate the intrapancreatic reactive oxygen species (ROS) responsible for acinar tumor necrosis factor-alpha (TNF-α) production. TNF-α released from both pancreatic acinar cells and leukocyte infiltrate, impact on pancreatic acinar cells producing disruption of the actin cytoskeleton (redistribution from apical to basolateral membrane), a protein kinase C delta (PKCδ)- and PKC epsilon (PKCε)-mediated activation of the transcription factor nuclear factor-kappa beta (NF-κB), and an increase in levels of active trypsin. Dysregulation of enzyme secretion, induction of inflammatory acinar response and premature intra-acinar protease activation associated to these pathological pathways sensitize the exocrine pancreas to pancreatic insults and increase the risk to develop pancreatitis.

bol ester independently of calcium; and the atypical PKC isozymes (-λ, -ι, and -ζ), which are calcium independent and not responsive to phorbol ester. Rat pancreatic acini express the α, δ, ε, and ζ PKC isozymes[76]. Changes in PKC activity are associated with inflammation in a variety of tissues, including skin, kidney, intestine, and pancreas[77-80]. Specifically, PKC-δ and PKC-ε regulate the signal transduction pathways implicated in the pathophysiological activation of NF-κB and trypsinogen in rat pancreatic acini[72,81]. TNF-α activates both PKC-δ and PKC-ε in rat pancreatic acini[72], which convert physiological CCK concentrations into phytopathogenic concentrations[20]. Different studies have consistently shown that modulation of PKC activity sensitizes acinar cells to physiological secretagogue treatments, resulting in harmful levels of NF-κB and trypsin activity[81,82]. In agreement, TNF-α plus submaximal CCK pathologically activates NF-κB and trypsinogen in rat pancreatic acini, and induced both apoptosis and necrosis[20]. However, pancreatic acini response from rats seems to differ from that observed in mice, since TNF-α by itself only induces trypsinogen activation and necrosis in mice, with an extent comparable to supramaximal cerulein stimulation[20,49]. This could be a concentration-dependent effect or relative to differences between species, which is well-documented for experimentally-induced pancreatitis in rodents[83-86], but further studies are required to address this disparity in pancreatic acinar response to exogenous TNF-α.

Summarizing this topic, chronic stress appears as a risk factor to develop pancreatitis by sensitizing the exocrine pancreas through TNF-α, which seems to exert its detrimental effects through different pathways (Figure 2).

CONCLUSION

Depending on the timing and duration, stress can result in beneficial or harmful consequences for the exocrine pancreas. Prior acute-short-term stress could be useful for high-risk procedures such as endoscopic retrograde cholangiopancreatography. Conversely, the management of chronic stress appears critical for patients with risk of pancreatitis. Nonetheless, the mechanisms underlying protection by previous-acute-short-term stress as well as burden by chronic stress, have to be further explored.

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REVIEW

Intervention on toll-like receptors in pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is a devastating disease with pronounced morbidity and a high mortality rate. Currently available treatments lack convincing cost-efficiency determinations and are in most cases not associated with relevant success rate. Experimental stimulation of the immune system in murine PDA models has revealed some promising results. Toll-like receptors (TLRs) are pillars of the immune system that have been linked to several forms of malignancy, including lung, breast and colon cancer. In humans, TLRs are expressed in the pancreatic cancer tissue and in several cancer cell lines, whereas they are not expressed in the normal pancreas. In the present review, we explore the current knowledge concerning the role of different TLRs associated to PDA. Even if almost all known TLRs are expressed in the pancreatic cancer microenvironment, there are only five TLRs suggested as possible therapeutic targets. Most data points at TLR2 and TLR9 as effective tumor markers and agonists could potentially be used as *e.g.* future adjuvant therapies. The elucidation of the role of TLR3 in PDA is only in its initial phase. The inhibition/blockage of TLR4-related pathways has shown some promising effects, but there are still many steps left before TLR4 inhibitors can be considered as possible therapeutic agents. Finally, TLR7 antagonists seem to be potential candidates for therapy. Independent of their potential in immunotherapies, all existing data indicate that TLRs are strongly involved in the pathophysiology and development of PDA.

Key words: Pancreatic cancer; Pathophysiological mechanism; Toll-like receptor; Intervention; Adjuvant therapy

Core tip: The combination of high mortality rates and a tremendously complex pathophysiology makes pancreatic ductal adenocarcinoma (PDA) an enormous challenge. We summarize the current knowledge about the importance of toll-like receptors (TLR) in PDA. Since both tumor and tumor-related cells express TLRs, intervention on TLR-related pathways may represent future candidates for therapy.

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INTRODUCTION

Disorders of the pancreas are leading causes of morbidity and mortality. Despite advanced surgical and/or oncological treatment strategies, pancreatic ductal adenocarcinoma (PDA) is still associated with an extremely poor prognosis with a median survival of 6 mo and a 5-year survival rate less than 1%-2%[1,2]. PDA represents the fourth cause of cancer-related deaths and its incidence is rising in most countries[3].

The causes of PDA are mainly unknown. A family history is found in up to 5%-10% of patients[4]. Known

risk factors for PDA are among others tobacco smoking, diabetes mellitus, obesity and chronic pancreatitis[5-7]. Pancreatic intraepithelial neoplasia (PanIN) in the ductal epithelium has been suggested as the primordial precursor of PDA[8]. As PanIN progress to carcinoma, accumulated mutations might result in the activation of the *KRAS2* oncogene, loss of CDKN2A/p16 and/or the inactivation of TP53 and SMAD4[9]. Likewise, stellate cells are major players in PDA, as they are fundamental for the development of the characteristic desmoplastic stroma found in PDA[10]. Pancreatic cancer stem cells might be important in treatment resistance and metastasis. A large range of cell populations, such as tumor-associated macrophages (TAMs), have been reported as central in PDA[11,12]. The current knowledge of the pathophysiology of PDA has elegantly been summarized by Hidalgo[13,14].

At the time of diagnosis, most patients have already developed locally advanced (stages Ⅱ or Ⅲ) or metastatic (stage Ⅳ) disease and palliative treatment is the only alternative. Gemcitabine is a nucleoside analogue with a broad-spectrum against solid tumors that for long has been used as first-line treatment. In PDA, gemcitabine increases the quality of life of many patients, but merely prolongs the mean survival by one month[15]. Furthermore, a majority of patients do not respond to gemcitabine due to lack of the necessary nucleoside transporter, and the total costs and side-effects related to gemcitabine overtreatment are high[16,17]. FOLFIRINOX (5FU/leucovorin, irinotecan and oxaliplatin) is currently a first-line treatment for metastatic PDA as the regime is more active than gemcitabine at overall survival, progression-free survival and response rate. Moreover, the degradation of the quality of life is also delayed by FOLFIRINOX[18]. However, the regime is more expensive than gemcitabine and not suitable for all patients due to its toxicity. Hence, in most developing countries, gemcitabine is still the gold standard. Thus, current chemotherapeutic strategies lack proper cost-efficiency determinations and are not effective in the vast majority of cases.

In order to increase survival rates in PDA, it is imperative to find novel therapies that specifically target tumor cells and/or associated cell populations and stroma. Toll-like receptors (TLRs) are pillars of the immune system that have been linked to major cancer forms, including lung, breast and colon cancer[19-21]. In humans, TLRs are expressed in the pancreatic cancer tissue and in several cancer cell lines, whereas they are not expressed in the normal pancreas[22,23] (Table 1). TLRs thus appear to play a role in the pathophysiology of PDA (Table 2, Figure 1) and may thereby also represent targets for intervention (Table 3). In the present review, we explore the current knowledge concerning the role of different TLRs associated to PDA.

TOLL-LIKE RECEPTORS

TLRs are pattern recognition receptors that recognize numerous pathogen-associated molecular patterns (PAMPs) derived from virus, pathogenic bacteria, pathogenic fungus and parasitic protozoa. Likewise, TLRs can start immunological reactions against endogenous molecules released into the extracellular compartment under *e.g.*, stress or tissue damage[24]. TLRs are type I integral membrane glycoproteins expressed in various cell compartments, and in humans the expression of ten different TLRs (TLR1 to TLR10) has been reported[25]. Upon activation, TLRs form heterodimers or homodimers, and an activating signal is started. After the recruitment of adaptor molecules, TLRs can activate two major intracellular signaling pathways. All TLRs, except TLR3, can activate a MyD88-dependent pathway, causing the transcription of pro-inflammatory genes through the activation of nuclear factor κβ (NFκB) and/or the activation of activating protein 1[24,26]. An alternative, non MyD88-dependent pathway, can be initiated by TLR3 and TLR4. In the TRIF-pathway, the activation of interferon-regulated factors (IRF) *via* TRIF results in the synthesis of interferon (IFN) and/or the activation of NFκB[24].

TLR2-PROMISING ADJUVANT THERAPY

Mainly expressed on the plasma membrane, TLR2 is found in a large diversity of cells of the immune system[27]. In addition to its role in infectious diseases, TLR2 has been associated to *e.g.*, atherosclerosis, asthma and renal disease[28-30].

Macrophage activating lipopeptide-2 (MALP-2) is a synthetic lipopeptide that activates immune responses through TLR2 and TLR6[31,32]. In syngeneic subcutaneous and in orthotopic murine models the local administration of MALP-2 results in significant tumor growth reduction and prolonged survival[33]. Furthermore, the MALP-2 anti-tumor effect is enhanced by co-treatment with gemcitabine. However, the metastatic potential of cancer cells is not reduced by MALP-2 administration. MALP-2 might exert its effects through CD8+ lymphocytes and NK-cells since the murine Panc-2 cell line used for this experiment do not express TLR2. Hypothetically, MALP-2 activates dendritic cells (DCs) in a TLR2/TLR6-dependent manner[34]. A subsequent phase Ⅰ / Ⅱ trial showed promising results[35]. Ten patients in different PDA disease stages were included, both with "radical" surgery or palliative procedures leaving the pancreatic tumor behind. MALP-2 was injected intratumorally during surgery and six patients received adjuvant chemotherapy. The drug was well tolerated and a mean survival of 17.1 mo was observed. The median survival was 9.3 mo and no metastases were reported during follow-up. Despite the limited number of patients, the reported mean survival was remarkably high. The local administration of MALP-2 appears to upregulate the activation of both the innate and the adaptive immune system, resulting in decreased tumor proliferation and metastasis. Still, it is unclear if MALP-2 has a future as adjuvant therapy in PDA, since no further trials have been reported up to date. In addition, several less expensive TLR2 agonists appear to have similar biochemical properties when compared to

Table 1 Toll-like receptors found in human pancreatic adenocarcinoma cell lines

Cell line	Source	Phenotype	Expressed TLR	Ref.
AsPC-1	Metastasis: ascites	Du	TLR3, TLR4, TLR9	[23,57,58]
BxPC-3	Primary tumor	Du	TLR2-4	[40,50,57]
CFPAC	Metastasis: liver	Du	TLR4	[57]
Colo357	Metastasis: lymph node	Un	TLR3, TLR7	[48]
GER	Primary tumor	An	TLR9	[72]
MIA PaCa-2	Primary tumor	An	TLR2-4, TLR7, TLR9	[23,40]
MDAPanc-28	Primary tumor	Du/Ac	TLR2-4, TLR7, TLR9	[23]
Panc-1	Primary tumor	Du/An	TLR2-4, TLR7, TLR9	[23,40,50,58,79]
Panc-89	Metastasis: lymph node	Du	TLR3 , TLR7	[48]
PancTu-1	Primary tumor	Du	TLR3, TLR7	[48]
Pt45P1	Primary tumor	Du	TLR3, TLR7	[48]
SU.8686	Metastasis: liver	Du	TLR2	[41]
SW-1990	Metastasis: spleen	Du	TLR2-4, TLR7, TLR9	[23]
T3M4	Metastasis: lymph node	Du	TLR9	[77]

TLR: Toll-like receptor; Du: Ductal; Ac: Acinar; An: Anaplastic; Un: Undefined.

Table 2 Toll-like receptors expressed in pancreatic ductal adenocarcinoma and their reported implications

	Pathophysiological significance	Ref.
TLR2	Cell growth	[33,40,43]
	Immunosuppression	[33,41,43]
	Mean survival	[33,35]
	Progression and metastasis	[43]
TLR3	Carcinogenesis	[47]
	Cell growth and migration	[50]
	Immune responses	[48]
TLR4	Angiogenesis	[63]
	Carcinogenesis	[49]
	Cell growth	[49,57,61]
	Epithelial-to-mesenchymal transition	[61]
	Leukocyte recruitment and genomic instability	[57]
	Mean survival	[62]
	Progression and metastasis	[49,58,61]
	Stromal expansion	[49,61]
TLR7	Carcinogenesis, stromal expansion, progression and metastasis	[67]
	Immune responses	[48]
TLR9	Cell growth	[77,79]
	Mean survival	[77]
	Metastasis	[72,77,79]

TLR: Toll-like receptor.

MALP-2.

Protein-bound polysaccharide-K (PSK, Krestin®) is a natural remedy derived from highly purified mushroom extracts (*Trametes versicolor*) that since decades has been used as adjuvant therapy in cancer[36]. Even if the mechanisms are only partially known, PSK is thought to be a novel TLR2 agonist and it has documented therapeutic effects in colorectal and lung cancer[37-39]. Moreover, PSK promotes apoptosis and inhibit tumor growth in various human PDA (hPDA) cell lines[40]. Even if PSK-related cancer cell apoptosis is unlikely to be mediated through TLR2, the inhibition of the later significantly reduced the positive effects of PSK in all cell lines challenged. Thus, TLR2-pathways might be (if only in part) involved in the tumor suppressor effect of PSK.

TLR2 is also a promising cell-surface target since its protein expression is specifically increased in hPDA tissue[22]. Designed, fully synthetic high affinity TLR2 agonists have been studied with encouraging outcome. Derived from natural TLR2 ligands and also from MALP-2, these new compounds are able to induce the immune system when given as vaccine adjuvants in murine PDA (mPDA) models[41]. These results imply a potential in developing high affinity tumor targeted therapies through TLR2. A particularly potent compound has been conjugated with a near-infrared fluorescent dye, the novel Dmt-Tic-Cy5. The combination of Dmt-Tic-Cy5 and 3D imaging methods was applied in the intraoperative detection of tumor masses in a mouse xenograft model[42]. Using Dmt-Tic-Cy5 as a tumor marker during surgery in mice, successful R0 resections were obtained. Future applications of this technic could include the detection of early tumors or the improvement of current surgical procedures in hPDA.

Pancreatic adenocarcinoma upregulated factor (PAUF) is a protein overexpressed in hPDA and other types of cancer[43]. PAUF appears to modulate the metastatic potential of cancer cells and it upregulates the expression of CXCR4, the later being related to increased cancer cell motility[44]. PAUF induce the expression of the cytokines RANTES and MIF *via* TLR2 and it is also associated with the inhibition of CXCR4-dependent and TLR2-mediated NFκB activation, with subsequent decreased tumor necrosis factor-α levels[45]. Theoretically, PAUF might contribute to tumor persistence *via* the disruption of TLR2-dependent anti-tumor pathways in cancer.

In summary, TLR2 is not only expressed in tumor tissue but also in several hPDA cell lines (Table 1). Since TLR2 is present in both primary tumor cell lines and in cell lines from metastases, the receptor may be a novel target for immunotherapy in hPDA. The clinical significance of TLR2-targeting can become important in the future since the marker is present in up to 70% of resected tumors[22] but mainly absent in the normal pancreas. While the pathophysiological role of TLR2 in mPDA seems to be complex (Table 2, Figure 1), TLR2 agonists have shown promising results in animal models and in a phase I / II clinical trial (Table 3).

TLR3-UNEXPLORED IMPLICATIONS

TLR3 is a nucleic acid-recognizing receptor expressed as dimers on endosomal membranes of DCs and mono-

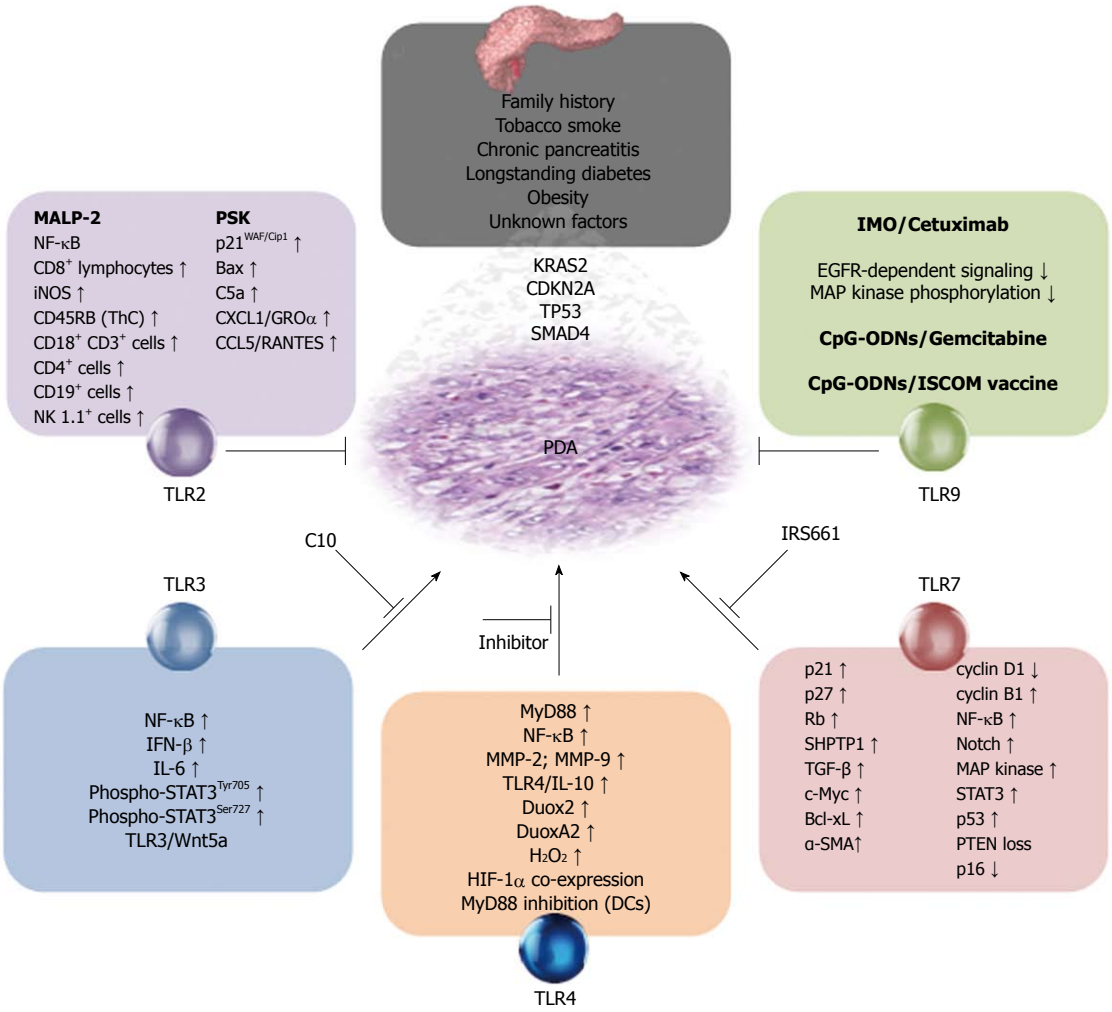

Figure 1 Toll-like receptors in the pathophysiology pancreatic ductal adenocarcinoma. TLR: Toll-like receptor; MALP-2: Macrophage activating lipopeptide-2; PSK: Polysaccharide-K; C10: Phenylmethimazole; IRS661: Immunoregulatory sequence 661; CpG-ODN: CpG oligodeoxynucleotide; IMO: Immunomodulatory nucleotides; TGF-β: Transforming growth factor-β; α-SMA: α-smooth-muscle antibody. NF-κB: The nuclear factor NF-κB.

cytes. Besides its role in viral infections, TLR3 has been linked to chronic pancreatitis and breast cancer[46,47].

Polycytidylic acid (Poly I:C) is a well-known TLR3 agonist capable of inducing cell lysis in hPDA cell lines by enhancing the cytotoxic activity of γδ T cells *in vitro*[48]. However, Poly I:C has also been reported to accelerate pancreatic carcinogenesis in KRAS-mutated mice[49].

TLR3 expression in hPDA cell lines is correlated with increased tumor cell growth and constitutive Wnt5a expression[50]. Wnt-associated pathways are related to a vast variety of cellular processes in embryogenesis and carcinogenesis[51]. Phenylmethimazole (C10) is a TLR3 inhibitor able to suppress the dsRNA induced, TLR3-mediated IRF3/IFN-pathway, independent of Wnt5a. The administration of C10 leads to less tumor development in a xenograft murine model. Importantly, C10 decreased TLR3 expression and significantly inhibited hPDA cell growth and motility/migration. The expression of TLR3 in tumor cells might result in increased interleukin (IL)-6 levels[52]. C10 effects could then be mediated by the inhibition of phosphorylated STAT3 *via* the disruption of TLR3/Wnt5a-related pro-inflammatory IL-6 expression in hPDA.

Even if TLR3 is constitutively expressed in primary hPDA cell lines (Table 1), it is unclear which role TLR3 plays in hPDA. Opposite results have been reported when TLR3 inhibitors have been tried. Hence, no conclusions can be made at this point.

TLR4-IS INHIBITION THE ANSWER?

Being the first TLR identified, TLR4 is widely expressed as homodimers or heterodimers with TLR6 on the plasma membranes of many immune cells. TLR4 has been linked to several diseases, including obesity, acute pancreatitis and breast cancer[18,53,54].

TLR4 is overexpressed both in mPDA and hPDA[49]. Stromal leukocytes from patients have increased TLR4 expression. Interestingly, the upregulation is also found both in epithelial and stromal cells in KRAS-mutated mice. Moreover, TLR4-inhibition in these mice had protective effects against tumorigenesis and TLR4$^{-/-}$ animals had a slower tumor growth. However, the inhibition of MyD88-dependent and TRIF-pathways had opposite

Table 3 Toll-like receptors and their intervention in pancreatic ductal adenocarcinoma

	Substance/compound	Intervention	Effects	Ref.
TLR2	MALP-2 (G)	Activation	Induce lymphocyte invasion and tumor necrosis	[33]
			Inhibit tumor growth	[33]
			Prolongs mean survival	[35]
			Reverse tumor-associated immunosuppression	[33]
	Polysaccharide-K (G)	Activation	Inhibit tumor growth and induce apoptosis in tumor cells	[40]
	Dmt-Tic-Cy5	Activation	Acts as vaccine adjuvant in pancreatic cancer	[41]
			Target imaging and therapy	[41,42]
	PAUF	Mixed	Facilitates tumor growth	[43]
			Promotes tumor immune-resistance	[43]
TLR3	Polycytidylic acid	Activation	Accelerates carcinogenesis	[49]
			Induces T cell invasion and tumor lysis	[48]
	Phenylmethimazole	Inhibition	Inhibits tumor growth and migration	[50]
TLR4	Lipopolysaccharide	Activation	Accelerates carcinogenesis	[49]
			Induce desmoplastic stroma	[49]
			Induce increased H_2O_2 extracellular production	[57]
			Increased invasiveness	[58,61]
			Induce M2-polarization in tumor-associated macrophages	[61]
TLR7	Imiquimod	Activation	Induce T cell invasion and tumor lysis	[48]
	IRS661	Inhibition	Prevent tumor progression and stromal expansion	[67]
			Regulates cell cycle in cancer cells	[67]
TLR9	CpG-ODN 1816/26 (G')	Activation	Delays tumor development, reduce invasiveness	[72]
			Prolongs mean survival	[72]
	IMO (C)	Activation	Prolongs mean survival, inhibit tumor growth and migration	[77]
			Reestablish cetuximab sensibility in cancer cells	[77]
	CpG-ODN 2216	Activation	Inhibits tumor growth and migration	[79]

TLR: Toll-like receptor; MALP-2: Macrophage activating lipopeptide-2; PAUF: Pancreatic adenocarcinoma upregulated factor; IRS661: Immunoregulatory sequence 661; CpG-ODN: CpG oligodeoxynucleotide; IMO: Immunomodulatory nucleotides. (G): Synergism when combined with gemcitabine; (G'): Effect mainly when combined with gemcitabine; (C): Effect merely when combined with cetuximab.

effects in mPDA. While MyD88-inhibition clearly accelerated tumor development and gave rise to highly aggressive TP53 mutated cancer cells, TRIF-inhibition had antitumor effects. MyD88-inhibition could induce aggressive cancer cells even in TRIF-deficiency co-existence.

Even if MyD88 blockage has been associated with a decreased tumor development in other cancer forms[55], the presence of DCs in PDA microenvironment appears to be the main factor for MyD88-dependent tumor-stimulating effects. Upon MyD88 blockage, DCs seem to induce pancreatic antigen-restricted Th2-deviated $CD4^+$ T cells[49]. Furthermore, the abundance of Th2 cells in hPDA is linked to a worsened prognosis[56].

Inflammatory cytokines can induce NFκB activation in mPDA. LPS and INF-γ challenge results in increased production of extracellular H_2O_2 in primary hPDA cell lines[57]. Through TLR4, the activation of NFκB might enhance the transcription of dual oxidase 2, trigger leukocyte recruitment and genetic instability. In hPDA cell lines, LPS challenge induced improved invasiveness *via* TLR4/MyD88-depending pathways[58]. Moreover, RNAi silencing TLR4 or MyD88 completely reversed the effects of LPS. NFκB activation might induce increased expression of matrix metalloproteinases (MMPs) in mPDA. MMP-2 and MMP-9 overexpression is related to the progression of hPDA, and its blockage has been subject of intensive research[59]. Thus, LPS may act through a TLR4-MyD88-NFκB axis that finally leads to MMP-9 overexpression and thereby to increased invasiveness *in vitro*[60].

The overexpression of MMPs has also been coupled to TAMs. M2-polarized TAMs mediate EMT, induce cancer cell proliferation and migration in hPDA cells *in vitro*[61]. These effects may partially be achieved through TLR4. TLR4 overexpression in M2-polarized macrophages could lead to IL-10 release with impact on the EMT and thereby on the metastatic potential of the cancer cells.

hPDA is characterized by a poor vascularization. Thus, the role of angiogenesis in hPDA remains controversial[9]. In humans, hypoxia-inducible transcription factor alpha (HIF-α) is overexpressed in resected pancreatic cancer tissue. Moreover, a positive correlation between mRNA/protein HIF-α levels and mRNA/protein TLR4 levels in primary tumors and metastases has been found. TLR4 was expressed in 69.2% of the analyzed tumor tissue. Besides, the expression of either TLR4 or HIF-α was related to a decreased survival rate and when both were expressed, an accumulative effect was observed[62]. Some data imply that hypoxia in solid tumors, such as hPDA, induces HIF-α overexpression, which might be responsible for the expression of TLR4 in hPDA cells *in vitro* and in a xenograft murine model[63]. Here, TLR4 was found in 76 % of the tumor tissue but no data on average survival or prognosis was presented.

The inhibition/blockage of TLR4-related pathways has shown some promising results, but there are still many steps left before TLR4 inhibitors can be considered as possible therapeutic agents. Since both stromal cells and primary tumor cells express TLR4, it is plausible

that TLR4 ligands found in the inflammatory tumor microenvironment initiate complex interactions between the different cell populations. This might in turn lead to the secretion of tumor stimulating cytokines and the recruitment of further cell populations into the tumor stroma. Since hypoxia and TLR4 ligands are common in the tumor stroma, the upregulation of TLR4 and HIF-α in hPDA could be auto-stimulatory. Poor prognosis can then be partially predicted, as a highly hypoxic tumor stroma is less sensitive for radiotherapy and disrupt the delivery of chemical agents into the primary cancer cells.

TLR7-PROMOTING CANCER PROGRESS

TLR7 is a nucleic acid-recognizing receptor expressed as dimers on endosomal membranes of APCs and leukocytes. TLR7 activation is currently used for the treatment of various malignancies, such as melanoma and breast cancer[64]. Like TLR3, TLR7 has also been used to enhance cytotoxic activity in γδ T cells *in vitro*[48].

The role of TLR7 in mPDA has been reported previously[65,66]. Upregulated TLR7 is found in epithelial cells and macrophages, DCs, neutrophils, and B- and T-cells of the tumor microenvironment. In hPDA, the expression of TLR7 is increased both in epithelial ductal cells and inflammatory cells within the tumor stroma.

Moreover, the administration of ssRNA40, a TLR7 agonist, results in pronounced tumor growth and stromal expansion in mice. In KRAS-mutated mice, the tumor-stimulating effects of TLR7 appears to be mediated by a complex array of events, including loss of expression of PTEN, p16 and cyclin D1 and upregulation of among others p21, p27, p53, c-Myc, SHPTP1, TGF-β, PPARγ and cyclin B1. Moreover, ssRNA40 challenge resulted in the activation of STAT3, MAP kinase, Notch and NFκB pathways. Notch target genes were downregulated, giving rise to the hypothesis that Notch, together with NFκB, might mediate inflammation in the tumor microenvironment, thus promoting tumor persistence and metastatic potential[67].

Importantly, TLR7 stimulation is not self-sufficient for malignant transition when KRAS mutations are absent. Equally important, mice with $TLR7^{-/-}$ phenotype seem to be protected against tumor progression. The administration of IRS661, an oligonucleotide inhibitor of TLR7, prevented tumor progression and stromal expansion in mice[67]. IRS661 treatment decreased the expression of p21, p27, p-p27, cyclin B1, CDK4 and p-STAT3 in mice with invasive PDA. Thus, TLR7 inhibition was able to affect cell cycle regulation in already formed pancreatic tumors. However, the expression of Rb or TP53 was not affected by IRS661.

The evidence of the importance of TLR7 in mPDA is strong and TLR7 antagonists are without doubt promising experimental adjuvant agents that must be further evaluated. Importantly, PanIN in humans do not express TLR7 with the same intensity as established hPDA tumors. Moreover, the expression of TLR7 appears to increase with tumor progression and it is found in nearly 50% of the advanced tumors[67]. TLR7 may induce tumor progression in a KRAS-dependent manner since the mutation must be present for TLR7-mediated tumor progression in mice. As KRAS2 is mutated in over 90% of hPDA[18], these may only be a minor obstacle for the future clinical use of TLR7-targeting.

TLR9-AGONISTS AS FUTURE ADJUVANT THERAPY?

As TLR3 and TLR7, TLR9 is expressed on endosomal membranes of several immune cells, including macrophages, B cells and DCs[68]. Besides its role in bacterial, viral or malaria infection, TLR9 has been linked to acute pancreatitis and cancer[54,69].

Synthetic TLR9 agonists (CpG-ODNs) are oligodeoxynucleotides containing CpG motifs that have been used as vaccine adjuvants or as antiallergic agents[70]. In combination with vaccines based on immune stimulatory complexes, a TLR9 agonist inhibits the tumor immune evasion in mPDA[71]. It is believed that CpG-ODNs can activate NK-cells, DCs and cytotoxic T cells, thus initiating anti-tumor immune responses. TLR9 is highly expressed in the tumor microenvironment and in circulating leukocytes in a murine xenograft PDA model. CpG-ODNs treated mice had a reduced tumor spread to the diaphragm, liver and spleen and the combination of gemcitabine and CpG-ODNs resulted in delayed development of bulky disease, less metastasis and improved survival, when compared to gemcitabine monotherapy[72].

The epidermal growth factor receptor (EGFR) is overexpressed in 50%-60% of hPDA[73]. Cetuximab is a monoclonal anti-EGFR antibody that has shown promising results experimentally, but not clinically in hPDA[74,75]. Immunomodulatory nucleotides (IMO) are second-generation CpG-ODNs with higher metabolic stability. IMO interferes with EGFR-dependent signaling and has thereby a synergistic effect with anti-EGFR agents[76]. In combination with cetuximab, IMO inhibits cell growth in hPDA and cancer progression in KRAS-mutated murine cell lines[77]. Importantly, in cetuximab-resistant cell lines, IMO potentiated the activity of cetuximab. The administration of IMO resulted in tumor growth inhibition and prolonged survival in a murine xenograft model. The associations between EGFR/TLRs interactions and carcinogenesis are slowly being elucidated. However, the impact on hPDA is still unexplored[78].

Another CpG-ODN (ODN2216) has shown antiproliferative properties in an hPDA cell[79]. Tumor cell growth, replication rate and migration ability were decreased in cells challenged with ODN2216. The effects seem to be time- and dose-dependent. Moreover, the expression of TLR9 is more pronounced in hPDA tissue than in peritumoral ones (73.3% *vs* 33.3%)[79].

As TLR2, TLR9 appears to be a promising tumor marker. Likewise, TLR9 agonists could be used as adjuvant therapy by themselves or in combination with al-

ready established chemotherapies (Table 3). Nonetheless, the pathophysiological role of TLR9 in hPDA is mainly unexplored.

CONCLUSION

The role of the immune system in cancer is an area of intensive research. Cancer cells have the ability to evade immune responses and promote tumor phenotypes and pathways in immune cells. TLRs are related to several cancer forms, and immunotherapies involving TLRs are a reality[27]. At least thirty new clinical trials evaluating TLRs agonist and cancer have started since May 2012[80].

The combination of high mortality rates and a tremendously complex pathophysiology makes PDA an enormous challenge. The role of inflammation and immune cells in PDA cannot be stressed enough[81]. Both MyD88-dependent cascades and TRIF-pathways have been associated with tumor growth, survival and metastatic potential in PDA[65]. Even if almost all known TLRs are expressed in the pancreatic cancer microenvironment, there are only five TLRs suggested as potential therapeutic targets.

Importantly, the effects of TLRs agonists and antagonists in PDA are presumably mediated by the inducement of anti-tumor immune response. This requires access to the primary tumor site. Moreover, TLR-targeting can theoretically disrupt important pathways in primary tumor cells with therapeutic effects. Thus, TLR-based agents must either be administered intratumorally or delivered through the tumor stroma. The recognition of specific tumor targets is then imperative for the application of TLRs intervention in PDA. In clinical practice, CA 19-9 is widely used as hPDA marker. CA 19-9 is a relatively specific marker useful as indicator for advanced disease or tumor recurrence after surgery. However, as pancreatic cancer progress and spreads beyond the pancreas, the accumulation of abnormalities might change the sensitivity and/or specificity of tumor markers since metastases may differ profoundly from the primary tumor[82]. We have recently propose mucin 4 (MUC4) as a novel tumor marker in hPDA. MUC4 is found in both primary and matched metastatic tumors with a high level of concordance (82 %)[83]. Specific tumor markers open the door for efficient drug delivery *via e.g.*, nanotechnology. For instance, targeted liposomal delivery of TLR9 ligands in cancer has already been evaluated with encouraging results[84].

Independently of their potential in immunotherapies, all existing data indicate that TLRs are strongly involved in the pathophysiology of PDA (Figure 1). The role of TLRs in PDA is not limited to the direct effect on tumors or associated cells. TLRs are also involved in the pathophysiology of several risk factors for hPDA, such as chronic pancreatitis, diabetes and obesity[47,85].

The present paper summarizes the current understanding of interventions on TLRs in PDA. Despite initial encouraging results, further research and elucidation of involved mechanisms is demanded.

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REVIEW

MicroRNAs: New therapeutic targets for intestinal barrier dysfunction

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Abstract

Defects in intestinal barrier function characterized by an increase in intestinal permeability contribute to intestinal inflammation. Growing evidence has shown that an increase in intestinal permeability has a pathogenic role in diseases such as inflammatory bowel disease (IBD) and celiac disease, and functional bowel disorders such as irritable bowel syndrome. Therefore, clarification of the inflammatory responses, the defense pathway and the corresponding regulatory system is essential and may lead to the development of new therapies. MicroRNAs (miRNAs) are small (19-22 nt) noncoding RNA molecules that regulate genes at the post-transcriptional level by base-pairing to specific messenger RNAs for degradation to repress translation. Recent studies suggested that miRNAs are important in the immune response and mediate a critical role in multiple immune response-related disorders. Based on these discoveries, attention has been focused on understanding the role of miRNAs in regulating intestinal barrier dysfunction, especially in IBD. Here, we provide a review of the most recent state-of-the-art research on miRNAs in intestinal barrier dysfunction.

Key words: MicroRNAs; Intestinal barrier dysfunction; Inflammatory bowel disease; Celiac disease; Therapeutic target

Core tip: This article summarizes the latest findings on the important roles of microRNAs (miRNAs) in regulating inflammation and autoimmune disorders in inflammatory bowel disease (IBD). Insight into miRNAs-21 as a novel biomarker is also provided, which shows that miRNAs-21 is a potential diagnostic and therapeutic target for IBD.

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INTRODUCTION

The intestinal barrier plays an important role in absorbing nutrients and secreting waste[1]. In addition to its abilities to support paracellular transport, the intestinal barrier can also prevent luminal microbes and their products reaching the internal milieu. Tight junctions and their associated proteins, including claudins, occludin, and zonula occludens, are the most adhesive apical junctional complexes and act as a structural and functional barrier against paracellular permeation of luminal substances[2-4]. Breakdown or disruption of the epithelial barrier is thought to be an essential determinant in the predisposition to

intestinal inflammation and a number of inflammatory disorders, such as Crohn's disease[5], ulcerative colitis[6,7], celiac disease[8], and a series of infectious diarrheal syndromes[9,10]. The phenomenon of intestinal tight junction (TJ) barrier disruption has previously been reported, but the intracellular mechanism is still poorly understood. Of the essential factors relating to this issue, two are most critical. The first is to identify the early signaling event which triggers the immune response and inflammatory cascade which will help us to understand induction of the disease, and the second is to identify the regulatory system which will lead to the discovery of the therapeutic target. MicroRNAs (miRNAs), which are small noncoding RNAs, were recently discovered to have a promising role in the treatment of immune-related diseases[11,12]. By regulating the degradation of mRNAs at the post-transcriptional level, miRNAs can affect various signaling pathways, and may be good candidates for the treatment of immune-related diseases. More recently, investigations have focused on the role of miRNAs in intestinal-related diseases. These studies may not only provide novel insights into understanding the pathological and physiological process of intestinal barrier dysfunction, especially in inflammatory bowel disease (IBD), but have also suggested the therapeutic role played by miRNAs. The major purpose of this review was to examine current research on the role of miRNAs in the regulation of intestinal barrier function and their therapeutic potential.

miRNA BIOGENESIS AND FUNCTION

MiRNAs, non-coding small endogenous RNAs of 19-22 nucleotides in length, were first identified in *C. elegans* by Lee *et al*[13] in a study on the function of gene *lin-14* as a sequence-specific regulator of gene expression[14]. MiRNAs which are encoded by eukaryotic nuclear DNA can target the 3' untranslated region (3'UTR) of specific mRNAs, usually resulting in gene downregulation *via* translational repression or target degradation[15]. More than 1500 miRNAs have been found to be encoded in the human genome and over 60% of human genes are targeted by miRNAs[16-18]. miRNAs play an important role in many different types of human cells[19].

RNA polymerase Ⅱ (*Pol Ⅱ*) is associated with the miRNA promoter and induces primary miRNAs (pre-miRNAs) to be transcribed in the nucleus[20,21]. These pre-miRNAs have a capped structure and a poly(A) tail[20,22]. After being transported to the cytoplasm, the pre-miRNAs are further processed by the RNase Ⅲ endonuclease, Dicer, in a complex with a trans-activator RNA binding protein into a double-stranded mature miRNA[23-25]. One strand of the mature miRNA is then incorporated into the RNA-induced silencing complex (RISC) and leads this complex to the untranslated region (3'UTR) of specific mRNAs, which causes repression of the corresponding protein[26-29]. In this way, miRNAs are thought to be fine-tune regulators in gene expression and disease control.

A number of biological processes are regulated by miRNAs, such as cell proliferation, apoptosis, differentiation, migration and cell cycle control[30-33]. miRNAs have also been reported to be involved in human diseases, including tumors[34-41], immune dysregulation[42-44], cardiovascular diseases[45-48], metabolic syndrome[49,50] and others[51].

EXPRESSION OF miRNAs IN INTESTINAL EPITHELIAL CELLS

In 2008, Wu *et al*[52] first reported miR-192 which was detected in the epithelial cells of colonic mucosa samples from healthy individuals, but not in patients with active UC using immunohistochemistry and *in situ* hybridization. These authors also found 11 differentially expressed miRNAs in active UC *vs* healthy samples and confirmed an inverse relationship between macrophage inflammatory peptide-2α (*MIP-2α*, previously shown to be involved in IBD[53]) and miR-192. Similarly, Bian *et al*[54] demonstrated that miR-150 was significantly increased in the epithelial cells of colonic mucosa in UC patients compared with controls, and suggested an inverse correlation between miR-150 and its target, *c-Myb*[55], a proto-oncogene involved in apoptosis. Consequently, these two pioneer studies have provided new insight into the pathogenesis of intestinal barrier dysfunction. A summary of the expression of miRNAs in IBD is shown in Figure 1.

ROLE OF miRNAs IN REGULATION OF TIGHT JUNCTION PROTEINS

Occludin and claudins are important transmembrane TJ proteins localized at the TJ strands and function in the TJ barrier[56-58]. Ye *et al*[59] demonstrated that miR-122a plays a central role in the regulation of intestinal TJ permeability by degrading the protein occludin. The regulation of claudins by miRNAs was reported in breast cancer[60,61] and HIV-associated neurological disorders[62], but not in the intestinal TJ barrier. Further studies on the relationship between claudins and miRNAs in the intestinal TJ barrier system are required. Zonula occludens 1 (*ZO-1*) is another major component of the TJ barrier which regulates intestinal permeability[63]. Tang *et al*[64] found miR-212 overexpression in colon biopsy samples from patients with alcoholic liver disease and in Caco-2 cells (a human intestinal epithelial cell line) treated with ethanol. Alcohol can induce miR-212 overexpression and leads to gut leakiness by down-regulating *ZO-1* translation.

miRNAs AND INFLAMMATION

Many groups have demonstrated that miRNAs play pivotal roles in both adaptive and innate immunity[65]. miRNAs regulate the development of various immune cells as well as their immunological functions. miRNAs are also essential in B- and T-cell functions. A deficiency

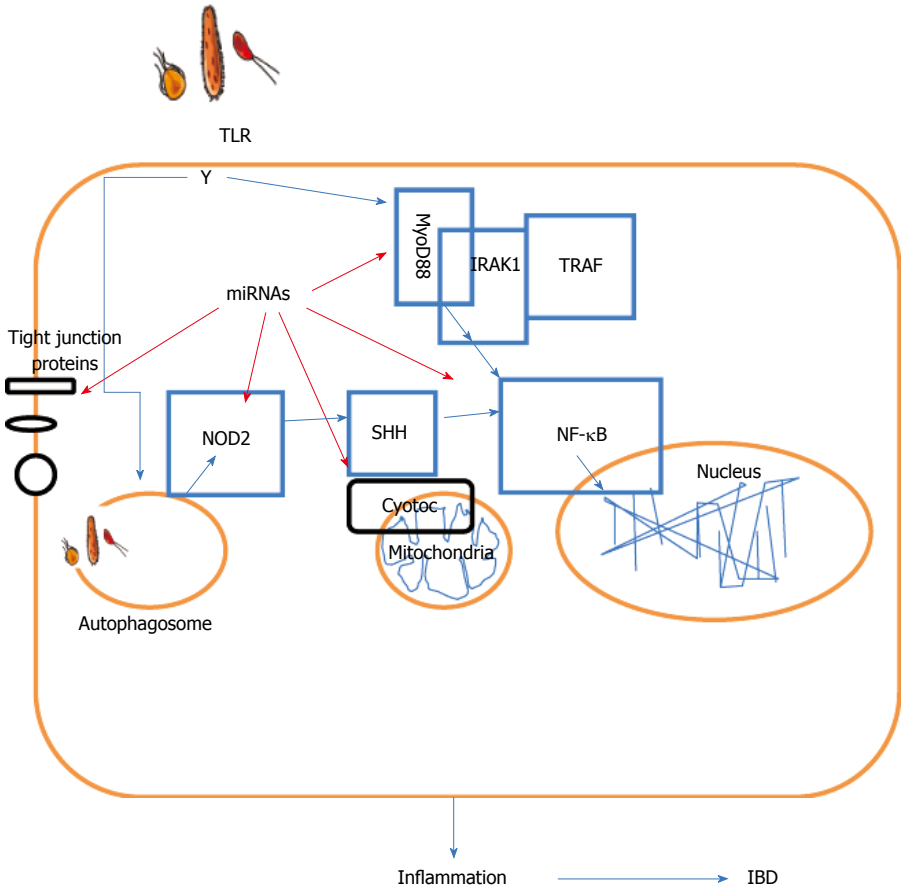

Figure 1 Summary of the role of microRNAs in inflammatory bowel disease. IBD: Inflammatory bowel disease. NF-κB: The nuclear factor NF-κB; TLR: Toll-like receptor; TRAF: TNF receptor-associated factor; NOD2: Nucleotide-binding oligomerization domain-containing protein 2.

in Dicer, the key enzyme in processing miRNAs, results in inhibition of T cell development[65]. In addition, several miRNAs, including miR-155, miR-181a, miR-150 and the miR-17-92 cluster, are also involved in B- and T-cell regulation[66]. Forced overexpression of miR-150 blocks B cell development[67]. Innate immune responses provide the initial defense against pathogens. Pattern recognition receptors, such as Toll-like receptor (TLR), expressed on macrophages and dendritic cells (DCs), are regulated by miRNAs. miRNAs have also been shown to be important in regulation of the TLR signaling cascade[68,69]. MiR-146a expression can be induced by exposure to TLR ligands, such as lipopolysaccharide (LPS), peptidoglycan, and flagellin[70]. miR-146a then functions in a negative feedback mechanism in the TLR signaling cascade by decreasing the expression of TNF-receptor-associated factor-*6* and IL-1 receptor associated kinase-*1*, two target genes of the TLR signaling cascade. Furthermore, loss of miR-155 in DCs impairs their antigen presenting capacity and costimulation activity. The target gene of miR-155 in DCs is *SOCS1* which negatively regulates antigen presenting capacity in DCs. Therefore, deregulation of *SOCS1* in the absence of miR-155 could account for impaired DC function[71]. In macrophages, the downregulation of miR-125b is required to ensure that the correct inflammatory response is produced[72]. The idea of miRNAs as regulators of IBD provides new insight into the development of appropriate therapies.

miRNAs AND AUTOIMMUNE RESPONSE IN IBD

Several studies have reported that miRNAs are involved in autoimmune diseases (AIDs)[42-44]. As miRNAs have been confirmed to play a role in immune cell development and have an impact on cell functions, it is reasonable to deduce that miRNAs are related to AIDs. Reports have shown that miRNAs take part in AIDs, such as rheumatoid arthritis[73], systemic lupus erythematosus[74], multiple sclerosis[75], primary biliary cirrhosis[76], inflammatory bowel disease[52], idiopathic thrombocytopenic purpura[77] and psoriasis[78].

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic gastrointestinal inflammatory disorder whose pathophysiology has been extensively studied over the past several years, but is still poorly understood.

Wu and colleagues[52] studied mucosal tissues from healthy subjects and UC patients and found that miR-192 was predominantly expressed in the intestinal epithelial tissue of healthy subjects and was significantly decreased in UC patients. Furthermore, they also observed that the

inflammatory protein, *MIP-2,* was mainly expressed in UC patients and was decreased in healthy subjects. The expression of *MIP-2α* had an inverse relationship with miR-192. Besides miR-192, they also identified several other differentially expressed miRNAs between UC patients and healthy subjects. MiR-21 was increased in UC patients compared with healthy subjects. miR-375, miR-422b and miR-23a were increased in healthy subjects and all three miRNAs had a similar level of expression in inactive UC[52]. A previous study also showed that *TGF-β* can induce miR-192[79]. This suggests that miR-192 may be the master regulator in the process of inflammation. These findings indicate that miRNAs are involved in the pathogenesis of IBD.

miRNAs AND MITOCHONRIAL STRESS IN IBD

Mitochondria are fundamental subcellular components that play a critical role in the maintenance of normal structure, function and survival of cells. Mitochondrial dysfunction is associated with metabolic diseases including insulin resistance, obesity, diabetes, and the cardiorenal metabolic syndrome[80-83]. Growing evidence suggests that miRNAs provide another layer of regulation with regard to mitochondrial function. MiR-338 can modulate mitochondrial function by targeting cytochrome c oxidase IV (*COX IV*) mRNA[84]. The miRNA-200 family is implicated in epithelial-to-mesenchymal transition which is accompanied by mitochondrial biogenesis and is involved in organ fibrosis and carcinoma progression[85]. Nishi *et al*[86] showed that miR-15b, miR-195 and miR-424 can down-regulate cellular ATP levels and affect mitochondrial integrity. In addition, miR-23a/b in hypertrophy acts in a compensatory mechanism to down-regulate mitochondrial glutaminase[87]. More recently, Yuan *et al*[88] suggested that prohibitin which can inhibit mitochondrial dysfunction may be a potential target in IBD. However, further studies are required to determine whether miRNAs can affect IBD by regulating mitochondrial function.

miRNAs AND AUTOPHAGY IN IBD

Autophagy is a unique cellular process of self-digestion, characterized by the engulfment of cytosolic macromolecules and organelles in a autophagosome, which are then transported to the lysosome for degradation[89,90]. Autophagy helps to recycle and store nutrients for stress conditions[89].

Recently, autophagy-related gene (*ATG*) *16L1* was reported to be involved in CD[91,92]. *ATG 16L1* shares some sequence homology with yeast *Apg16L* and was originally identified in the protein complex *ATG5-ATG12*[93]. In autophagy, *ATG16L* plays an important role in autophagosome formation and functions as an E3-like enzyme to mediate lipidation[94]. Lu *et al*[95] recently provided evidence to show that miR106b and miR93 suppress autophagy-mediated removal of bacteria in epithelial cells by targeting *ATG16L1*. Furthermore, *NOD2*, an intracellular bacterial sensor of the nucleotide-binding and oligomerization domain (NOD)-like receptor family, can sense the presence of muramyl dipeptide, a component of the peptidoglycan cell wall from both Gram-positive and -negative bacteria. *NOD2* activation results in pro-inflammatory and anti-bacterial molecule production dependent on cell signaling pathways mediated by *RICK/RIP2*, *NF-κB* and *MAPKs*. More recently, Ghorpade *et al*[96] found that miR-146a-mediated *NOD2-SHH* signaling regulated gut inflammation in a mouse model of IBD. In addition, Brest *et al*[97] demonstrated that the miR-196 family of miRNAs downregulates the CD protective variant (*c.313C*) of the immunity-related GTPase family M protein 1 gene in CD patients. Consequently, the control of intracellular replication of CD-associated adherent invasive *E. coli* by autophagy was lost due to a decrease in *IRGM1*[97]. By targeting the related gene, miRNAs may eventually contribute to the improvement of IBD.

NOVEL BIOMARKERES AND THERAPEUTIC TARGETS IN IBD

With regard to the involvement of miRNAs in the pathogenesis of IBD, it is vital to identify which miRNAs are consistently dysregulated in IBD and the target genes of the miRNAs. miR-21, the most investigated and well-described miRNA, also known as the "oncomiR", has been shown to have potential clinical application[98]. According to published data[52,53,99,100], miR-21 is the only miRNA usually upregulated in inflamed tissue or serum in IBD patients. The expression of miR-21 is regulated by *NF-κB* which is a master gene in multiple immune diseases (including IBD)[101]. Thus, miR-21 has the potential as a biomarker. Iborra *et al*[102] recently conducted a study to establish the specific expression patterns of miRNAs in the serum and mucosa of IBD patients. They identified six and five differentially expressed miRNAs in the serum and mucosa of patients with active CD compared with those with inactive CD, respectively. Their study again suggested the utility of miRNAs as possible biomarkers. The actual role of miRNAs in IBD still need to be confirmed by functional studies, however, miRNAs have shown promise in the treatment of IBD.

CONCLUSION

Recently, increasing attention has been paid to the gene expression of miRNAs in IBD. Clinical trials have been carried out to test the therapeutic efficacy of miRNA-based therapies. "Miravirsen", a specific inhibitor of miR-122, is now being evaluated in a phase Ⅱ clinical trial[103]. Furthermore, a recent review[104] suggested the use of an miRNA inhibitor or synthetic miRNA targeting the *PI3K* and *Ras/MAPK* pathways in multiple my-

eloma (MM) treatment. MiR-29b is a promising target in MM treatment by multiple mechanisms, including the regulation of osteoclastic differentiation[105] and epigenetic regulation of the cell cycle[106,107]. Future studies will provide a basis for more clinical trials and shed light on miRNA-based therapies in IBD.

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ORIGINAL ARTICLE

Identification of biomarkers for hepatocellular carcinoma by semiquantitative immunocytochemistry

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Abstract

AIM: To investigate the expression of key biomarkers in hepatoma cell lines, tumor cells from patients' blood samples, and tumor tissues.

METHODS: We performed the biomarker tests in two steps. First, cells plated on coverslips were used to assess biomarkers, and fluorescence intensities were calculated using the NIH Image J software. The measured values were analyzed using the SPSS 19.0 software to make comparisons among eight cell lines. Second, eighty-four individual samples were used to assess the biomarkers' expression. Negative enrichment of the blood samples was performed, and karyocytes were isolated and dropped onto pretreated glass slides for further analysis by immunofluorescence staining. Fluorescence intensities were compared among hepatocellular carcinoma (HCC) patients, chronic HBV-infected patients, and healthy controls following methods similar to those used for cell lines. The relationships between the expression of biomarkers and clinical pathological parameters were analyzed by Spearman rank correlation tests. In addition, we studied the distinct biomarkers' expression with three-dimensional laser confocal microscopy reconstructions, and Kaplan-Meier survival analysis was performed to understand the clinical significance of these biomarkers.

RESULTS: Microscopic examination and fluorescence intensity calculations indicated that cytokeratin 8/18/19 (CK) expression was significantly higher in six of the seven HCC cell lines examined than in the control cells, and the expression levels of asialoglycoprotein receptor (ASGPR) and glypican-3 (GPC3) were higher in all seven HCC cell lines than in the control. Cells obtained from HCC patients' blood samples also displayed sig-

nificantly higher expression levels of ASGPR, GPC3, and CK than cells from chronic HBV-infected patients or healthy controls; these proteins may be valuable surface biomarkers for identifying HCC circulating tumor cells isolated and enriched from the blood samples. The stem cell-like and epithelial-mesenchymal transition-related biomarkers could be detected on the karyocyte slides. ASGPR and GPC3 were expressed at high levels, and thus three-dimensional reconstructions were used to observe their expression in detail. This analysis indicated that GPC3 was localized in the cytoplasm and membrane, but that ASGPR had a polar localization. Survival analyses showed that expression of GPC3 and ASGPR is associated with a patient's overall survival (OS).

CONCLUSION: ASGPR, GPC3, and CK may be valuable HCC biomarkers for CTC detection; the expression of ASGPR and GPC3 might be helpful for understanding patients' OS.

Key words: Hepatocellular carcinoma; Biomarker; Immunocytochemistry; Semiquantitative analysis; Three-dimensional reconstruction

Core tip: We report a novel workflow to detect potentially valuable biomarkers for hepatocellular carcinoma (HCC). We measured immunofluorescence intensity and performed statistical analyses to assess the expression of biomarkers in cell lines and patient blood samples. Furthermore, we determined the expression of biomarkers via three-dimensional reconstructions. These analyses indicated that asialoglycoprotein receptor (ASGPR), glypican-3 (GPC3), and cytokeratin (CK) may be valuable HCC biomarkers for detecting circulating tumor cells (CTCs). In addition, the expression of ASGPR and GPC3 might correlate with patients' prognoses, and our CTC detection method can include epithelial cell adhesion molecule- and vimentin-positive tumor cells, and will thus supplement previous studies and potentially help predict future tumor recurrence and metastasis.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world, ranking seventh and third in morbidity and mortality, respectively, for all cancers[1,2]. Although most of the burden lies in developing countries, where almost 85% of HCC cases occur, large increases in incidence, particularly in younger age groups, have been reported in the United States and Europe over the past two decades[2]. Currently, approximately 50% of the annual incidence occurs in China, making HCC the second leading cause of cancer-related deaths. This trend is likely to persist in the near future, given that 93 million hepatitis B virus (HBV) carriers and 980000 HBV patients live in China[3,4]. At present, hepatectomy is the main curative treatment, but postoperative risks can only be predicted by common clinical and pathological parameters, such as alpha fetoprotein (AFP) and tumor differentiation; prognosis remains poor due to the high incidence of recurrence and metastasis[5,6]. Considering that there are no reliable diagnostic and prognostic biomarkers for HCC patients, a variety of studies have been performed to identify new biomarkers, mainly using tumor sections or patients' serum for the analyses[7-10].

In recent years, the number of circulating tumor cells (CTCs) has been reported to be associated with a poor prognosis[11-13]. The detection of CTCs may provide a method for assessing disease prognosis and a model for the biology of cancer metastasis. The CellSearch™ system (Veridex LLC, Warren, NJ, United States), based on the positive capture of epithelial cell adhesion molecule (EpCAM), has been approved by the United States Food and Drug Administration (FDA) for the detection of CTCs in patients with metastatic breast, prostate, and colorectal cancers[14]. In 2012, this system was approved by the China Food and Drug Administration for assessing patients with breast cancer[15]; however, few studies have examined HCC CTCs. We argued that the expression of EpCAM in HCC tissues was as low as 30%, and could be considered a cancer stem cell-like biomarker for HCC. The epithelial-mesenchymal transition (EMT), considered an initiation process for cancer metastasis, involves the loss of epithelial biomarkers such as EpCAM, which means that the CellSearch™ system may overlook HCC CTCs[16,17]. Although several studies have investigated a suitable method for identifying HCC CTCs[18,19], further efforts are required. Here, we analyzed the expression of potential cellular biomarkers for the identification of HCC CTCs in seven cell lines and in karyocytes isolated from HCC patients' peripheral blood. We included HCC-related biomarkers, such as cytokeratin 8/18/19 (CK), glypican-3 (GPC3), and asialoglycoprotein receptor (ASGPR); stem cell-related biomarkers, such as EpCAM and CD133; and EMT-related biomarkers, such as vimentin. Cytokeratins are proteins in the intracytoplasmic cytoskeleton, and CK expression has been used as a biomarker in hepatoma histopathology[20,21]. GPC3 attached to the cell surface is overexpressed in most HCC foci and undetectable in normal livers and benign liver diseases[7]. ASGPR, as a membrane receptor, can specifically interact with the pre-S1 domain of HBV[22]. EpCAM was reported as a

Table 1 Clinical characteristics of individuals enrolled in the study *n* (%)

Clinical characteristics	HCC number	Control number
Age at baseline, years		
⩾ 60	20 (32.3)	6 (27.3)
< 60	42 (67.7)	16 (72.7)
Median	55	51
Range	29-76	20-72
Sex		
Male	53 (85.5)	11 (50.0)
Female	9 (14.5)	11 (50.0)
KPS score		
> 80	31 (50.0)	22 (100.0)
60-80	31 (50.0)	0 (0.0)
Hepatitis infection		
HBV positive	59 (95.2)	7 (31.2)
HBV negative	3 (4.8)	15 (68.2)
Tumor size (cm)		NA
⩽ 3.0	21 (33.9)	
> 3.0	41 (66.1)	
Primary foci		NA
Single	54 (87.1)	
Multiple	8 (12.9)	
Carcinoma cell embolus		NA
Yes	1 (1.6)	
No	61 (98.4)	
AJCC stage at enrollment		NA
Early (stage 1 + 2)	55 (88.7)	
Late (stage 3 + 4)	7 (11.3)	

HCC: Hepatocellular carcinoma; KPS: Karnofsky performance status; HBV: Hepatitis B virus; AJCC: American Joint Committee on Cancer; NA: Not applicable.

HCC stem cell-like biomarker, and CD133 is a common stem cell biomarker. EpCAM- and CD133-positive cells can potentially undergo self-renewal and differentiation[23,24]. Vimentin is a predominant mesenchymal marker in the EMT[25]. Given that normal epithelial cells and hepatocytes in the circulation of healthy adults are rare, any of these cells identified by CK, GPC3, or ASGPR are likely to be tumor cells[7,19,26]. Stem cell-related and EMT marker-positive tumor cells may be associated with recurrence and metastasis, which corresponds with the clinical significance of CTCs in several cancers[27-30].

The primary aim of this study was to assess biomarker expression in hepatoma cell lines and enriched patient blood cells using a semiquantitative cytopathological workflow. Secondly, the present study analyzes if exceptional biomarker expression in HCC patients could be valuable for determining cancer prognosis.

MATERIALS AND METHODS

Cell culture and preparation of cells plated on coverslips

Human hepatoma cell lines HepG2 (ATCC HB-8065), Hep3B (ATCC HB-8064), and SK-HEP-I (ATCC HTB-52) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States), and Bel7402, Bel7404, SMMC7721, and the human hepatic cell line L02 cells were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China. Huh7 cells were obtained from the Human Science Research Resources Bank (Osaka, Japan). HepG2, Hep3B, and SK-Hep-I cells were cultured in MEM (Hyclone, Logan, UT, United States) supplemented with 10% FBS (Hyclone, Logan, UT, United States), 1% 200 mmol glutamine, and 1% 100 mmol pyruvic acid sodium. Huh7 cells were cultured in Dulbecco's modified Eagle medium (Hyclone, Logan, UT, United States) supplemented with 10% FBS. Bel7402, Bel7404, and SMMC7721 cells were cultured in RPMI-1640 (Hyclone, Logan, UT, United States) supplemented with 10% FBS. Cells were maintained at 37 ℃ in a humidified atmosphere containing 5% CO_2, and were harvested with trypsin before plating for experiments in tissue culture plates and on coverslips. The cells were seeded onto coverslips pretreated with 0.01% poly-L-lysine and fixed with 4% paraformaldehyde when the confluence reached approximately 60%-70%.

Blood sample preparation

Eighty-four individuals, including 62 HCC patients, 7 chronic HBV-infected patients, and 15 healthy individuals, were recruited in the present study. A total of 7.5 mL of peripheral blood was collected in BD vacutainer tubes containing acid citrate dextrose (Becton Dickinson, Franklin Lakes, NJ, United States). Written informed consent was obtained from each participant, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the Review Board at the Cancer Hospital affiliated with the Chinese Academy of Medical Sciences, Peking Union Medial College, and Navy General Hospital (Beijing, China). To avoid epithelial cell contamination during venous puncture, all samples were collected after discarding the first 2 mL of blood. Samples were processed within 24 h of collection. Diagnoses were pathologically confirmed using surgical specimens. The clinical characteristics of HCC patients are summarized in Table 1. HCC patients were classified according to the seventh edition of the cancer staging system published by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC).

The karyocyte enrichment method was similar to previous descriptions[31]. Blood was transferred to a 50 mL centrifuge tube. The collecting tubes were rinsed twice with wash buffer (137 mmol/L NaCl, 2.7 mmol KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, 2 mmol/L EDTA, 0.5% BSA, pH = 7.4) to a combined volume of 45 mL. Blood samples were centrifuged at 1400 rpm for 5 min, and the supernatant was aspirated. Red blood cells (RBCs) were mixed with 37.5 mL of lysis buffer (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L EDTA), rotated for 8 min, and centrifuged at 1400 rpm for 5 min. The procedure was repeated twice. The resulting cell pellet was resuspended, washed, and incubated with CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) at a proportion of 20 μL per 10^7 total white blood cells (WBCs) for 15 min. WBCs bound to microbeads were removed with an LS column in a Midi-

MACS™ separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Supernatants were transferred into a new tube and centrifuged at 1400 rpm for 5 min. Cell pellets were fixed with 4% paraformaldehyde on SuperFrost Plus slides (Thermo Fisher Scientific, Pittsburgh, PA, United States), with immunofluorescence (IF) staining then being performed.

Immunofluorescence staining and microscopic examination

Cells on coverslips and karyocyte slides enriched from blood samples were blocked using 2% BSA (Sigma-Aldrich, St. Louis, MO, United States) for 45 min. Direct and indirect IF staining was performed at room temperature with the following HCC-related biomarkers (1:100 diluted in 2% BSA): anti-CK8/18/19-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-GPC3 (Santa Cruz Biotechnology Inc, Dallas, TX, United States), anti-ASGPR (Sigma-Aldrich, St. Louis, MO, United States), anti-AFP (Zymed Laboratory Inc., South San Francisco, CA, United States), and anti-CD45-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). EMT-related and stem cell-related biomarkers (1:100 diluted in 2% BSA) were also tested, including anti-vimentin (Thermo Fisher Scientific, Pittsburgh, PA, United States), anti-EpCAM-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany), and anti-CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). Coverslips and slides were incubated for 60 min with primary antibodies for IF staining and with Alexa Fluor-labeled secondary antibody (Life Technologies, Grand Island, NY, United States) for another 60 min for indirect IF staining. They were then washed three times with 0.2% BSA for 3 min. Cells were mounted with medium containing the nuclear dye 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, United States). Blinded review of the fluorescent images was performed by three technicians using a Nikon 80i 3-color fluorescent microscope (Nikon Co, Tokyo, Japan) and a Leica TCS SP2 confocal microscope (Leica Co, Berlin, Germany). Fluorescence intensities were measured using the Image J software (NIH, Bethesda, MD, United States). Relative biomarker intensities on cells were assessed using the following formula that calculates how many times higher the biomarker staining intensity of the cell is than the background, which was based on the CellSearch™ model for HER2 staining of CTCs[32,33]:

Biomarker Relative Intensity = [Foreground Intensity (Cell staining)/Surface Area]/[Background Intensity/Surface Area]

The experiments were performed in triplicate. Representative images of 5 cells from each cell line and 3 cells from each patient were analyzed, and the mean intensity was calculated. Only cells from patients who met the following stringent criteria were analyzed: intact cells with diameters above 10 μm, biomarker-positive, CD45-negative, and DAPI-positive. The positive ratios of biomarker expression were calculated from the observation of 500 cells.

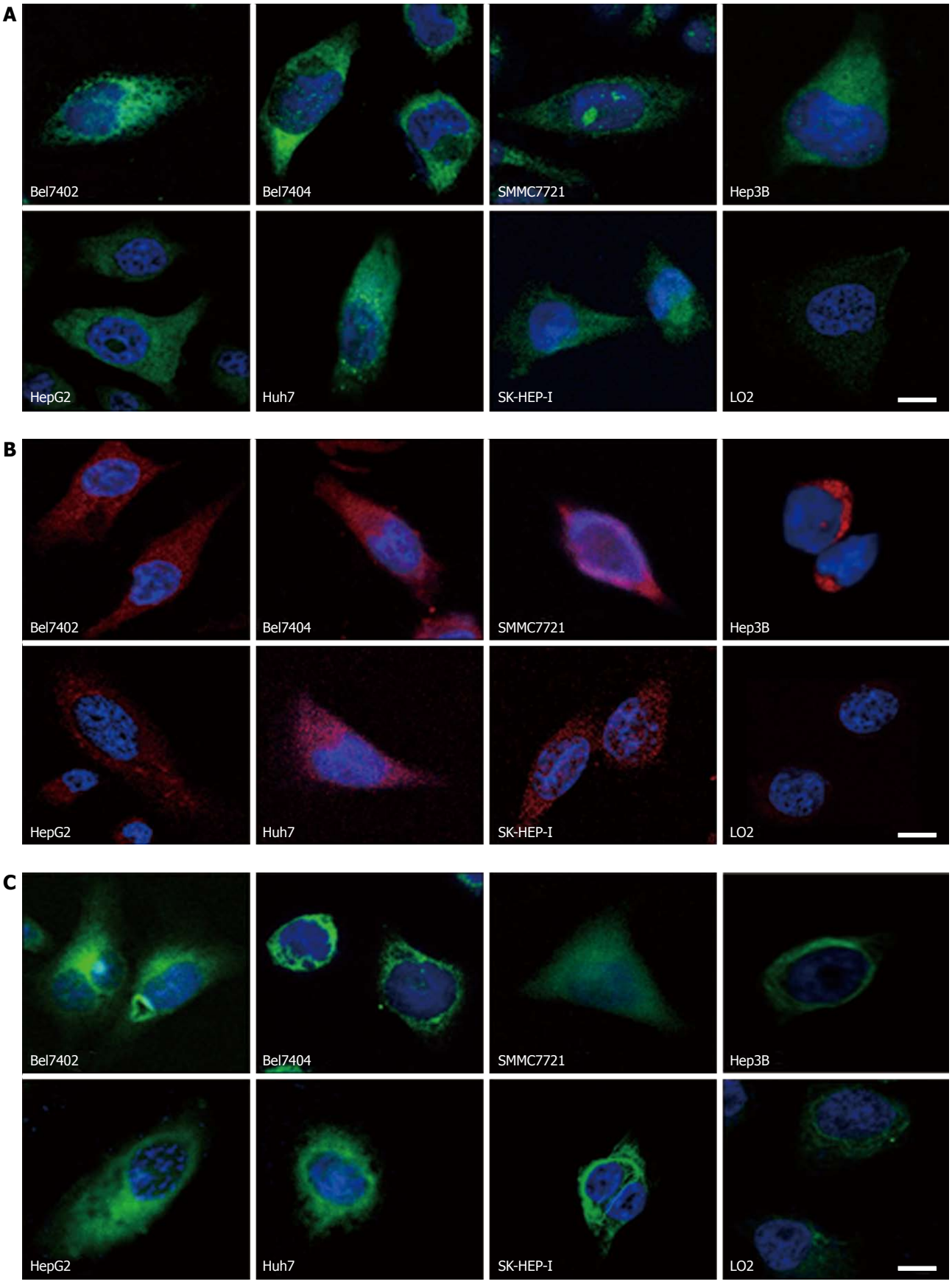
Immunohistochemical staining of liver cancer samples

Tissue arrays were composed of tumor samples and para-carcinoma liver tissues collected from 32 patients who were diagnosed with HCC and underwent surgical resection (Shanghai National Engineering Research Center for Biochip, Shanghai, China). From each paraffin block, 4-μm-thick sections were cut, cleared in xylene, and rehydrated. Slides were then moved from water to plastic slide holders, fully immersed in 10 mmol sodium citrate buffer (pH = 6.0), and heated for 20 min at 120 ℃ in a commercially available pressure cooker. After cooling to room temperature in the sodium citrate buffer, slides were treated with a solution of 3% hydrogen peroxide (H_2O_2) for 30 min at room temperature to abolish endogenous peroxidase activity. Sections were then incubated for 10 min in a moist chamber with non-immune goat serum diluted to 5% in PBS (pH = 7.4) to reduce non-specific background staining. Sections were then incubated overnight at 4 ℃ with the primary antibody (anti-GPC3 or anti-ASGPR) diluted to 1:100. Sections were subsequently incubated for 30 min with biotin-labeled secondary antibody (Santa Cruz Biotechnology Inc, Dallas, TX, United States) diluted to 1:600 in PBS, and then incubated in a streptavidin-biotin-peroxidase preformed complex (Zhongshan Jinqiao Biotechnology Co, Beijing, China) for 30 min. The immunologic reaction was visualized using 3,3'-diaminobenzidine substrate, and samples were counterstained with hematoxylin, dehydrated, and mounted with Canada balsam (Sigma-Aldrich, St. Louis, MO, United States). A negative control was performed by omitting the primary antibody.

The tumor expression of ASGPR and GPC3 was evaluated by two pathologists (HM and HZL) blinded to clinical data, and discrepancies were resolved by consensus. Images were visualized using an Olympus BX40 microscope (Olympus Co, Tokyo, Japan). Ten random fields per biomarker were selected, and the percentage of immunoreactive cells in a total of 1000 tumor cells was determined[34]. As described previously, the intensity of a biomarker and the percentage of positive cells were both graded with different scores. The product of the two scores was used to evaluate the biomarker's staining[35,36]. An intensity score representing the average intensity of positive cells (0, none; 1, weak; 2, intermediate; 3, strong) was assigned. A proportion score representing the estimated proportion of positive-staining cells (0, 0-5% positive cells; 1, 6%-25%; 2, 26%-50%; 3, 51%-75%; 4, 76%-100%) was assigned. The proportion and intensity scores were multiplied to create an immunoreactivity score (IS). The IS was further divided as follows: 0-1 (−); 2-4 (+); 5-7 (++); > 8 (+++). "-" and "+" were considered low levels of immunoreactivity; "++" and "+++" were considered high levels of immunoreactivity.

Statistical analysis

Analyses were performed using SPSS version 19.0 (IBM, New York, NY, United States). The Mann-Whitney test was used to compare continuous variables between the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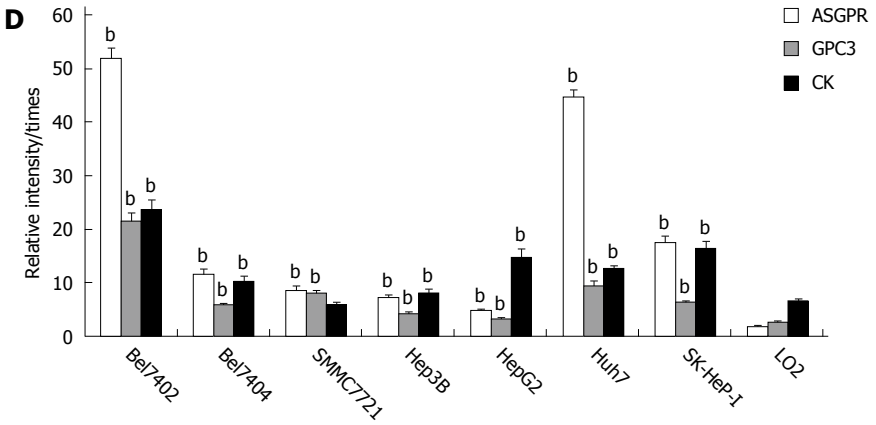

Figure 1 Asialoglycoprotein receptor, glypican-3 and cytokeratin expression, and fluorescence intensities in eight cell lines. A-C: Representative images of cells on the coverslips with DAPI-stained nuclei (blue) and asialoglycoprotein receptor (ASGPR) staining (green)/glypican-3 (GPC3) staining (red)/cytokeratin (CK) staining (green). The scale bar is 5 μm. D: The bar graph of fluorescence intensities in eight cell lines. Fluorescence intensities were measured using NIH Image J software. Biomarker relative intensities were calculated as the difference between the biomarker staining intensity of the cell and the background intensity. The comparisons between HCC cell lines and the control cell line were analyzed using the Mann-Whitney test ($^bP < 0.01$ *vs* control).

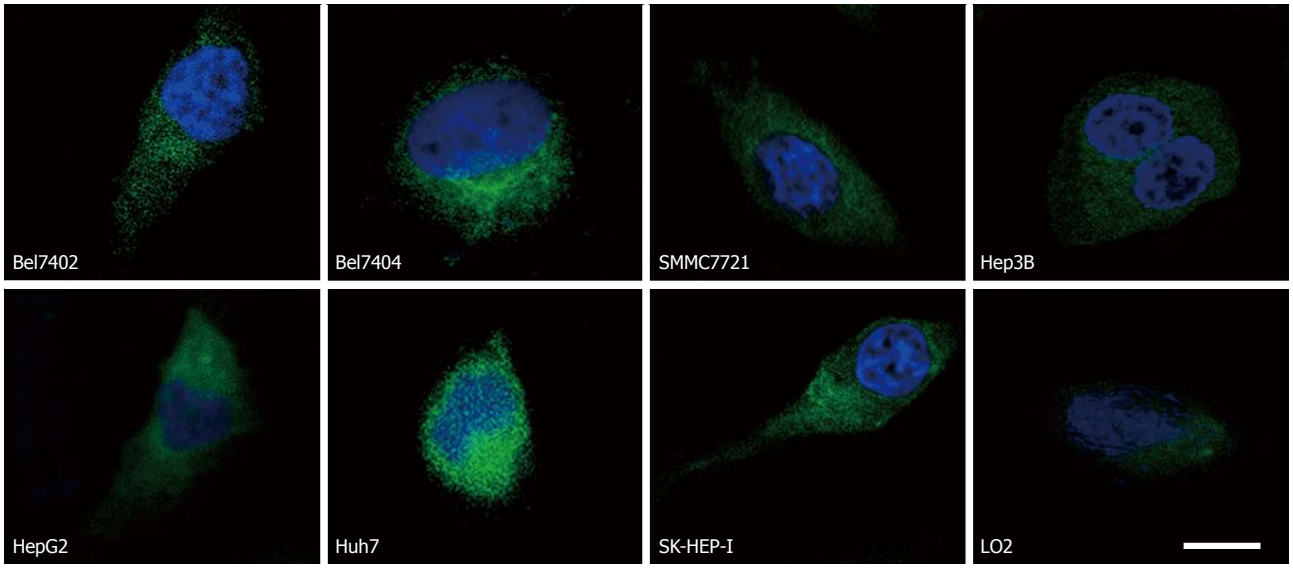

Figure 2 Epithelial cell adhesion molecule expression in eight cell lines. Representative images of cells on the coverslips with DAPI-stained nuclei (blue) and EpCAM staining (green). The scale bar is 5 μm.

two groups. Fluorescence intensities and clinical characteristics, such as age, AFP, ALT, AST, tumor size, differentiation, and AJCC stage, were subjected to Spearman rank correlation analysis. Survival analysis was performed using the Kaplan-Meier method. *P* values less than 0.05 were considered statistically significant, and *P* values less than 0.1 were considered potentially significant.

RESULTS

Expression of HCC-related biomarkers in cell lines

IF staining indicated that ASGPR (Figure 1A) and GPC3 (Figure 1B) were expressed in all liver cancer-related cell lines, and fluorescence intensity was significantly higher in the seven HCC cell lines than in L02, the control cells (Figure 1D). Confocal microscopic examination suggested that ASGPR is located in the nuclei or nucleoli of the Bel7404 and SMMC7721 cells. In addition, GPC3 expression was significantly stronger in cell lines derived from Asians than from Westerners (10.41 ± 6.67 *vs* 5.61 ± 2.89, $P < 0.05$). CK was expressed in all cell lines and located in the cytoplasm (Figure 1C). When the fluorescence intensities were compared, CK expression in six of the seven HCC cell lines was significantly higher than in the control cell line, L02 (Figure 1D). AFP was only expressed in Hep3B, HepG2, and SK-HEP-I cell lines.

Expression of EMT- and stem cell-related biomarkers in cell lines

IF staining indicated that there were considerably fewer cells expressing EpCAM than there were cells expressing the above three biomarkers (Figure 2). The cells expressing CD133 were even rarer than those expressing EpCAM. Table 2 presents the positive ratios in the cell lines

Table 2 Positive ratios of epithelial cell adhesion molecule/CD133 expression in hepatocellular carcinoma cells

%	Bel7402	Bel7404	SMMC7721	Huh7	HepG2	Hep3B	SK-Hep-I	L02
EpCAM	0.4	1.2	0.2	1	0.6	0.8	2	0
CD133	0.4	0.4	0.2	0.2	0.2	0.4	0.6	0

EpCAM: Epithelial cell adhesion molecule.

tested. Vimentin was expressed at very low levels, and ratios in the cell lines determined by IF staining.

Expression of biomarkers in blood samples

Based on the above results, three HCC-related biomarkers were further detected in patient samples. IF staining indicated that GPC3, ASGPR, and CK were expressed in most HCC patients (Figure 3), independent of HBV infection. Positive staining was rarely observed on the karyocyte slides from chronic HBV-infected patients or healthy controls.

We compared the fluorescence intensities of three biomarkers between HCC patients, chronic HBV-infected patients, and healthy controls. The expression of the three biomarkers was significantly increased in HCC patients compared to chronic HBV-infected patients and healthy controls. The fluorescence intensities of GPC3 and ASGPR were considerably higher than that of CK. The concordance rates between a biomarker's expression and pathological diagnosis were 90% for GPC3, 93.3% for ASGPR, and 63.3% for CK, whereas the concordance rate between AFP serum detection and pathological diagnosis was 46.7%. The positive and negative predictive values of these three biomarkers were 90% and 71.4% for GPC3, 93.1% and 75% for ASGPR, and 82.6% and 28.6% for CK, respectively.

To understand the clinical significance of the biomarkers' expression levels, we used Spearman rank correlation analysis to assess the relationship between fluorescence intensities and clinical pathological parameters. No significant correlations were found.

Vimentin was positively expressed on some of the karyocyte slides and was accompanied by the expression of EpCAM (Figure 4). Because EpCAM is associated with stem cell-like properties in HCC patients, it was unnecessary to compare its fluorescence intensities in the manner performed for the HCC-related biomarkers discussed previously. EpCAM was expressed in 34.4% and vimentin was expressed in 24.1% of the cells on the karyocyte slides.

Three-dimensional reconstruction for GPC3 and ASGPR

Three-dimensional reconstructions were generated to evaluate ASGPR and GPC3 localization. The cells were revolved around the X-, Y-, and Z-axes, which indicated that GPC3 was localized in the cytoplasm and on the membrane (mov 1), whereas ASGPR had a polar localization (mov 2).

Survival analysis for GPC3 and ASGPR

The expression of GPC3 and ASGPR is shown in Figure 5. Kaplan-Meier survival analyses indicated that ASGPR expression was significantly associated with the overall survival (OS) (P = 0.017), and GPC3 expression exhibited a trend toward significant association with OS (P = 0.068). The mean OS of patients with high and low levels of ASGPR was 24.1 and 48.5 mo, respectively. The mean OS of patients with high and low levels of GPC3 was 31.0 and 48.9 mo, respectively.

DISCUSSION

HCC ranks as the third most frequent cause of cancer-related death worldwide[2]. In China, it is the third most prevalent cancer, the second leading cause of cancer-related death, and new cases in the country account for 55% of those reported globally[4]. Currently serum AFP, a secretory protein, is widely used for detecting HCC patients and monitoring disease progression, but it has a sensitivity ranging from 39% to 97% and a specificity ranging from 76% to 95%, even when used to screen high-risk populations[37-39]. In a previous study, AFP was used to identify CTCs in patients with liver cancer[18]. However, given the shortcomings of AFP, there may be biomarkers that can detect CTCs with a higher sensitivity.

In this study, and based on our previous tests, we assessed the expression of potential key biomarkers in seven HCC cell lines and karyocytes isolated from patient blood samples. Cytokeratins are proteins that consist of keratin-containing intermediate filaments in the intracytoplasmic cytoskeleton, and their expression primarily depends on the type of epithelia, the moment of terminal differentiation, and the stage of development[40]. In many cases, cytokeratin expression in tumors and peripheral blood has prognostic significance for cancer patients, and the levels of CK8/18/19 expression in HCC have been used as biomarkers in histopathology[20,21]. In addition to conventional histopathology, CK8/18/19 have been used as diagnostic biomarkers in CTCs for breast, prostate, and colorectal cancers[11-13]. Expression in HCC cells obtained from peripheral blood was explored in this study in order to broaden knowledge regarding cytopathology. Positive results from cell lines and blood samples provide evidence for potential applications of biomarkers, although additional investigation may be needed in the future.

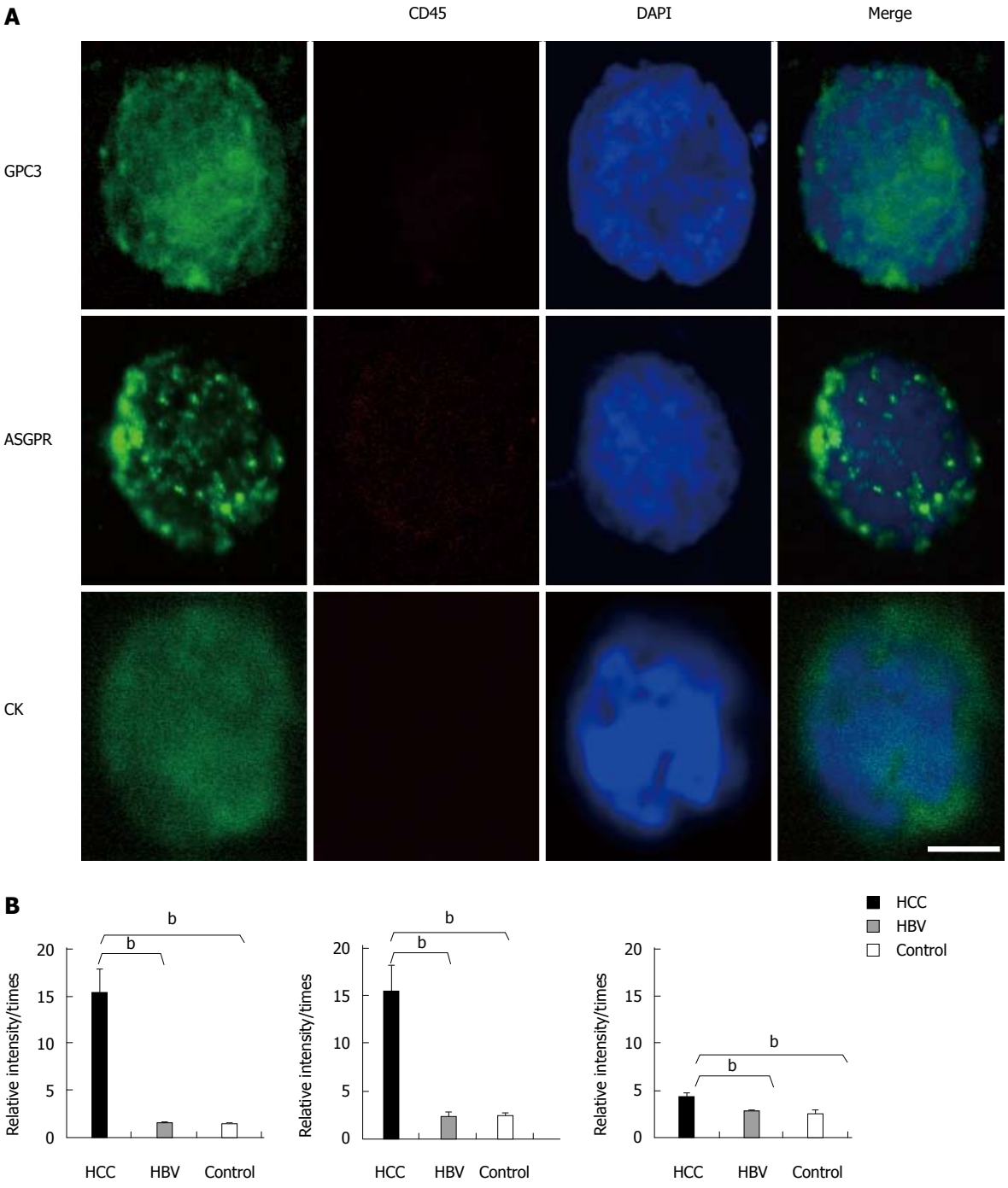

Figure 3 Representative images and fluorescence intensities of cells obtained from patients. A: Representative image of circulating tumor cells (CTCs) with DAPI-stained nuclei (blue), positive staining of ASGPR/GPC3/CK (green), and negative staining for CD45. The scale bar is 5 μm. A total of 7.5 mL of peripheral blood was collected from each individual, mixed with red blood cell lysis buffer, and incubated with CD45 microbeads to deplete the white blood cells. The remaining cell pellets were fixed on SuperFrost Plus slides for immunofluorescence staining; B: The bar graph of ASGPR/GPC3/CK (from left to right) fluorescence intensities in 30 HCC patients, 7 chronic HBV-infected patients, and 15 healthy controls. Fluorescence intensities were measured using NIH Image J software. Biomarker relative intensities were calculated as the difference between the biomarker staining intensity of the cell and the background intensity. The comparisons between HCC patients and healthy controls were analyzed by the Mann-Whitney test ($^bP < 0.01$ *vs* control).

ASGPR is located on liver cells and was previously known as a hepatic galactose/N-acetylglucosamine (GlcNAc) receptor. Its primary physiological function is to bind, internalize, and subsequently clear glycoproteins containing terminal galactose or GlcNAc residues from circulation[41-43]. Several investigations have supported ASGPR as a multifunctional membrane receptor involved in the removal of apoptotic cells[44], a carrier of low-density lipoprotein (LDL)[45], and an entry point for hepatotropic viruses[46,47]. HBV DNA and virions can reportedly be transferred into hepatocytes via ASGPR[48]. Zhang *et al*[22] found that ASGPR on human hepatocytes

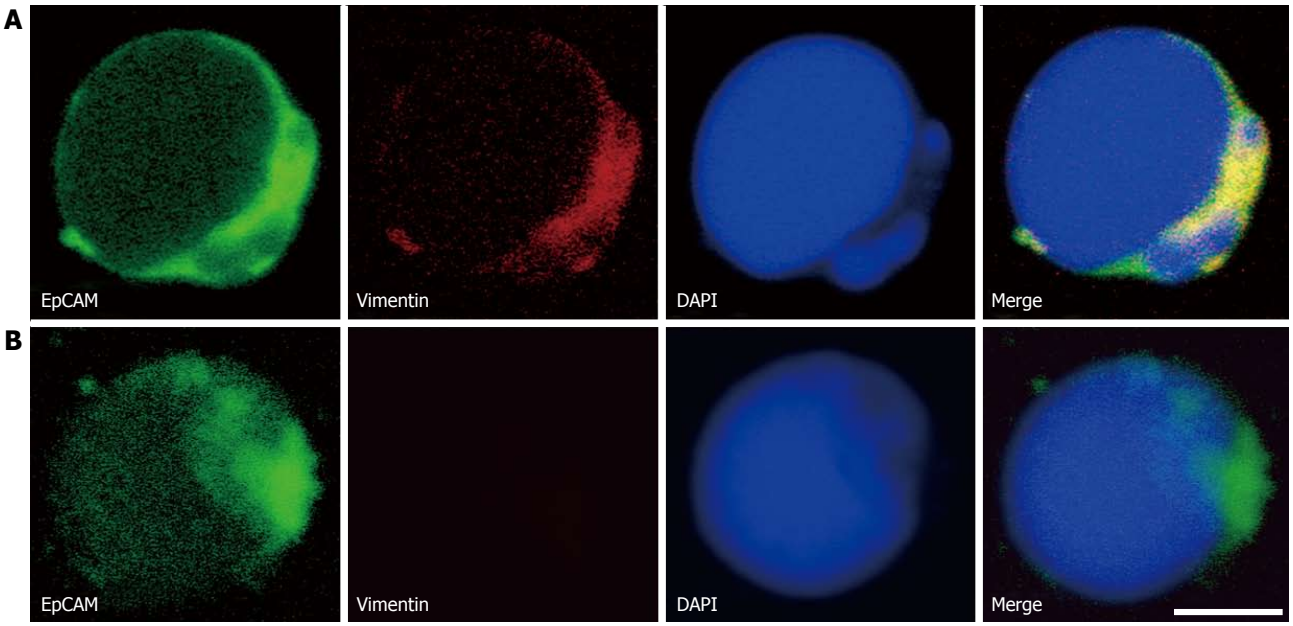

Figure 4 Representative images of epithelial-mesenchymal transition marker-related cells obtained from hepatocellular carcinoma patients. A: CTCs with dye 4',6-diamidino-2-phenylindole, (DAPI) -stained nuclei (blue), positive staining of epithelial cell adhesion molecule (EpCAM) (green), and positive staining of vimentin (red). B: CTCs with DAPI-stained nuclei (blue), positive staining of EpCAM (green), and negative staining of vimentin. The scale bar is 5 μm.

can interact specifically and directly with the pre-S1 domain of HBV in vitro and *in vivo*[22]. The IF images of liver cancer cell lines and patient CTCs in this study indicated strong ASGPR expression, which suggested its potential use for identifying HCC cells in circulation, especially for HBV-related HCC.

GPC3 is a proteoglycan attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor[7,49]. GPC3 mRNA levels have been reported to be significantly elevated in most HCC foci compared with normal liver and non-malignant liver lesions, and they are elevated more frequently than AFP in HCC[50]. Similar results have been obtained at the protein level. Using immunohistochemical techniques, Capurro *et al*[7] found that GPC3 was specifically overexpressed in most HCC foci, but was undetectable in hepatocytes from normal liver and benign liver diseases. Staining was localized to the cell membrane and/or the cytoplasm. Zhang *et al*[8] found that 87.1% (128/147) of surgically excised HCC samples were GPC3 positive, and did not observed GPC3 expression in para-carcinoma or cirrhotic tissues. The detection of HCC cell lines and karyocytes in patients' blood in our studies was consistent with the above results for both mRNA and protein expression, displaying positive results compared to the controls. Additionally, tumor cells can be identified by GPC3 in patients' blood, in which AFP tests could be positive or negative. This result was aligned with a previous report that tested for GPC3 in HCC patients' serum[51]. The GPC3 biomarker test results corresponded better to the pathological diagnosis in our study than the serum AFP test.

The results from Spearman rank correlation analysis (Table 3) indicated that there was no significant correlation between fluorescence intensities and clinical pathological parameters, which suggested that fluorescence intensity may be unaffected by clinical pathological parameters. From our analyses of the blood samples, the fluorescence intensities of the above three biomarkers were significantly increased in HCC patients compared to chronic HBV-infected patients and healthy controls, although the elevation of CK expression was not as pronounced as the other two biomarkers. This finding indicates that CK expression may be perturbed in chronic liver disease, whereas GPC3 and ASGPR could provide a better distinction between HCC and chronic liver disease. AFP was only expressed in the cell lines derived from Westerners in our study. AFP was reported to be expressed in 52.3% of HCC patients (23/44) for the identification of CTCs, whereas the three biomarkers that we considered were more commonly detected than positive changes in AFP in the serum[18]. In this study, survival analysis indicated that patients with high ASGPR expression levels had poorer OS, and a similar trend was observed for GPC3 expression. These results suggest that GPC3 and ASGPR may correlate with patient prognosis.

EMT is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells[17]. This transition is considered one of the mechanisms underlying cancer metastasis and recurrence. EMT and MET form the initiation and completion of the invasion-metastasis cascade[27], and CTCs in the vessels act as "intermediates" between primary tumors and metastatic foci. We measured the expression of an EMT-related biomarker on the patients' karyocyte slides to understand the biology of CTCs. Vimentin, a widely used biomarker

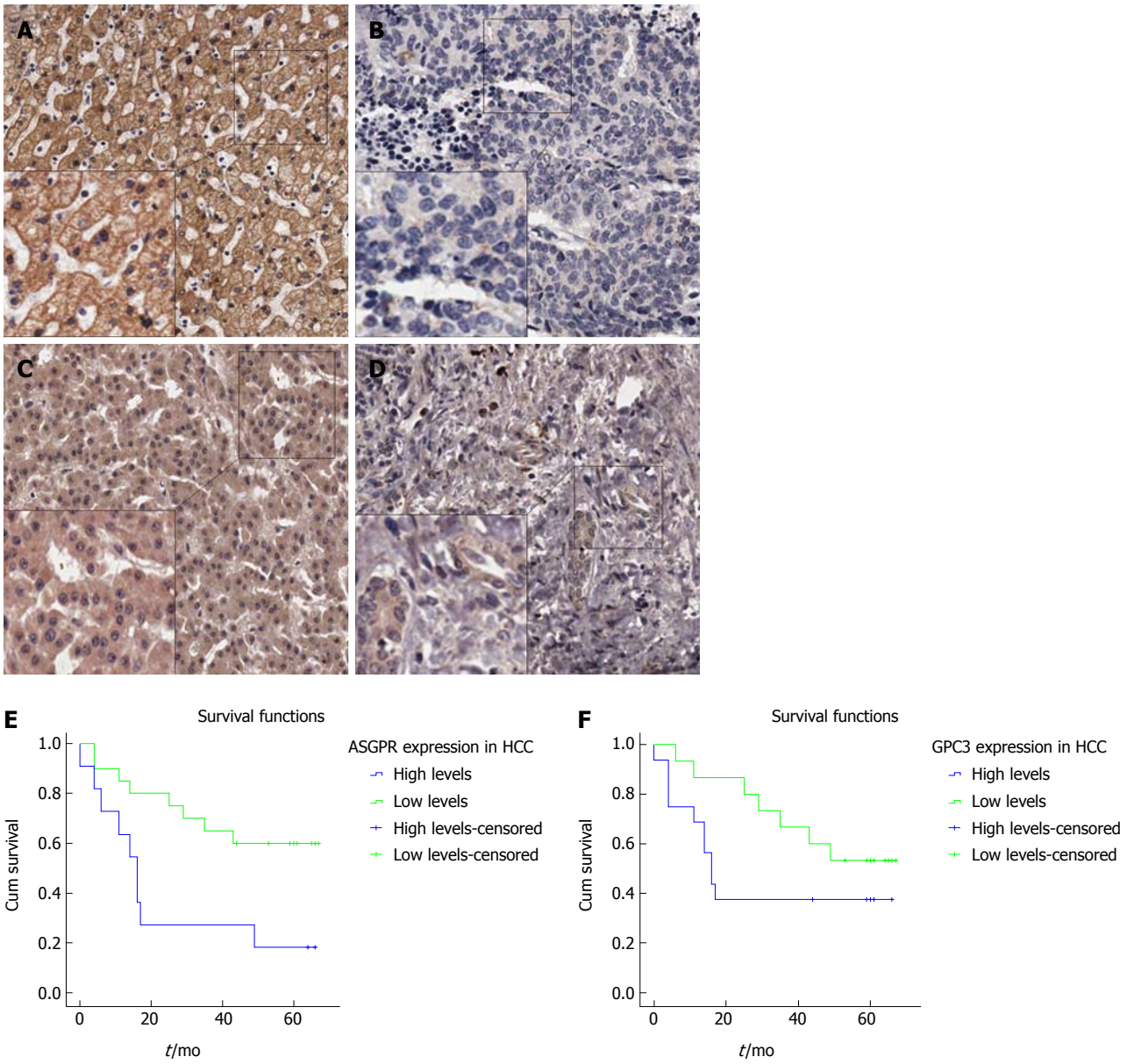

Figure 5 Representative images of asialoglycoprotein receptor / glypican-3 expression on tissue arrays, and Kaplan-Meier survival curves from follow-up studies. A-B: The expression of ASGPR in HCC (A) and paracarcinomatous (B) tissues. C-D: The expression of GPC3 in HCC (C) and matched paracarcinomatous (D) tissues (A-D: × 100; Inner × 200). The survival curves from six and a half years of follow-up indicate a significant difference between 32 HCC patients with high and low levels of ASGPR (E) and exhibit a trend toward significance between 32 HCC patients with high and low levels of GPC3 (F).

Table 3 Spearman rank correlation analysis for fluorescence intensities

P value	Age	AFP	ALT	AST	TBIL	Size	Differentiation	AJCC stage
ASGPR	0.945	0.909	0.131	0.281	0.810	0.537	0.418	0.796
GPC3	0.863	0.911	0.549	0.846	0.288	0.357	0.623	0.849
CK	0.889	0.173	0.573	0.771	0.970	0.173	0.974	0.425

ASGPR: Asialoglycoprotein receptor; GPC3: Glypican-3; CK: Cytokeratin 8/18/19; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; AJCC: American Joint Committee on Cancer.

for EMT, was expressed on some CTCs, whereas EpCAM, an epithelium-specific biomarker, was expressed at various levels. These results suggest that CTCs may be associated with cancer metastasis and recurrence via EMT mechanisms. Some recent evidence indicates that tumor cells undergoing EMT might gain stem cell-like properties, therefore giving rise to cancer stem cells (CSCs)[27,30], and EpCAM-positive HCC cells have been reported to have stem cell-like features, including self-renewal and differentiation capacities[23,29,52]. Previously,

EpCAM was only known as an epithelial biomarker in many human cancers with an epithelium origin[53], and it has thus been used as a target to capture CTCs by the CellSearch™ system. Our results demonstrate that EpCAM was not widely expressed in the HCC cell lines, and the expression ratio on the karyocyte slides was consistent with histological reports[35], which implied that the CellSearch™ system may not be ideal for the detection of HCC CTCs[52]. The expression levels of vimentin and EpCAM were approximately 20% and 30%, respectively, of the total CTCs identified by GPC3, ASGPR, and CK, which indicates that our selected biomarkers could detect more tumor cells, including the EpCAM-positive and EMT-related cells.

Of the biomarkers detected, GPC3, ASGPR, and CK were all significantly expressed in HCC cell lines and karyocytes from patient blood samples, suggesting that these proteins could be used as valuable biomarkers of circulating HCC cells. Considering that GPC3 and ASGPR may be specific to HCC from tests on clinical samples, we applied three-dimensional reconstruction for further observation, revealing their localization. Additionally, the survival analysis indicated that GPC3 and ASGPR expression correlate with patient prognoses. To our knowledge, few studies have evaluated biomarker expression using a similar workflow. The results presented in this study improve the understanding of HCC biomarkers from a cytopathological perspective given that serum and tissues are widely used in other clinical examinations for biomarkers. The HCC-related biomarkers may help to identify tumor cells in the peripheral blood, which may be relevant to patient prognosis[11-13,54]. In addition, the expression of EMT-related biomarkers suggests that CTCs may help elucidate metastatic mechanisms, and the expression of EpCAM supports its novel function associated with stem cell-like properties. Although our study was limited by the small sample size of 84 individuals in the cohort, the biomarkers tested here may be used as capturing targets for HCC CTCs and may assist in the development of effective HCC therapies and individually targeted treatments based on biomarker expression and localization.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is currently one of the most common malignant tumors in the world. Although the detection of circulating tumor cells (CTCs) will provide us with prognostic information for several tumors, it is difficult to identify HCC CTCs by cellular surface biomarkers, thus making it crucial to detect the expression of biomarkers in a novel workflow.

Research frontiers

The study of CTCs is an increasingly important field in several cancers, and may be relevant to metastasis and recurrence. However, the search for biomarkers for HCC CTC identification has not been unequivocally addressed. In this study, the authors demonstrate that asialoglycoprotein receptor (ASGPR), glypican-3 (GPC3), and cytokeratin (CK) can identify CTCs on karyocyte slides. The expression of GPC3 and ASGPR is clinically significant.

Innovations and breakthroughs

Recent reports have highlighted the importance of CTC detection. For HCC in particular, it is important to identify CTCs in the blood. This study is the first to detect several biomarkers of HCC in a semiquantitative immunocytochemical workflow. Furthermore, the results suggest that GPC3, ASGPR, and CK can identify CTCs on karyocyte slides and that GPC3 and ASGPR expression correlates to tumor prognosis.

Applications

By identifying HCC CTCs *via* the biomarkers that the authors detected, this study may represent a future strategy for monitoring CTC changes and assessing the therapeutic prognosis of HCC.

Terminology

CTCs are cells that have dislocated from a primary tumor to circulate in the blood, and they may be able to seed in distant organs to become a metastatic focus. Thus, they are thought to be relevant to metastasis and recurrence. However, potential biomarkers to identify HCC CTCs must be identified.

Peer review

The authors detected the expression levels of potential cellular biomarkers for HCC and demonstrated that GPC3, ASGPR, and CK may be valuable surface biomarkers for the identification of HCC CTCs. Furthermore, GPC3 and ASGPR can better distinguish HCC patients from chronic HBV patients and healthy controls, and their expression is clinically significant. In addition, the expression of EMT- and stem cell-related biomarkers might provide us with helpful information regarding metastasis and recurrence. The results are interesting and may represent a strategy for identifying HCC CTCs and developing effective targeted treatments.

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ORIGINAL ARTICLE

Down-regulated γ-catenin expression is associated with tumor aggressiveness in esophageal cancer

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Abstract

AIM: To evaluate the significance of γ-catenin in clinical pathology, cellular function and signaling mechanism in esophageal squamous cell carcinoma (ESCC).

METHODS: The mRNA expression of γ-catenin was detected by real-time quantitative reverse transcription-polymerase chain reaction in 95 tissue specimens and evaluated for association with the clinicopathologic characteristics and survival time of patients with ESCC. siRNAs against human γ-catenin were used to inhibit γ-catenin expression. Hanging drop aggregation assay and dispase-based dissociation assay were performed to detect the effect of γ-catenin on ESCC cell-cell adhesion. Transwell assay was performed to determine cell migration. Luciferase-based transcriptional reporter assay (TOPflash) was used to measure β-catenin-dependent transcription in cells with reduced γ-catenin expression. The expression and subcellular localizations of β-catenin and E-cadherin were examined using Western blot and immunofluorescence analysis.

RESULTS: γ-catenin mRNA expression was significantly associated with tumor histological grade (P = 0.017) in ESCC. Kaplan-Meier survival analysis showed that γ-catenin expression levels had an impact on the survival curve, with low γ-catenin indicating worse survival (P = 0.003). The multivariate Cox regression analysis demonstrated that γ-catenin was an independent prognostic factor for survival. Experimentally, silencing γ-catenin caused defects in cell-cell adhesion and a concomitant increase in cell migration in both KYSE150 and TE3 ESCC cells. Analysis of Wnt signaling revealed no activation event associated with γ-catenin expression. Total β-catenin and Triton X-100-insoluble β-catenin were significantly reduced in the γ-catenin-specific siRNA-transfected KYSE150 and TE3 cells, whereas Triton X-100-soluble β-catenin was not altered. Moreover, knocking down γ-catenin expression resulted in a significant decrease of E-cadherin and Triton X-100-insoluble desmocollin-2, along with reduced β-catenin and E-cadherin membrane localization in ESCC cells.

CONCLUSION: γ-catenin is a tumor suppressor in ESCC and may serve as a prognostic marker. Dysregulated expression of γ-catenin may play important roles in ESCC progression.

Key words: γ-catenin; Esophageal squamous cell carcinoma; Independent prognostic factor; Cell-cell adhesion; Cell migration

Core tip: We, like others, have shown in previous work that reduced classic and desmosomal cadherin expression correlated with increased metastasis both in laboratory models and in clinical esophageal squamous cell carcinoma (ESCC) samples; however, a direct functional role of γ-catenin in this process has not been shown. In this study, we report that γ-catenin plays a critical role in ESCC progression. Our work demonstrates that γ-catenin is a tumor metastasis suppressor in ESCC and its expression may serve as a prognostic marker. Loss of γ-catenin leads to significant changes in esophageal cancer cell phenotypes.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is the most common malignancy in China, and has high mortality[1]. It is difficult to diagnose ESCC during early stages of disease development, and advanced ESCC is frequently associated with local invasion and lymph node metastasis[2]. Therefore, a better understanding of tumor dissemination and growth is paramount, and identification of genes crucial for tumor metastasis is urgently needed for the development of ESCC diagnostics and therapeutics.

Adhering junctions, such as adherens junctions and desmosomes, are vital for the unity of cells in esophageal epithelial sheets[3]. Generally, adhering junctions are comprised of a transmembrane component and a variety of cytoplasmic adapter proteins that in turn link cytoskeletal structures to sites of cell-cell contact. Alterations in expression of these cytoplasmic adapter proteins have been linked to tumor progression and/or suppression[4]. One such protein is a close relative of β-catenin called γ-catenin (also known as junction plakoglobin), which can bind to both classic and desmosomal cadherins, but is found primarily in desmosomes and is critically important in the maintenance of normal epithelial tissue architecture[5,6]. Reduction of γ-catenin expression has been reported in numerous human carcinomas such as oral, lung, colorectal, prostate, ovarian, and bladder cancers[7-12]. In esophageal cancer, loss or reduced γ-catenin expression has been correlated with poor survival[13].

Despite the growing number of correlative studies, the possible role or mechanism by which depletion of γ-catenin may contribute to neoplastic progression is not fully understood. Previous study shows that γ-catenin can function as an inhibitor of β-catenin/Tcf-dependent gene transcription and highlights γ-catenin as a potentially novel tumor suppressor protein in a subset of human lung cancers[14]. However, another study reveals no activated Wnt signaling event associated with γ-catenin expression in the bladder model[15]. In oral squamous cell carcinoma, γ-catenin has tumor metastasis suppressor function by regulating the metastasis suppressor activity of Nm23[16]. In keratinocytes, γ-catenin deficiency results in extracellular matrix (ECM)-dependent disruption of mature focal adhesions and actin organization, through distinct ECM-Src and RhoGTPase-dependent pathways[17]. Recently, we have shown that desmocollin-2 (DSC2), the most widely distributed desmosomal cadherin family member, plays a causal role in esophageal cellular invasion and metastasis. The loss of DSC2 initiates tumor cell metastasis by affecting the subcellular localization of γ-catenin, activating the β-catenin pathway, and eventually inducing an epithelial-mesenchymal transition-like process[18,19]. However, from these experiments we can not assess the contribution of γ-catenin to esophageal cancer suppression. Whether these observed changes in γ-catenin play a causal role in ESCC progression has not been determined.

In this study, using a real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) technique, we identified differential expression of γ-catenin in ESCC. The significance of γ-catenin in clinical pathology, cellular function and the signaling mechanism by which γ-catenin exerts its tumor suppressor activity in ESCC cells was evaluated. Results of these studies may provide a foundation for further study of potential clinical applications in diagnosis, prognosis or therapeutics.

MATERIALS AND METHODS

Ethics

This study was approved by the ethical committee of the Central Hospital of Shantou City and the Medical College of Shantou University, and written informed consent was obtained from all surgical patients to use resected samples for research.

Patients and samples

ESCC tissue specimens and paired adjacent normal epithelial tissues were obtained from 95 patients (median age, 55 years, range 40-88 years) who underwent surgery in the Department of Pathology of Shantou Central Hospital from 2007 to 2008. The specimens were immediately frozen in liquid nitrogen following surgery and stored at -70 ℃ until RNA isolation. All of the tumors were confirmed as ESCC by the Clinical Pathology Department of the Hospital, and the cases were classified according to the 7th edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer and were included in this study only if a follow-

Table 1 Survival information of the patients by clinical characteristics *n* (%)

Parameter	Five year survival	*P* value
Age (yr)		
< 55	38 (63.2)	0.225
⩾ 55	57 (52.6)	
Gender		
Female	24 (54.2)	1.000
Male	71 (57.7)	
Tumor size		
⩽ 3 cm	30 (56.7)	0.078
3-5 cm	49 (62.5)	
> 5 cm	16 (37.5)	
Differentiation grade		
G1	23 (73.9)	0.060
G2	55 (58.2)	
G3	17 (38.5)	
Invasive depth		
T1 + T2	24 (70.8)	0.169
T3 + T4	71 (52.1)	
Regional lymph node metastasis		
N0	57 (64.9)	0.034
N1	38 (44.7)	
pTNM stage		
ⅠA + ⅠB + ⅡA + ⅡB	61 (68.9)	0.007
ⅢA + ⅢB + ⅢC + Ⅳ	29 (41.4)	

Kaplan-Meier curves (log-rank test).

up was obtained. Patients' data are summarized in Table 1.

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was extracted from frozen stored tissues with TRIzol reagent (Invitrogen, United States) in accordance with the manufacturer's instructions. Reverse transcription was performed in a total volume of 20 μL using 1 μg of total RNA by using the Reverse Transcription System (Promega, United States). Real-time quantitative PCR was carried out on the Rotor-Gene 6000 system (Corbett Life Science, Sydney, Australia). SYBR® Premix Ex Taq™ (TaKaRa) was used according to the manufacturer's instructions. γ-catenin PCR primers were designed based on human γ-catenin mRNA sequence (GenBank accession number NM_002230.1). The primer sequences are as follows: forward 5'-CCC ATC AAT GAG CCC TAT GGA G-3' and reverse 5'-GGG CAC ATC GCT GGA GTA CA-3'. As an internal control, a fragment of human β-actin was amplified using the following primers: forward 5'-CAA CTG GGA CGA CAT GGA GAA A-3' and reverse 5'-GAT AGC AAC GTA CAT GGC TGG G-3'. PCR conditions were an initial denaturation step of 10 s at 95 ℃, followed by 40 cycles consisting of 5 s at 95 ℃, 20 s at 60 ℃ and 15 sec at 72 ℃. Quantification was performed using the $2^{-\Delta\Delta CT}$ method. The absolute levels of γ-catenin mRNA were normalized to that of β-actin mRNA. The status of differentially expressed γ-catenin gene in the ESCC tissue was defined as "positive expression" if $2^{-\Delta\Delta CT}$ was > 0.5-fold or "negative expression" if $2^{-\Delta\Delta CT}$ was ⩽ 0.5-fold, when compared to that detected in the adjacent normal tissue.

Cell culture and transfection

The human esophageal squamous carcinoma cell lines KYSE150 and TE3[20] were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal calf serum. For siRNA transfection, approximately 5×10^4 cells per well were inoculated into 6-well plates, cultured for 24 h and then transfected with the relevant siRNA (50 nmol/L) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, United States). siRNAs were synthesized by Shanghai GenePharma Co., Ltd and contained two siRNAs against human γ-catenin (siRNA-1: 5'-GCU GAU CAU CCU GGC CAA U-3' and siRNA-2: 5'-AGU CGG CCA UUG UGC AUC U-3'). A control siRNA oligonucleotide, which does not match any known human coding cDNA, was used as a control.

Transwell migration assay

Transwell migration assay was conducted as previously described[19]. Cells were placed into the upper chamber of an insert in serum-free medium (BD Biosciences, NJ, United States). After 24 h of incubation, cells remaining in the upper chamber were carefully removed. Cells adhering to the lower membrane were fixed and stained with Giemsa reagent, imaged, and counted using an inverted microscope (Olympus, Tokyo, Japan). The mean value was calculated from data obtained from three separate chambers.

Hanging drop assay

Hanging drop cultures of aggregated cells were generated from 1×10^3 cells. Cells were allowed to aggregate overnight on the underside of a culture dish as previously described[21]. Resulting cell clusters were subjected to 30 rounds of pipetting through a 200 μL Gilson pipette, and the degree of dissociation was quantified by counting the particles after trituration.

Dispase-based dissociation assay

Cell cultures were seeded in triplicate onto 6-well plates. Twenty-four hours after reaching confluency, cultures were washed twice in phosphate buffered saline (PBS) and then incubated in 1 mL of dispase (2.4 U/mL; Sigma) for more than 30 min as previously described[21]. Released monolayers were carefully washed twice with PBS and transferred to 15 mL conical tubes. Enough PBS was added to a final volume of 2 mL. Tubes were secured to a rocker and subjected to 50 inversion cycles. Fragments were counted using an inverted microscope (Olympus, Tokyo, Japan).

β-catenin/Tcf transcriptional reporter assay

Cells were co-transfected with either TOPflash or FOPflash reporter plasmids along with the siRNA-1/-2 or control siRNA. The TOPflash reporter plasmid contained β-catenin/TCF binding motifs, whereas the FOPflash reporter plasmid contained mutant β-catenin/Tcf-binding sites and was used as a control. The level of

Table 2 Relationship between clinicopathological features and γ-catenin mRNA expression *n* (%)

Parameter	γ-catenin status		*P* value
	$2^{-\Delta\Delta CT} \leq 0.5$	$2^{-\Delta\Delta CT} > 0.5$	
Age (yr)			
≤ 55	18 (36)	32 (64)	0.216
> 55	11 (24)	34 (76)	
Gender			
Female	11 (46)	13 (54)	0.079
Male	18 (25)	53 (75)	
Tumor size			
≤ 3 cm	11 (37)	19 (63)	0.522
3-5 cm	12 (25)	36 (75)	
> 5 cm	5 (31)	11 (69)	
Differentiation grade			
G1	5 (22)	18 (78)	0.017
G2	13 (24)	42 (76)	
G3	8 (62)	5 (38)	
Invasive depth			
T1 + T2	8 (33)	16 (67)	0.734
T3 + T4	21 (30)	50 (70)	
Regional lymph node metastasis			
N0	16 (28)	41 (72)	0.528
N1	13 (34)	25 (66)	
pTNM stage			
ⅠA + ⅠB + ⅡA + ⅡB	16 (26)	45 (74)	0.640
ⅢA + ⅢB + ⅢC + Ⅳ	9 (31)	20 (69)	

β-catenin-dependent transcription was determined by a TOPflash luciferase activity assay[19]. Luciferase reporter activities were normalized to the activity of the Renilla internal control. Data represent the results of triplicate dishes from two independent experiments.

Western blot

Western blot was performed as described previously[19]. Total cell lysates were prepared in RIPA buffer (Paragon Biotech, China). For analysis of the Triton X-100-insoluble pool, cells were lysed in 1% Triton X-100 buffer (1% Triton X-100, 145 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 2 mmol/L EGTA, and 1 mmol/L PMSF) followed by centrifugation (16000 *g*, 10 min). The Triton X-100-insoluble pellet was solubilized in Laemmli sample buffer, resolved by SDS/PAGE (12% gels) and immunoblotted. Experiments were repeated two to three times with similar results.

Confocal laser scanning microscopy

The staining procedure was performed as previously described[19]. After fixation in 4% paraformaldehyde solution for 15 min, cells were incubated with donkey serum blocking buffer for 30 min and a primary antibody overnight at 4 ℃, followed by donkey anti-mouse IgG (DyLight 488) and/or donkey anti-rabbit IgG (DyLight 594) (Jackson, Germany) for 30 min at 37 ℃. Samples were counterstained with DAPI (Sigma, St. Louis, MO, United States) for 10 min. Finally, the cells were examined under a confocal microscope (OLYMPUS, FV-1000).

Statistical analysis

Associations of γ-catenin with clinicopathological characteristics including age, gender, tumor size, differentiation grade, invasive depth, lymph node metastasis, and TNM classification were assessed by the χ^2 test. Kaplan-Meier curves were constructed for overall survival analysis by a log-rank test. All statistical analyses were performed with SPSS 13.0 software (version 13.0; SPSS, Inc., Chicago, IL). Each *P*-value is two-tailed and significance level is 0.05.

RESULTS

Expression of γ-catenin in ESCC tissues

To assess the clinical implications of γ-catenin mRNA expression in ESCC, we analyzed expression of this gene by real-time quantitative PCR. With a median follow-up of 39.6 mo for the 95 patients analyzed in this study, the mean survival was 41.4 mo (range 37.0-45.8 mo), and that the 5-year survival rate was 48.9%. The absolute levels of γ-catenin mRNA were normalized to that of β-actin mRNA. The status of differentially expressed γ-catenin gene in the ESCC tissue was defined as "positive expression" if $2^{-\Delta\Delta CT}$ was > 0.5-fold or "negative expression" if $2^{-\Delta\Delta CT}$ was ≤ 0.5-fold, when compared to that detected in the adjacent normal tissue. By this criterion, 29 (31%) patients had a negative γ-catenin expression status and 66 (69%) patients had a positive γ-catenin expression status. Table 2 shows associations between the clinicopathologic characteristics of patients with ESCC and γ-catenin expression status. A significant correlation was observed between γ-catenin expression status and histologic grade of tumors ($P = 0.017$). Positive γ-catenin expression cases ($2^{-\Delta\Delta CT} > 0.5$) were found in 78% of grade Ⅰ tumors, 76% of grade Ⅱ, and 38% of grade Ⅲ. ESCC patients with histologic grade Ⅲ tumors were more likely to have low levels of γ-catenin expression ($2^{-\Delta\Delta CT} \leq 0.5$). There were no significant correlations between γ-catenin expression levels and other clinical parameters, such as pTNM classification, in patients with ESCC.

The expression level of γ-catenin was next evaluated for association with survival time using the Kaplan-Meier method. The results showed that patient survival time was positively correlated with γ-catenin expression. ESCC patients with tumors demonstrating negative expression of γ-catenin ($2^{-\Delta\Delta CT} \leq 0.5$) exhibited poor prognosis. Among 95 ESCC patients, in 29 cases of negative γ-catenin expression ($2^{-\Delta\Delta CT} \leq 0.5$), the median survival time was 25.0 mo and the 5-year survival rate was 15.5%, whereas in 66 cases of positive γ-catenin expression ($2^{-\Delta\Delta CT} > 0.5$), the median survival time was 42.1 mo and the 5-year survival rate was 62.6% ($P = 0.003$, Figure 1). The use of the Cox regression model in multivariate analysis showed that γ-catenin expression status was an independent prognostic predictor ($P = 0.004$) (Table 3).

Expression of γ-catenin in ESCC cells

We next explored the expression of γ-catenin in ESCC cell lines. As shown in Figure 2A, γ-catenin expression was detected in all cell lines evaluated, with KYSE150

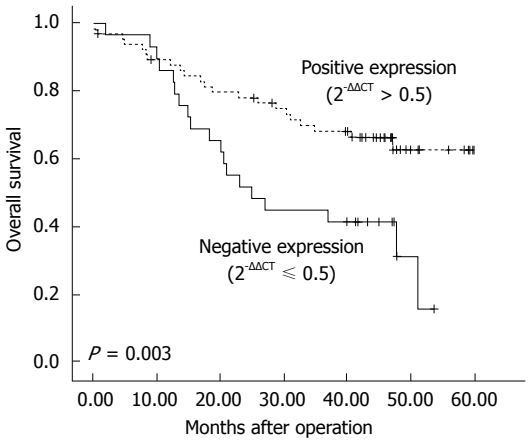

Figure 1 Kaplan-Meier estimates of overall survival by γ-catenin status in esophageal squamous cell carcinoma samples. The Kaplan-Meier analyses of γ-catenin mRNA expression in 95 patients with esophageal squamous cell carcinoma illustrate that γ-catenin expression levels had an impact on the survival curve, with low γ-catenin ($2^{-\Delta\Delta CT} \leq 0.5$) indicating worse survival (P = 0.003).

and TE3 cells contained higher γ-catenin expression levels. These cell lines were selected as the model for the subsequent function studies.

γ-catenin knockdown by transient siRNA transfection in ESCC cells

Two double-stranded siRNAs (siRNA-1 and -2) targeting the human γ-catenin gene were synthesized. These sequences are specific to γ-catenin mRNA and there is no match to other genes in the NCBI nucleotide database, in particular to other catenin family members, by BLAST searching. Western blot analysis revealed that γ-catenin expression decreased markedly in treated cells compared with the control. Transfection with siRNAs resulted in a 38%-54% reduction in γ-catenin expression in KYSE150 cells (Figure 2B, left panel) and a 25% to 44% reduction in expression in TE3 cells (Figure 2B, right panel), when compared to that transfected with the scramble sequence.

γ-catenin knockdown reduces cell-cell adhesion

In view of the fact that the malignant transformation of epithelial cells involves alteration of cell-cell adhesion that enables these cells to adopt a phenotype of migration leading to tumor invasion and metastasis[22], we next investigated whether reduced γ-catenin expression levels affected cell-cell adhesion in ESCC cells. We first employed a previously described hanging drop aggregation assay[21]. Cells allowed to aggregate for 24 h in a hanging drop on the underside of a culture dish were subjected to trituration through a 200 μL-Gilson pipette tip in an attempt to disrupt intercellular adhesion. The degree of dissociation was quantified by counting the particles that dissociated from the original cluster. KYSE150 and TE3 cells transfected with siRNA against γ-catenin were more susceptible to dissociation than control cells (Figure 2C), which exhibited on average an approximately threefold increase in particle number when compared with the control ($P < 0.01$).

Table 3 Univariate and multivariate Cox regression analyses by γ-catenin expression status

Cox regression analysis		Percentage of survival (95%CI)	*P* value
Univariate	Time to survival (mo)		
$2^{-\Delta\Delta CT} \leq 0.5$	31.309 ± 3.343	34.5 (24.757-37.860)	0.007
$2^{-\Delta\Delta CT} > 0.5$	45.463 ± 2.609	66.7 (40.349-50.576)	
Multivariate	Relative risk		
Tumor size	1.256	0.751-2.101	0.385
Differentiation grade	1.651	0.914-2.981	0.097
Regional lymph node metastasis	1.846	0.963-3.539	0.065
γ-catenin	0.370	0.189-0.723	0.004

To further confirm the effect of γ-catenin on ESCC cell-cell adhesion, we performed a Dispase-based dissociation assay. This assay was previously used to quantify the adhesion strength of a variety of epithelial cells[21,23]. Confluent monolayers of siRNA transfected KYSE150 and TE3 cells were harvested from tissue culture dishes by incubation with dispase. Monolayers were then transferred to 15 mL conical tubes. After inverting the tubes 50 times on a rocker, monolayer fragments were counted (Figure 2D). γ-catenin-specific siRNA-transfected monolayers dissociated into numerous smaller fragments, whereas control cell monolayers exhibited only minimal dissociation. Quantification of the number of fragments showed a four-fold increase in fragmentation ($P < 0.01$), suggesting that knocking down γ-catenin led to a significant decrease in cell-cell adhesion.

γ-catenin inhibits motility of ESCC cells

As weakening of cell-cell adhesions has been reported to increase cell motility[22], we next investigated whether γ-catenin status in cells correlated with their relative motility. Towards this end, we performed a transwell migration assay to compare cell motility. The assay revealed that γ-catenin knockdown cells were significantly more migratory than control siRNA-transfected cells over a 24-hperiod. The transfection of KYSE150 and TE3 cells with a γ-catenin-specific siRNA increased cell migration up to 25% compared with the RNAi control transfection ($P < 0.01$; Figure 2E). These data imply that reduced expression of γ-catenin promotes cell motility.

Effects of γ-catenin on expression of adherens junction molecule and β-catenin signaling

Although the role of β-catenin in Wnt signaling has been well established, the role of γ-catenin in this pathway is less clear[24]. To investigate whether γ-catenin influences β-catenin signaling in esophageal carcinoma cells, we first used a luciferase-based transcriptional reporter assay (TOPflash) to measure β-catenin-dependent transcription in cells with reduced γ-catenin expression. As illustrated in Figure 3A, KYSE150 and TE3 cells transfected with

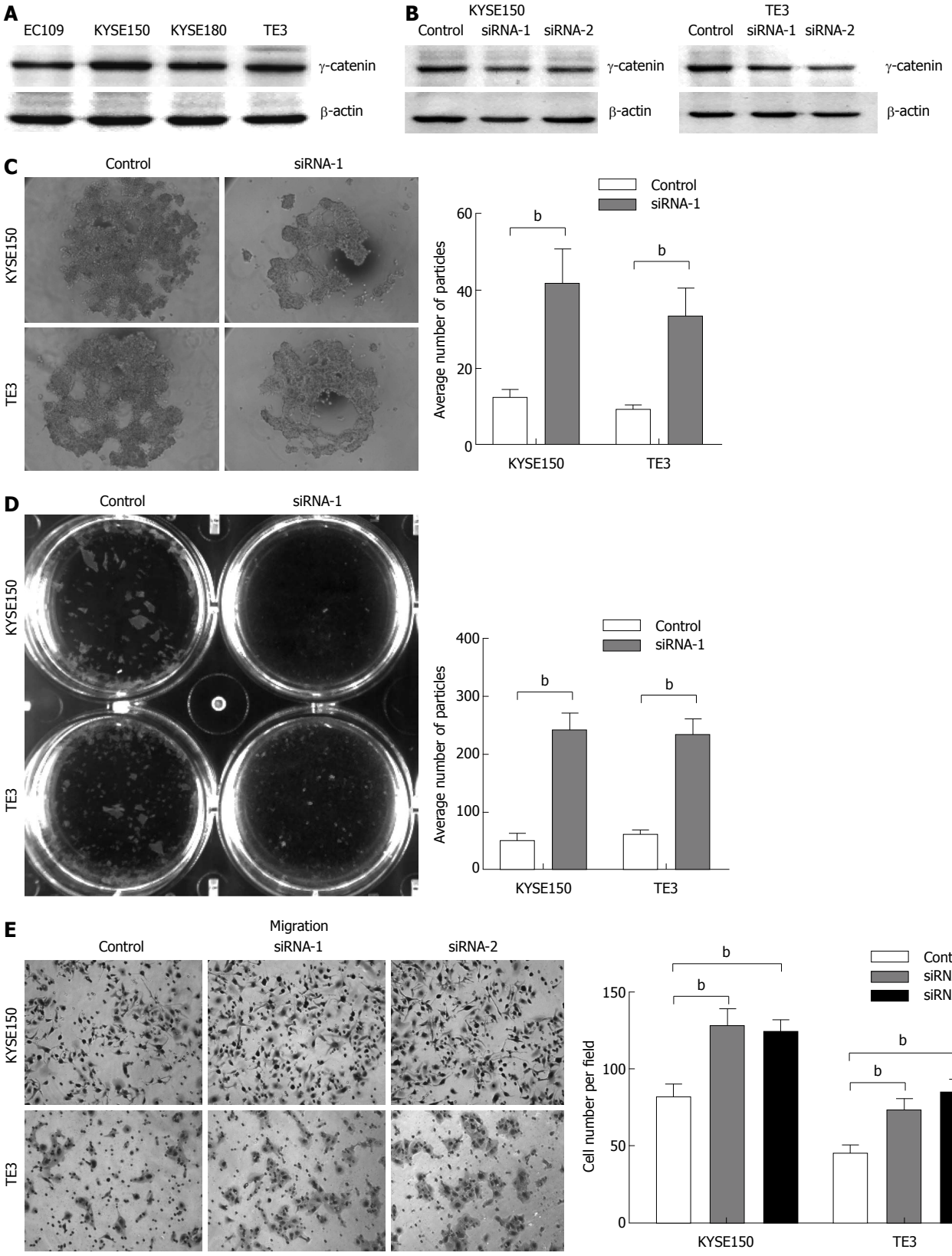

Figure 2 Alterations of cell-cell adhesion and cell migration exerted by RNAi-mediated knockdown of γ-catenin. A: Representative Western blot of γ-catenin in 4 esophageal cancer cell lines; B: KYSE150 and TE3 cells were transfected with γ-catenin siRNAs (siRNA-1 and -2), or a negative control siRNA (control). Western blot analysis was used to show RNAi-mediated knockdown. Equal loading was ascertained using β-actin as an internal control; C: Hanging drop assay. Cells were seeded into hanging drop cultures and allowed to aggregate for 24 h. After trituration by passing the cell cluster 30 times through a 200 μL pipette tip, the degree of dissociation of the cell cluster was visualized by microscopy and quantified by manual counting under a dissecting microscope; D: Dispase-based dissociation assay. Cell monolayers were separated from culture dishes via incubation with dispase. Monolayers were transferred to 15 mL conical tubes containing 2 mL of phosphate buffered saline. After 50 inversions, the degree of fragmentation of the monolayers was observed. The dissociation assay was quantified by counting the number of total particles; E: Transwell assay was performed to determine cell migration. Migrated cells were fixed and stained, and representative fields were photographed. The cells were quantified in 10 random fields with a light microscope (× 200). The mean value was calculated from data obtained from three separate chambers. $^{b}P < 0.01$ *vs* the control group.

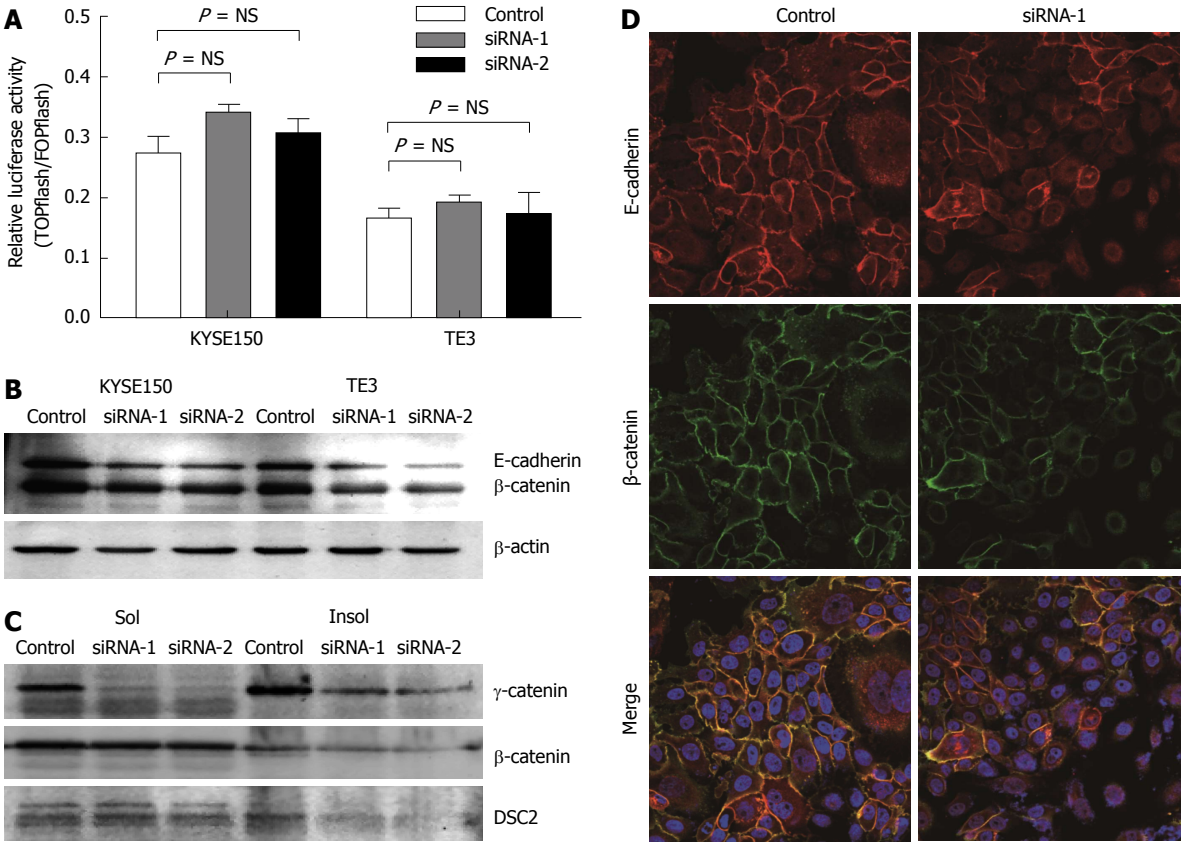

Figure 3 γ-catenin affects cell motility through cell-cell adhesion-dependent mechanisms. A: TOPflash reporter activity in KYSE150 and TE3 cells. Cells were co-transfected with either TOPflash or FOPflash reporter plasmids along with siRNA-1/-2 or control siRNA. The level of β-catenin-dependent transcription was determined by TOPflash luciferase activity. FOPflash reporter plasmids containing mutant β-catenin/ TCF-binding sites were used as controls. Data represent the results of triplicate dishes from two independent experiments; B: Western blot analyses of E-cadherin and total β-catenin in γ-catenin-specific siRNA or control-transfected esophageal squamous cell carcinoma (ESCC) cells. β-actin served as a loading control; C: Western blot analyses of triton X-100-soluble and -insoluble γ-catenin, β-catenin and DSC2 proteins in γ-catenin-specific siRNA or control-transfected ESCC cells; D: The subcellular localizations of β-catenin and E-cadherin was examined using immunofluorescence analysis. Of note, knocking down γ-catenin expression caused reduced β-catenin and E-cadherin membrane localization.

γ-catenin-specific siRNA showed no significant Wnt activation ($P > 0.05$), when compared with the RNAi control transfected cells. We next examined β-catenin protein expression levels by Western blot. Total β-catenin and Triton X-100-insoluble β-catenin were significantly reduced in the γ-catenin-specific siRNA-transfected KYSE150 and TE3 cells, whereas Triton X-100-soluble β-catenin was not altered (Figure 3B and C). These results suggest that γ-catenin does not mediate its effects through the Wnt pathway in this model.

As γ-catenin is a member of the Armadillo family of proteins and binds directly to both classic and desmosomal cadherins[5]. We therefore investigated the effects of γ-catenin knockdown on expression of E-cadherin and DSC2. As shown in Figure 3, reducing expression of γ-catenin resulted in a significant decrease of E-cadherin and Triton X-100-insoluble DSC2 in ESCC cells. To confirm this result, the subcellular localizations of β-catenin and E-cadherin were examined using immunofluorescence. Consistent with the Western blot results, knocking down γ-catenin expression caused reduced β-catenin and E-cadherin membrane localization (Figure 3D).

DISCUSSION

Accumulating evidence suggests that adhesive interactions are critical in the process of metastatic tumor migration and that adhesion molecules can act as both positive and negative modulators of the metastatic process[25]. The cadherin-catenin complex promotes homotypic tumor cell adhesion, maintaining intercellular contacts that confine cells to the primary tumor site. Previous studies have shown a correlation between reduced classic and desmosomal cadherin expression and increased metastasis both in laboratory models and in clinical ESCC samples[18,19,26]; however, a direct functional role of γ-catenin in this process has not been shown. In this study, we report that γ-catenin plays a critical role in ESCC progression. Our work demonstrates that γ-catenin is a tumor metastasis suppressor in ESCC and its expression may serve as a prognostic marker. Loss of γ-catenin leads to significant changes in esophageal cancer cell phenotypes.

Evidence from clinical studies suggests that reduced expression of γ-catenin in human cancers is associated with increased tumor progression and adverse clinical

outcome[7-13]. To confirm whether these observed changes in γ-catenin play a causal role in ESCC progression, we first use a real-time quantitative RT-PCR method to analyze the prognostic importance of γ-catenin expression in ESCC. Reduced γ-catenin mRNA expression is associated with an unfavorable prognosis of ESCC patients. Patients with positive γ-catenin- expressing tumors have a better prognosis than those with reduced expression of γ-catenin. Moreover, γ-catenin expression level is an independent prognostic predictor. Our findings are consistent with earlier immunohistochemistry studies revealing that decreased γ-catenin expression was significantly associated with poorer prognosis[13], which implies more aggressive malignant behaviors of esophageal carcinoma cells.

Several studies have discovered that γ-catenin was involved in cell invasion and metastasis of human oral squamous cell cancer, lung cancer, and bladder cancer[14-16]. In our clinical investigation, expression of γ-catenin does not correlate with the degree of lymph node metastasis. However, Lin *et al*[13] have reported that reduced protein expression of γ-catenin is associated with lymph node metastasis in human ESCC. It seems that this confusing result conducted from the statistical analysis might be due to the insufficient ESCC specimens, or the discordance between protein and mRNA expression levels of γ-catenin. In *in vitro* experiments, we show that γ-catenin expression enhances cell-cell adhesion and suppresses the motility of ESCC cells, suggesting that γ-catenin might play as a metastasis suppressor in ESCC. As a junctional protein, γ-catenin interacts with both desmocollin and E-cadherin in both the soluble and cytoskeleton associated pools of cellular proteins[5]. Recently, it has been suggest that γ-catenin may play a central role in desmosome organization and is required for effective intermediate filament anchorage to desmosomes[27]. Consistent with these observations, the data presented in this work suggest that a decreased level of γ-catenin in the cell leads to a decrease in E-cadherin and desmocollin protein expression, which is accompanied by a decrease in cell-cell adhesion and an increase in cell migration. These results are consistent with data from Simpson *et al*[28] that show that increasing γ-catenin levels rescue cadherin expression, desmosome organization, and functional adhesion, leading us to conclude that knocking down γ-catenin expression with siRNA destabilizes other cadherins. Although we did not determine whether this represents transcriptional downregulation or increased turnover of protein possibly complexed with γ-catenin, the reduction of cell adhesion protein suggests that the inhibition of cell migration is partly dependent upon γ-catenin interactions with the classic and desmosomal cadherin proteins.

The signaling mechanism(s) by which γ-catenin exerts its tumour suppressor activity in ESCC cells remains unclear. γ-catenin localizes to the desmosomal plaque and seems to be required for desmosome organization as discussed earlier. Alternatively, desmosomes have been postulated to serve as signaling centers[29], and it is possible that an alteration in desmosome composition results in an alteration in signal transduction resulting in increased transformation. In addition, γ-catenin may directly modify intracellular signaling, thus altering cell behavior, such as cell migration under certain circumstances[17,30]. In numerous human cancers including ESCC, the Wnt/β-catenin signaling pathway is constitutively activated[31]. The previous study on human lung cancer cells shows that re-expression of γ-catenin reduces TCF activity[14]. However, it is interesting that we report here that γ-catenin does not affect β-catenin/TCF-dependent gene transcription. Our observations support the work of Rieger-Christ *et al*[15], who revealed no activated Wnt signaling event associated with γ-catenin expression in the bladder model. Hence, in esophageal carcinoma cells, γ-catenin might be exerting a tumor suppressor role *via* alternative signaling pathways from those established in other carcinoma cell models, which will require extensive study to determine.

In summary, this study provides both clinical and mechanistic evidence supporting the critical role of γ-catenin in ESCC progression. Our data demonstrate that γ-catenin is a tumor suppressor in ESCC and its expression may serve as a prognostic marker. Results of this study may provide a foundation for further study of potential clinical applications in diagnosis, prognosis or therapeutics.

COMMENTS

Background

γ-catenin is a member of the armadillo family of proteins, which can bind to both classic and desmosomal cadherins, but is found primarily in desmosomes and is critically important in the maintenance of normal epithelial tissue architecture. Reduction of γ-catenin expression has been reported in numerous human carcinomas.

Research frontiers

Accumulating evidence suggests that adhesive interactions are critical in the process of metastatic tumor migration and that adhesion molecules can act as both positive and negative modulators of the metastatic process. Previous studies have shown a correlation between reduced desmosomal cadherin expression and increased metastasis both in laboratory models and in clinical esophageal squamous cell carcinoma (ESCC) samples. However, a direct functional role of γ-catenin in ESCC progression has not been determined.

Innovations and breakthroughs

The significance of γ-catenin in clinical pathology, cellular function and the signaling mechanism by which γ-catenin exerts its tumor suppressor activity in ESCC cells was evaluated. The work demonstrates that γ-catenin is a tumor metastasis suppressor in ESCC and its expression may serve as a prognostic marker. Loss of γ-catenin leads to significant changes in esophageal cancer cell phenotypes.

Applications

This study may provide a foundation for further study of potential clinical applications of γ-catenin in ESCC diagnosis, prognosis or therapeutics.

Peer review

This is a good descriptive study in which the authors analyzed the relationship between γ-catenin mRNA expression and disease prognosis in patients with ESCC, and the cellular function and signaling mechanism by which γ-catenin exerts its tumor suppressor activity in ESCC cells. They conclude that γ-catenin is a tumor metastasis suppressor in ESCC and its expression may serve as a prognostic marker. Dysregulated expression of γ-catenin may play important roles in ESCC progression. The results are interesting and provide insights into esophageal cancer.

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ORIGINAL ARTICLE

Changes in intestinal microflora in rats with acute respiratory distress syndrome

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Abstract

AIM: To implement high-throughput 16S rDNA sequencing to study microbial diversity in the fecal matter of rats with acute lung injury/acute respiratory distress syndrome (ALI/ARDS).

METHODS: Intratracheal instillation of lipopolysaccharide was used to induce ALI, and the pathological changes in the lungs and intestines were observed. D-lactate levels and diamine oxidase (DAO) activities were determined by enzymatic spectrophotometry. The fragments encompassing V4 16S rDNA hypervariable regions were PCR amplified from fecal samples, and the PCR products of V4 were sequenced by Illumina MiSeq.

RESULTS: Increased D-lactate levels and DAO activities were observed in the model group ($P < 0.01$). Sequencing results revealed the presence of 3780 and 4142 species in the control and model groups, respectively. The percentage of shared species was 18.8419%. Compared with the control group, the model group had a higher diversity index and a lower number of species of *Fusobacteria* (at the phylum level), *Helicobacter* and *Roseburia* (at the genus level) ($P < 0.01$). Differences in species diversity, structure, distribution and composition were found between the control group and early ARDS group.

CONCLUSION: The detection of specific bacteria allows early detection and diagnosis of ALI/ARDS.

Key words: Lipopolysaccharide; Acute lung injury; Acute respiratory distress syndrome; Intestinal microflora; High-throughput sequencing

Core tip: This experimental study evaluated the possible association between acute respiratory distress syndrome (ARDS) and intestinal microflora using an animal model and high-throughput sequencing analysis. Differences in species diversity, structure, distribution and composition were found between the control group and early ARDS group. This study contributes to a better understanding of the mechanisms by which changes in the intestinal mucosal barrier and host microflora may be involved in the pathogenesis of ARDS.

Li Y, Liu XY, Ma MM, Qi ZJ, Zhang XQ, Li Z, Cao GH, Li J, Zhu WW, Wang XZ. Changes in intestinal microflora in rats with acute respiratory distress syndrome. *World J Gastroenterol* 2014; 20(19): 5849-5858 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5849.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5849

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a continuous pathological process, with acute lung injury (ALI) at its early stage. According to Ashbaugh *et al*[1], ARDS is characterized by alveolar epithelial and endothelial barrier disruption, significant inflammation, gas exchange dysfunction and severe respiratory failure. Despite remarkable progress in the pathophysiology and standard treatment (*i.e.*, supportive mechanical ventilation) of ARDS, the mortality rate remains high at approximately 40%-60%[2,3], making it the leading factor contributing to the high morbidity and mortality of patients in the intensive care unit[4,5]. ARDS results from sepsis, trauma, gastric aspiration, and pneumonia[6]. The most common precipitating factor of ARDS is sepsis. In 1988, Wilmore *et al*[7] proposed that the intestine is the target organ of ARDS. The intestine is considered to be the largest repository of bacteria and endotoxins. A weak or damaged intestinal barrier triggers the translocation of intestinal bacteria or endotoxins, leading to sepsis. Multiple organ dysfunction syndrome (MODS) is triggered by the chain reaction of cytokines and other inflammatory mediators. The serum level of endotoxic lipopolysaccharides (LPS) from Gram-negative bacteria is a predictive or prognostic factor for ARDS. Intratracheal LPS instillation has been used to induce sepsis-related ALI/ARDS[8] in animal models.

Approximately 10^{13}-10^{14} microbes thrive in the intestine and cohabit discretely with the mucosal immune system[9]. About 1000 species of bacteria live in the human intestine. This number of bacteria is 10 times that found in cells in the body; in addition, the number of bacterial genes is 100 times higher than the genes of the human host[10,11]. The effects of bacterial genes in the large intestine have become a central issue among microbiologists. Normal intestinal flora can prevent infections caused by pathogens, provide nutrients, such as vitamin B and vitamin K, participate in the metabolism of carbohydrates and proteins, shape the mucosal immune system and serve as a biological barrier[12-14]. High-throughput sequencing greatly contributes to research on the diversity of environmental microbes, including uncultured microorganisms and trace amounts of bacteria.

Intestinal damage triggers or aggravates ARDS or MODS. However, the mechanism by which the intestine changes its mucosal barrier and microflora during ARDS remains unclear. High-throughput sequencing has contributed greatly to the research on intestinal flora. Recent studies have employed high-throughput sequencing to reveal the specific relationship between diseases and intestinal flora[15-18]. The present study aimed to determine the relationship between ARDS and intestinal flora using high-throughput sequencing. This study will provide new insights and a possible experimental basis for the early detection and diagnosis of ARDS in the future.

MATERIALS AND METHODS

Animals and reagents

Sixteen male Sprague-Dawley rats weighing approximately 230 g were randomly divided into two groups (n = 8 for each group): a control/normal group and a LPS/model group. All animals were housed in autoclaved cages with free access to laboratory food and water, and were exposed to alternate cycles of 12 h of light and darkness at room temperature (25 ℃). All experimental procedures complied with the Declaration of Helsinki of the World Medical Association and the protocols were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University. LPS (*Escherichia coli* LPS, 055:B5), D-lactate and diamine oxidase (DAO) kits were purchased from the Sigma Chemical Company (St. Louis, MO, United States).

LPS-induced ALI animal model

The rats were fasted overnight and given ad libitum access to water. The rats were anesthetized with 40 mg/kg of chloral hydrate and then fixed on an operating table. LPS (10 mg/kg body weight) in phosphate-buffered saline (PBS) was instilled intratracheally to induce ALI[19]. The normal group underwent the same procedure, but with intratracheal instillation of PBS. All rats were anesthetized and killed after 24 h.

Wet/dry ratio

The water content of the lungs was evaluated by calculating the wet/dry weight ratio. The left cranial lobe was excised, rinsed in PBS, blotted and then weighed to obtain the wet weight. The lung was dried at 80 ℃ for 72 h to constant weight to obtain the dry weight. The wet/dry ratio was calculated by dividing the wet weight by the dry weight.

Pulmonary histopathology

The rats were perfused with PBS *via* the pulmonary artery. As soon as the chest and abdominal cavities were excised, portions of the lungs were immediately removed and immersed in 4% paraformaldehyde for 72 h at room temperature. These portions were then processed and embedded in paraffin. Tissue sections (4 μm thick) were prepared by embedding in paraffin. After hematoxylin and eosin (HE) staining, the slides were observed under a light microscope. Six visual fields were randomly observed on a slide under × 400 magnification. The lung injury score (LIS) was assessed using the method described by Nishina *et al*[20]. Lung injury was assessed by alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall and thickness of the alveolar wall or hyaline membrane. The severity of lung injury was scored as follows: 0, minimum; 1, mild; 2, moderate; 3, severe; and 4, maximum. Six high-magnification fields were randomly selected and graded for the average LIS for each stained sample.

Intestinal histopathology and electron microscopy

The intestines, from the ileum to 5 cm above the cecum, were acquired immediately after the rats were killed. Tissues for histopathology were fixed with 4% formaldehyde. Paraffin-embedded samples were cut and stained

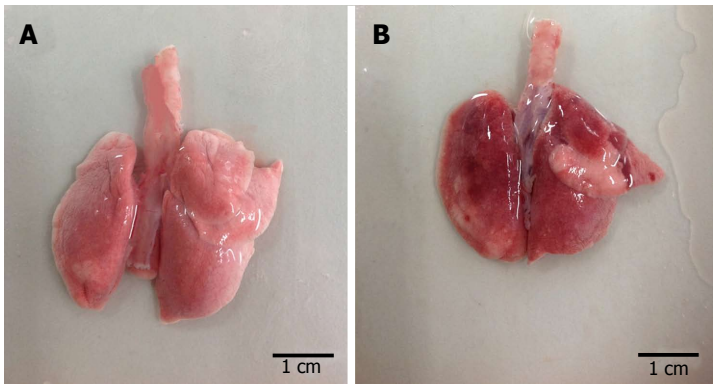

Figure 1 Macroscopic observation of lung morphology in rats. The chest was opened and the lungs were dissociated 24 h after phosphate buffer saline or lipopolysaccharide instillation. A: Lung of normal rats; B: Lung of model rats.

using HE to detect histopathological changes. Another set of paraffin-embedded samples was used to observe ultrastructural changes. The samples were cut into 1 mm × 1 mm × 1 mm sections, pre-fixed with 3% glutaraldehyde, fixed with 1% osmium tetroxide, dehydrated in acetone (50%, 70%, 90% and 100%) and then embedded in Epon 812. Semi-thin sections were used for optical positioning, whereas ultra-thin sections were used for double staining with uranyl acetate and lead citrate. The sections were observed by electron microscopy.

DAO activity and D-lactate levels in serum

Plasma was harvested from the collected abdominal aortic blood and kept at -20 ℃. Permeability of the intestinal mucosa was assayed by measuring D-lactate and DAO levels in the plasma. Plasma D-lactate levels were measured by enzymatic spectrophotometric assay as previously described[21]. Plasma DAO activities were also determined by enzymatic spectrophotometry as previously described[22].

Fecal collection and bacterial DNA extraction

Rat colons were immediately excised and fecal samples were harvested for microbial DNA extraction using a QIAamp DNA stool minikit (Qiagen, West Sussex, United Kingdom) following the manufacturer's instructions. The quality and quantity of genomic DNA were assessed with a Nanodrop spectrophotometer, with the A260/A280 ratio between 1.8 and 2.0 considered a criterion for quality control. No obvious RNA banding was shown by gel electrophoresis, and genomic bands were clear and complete. DNA was frozen at -80 ℃ prior to PCR amplification.

Partido comunista revolucionario amplification of 16S rDNA V4 hypervariable regions

Fragments encompassing V4 16S rDNA hypervariable regions were PCR amplified from each of the 6 DNA samples using fusion primers (forward: 5'-Index + AYTGGGYDTAAAGNG-3', reverse: 5'-TACNVGGGTATCTAATCC-3') and universal primers (forward: 5'-AYTGGGYDTAAAGNG-3', reverse: 5'-TACNVGGGTATCTAATCC-3').

The annealing temperature and extension time were 50 ℃ and 30 s, respectively. The PCR conditions were as follows: 94 ℃ for 5 min, 25 cycles of 94 ℃ for 30 s, annealing temperature for 30 s and 72 ℃ for 30 s, 72 ℃ for 7 min and holding at 4 ℃. PCR products were excised from 1% agarose gels and purified using a QIA quick Gel Extraction Kit. The PCR products of V4 were sequenced by Illumina MiSeq.

Statistical analysis

Quantitative data are reported as mean ± standard error of the mean. Statistical differences in basal characteristics between the groups were calculated by one-way analysis of variance and *t* test for continuous variables. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS 16.0 software.

RESULTS

Lung macroscopic view and histopathology

The lung tissue in the control group was pale pink with a smooth surface, good expansion, soft texture and no obvious abnormalities (Figure 1A). The lung tissue in the injury group appeared dark with sub-capsular hemorrhage, reddish liquid overflowing from the tangent plane and atelectasis (Figure 1B). The rats in the control group had slight pulmonary histological changes (Figure 2A), whereas those in the LPS group had the typical symptoms of ALI, with marked recruitment of neutrophils, alveolar hemorrhage and obvious alveolar wall thickening (Figure 2B).

Intestinal histopathology and electron microscopy

The epithelial cells of rats in the control group showed normal morphological characteristics, including a neatly arranged brush border, alternating cup-shaped cells and uniform cell size as observed under an optical microscope (Figure 3A). Ultrastructure was visible with normal mitochondria and endoplasmic reticulum (Figure 4A). In contrast, the epithelial cells of rats in the model group showed disorganized intestinal villi under a light microscope (Figure 3B) and slightly swollen mitochondria with a non-compact structure, expanded endoplasmic reticulum, structurally disordered cells and other cell damage, as observed by electron microscopy (Figure 4B).

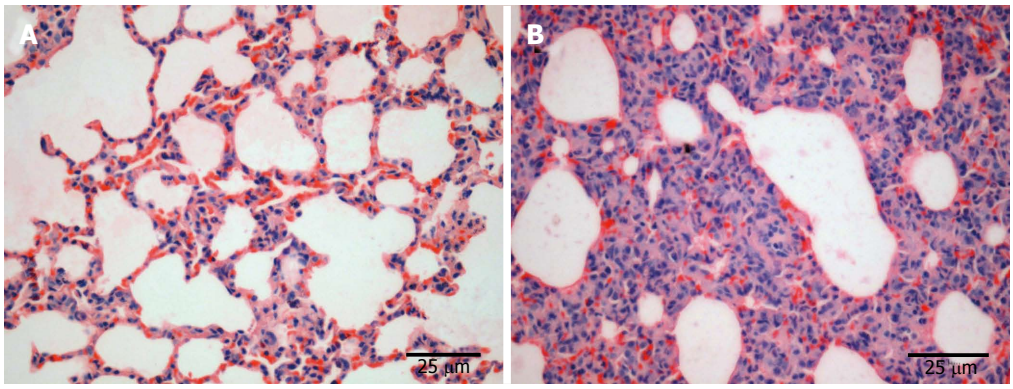

Figure 2 Pulmonary histopathological changes in rats. Lung tissue specimens were obtained from the normal group (A) and model group (B). Magnification × 400.

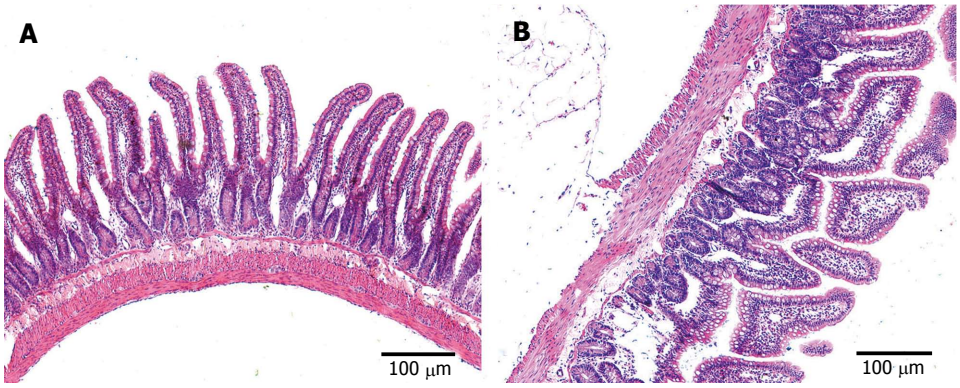

Figure 3 Intestinal histopathological changes in rats. Intestinal tissue specimens were obtained from the ileum to 5 cm above the cecum in the normal group (A) and model group (B). Magnification × 100.

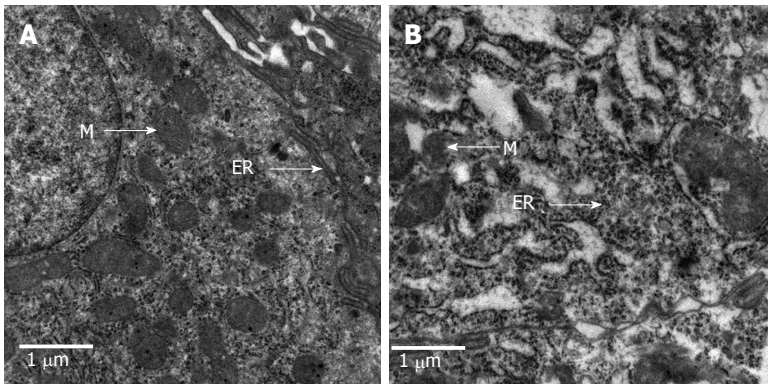

Figure 4 Ultra-structural analysis of intestinal mucosa in rats. Tissue samples were fixed, cut, stained and examined by transmission electron microscopy. Images are representative electron micrographs of the normal (A) and the model (B) groups. Cell organelles: ER: Endoplasmic reticulum; M: Mitochondria.

Table 1 Indicators of injury in the lung and intestinal tissues (n = 16)

Group	LIS	Wet-dry ratio	Vitality of DAO (U/L)	Content of D-lactic acid (μg/L)
Normal	2.33 ± 0.52	2.62 ± 0.316	3.14 ± 1.193	561 ± 18.22
Model	9.00 ± 0.28[b]	3.38 ± 0.399[a]	11.79 ± 2.542[b]	619 ± 31.46[b]

[a]$P < 0.05$ for Normal group *vs* Model group; [b]$P < 0.01$ for Normal group *vs* Model group.

LIS and wet-dry ratio

The average LIS in the model group was significantly higher than that in the control group ($P < 0.01$) (Table 1). The wet/dry ratio significantly increased in the model group compared with the control group ($P < 0.05$) (Table 1). These two factors, together with pulmonary histopathology, demonstrated the successful ALI modeling.

DAO and D-lactate in serum

Plasma D-lactate and DAO levels can reflect the presence of intestinal injuries, including intestinal mucosal barrier damage after ARDS. The levels of DAO and D-lactate significantly increased in the ALI group (11.79 ± 2.542 U/L and 619 ± 31.46 μg/L) compared with those in the normal group (3.14 ± 1.193 U/L and 561 ± 18.22 μg/L) ($P < 0.01$) (Table 1). These data suggest that the mucosal

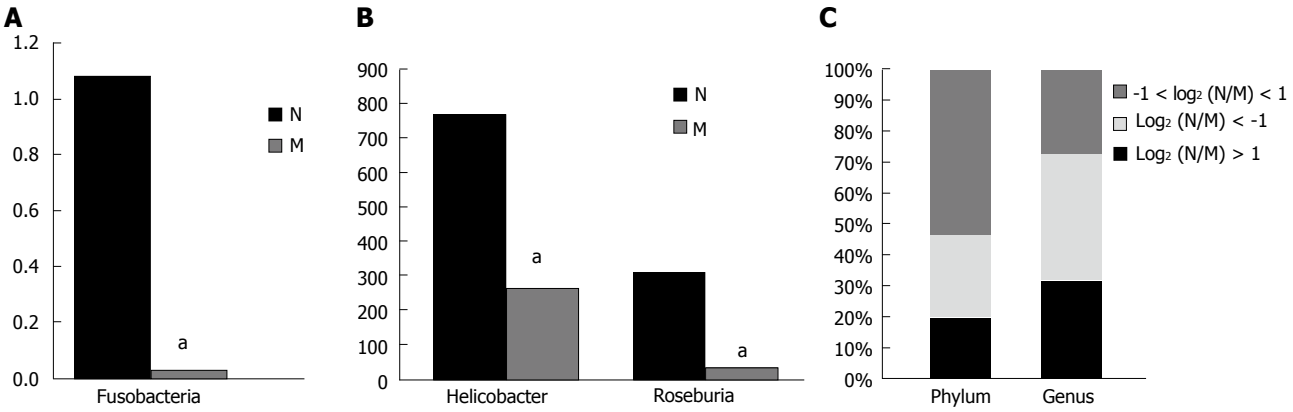

Figure 5 Statistically significant flora and percentage of more than twice the number of species are shown at phylum and genus levels. A: At the phylum level, *Fusobacteria* was significantly different between the two groups. B: At the genus level, *Helicobacter* and *Roseburia* were significantly different between the two groups; Vertical axis represents sequence reads. C: Percentage of all parts at the phylum and genus levels. $^{a}P < 0.05$ for normal group *vs* model group. M: Model group; N: Normal group.

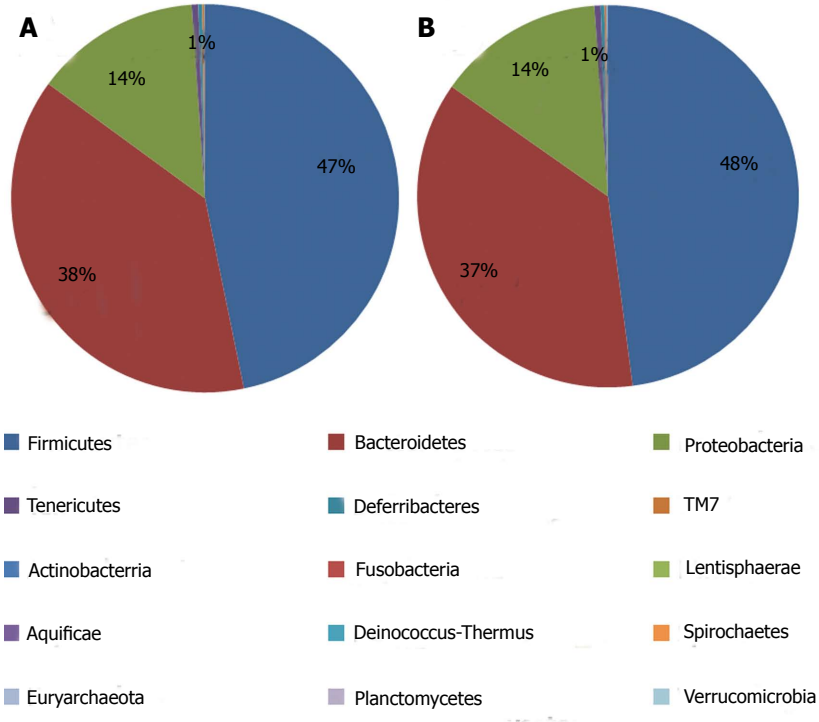

Figure 6 Microbial distributions at phylum level in the samples from the normal and model groups. Percentages are based on proportions of assignable tags.

barrier was damaged.

Filtering and quality evaluation of original data

Flash software was used to control the quality of the raw data by truncating or abandoning low-quality sequences. The ends of the corresponding sequences were connected. The sequences which were unable to connect were abandoned. According to the experimental requirements, the connected sequences were filtered for analysis. A total of 104 742 V4 16S rDNA sequence reads from the six samples, with an average of 17 457 sequence reads for each sample (the minimum and maximum numbers of reads per sample were 9170 and 29700, respectively), were used in this analysis. The average length of the sequence reads was 225 bp, and they were classified into different taxonomic categories using MGRAST[15].

Operational taxonomic unit

According to the sequence similarity (> 97%), high-quality sequences were classified into multiple operational taxonomic units (OTUs) using Qiime to facilitate analysis. The OTU in each sample and the number of sequences in each OTU were counted to obtain the taxonomic information of the OTU. The taxon abundance of each sample was generated into 15 phyla, 27 classes, 48 orders, 71 families and 120 genera using mainly the RDP, Greengenes and SSU databases. Up to 3780 and 4142 species were found in the normal and model groups, respectively. The two groups shared 1256 (18.8419%) species, and the total richness for all groups was 6666.

Alpha diversity analysis

Alpha diversity refers to the diversity in a specific area

Table 2 Estimation of diversity within the normal and model groups

Group	Chao	Ace	Shannon	Coverage
Normal	4860.3	8761.6	5.09	0.921
Model	4940.6	9400.3	4.97	0.927

or ecosystem in terms of species richness. According to species richness in the list of OTUs in the sample, diversity, richness, coverage and evenness estimations were calculated for all data sets using Mother software. The Chao/Ace calculation is an estimator of phylotype richness, and the Shannon index of diversity reflects both the richness and community evenness. The model group had a higher richness index (Chao, 4940.6 and ACE, 9400.3) and lower diversity index (4.97) than the normal group (Chao, 4860.3, ACE, 8761.6 and Shannon, 5.09). These results suggested that the model group had higher levels of biodiversity and unevenness estimations than the normal group. Good's coverage, a measure of sampling completeness, ranged between 92.1% and 92.7% for the data sets at a 97% similarity level (Table 2).

Variance analysis of species abundance

Fifteen phyla and 120 genera in total were found between the two groups. Compared with the normal group, the model group had a two-fold lower number of species of *Spirochaetes*, *Fusobacteria* and *Deinococcus-Thermus* [$\log_2$(DN/DM) > 1], but a higher number of species of *Verrucomicrobia*, *Actinobacteria*, *Planctomycetes* and *Euryarchaeota* [$\log_2$(DN/DM)< -1]. A significant difference in the number of species of *Fusobacteria* was found between the two groups ($P < 0.05$) (Table 3 and Figure 5A). Compared with the control group, the model group had more than a two-fold lower number of species of 25 genera, including *Anaeroplasma*, *Desulfovibrio*, *Fusobacterium*, *Helicobacter*, *Roseburia* and *Sporobacter*, but more than twice the number of species of 32 genera, including *Aerococcus*, *Bifidobacterium, Coprococcus*, *Escherichia*, *Lactobacillus* and *Proteus*. Significant differences in the number of species of *Helicobacter* and *Roseburia* were found between the two groups ($P < 0.05$) (Table 4 and Figure 5B). In the model group, the percentages of species that was more than two-fold lower and higher were 20% and 27% at the phylum level and 32% and 41% at the genus level compared with the normal group, while the percentages of species that was less than twice were 53% and 27% at the phylum and genus levels, respectively (Figure 5C).

Single sample species distribution

The information on classification and abundance in the OTU list and the map pie chart on each species distribution were sorted. At the phylum level, 15 phyla were found in both groups. In the normal group, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Tenericutes*, *Deferribacteres*, *TM7*, *Actinobacteria*, *Lentisphaerae*, *Aquificae*, *Fusobacteria*, *Deinococcus-Thermus* and *Spirochaetes* were found, whereas in the model group, *Euryarchaeota, Verrucomicrobia* and *Planctomycetes* with trace amounts of *Fusobacteria*, *Deinococcus-Thermus* and *Spirochaetes* were found. The percentage distributions of the microbiome community in the normal and model rats were 47 and 48 for *Firmicutes*, 38 and 37 for *Bacteroidetes* and 14 and 14 for *Proteobacteria*, respectively. Both groups had less than 1 for *Tenericutes, Actinobacteria*, *Deinococcus-Thermus*, *Euryarchaeota* and *Fusobacteria* (Figure 6). At the genus level, the percentage distributions of the microbiome community in the normal and model rats were 30 and 6 for *Helicobacter*, 12 and 1 for *Roseburia*, 10 and 13 for *Lactobacillus*, 8 and 7 for *Parasutterella*, 7 and 7 for *Oscillibacter*, 4 and 1 for *Desulfovibrio*, 4 and 19 for *Alistipes*, 2 and 9 for *Ruminococcus* and 1 and 21 for *Escherichia*, respectively (Figure 7).

Table3 Variance analysis of species abundance at the phylum level

$\log_2$ (N/M)	Taxon
$\log_2$ (N/M)> 1	Bacteria; Deinococcus-Thermus
3	Bacteria; Fusobacteria[a]
	Bacteria; Spirochaetes
$\log_2$ (N/M)<-1	Archaea; Euryarchaeota
4	Bacteria; Actinobacteria
	Bacteria; Planctomycetes
	Bacteria; Verrucomicrobia

For bacteria; Fusobacteria; [a]$P < 0.05$ *vs* control ($P = 0.0222$).

β-diversity analysis

UniFrac β-diversity analysis represents the extent of similarity between different microbial communities. UniFrac PCoA (principal co-ordinate analysis) of 6666 OTUs (grouped at 97% sequence identity) showed a clear separation between the normal and model samples using weighted analysis (Figure 8). Percentage values at the axes indicate contribution of the principal components to the explanation of total variance in the dataset. The figure showed that the percentages of variation explained by PC1 and PC2 were 48.41% and 23.35%, respectively. The samples in the model group were well separated from those in the normal group based on the weighted UniFrac distances measured at the OTU level.

DISCUSSION

Intestinal barrier dysfunction can cause bacterial and endotoxin translocation, resulting in sepsis and eventually lung injury, the most important factor that initiates ARDS. However, the mechanism by which the intestine changes its mucosal barrier and microflora during ARDS remains unclear. The mucosa is an important aspect of bowel function, and the intestinal mucosal epithelial cells form a critical defensive barrier system against foreign bacteria. Disruption of the epithelial barrier results in bacterial recruitment and activation of mucosal immune cells, which initiate acute inflammation[23]. DAO activity is particularly high in the upper portion of the small intestinal villi; therefore, DAO level has been used as a marker of intestinal mucosal integrity[24]. Mammals only

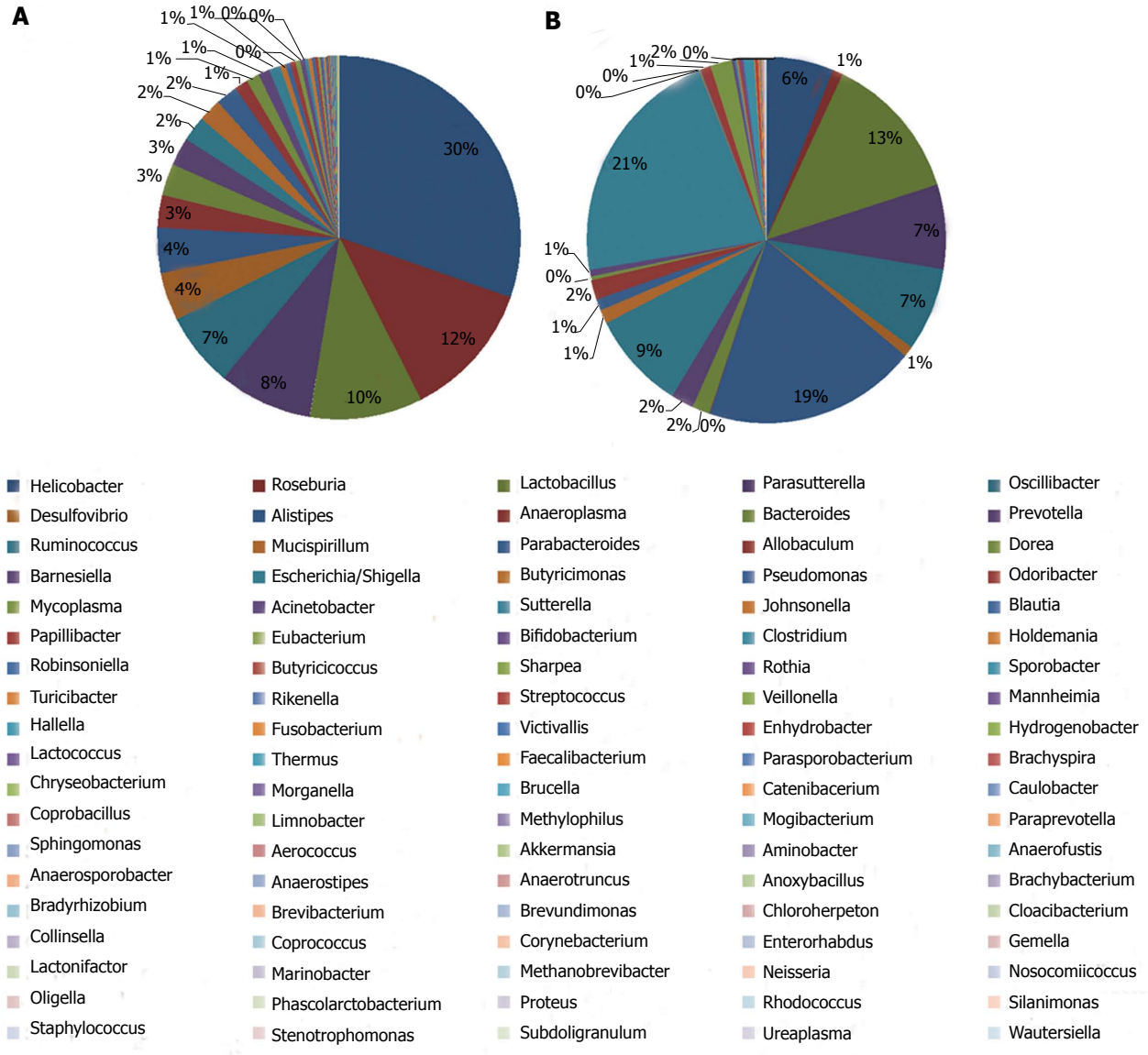

Figure 7 Microbial distributions at genus level in the samples from the normal and model groups. Percentages are based on proportions of assignable reads.

have L-lactate dehydrogenase and L-lactate as markers of cell hypoxemia, and the levels of these markers have been shown to correlate with the survival of septic shock patients[25,26]. However, bacteria have D-lactate dehydrogenase and produce D-lactate during fermentation. Thus, D-lactate levels in the blood not only reflect the status of intestinal mucosal damage, but also correspond to changes in intestinal mucosa permeability[27-28].

Intestinal flora generally does not change in healthy individuals, but can be affected by diseases. Scientists have studied the abundance and biodiversity of bacteria under healthy and disease conditions using high-throughput sequencing. An imbalance in gut microflora is also evident in diabetes[29], cancer[30-31] and obese patients[32]. Animal models provide the possibility of controlling these factors, a mechanistic understanding of host-microbial interactions and potentially allowing the rational design of future human studies. In this study, rats were killed within 24 h after intratracheal instillation of LPS. We investigated the characteristics of microflora in the fecal matter of rats using 16S rDNA-based molecular sequencing and found significant differences in the species and distribution between the model and control groups. The results showed higher biodiversity and species richness in the model group than in the control group. Compared with the model group, the control group had a significantly higher number of species of *Fusobacteria* (at the phylum level), *Helicobacter* and *Roseburia*, as determined by abundance difference analysis. This study is the first to reveal that the abundance of *Helicobacter* and *Roseburia* species significantly decreased in early ARDS, which may be the result of stress response. Increased microbial diversity and abundance were observed in the model group, but it is unknown whether these changes are due to passive damage to the body or a positive protective response mechanism. However, a close relationship was confirmed between the microflora and ARDS. ARDS can occur due to hypoxemia, activation of the systemic inflammatory response, injury to inflammatory mediators, damage by free radicals, immune destruction and irritation of the

Table 4 Variance analysis of species abundance at the genus level

Log_2 (N/M)	Taxon
Log_2 (N/M) > 1	Bacteria; Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma
25	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Brachyspiraceae; Brachyspira
	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Butyricimonas
	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Caulobacter
	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Chryseobacterium
	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio
	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Enhydrobacter
	Bacteria; Firmicutes; Clostridia; Clostridiales; Eubacteriaceae; Eubacterium
	Bacteria; Fusobacteria; Fusobacteria; Fusobacteriales; Fusobacteriaceae; Fusobacterium
	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Helicobacteraceae; Helicobacter[a]
	Bacteria; Aquificae; Aquificae; Aquificales; Aquificaceae; Hydrogenobacter
	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Limnobacter
	Bacteria; Proteobacteria; Betaproteobacteria; Methylophilales; Methylophilaceae; Methylophilus
	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Morganella
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Parasporobacterium
	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Robinsoniella
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Roseburia[c]
	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Rothia
	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Sharpea
	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas
	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Sporobacter
	Bacteria; Deinococcus-Thermus; Deinococci; Thermales; Thermaceae; Thermus
Log_2(N/M) <-1	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella
32	Bacteria; Firmicutes; Bacilli; Lactobacillales; Aerococcaceae; Aerococcus
	Bacteria; Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales; Verrucomicrobiaceae; Akkermansia
	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; Alistipes
	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum
	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; Aminobacter
	Bacteria; Firmicutes; Clostridia; Clostridiales; Eubacteriaceae; Anaerofustis
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Anaerosporobacter
	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Anaerotruncus
	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium
	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bradyrhizobium
	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Brevibacteriaceae; Brevibacterium
	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas
	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Butyricicoccus
	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium
	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Coprobacillus
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus
	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; Corynebacterium
	Bacteria; Actinobacteria; Actinobacteria; Coriobacteriales; Coriobacteriaceae; Enterorhabdus
	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia/Shigella
	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Faecalibacterium
	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus
	Archaea; Euryarchaeota; Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanobrevibacter
	Bacteria; Firmicutes; Clostridia; Clostridiales; Incertae Sedis XIII; Mogibacterium
	Bacteria; Tenericutes; Mollicutes; Mycoplasmatales; Mycoplasmataceae; Mycoplasma
	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria
	Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Nosocomiicoccus
	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Odoribacter
	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Paraprevotella
	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Proteus
	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Ruminococcus
	Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus
	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Turicibacter

For Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Helicobacteraceae; Helicobacter, [a]$P < 0.05$ *vs* control ($P = 0.0128$) and Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Roseburia [c]$P < 0.05$ *vs* control ($P = 0.0164$).

intestinal mucosa barrier, leading to intestinal bacterial translocation and changes in the intestinal environment. These mechanisms affect the distribution of intestinal flora.

In conclusion, research on the structure and function of intestinal microflora in ALI/ARDS can help to understand the close relationship between the lungs and the intestines and to provide a valid experimental basis for the important function of intestinal microflora in preventing and treating ALI/ARDS. We found that these microbes were involved in the pathogenesis of ARDS. This study

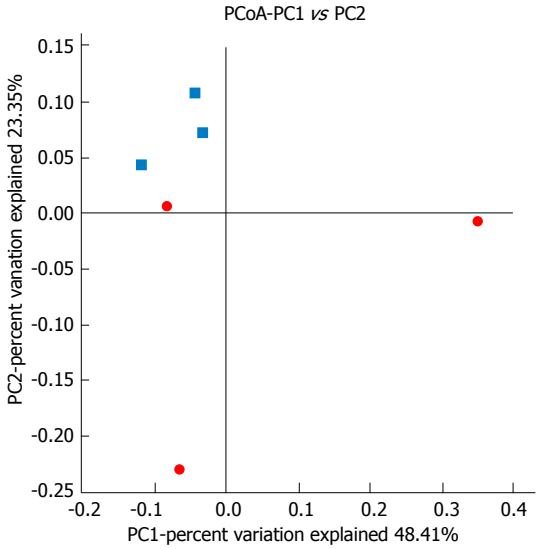

Figure 8 16S rDNA high-throughput sequencing revealed changes in microbial diversity in normal and acute respiratory distress syndrome rats. Clustering of microbial communities using PCoA of the weighted UniFrac matrix from six samples. The percentage of variation explained by the principal coordinates is indicated on the axes. Subject color coding: blue, samples from the normal group; red, samples from the model group. PCoA: Principal co-ordinate analysis.

is the first to employ high-throughput sequencing technology to determine changes in gut microbes in a rat ARDS model, and to reveal the pathogenesis of ARDS in this model. The results of this study may be used as a basis for improving the clinical treatment of ARDS. The detection of specific bacteria allows early detection and diagnosis of ALI/ARDS.

COMMENTS

COMMENTS

resulting in sepsis and eventually lung injury, the most important factor that initiates acute respiratory distress syndrome (ARDS). However, the mechanism by which the intestine changes its mucosal barrier and microflora during ARDS remains unclear. The intestinal flora generally does not change in healthy individuals, but it can be affected by diseases, such as ARDS.

Research frontiers

Scientists have studied the abundance and biodiversity of bacteria under healthy and disease conditions using high-throughput sequencing. Recent studies have employed high-throughput sequencing to reveal the specific relationship between diseases and intestinal flora. Imbalance of gut microflora is also evident in diabetes, cancer and obese patients.

Innovations and breakthroughs

The results of this study may serve as a reference for elucidating the relationship between the lungs and intestines. This study is the first to employ high-throughput sequencing technology to determine changes in the gut microbes of rat models with ARDS and to further reveal the pathogenesis of ARDS.

Applications

The results of this study may be used as a basis for improving the clinical treatment of ARDS. Overall, the detection of specific bacteria allows early detection and diagnosis of acute lung injury (ALI)/ARDS in the future.

Terminology

ARDS is a continuous pathological process, with ALI at its early stage. High-throughput sequencing is a technology used to sequence thousands to millions of DNA molecules, thus making a detailed and overall analysis of the transcriptome and the genome of a species possible, therefore it is also known as deep sequencing. High-throughput sequencing greatly contributes to the research on the diversity of environmental microbes, including uncultured microorganisms and trace amounts of bacteria.

Peer review

This is an experimental study for evaluation of the possible association between ARDS and intestinal microflora using an animal model and the high-throughput sequencing analysis. The study contributes to a better understanding of mechanisms by which the changes in intestinal mucosa barrier and host microflora could be involved in the pathogenesis of ARDS. The investigational significance is high due to very strong clinical and translational potential.

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RESEARCH REPORT

A pilot proof-of-concept study of a modified device for one-step endoscopic ultrasound-guided biliary drainage in a new experimental biliary dilatation animal model

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Abstract

AIM: To evaluate the technical feasibility of a modified tapered metal tip and low profile introducer for one-step endoscopic ultrasound (EUS)-guided biliary drainage (EUS-BD) in a new experimental biliary dilatation porcine model.

METHODS: A novel dedicated device for one-step EUS-guided biliary drainage system (DEUS) introducer has size 3F tapered catheter with size 4F metal tip for simple puncture of the intestinal wall and liver parenchyma without graded dilation. A self-expandable metal stent, consisting of both uncovered and nitinol-covered portions, was preloaded into DEUS introducer. After establishment of a biliary dilatation model using endoscopic hemoclips or band ligation with argon plasma coagulation in 9 mini-pigs, EUS-BD using a DEUS was performed following 19-G needle puncture without the use of fistula dilation devices.

RESULTS: One-step EUS-BD was technically successful in seven pigs [7/9 (77.8%) as intention to treat] without the aid of devices for fistula dilation from the high body of stomach or far distal esophagus to the intrahepatic (n = 2) or common hepatic (n = 5) duct. Primary technical failure occurred in two cases that did not show adequate biliary dilatation. In seven pigs with a successful bile duct dilatation, the technical success rate was 100% (7/7 as per protocol). Median procedure time from confirmation of the dilated bile duct to successful placement of a metallic stent was 10 min (IQR; 8.9-18.1). There were no immediate procedure-related complications.

CONCLUSION: Modified tapered metal tip and low profile introducer may be technically feasible for one-step EUS-BD in experimental porcine model.

Key words: Endoscopic ultrasound; Biliary drainage; Biliary dilation; Feasibility; Complications

Core tip: Endoscopic ultrasound (EUS)-guided biliary drainage (EUS-BD) has been restricted due to limited data and dedicated devices to perform safely without multi-step processes. We report a pilot study of a modified tapered metal tip and low profile introducer for one-step EUS-BD in a new experimental biliary dilatation porcine model. EUS-guided hepaticoenterostomy using a novel dedicated device for one-step EUS-BD system (DEUS) in an experimental bile duct dilatation porcine model might be technically feasible and effective. One-step stent insertion and deployment precluded the need for additional fistula dilation devices for graded dilation or needle cautery incision, without involving complex procedures or DEUS-related complications.

Lee TH, Choi JH, Lee SS, Cho HD, Seo DW, Park SH, Lee SK, Kim MH, Park DH. A pilot proof-of-concept study of a modified device for one-step endoscopic ultrasound-guided biliary drainage in a new experimental biliary dilatation animal model. *World J Gastroenterol* 2014; 20(19): 5859-5866 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5859.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5859

INTRODUCTION

Percutaneous transhepatic biliary drainage (PTBD) is most predominant salvage procedure used to access the biliary tract after failed endoscopic retrograde cholangiopancreatography (ERCP). However, PTBD-related adverse event rates of 9%-33% and mortality rates of 2%-15% have been reported[1-4]. Furthermore, in terms of the quality of life, long-term placement of external catheters is very uncomfortable for the patient.

Endoscopic ultrasound-guided biliary drainage (EUS-BD) has been introduced as an effective alternative to PTBD after failed ERCP. The potential benefits of EUS-BD include internal drainage, thereby avoiding long term external drainage in cases where external PTBD drainage catheters cannot be internalized. EUS-guided hepaticogastrostomy (EUS-HG), which is a specialized form of EUS-BD that allows biliary drainage from the intrahepatic bile duct to the stomach, can also be used as an additional and effective treatment alternative after failed ERCP. However, EUS-HG with transluminal stenting has inherent procedural complexity, as it requires fistula dilation prior to stenting, resulting in prolonged procedure time, loss of the guidewire, and several adverse events, including stent migration or bile peritonitis[5-10]. Clinical practice in this area has been restricted due to limited data, and specialized devices to perform this procedure safely are needed.

At present, dedicated endoscopic devices to EUS-BD/HG with transluminal stenting are limited. Therefore, we developed a novel device dedicated to one-step EUS-guided biliary drainage system (DEUS), which has a modified tapered metal tip and low profile introducer including partially covered self-expandable metal stent for one-step transluminal stenting after EUS-guided needle puncture without graded dilation or use of cautery incision. To tailor treatment to individual patients clinical training with this stent should be simple without serious complications. Furthermore, there is no proven biliary dilatation animal model suitable for evaluation of the efficacy of this specialized stent. This pilot animal study was performed to evaluate the technical feasibility and clinical outcomes of DEUS in an experimental biliary dilatation porcine model.

MATERIALS AND METHODS

This pilot proof-of-concept study was performed in accordance with the rules of the Institutional Animal Care Committee and the National Center of Efficacy Evaluation for the Development of Health Products Targeting Digestive Disorders. This study was approved by the Committee on Animal Research at Inha University and the Animal Protection Committee of the Korean government.

In vivo study using a porcine animal model

The *in vivo* experiments were performed using nine domesticated female mini-pigs with a mean weight of 40 kg (range 38-42 kg). Before endoscopic procedures, the animals were fasted for 48 h. On the day of the procedure, animals were sedated by intramuscular injection of tiletamine/zolazepam (Zoletil; 5 mg/kg) and xylazine (Narcoxyl; 2 mg/kg). Anesthesia was administered by intravenous injection (0.1 mg/kg) followed by continuous infusion (4-6 mg/h) of vecuronium. After tracheal intubation, the animals were ventilated with a 1:1 mixture of 1.0%-2.0% inspired isoflurane (Ifran®, Hana Pharm Co Ltd) and oxygen (5-10 mL/kg per minute). The animals were placed recumbently on their left sides on a fluoroscopy table. Vital signs were continuously monitored during the procedure. Prophylactic antibiotics were administered 3 d before and after EUS-guided intervention. The animals were maintained on their usual diet for 7 d following successful transluminal stent insertion. All animals were euthanized by potassium chloride overdose 7 d post-stent insertion.

Preparation of the experimental biliary dilatation porcine model

To date, no suitable biliary dilatation model or adequate dilatation training in animal studies has been available. Adequate biliary dilatation is essential for successful EUS-HE in porcine models. In preliminary to EUS-HE, we established a biliary dilatation model using endoclip closure or endoscopic band ligation (EBL) of the major papilla followed by argon plasma coagulation (APC System, power setting 40 W, gas flow 2 L/min; ERBE Elektromedizin GmbH, Tubingen, Germany) in the ampullary orifice (Figure 1). First, we made biliary dilatation model using endoclip closure with APC. After 2 wk, we

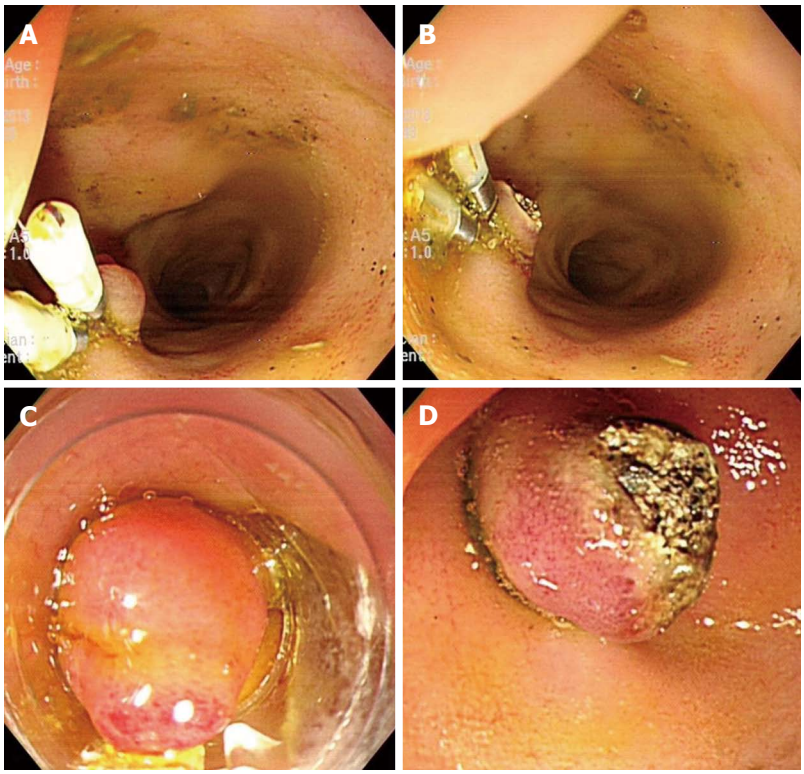

Figure 1 Experimental biliary dilatation model using endoclips and endoscopic band ligation with argon plasma coagulation. A, B: Argon plasma coagulation (APC) was performed on the orifice of ampulla on the duodenal bulb following multiple rounds of hemoclipping on the ampulla of Vater; C, D: In an alternate model, endoscopic band ligation was performed on the ampulla of Vater, again followed by APC in the same manner.

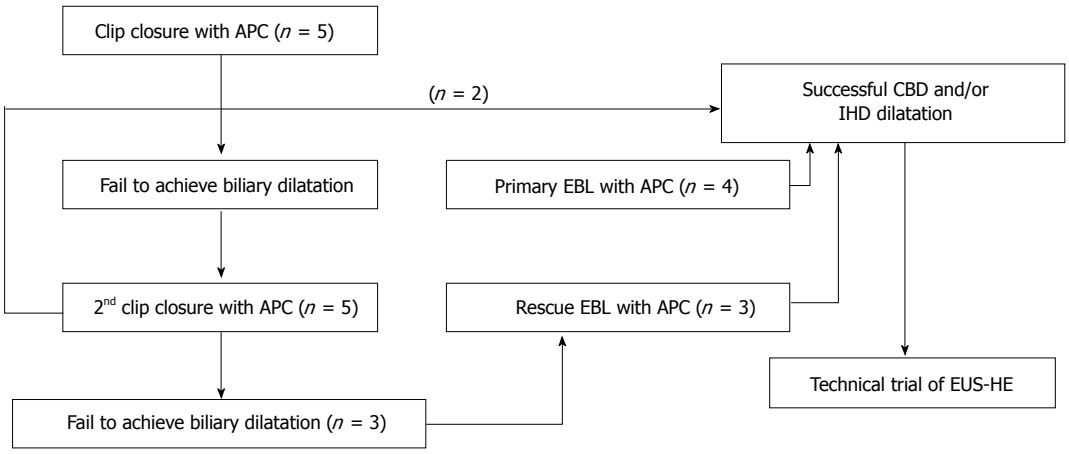

Figure 2 Flow of experimental endoscopic biliary dilatation using clip closure and/or endoscopic band ligation with argon plasma coagulation in a porcine model. APC: Argon plasma coagulation; EBL: Endoscopic band ligation; IHD: Intrahepatic bile duct; CBD: Common bile duct; EUS-HE: Endoscopic ultrasound-guided hepaticoenterostomy.

evaluated the efficacy of this first biliary dilatation model by EUS. Depending on the intra-/extra-hepatic bile duct dilatation, we attempted EUS-HE or switched to a second dilatation model using repeated clipping or EBL with APC (Figure 2). We evaluated duct dilatation by EUS at the same time intervals and attempted EUS-HE. This experimental animal model was created by three endoscopists (Park DH, Lee TH, Choi JH).

A novel dedicated DEUS

The DEUS introducer (Standard Sci Tech Inc., Seoul, South Korea) has 3F catheter with 4F tapered pentagonal metal tip for simple puncture of the intestinal wall and liver parenchyma without the need of graded dilation devices (Figure 3). The tapered metal tip and catheter may reduce wall resistance and enhance pushability during one-step tract puncture *via* a guidewire. The outer sheath of the delivery catheter is size 7F, which provides good pushability and adequate resistance. The concept of this 3F distal/4F metal tip device was inspired by a 4F tip balloon catheter with stainless steel stylet (Hurricane Balloon, Boston Scientific, Natick, Mass) that we found

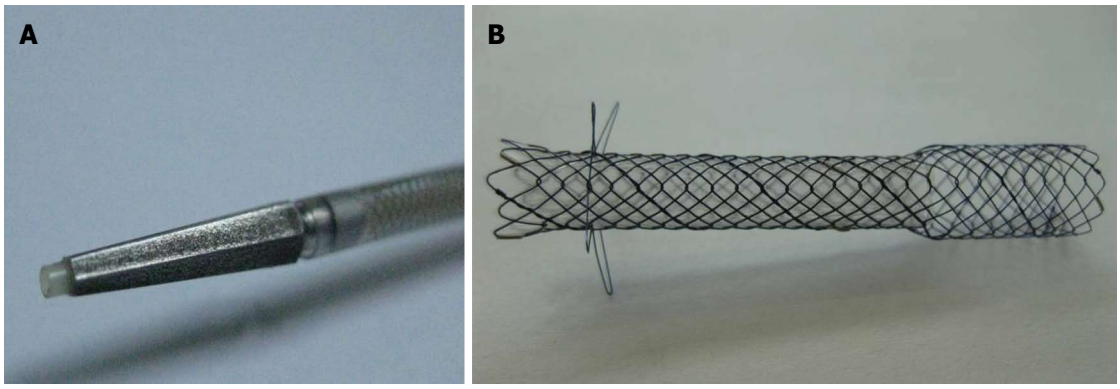

Figure 3 Gross finding of dedicated device for one-step endoscopic ultrasound-guided biliary drainage system. A: The 7F delivery sheath and 3F catheter with 4F tapered pentagonal metal tip; B: Gross finding of the metallic stent. The uncovered proximal end of the stent (8 mm in diameter and 15 mm in length), the body and distal portions of the covered stent with a silicone membrane (6 mm in diameter and 35 or 45 mm in length), and the distal end with four flaps.

to have high primary fistula puncture and dilation performance during EUS-HG on our institutional experiences. A self-expandable metal stent, consisting of both uncovered and nitinol-covered portions, was preloaded into the catheter. The uncovered proximal end of the stent (8 mm in diameter and 15 mm in length), which is funnel shaped to prevent small-branched bile duct obstruction and distal migration, was placed into the bile duct. The body and distal portions of the stent were covered with a silicone membrane (6 mm in diameter and 35 or 45 mm in length) for the prevention of bile leaks, and the distal end was equipped with four flaps for the prevention of inward stent migration (Figure 3).

One-step EUS-HE technique

EUS-HE was performed using a GF-UCT 240 linear-array echoendoscope together with the EU-ME-1 Ultrasound System (Olympus Medical Systems, Tokyo, Japan) by two experienced endoscopists (Park DH, Lee TH). We performed EUS-HE using the porcine biliary dilatation model after confirming the route and dilatation of the biliary duct *via* EUS-guided contrast injection into the intrahepatic bile duct (IHD) or common bile duct (CBD) with the endoscope positioned in the stomach high body or the duodenal bulb. If the IHD was dilatated > 5 mm, then it was punctured directly *via* the liver parenchyma. However, in cases of inadequate IHD dilatation (< 5 mm), despite adequate CBD dilatation (> 7 mm), the puncture was made near the hilum through the liver parenchyma. Since the porcine ampulla of Vater is anatomically much closer to the duodenal bulb, choledochoduodenostomy is technically restricted; consequently, we initially planned to perform a hepaticoenterostomy; hepatic gastrostomy on the high body of stomach or hepaticoesophagostomy on the far distal esophagus. We punctured the bile duct using a 19-G fine aspiration needle (EZ Shot2TM, Olympus Medical Systems, Tokyo, Japan) followed by bile aspiration and radiopaque contrast injection to delineate the CBD and IHD under fluoroscopy. Next, we inserted a 0.025-inch guidewire (VisiGlide, Olympus Medical Systems, Tokyo, Japan) through the EUS-guided 19-G needle and coiled it into the bile duct lumen for transluminal stent placement. EUS-HE was performed according to the direction that the guidewire was inserted into the CBD/IHD. Without using a graded dilation catheter, bougie, or needle-knife cautery, the DEUS was directly inserted and the metallic stent then sequentially deployed over the guidewire (Figure 4). Technical success was defined as metal stent placement along the intestine to the bile duct without the use of fistula dilation methods, such as a catheter, bougie, balloon dilator or needle-knife, and by the flow of contrast medium and/or bile through the stent. Early adverse events were defined as any procedure or stent-related complications that occurred within 48 h.

Pathologic examination

The liver and intestine from esophagus to duodenum were excised for gross examination. Following examination for bile leakage, perforation, or stent dislocation, the tissue specimens near the stent location were fixed for 72 h in formalin and then sectioned. Sectioned tissues were stained with hematoxylin-eosin and Wheatley's trichrome. The severity of inflammation, fibrosis, mucosal sloughing, or hyperplasia were evaluated in each section. All pathologic examinations were performed by a single experienced gastrointestinal pathologist (Cho HD).

RESULTS

Technical feasibility of DEUS

One-step EUS-HE was successful in seven porcine subjects [7/9 (77.8%) as intention to treat], all of which underwent transluminal stenting following EUS-guided 19G needle puncture without graded fistula dilation. The route of EUS-HE was from the high body of stomach (n = 4) or far distal esophagus (n = 3) to the IHD (n = 2) or CHD (n = 5). The median procedure time from the time of confirmation of dilated IHD and/or CHD to that of successful stent placement was 10 minutes (IQR; 8.9-18.1). The stent used was either 5 cm (n = 2) or 6 cm (n = 4) in length. In one case both 5- and 6-cm stents were

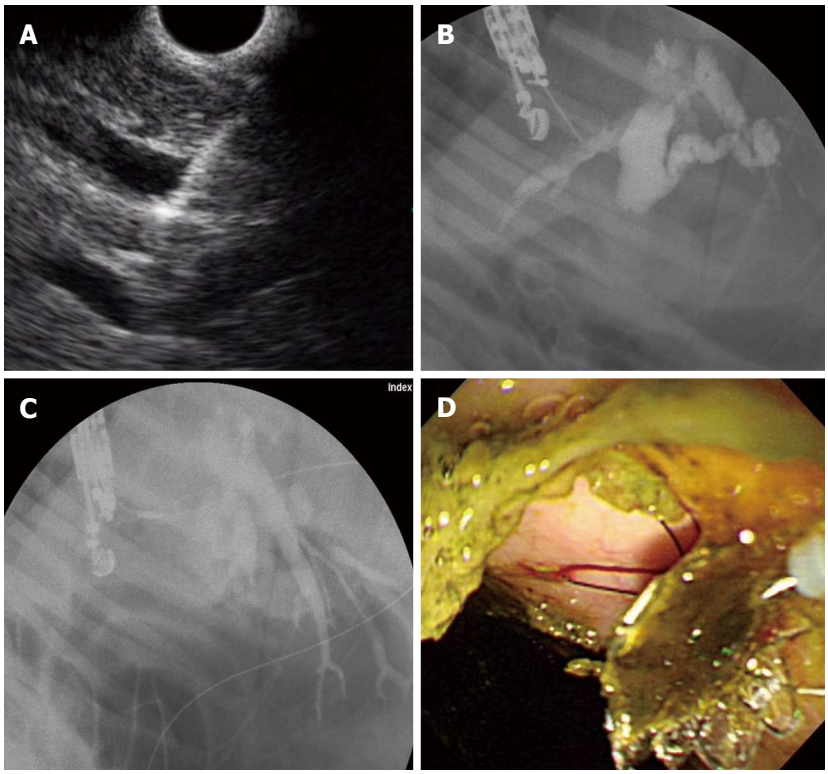

Figure 4 Endoscopic ultrasound-guided hepaticoenterostomy. A: Linear endoscopic ultrasound (EUS) image shows a 19-G needle puncture targeting the intrahepatic bile duct (IHD; B: Fluoroscopic image reveals marked IHD and common bile duct dilatation with ampullary obstruction; C: After EUS-guided 19-G needle puncture of the IHD, the device for one-step endoscopic ultrasound-guided biliary drainage system was inserted and the stent released under the guidance of EUS and fluoroscopy; D: Following successful deployment of the stent, the bile passage was seen in the high body of stomach.

used due to underestimation of the tract length (Table 1). Six of seven pigs underwent successful EBL plus APC (n = 4, primary band ligation with APC; n = 3, band ligation after failure of second clipping with APC). In the two cases of primary clipping with APC, only one had successful stent placement following the repeated puncture and insertion trial. Primary technical failure occurred in two cases due to inadequate dilatation of the CBD or IHD as compared with the successfully dilated cases (7.3 mm *vs* 11.7 mm in CBD, P = 0.013; 1.8 mm *vs* 4.3 mm in IHD, P = 0.037). In the seven pigs with adequate bile duct dilatation, the technical success of DEUS was 100% (7/7 as per protocol).

Procedure-related complications and pathology

There were no immediate post-procedure or stent-related complications. One pig died 2 d after the procedure from bile leakage, according to autopsy. This subject underwent numerous needle punctures (5 attempts) and contrast injections prior to stent insertion. Necropsy findings in the other pigs showed no signs of bile peritonitis, major vessel injuries, or adjacent organ damage. The gross pathology specimens showed a well-demarcated lesion from the distal esophagus to the IHD through the surrounding liver parenchyma. Microscopic examination revealed mild inflammation and necrotic tissues around the metallic stent location but without complications (Figure 5).

DISCUSSION

EUS-BD with transluminal stenting has an inherent procedural complexity, especially in the case of graded dilation prior to stent insertion. Repeated dilation can be performed with sequential use of 4F ERCP catheters followed by 6F and 7F bougie dilators. Alternatively, needle-knives or cystotome cautery incision followed by stent placement can be used[5-17]. However, the drawback of a needle knife is the higher probability of adverse events as compared with graded dilation[6,7]. In addition, these multi-step procedures may be time consuming and increase the risk of procedure-related complications, such as bile leak, pneumoperitoneum, or bleeding. At present, no dedicated endoscopic devices for EUS-BD with transluminal stenting are available, except for a pseudocyst or gallbladder drainage[18].

To overcome these problems, we explored a modified delivery system involving deployment of a self-expandable metal stent for one-step EUS-guided HE. First, the DEUS delivery catheter has a 3F catheter tip and tapered pentagonal 4F metal tip for simple puncture, precluding the need for dilation devices, such as bougies, catheters, needle-knives, or balloon dilations. Furthermore, the stent introducer is also composed of a 7F introducer sheath, which may have reduced wall resistance and good pushability compared with conventional 8F, or 8.5F stent introducers for EUS-HE. Metallic stent in DEUS

Table 1 Outcomes of a novel dedicated device for one-step EUS-guided biliary drainage system and associated complications

CBD diameter, mm	IHD dilatation, mm	Route	Stent length, cm	Technical success	Procedure time, min	Adverse events	Mortality
6.2	1.6	-	-	Failure	20	-	-
13.2	5.2	S to IHD	6	Success	10	-	-
8.4	2.0	-	-	Failure	31	-	-
12.5	6.2	S to CHD	5, 6	Success	8.4	-	-
13.6	5.8	E to IHD	5	Success	16.2	Bile leakage[1]	Expire, day 3
11	3.3	E to CHD	5	Success	8	-	-
9.2	3.5	S to CHD	6	Success	9.4	-	-
12.8	3.8	E to CHD	6	Success	13.2	-	-
10	2.8	S to CHD	6	Success	10	-	-

[1]Related to multiple needle puncture, no obvious bile leakage on persistent areas on necropsy. CBD: Common bile duct; CHD: Common hepatic duct; IHD: Intrahepatic bile duct; S: The high body of stomach; E: Far distal esophagus.

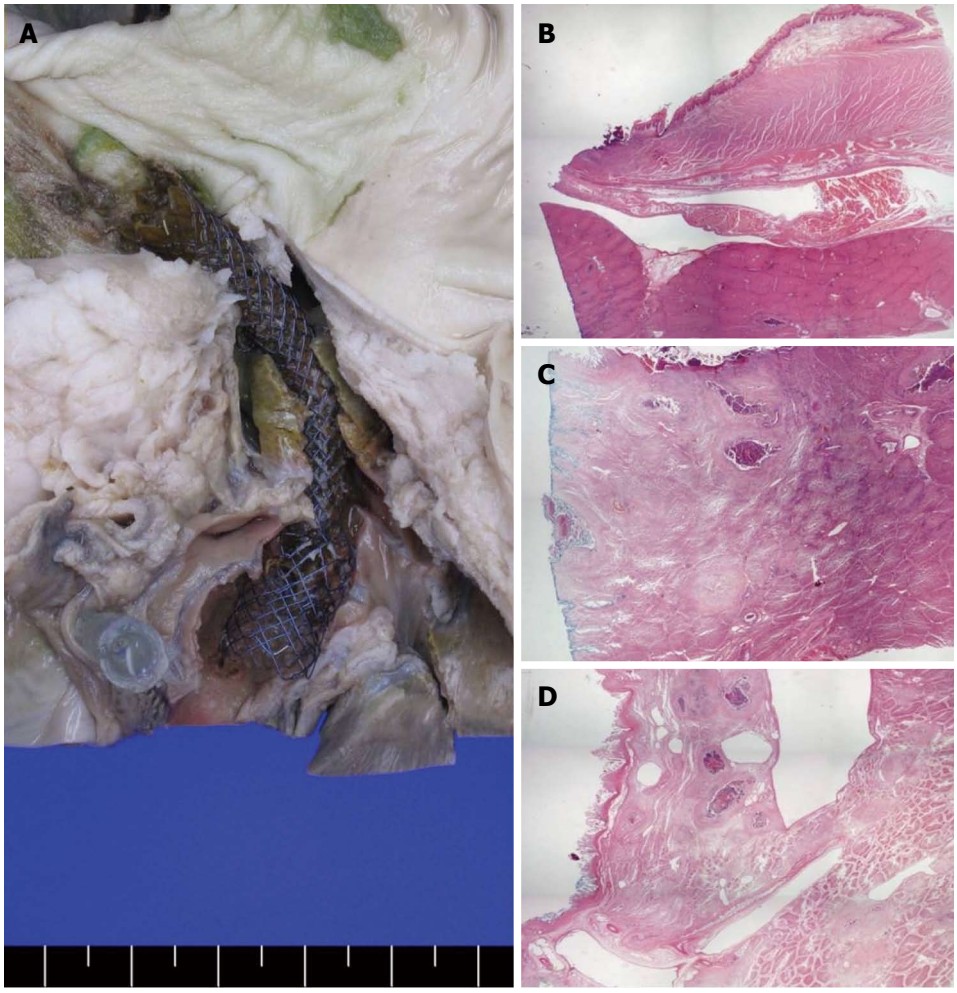

Figure 5 Gross and microscopic findings in the autopsy specimens. A: Gross hepaticoenterostomy specimens showed that stent insertion along the far distal esophagus to the intrahepatic bile duct (IHD) *via* the liver parenchyma did not cause complications, such as stent dislocation or migration (left); B-D: Microscopic findings showed the distal esophagus (B), liver parenchyma (C), and IHD (D) sections adjacent to the inserted metallic stent. Surrounding mild inflammation and necrotic tissue were seen but without any other complications, such as abscess or perforation.

is the first device targeted by EUS-HE. DEUS exhibited high technical feasibility in the biliary dilatation model without use of any dilation processes despite the small number of cases. In the seven successful stent placement cases, puncture of the high body of stomach or far distal esophagus was relatively easy and potentially reduces procedure time, since DEUS does not require sequential dilation. Second, the design of the newly developed stent comprises an uncovered proximal segment to minimize obstruction of the bile duct side branches, and the remaining longer portion is covered with silicone to prevent bile leakage; it also has multiple flaps on the distal portion to prevent inward migration. No stent migration was evident in any of the successful cases. According to

pathologic evaluation, hepaticoenterostomy caused well-demarcated tract maturation without bile leakage and peri-fistula abscess formation according to the short-term follow-up. This suggests that EUS-HE with metal stenting may cause minimal tissue damage around the fistula.

Regarding complications, there were no immediate DEUS-related complications during the procedure. In the success groups of the initial trial, stent insertion on the first attempt, deployment, and bile drainage were all successful. The one mortality case underwent multiple rounds of clipping for duct dilatation, and we attempted several IHD and CBD punctures to estimate duct dilatation and obstruction. Although successful placement of the stent was achieved, the repeated needle punctures (5 attempts) and contrast injections required may have induced delayed bile leakage or microperforations. At autopsy, large amounts of bile were detected in the peritoneum, but neither migration nor inappropriate positioning of the stent was detected. In addition, no obvious bile leakage was noted on necropsy. Relatively short duration of and inadequate bile duct dilatation may cause technical difficulties and even mortality. CBD and IHD dilatations in the two failed cases were less than those in the successful cases. If adequate dilatation is achieved during long-term periods, needle puncture and stent insertion may be more technically feasible.

In addition, there was no available bile duct dilatation porcine model for EUS-BD. In our experimental model, we found that EBL plus APC showed sufficient IHD and CBD dilatation compared with multiple rounds of endoclipping plus APC. In the EBL model, we were able to insert DEUS directly after 2 wk in four of the cases, and after 1 wk in the other three cases, which had initially failed the second round of endoclipping. As a training model for porcine biliary dilatation, EBL with APC may be effective for EUS-guided biliary intervention. However further evaluation of the use of APC and the time period necessary for ideal biliary dilatation should be performed, especially for IHD dilatation, which was not as great as that of the CBD during the observational period.

The limitations of this study included the small sample size and a non-comparative design, as we did not make comparisons with other current metal or plastic stents. Second, our result regarding procedure time may be exaggerated in this porcine model. The procedure time was relatively short in this study. Despite of the advance of devices, it may be due to the relatively thin gastric and esophageal wall in pigs. Third, we autopsied all pigs after short-term follow-up. Even though the live pigs were healthy until the time of autopsy, long-term follow up is necessary to evaluate stent patency and complications. Fourth, bile duct dilatation or obstruction by clipping/EBL plus APC was estimated by serial EUS prior to endoscopy. Less invasive methods and effective dilatation models with respect to technique and timing should be developed for more advanced training.

In conclusion, this modified tapered metal tip and low profile 7F introducer may be technically feasible for EUS-HE in an experimental bile duct dilatation porcine model. One-step EUS-HE using DEUS did not require additional fistula dilation devices for graded dilation or needle knife cautery or involve complex procedures. However, this *in vivo* study was limited by the small number of subjects. Based on this pilot study results, human clinical trials may be warranted to confirm technical feasibility and safety.

COMMENTS

Background

Endoscopic ultrasound (EUS)-guided biliary drainage (EUS-BD) may be an effective alternative to percutaneous transhepatic biliary drainage after failed endoscopic retrograde cholangiopancreatography (ERCP). However, its clinical practice has been restricted due to limited data and devices to perform the procedure safely.

Research frontiers

Although EUS-BD may be a useful alternative, there have been few studies proving the feasibility and safety of dedicated devices to EUS-BD. In this study, the authors demonstrated the technical feasibility of a novel dedicated device for one-step EUS-BD in a new experimental biliary dilatation porcine model.

Innovations and breakthroughs

EUS-BD with transluminal stenting has inherent procedural complexity, such as sequential dilation of the fistula after needle puncture prior to stenting, resulting in a prolonged procedure time, loss of the guidewire, or several adverse events. This is the first pilot study to report that a novel dedicated device for one-step EUS-BD in an experimental bile duct dilatation porcine model was technically feasible and relatively safe.

Applications

By experimental animal study, EUS-BD using this device may represent a future strategy for therapeutic intervention in the treatment of patients with malignant biliary obstruction and/or who failed ERCP.

Terminology

One-step EUS-guided hepaticoenterostomy (EUS-HE): One-step EUS-HE is the placement of biliary stents without the aid of devices for fistula dilation from the high body of stomach or far distal esophagus to the intrahepatic or common hepatic duct.

Peer review

The authors examined the feasibility of a modified delivery system for one-step EUS-BD. It is an *in vivo* pilot study in a biliary dilatation porcine model and, despite the small number of cases, the device developed by the authors could be an alternative to the usual technique, less complicated and offering reduction of the procedure time.

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ABCB4 mutations underlie hormonal cholestasis but not pediatric idiopathic gallstones

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Abstract

AIM: To investigate the contribution of *ABCB4* mutations to pediatric idiopathic gallstone disease and the potential of hormonal contraceptives to prompt clinical manifestations of multidrug resistance protein 3 deficiency.

METHODS: Mutational analysis of *ABCB4*, screening for copy number variations by multiplex ligation-dependent probe amplification, genotyping for low expression allele c.1331T>C of *ABCB11* and genotyping for variation c.55G>C in *ABCG8* previously associated with cholesterol gallstones in adults was performed in 35 pediatric subjects with idiopathic gallstones who fulfilled the clinical criteria for low phospholipid-associated cholelithiasis syndrome (LPAC, OMIM #600803) and in 5 young females with suspected LPAC and their families (5 probands, 15 additional family members). The probands came to medical attention for contraceptive-associated intrahepatic cholestasis.

RESULTS: A possibly pathogenic variant of *ABCB4* was found only in one of the 35 pediatric subjects with idiopathic cholesterol gallstones whereas 15 members of the studied 5 LPAC kindreds were confirmed and another one was highly suspected to carry predictably pathogenic mutations in *ABCB4*. Among these 16, however, none developed gallstones in childhood. In 5 index patients, all young females carrying at least one pathogenic mutation in one allele of *ABCB4*, manifestation of LPAC as intrahepatic cholestasis with elevated serum activity of gamma-glutamyltransferase was induced by hormonal contraceptives. Variants *ABCB11* c.1331T>C and *ABCG8* c.55G>C were not significantly overrepresented in the 35 examined patients with suspect LPAC.

CONCLUSION: Clinical criteria for LPAC syndrome

caused by mutations in *ABCB4* cannot be applied to pediatric patients with idiopathic gallstones. Sexual immaturity even prevents manifestation of LPAC.

Key words: Idiopathic cholelithiasis; Intrahepatic cholestasis; Oral contraceptives; Low phospholipid-associated cholelithiasis; Gallbladder disease 1

Core tip: Mutations in *ABCB4* are not overrepresented in children with idiopathic gallstones who fulfill the clinical and laboratory criteria for low phospholipid-associated cholelithiasis syndrome (Gallbladder Disease 1, OMIM #600803). Sexual immaturity prevents manifestation of low phospholipid-associated cholelithiasis. In young females, manifestation of low phospholipid-associated cholelithiasis syndrome such as intrahepatic cholestasis with elevated serum activity of gamma-glutamyltransferase may be induced by hormonal contraceptives.

Jirsa M, Bronský J, Dvořáková L, Šperl J, Šmajstrla V, Horák J, Nevoral J, Hřebíček M. *ABCB4* mutations underlie hormonal cholestasis but not pediatric idiopathic gallstones. *World J Gastroenterol* 2014; 20(19): 5867-5874 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5867.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5867

INTRODUCTION

Low phospholipid-associated cholelithiasis syndrome (LPAC, synonym Gallbladder disease 1, OMIM #600803) has been defined as symptomatic and recurring cholelithiasis associated with mutations in *ABCB4* encoding multidrug resistance protein 3 (MDR3), the canalicular phospholipid export pump[1,2]. LPAC should be suspected in patients with symptomatic cholelithiasis in whom at least one minor criterion is present. These minor criteria are proposed as: (a) age below 40 years at the onset of symptoms; (b) recurrence after cholecystectomy; (c) intrahepatic hyperechoic foci with a topography compatible with lipid deposits along the luminal surface of the intrahepatic biliary tree; (d) intrahepatic sludge; (e) microlithiasis; (f) history of gallstones in first-degree relatives; or (g) history of intrahepatic cholestasis of pregnancy[3]. The distribution of associated *ABCB4* mutations in conserved regions of the gene, as well as their type, strongly support the role of partial MDR3 deficiency in LPAC, with decreased MDR3 activity and/or expression altering biliary lipid composition.

Apart from LPAC, mutations in *ABCB4* that reduce but do not abrogate the activity of MDR3 can cause a variety of milder forms of familial intrahepatic cholestasis type 3 (OMIM #602347), with slowly progressive or non-progressive hepatobiliary disease or anicteric cholestasis with varying liver fibrosis in adulthood[4]. Several reports[5-7] have shown that intrahepatic cholestasis of pregnancy is associated with *ABCB4* mutations in some women. Finally, the idea that contraceptive-induced cholestasis (CIC) may be associated with mutations in *ABCB4* has also been proposed. Asymptomatic gallstones and clinically silent cirrhosis, diagnosed later as progressive familial intrahepatic cholestasis type 3, became manifest in a 17-year-old girl when cholestasis developed on ingestion of contraceptive pills containing ethinylestradiol 30 μg, and levonorgestrol 150 μg[8], and isolated gallstone disease unmasked by oral contraception and associated with *ABCB4* mutation has been reported[1]. In contrast, no mutations in *ABCB4* were found in 5 subjects with CIC studied by Lang *et al*[9].

In our previous study[10] we focused on the role of the common variants c.523A>G (p.Thr175Ala) and c.1954A>G (p.Arg652Gly) in *ABCB4*, c.1331T>C (p.Val444Ala) in *ABCB11* and c.55 G>C (p.Asp19His) in *ABCG8* in pediatric gallstone disease. These variants are considered either as potentially pathogenic or as susceptibility alleles for cholesterol cholelithiasis in adults; however, they were not observed to contribute to genetic predisposition to gallstones in childhood[10].

In this study we investigated: (1) the role of *ABCB4* mutations in the etiology of pediatric idiopathic gallstones; and (2) the capability of hormonal contraceptives to unmask hitherto clinically silent MDR3 deficiency.

MATERIALS AND METHODS

Pediatric patients with gallstones

Pediatric patients with gallstones were selected as described[10] (see Figure 1 for the selection algorithm). Briefly, 109 children (53 males and 56 females) with gallbladder gallstones who had been hospitalized at the Department of Pediatrics, Faculty Hospital Motol, Prague, between 1995-2004, were considered. In 22 patients, gallstones were clearly associated with another disease such as Down syndrome, Gaucher disease, cystic fibrosis, hemolytic anemia, inflammatory bowel disease, immune deficiency and Gilbert syndrome. Thirty-three of the 87 invited patients did not respond. In 13 of 54 patients, the etiology of gallstones was uncertain. However, as these 13 patients had at least one of the following: long-term parenteral nutrition, treatment with cephalosporins or furosemide, dyslipidemia, hepatobiliary infectious disease or obesity (BMI > 27), *e.g.* conditions that could promote gallstone formation, they were not enrolled. In 41 patients, gallstones were most likely idiopathic. For *ABCB4* mutation testing, only 35 of these 41 patients (including only one of the monozygous twins) with idiopathic gallstones were selected who had at least one parent or grandparent with gallstones. These subjects (15 males and 20 females with positive family history), all unrelated Caucasians of Czech origin, met the major criterion and minor criteria (a) and (f) of Rosmorduc and Poupon[3]. The mean age at diagnosis of cholelithiasis was 10.7 ± 5.0 years (range 1-17). Nineteen of these 35

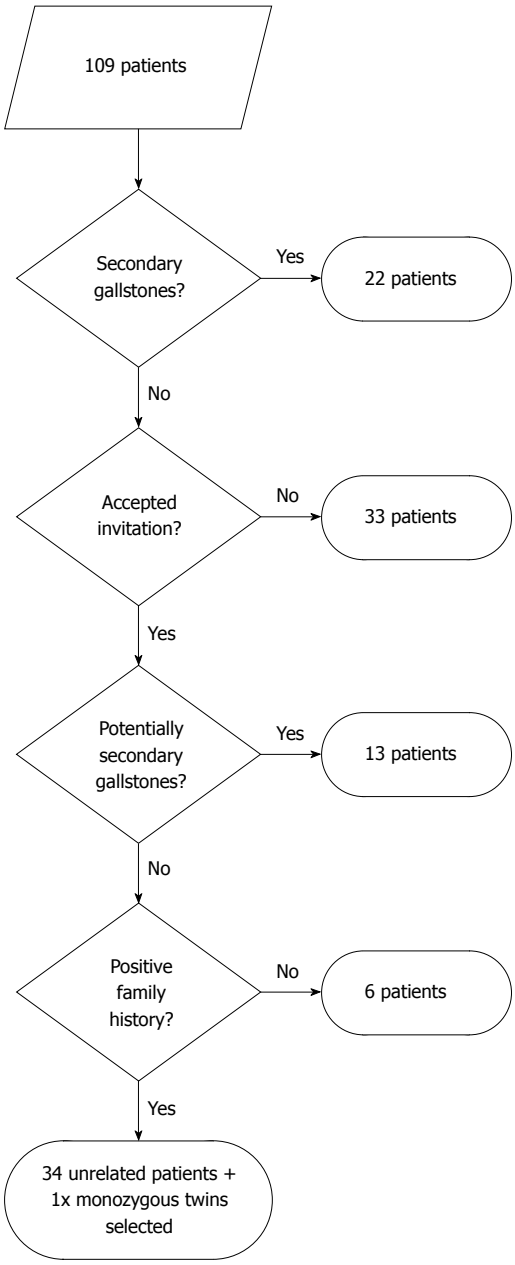

Figure 1 Flowchart describing the algorithm for selection of pediatric patients with low phospholipid-associated cholelithiasis syndrome-like idiopathic gallbladder gallstones.

patients (13 girls and 6 boys) underwent cholecystectomy with no recurrence after surgery. As of this writing, all cholecystectomized patients are well, without abdominal pain or jaundice.

Young adults with suspect LPAC

Five young adult female patients with symptomatic gallstones (age below 40 years at the onset of symptoms), a history of intrahepatic cholestasis, and a family history of gallstones in first-degree relatives were referred for *ABCB4* analysis. Their clinical characteristics are summarized in Table 1. None of the patients had hyperechoic foci in the liver parenchyma or proven intrahepatic sludge; duodenal bile was not investigated for microlithiasis. Nonethless, all met the proposed criteria for LPAC[3]. In patient I , intrahepatic cholestasis associated with exposure to an oral contraceptive containing ethinylestradiol 0.030 mg, and levonorgestrel 0.125 mg (Minisiston; Jenapharm, Jena, Germany) was the first clinical symptom of LPAC. Cholestasis resolved rapidly after withdrawal of the contraceptive. However, the patient developed cholecystolithiasis within one year despite ursodeoxycholic acid administration (15 mg/kg) and underwent cholecystectomy. One year later, ursodeoxycholic acid was withdrawn because the patient was completely asymptomatic with normal clinical/laboratory test results. Rechallenge with another oral contraceptive containing ethinylestradiol 0.020 mg and desogestrel 0.150 mg (Mercilon; Organon, Oss, The Netherlands) two years after cholecystectomy was followed within several weeks by a second attack of cholestasis. Clinical and laboratory findings improved again rapidly when the medication was withdrawn. None of the other 4 index patients mentioned any problems associated with the use of contraceptives; the data on contraceptives presented in Table 1 were obtained in part from clinical records and in part by specific questioning.

The patient studies were approved by the Institutional Review Board of the Faculty Hospital Motol. Either both parents or the examined subjects, when aged over 15 years, gave written informed consent before blood sampling.

Mutational analysis

Twenty-seven fragments covering all exonic (protein-coding) regions of *ABCB4* and including portions of adjacent intronic sequences were amplified from genomic DNA by PCR (primer sequences are available from the corresponding author). The DNA sequence of purified PCR products was analyzed on an ABI-PRISM 3100-Avant automated DNA sequencer (Applied Biosystems, Foster City, CA). Ensembl Acc. No. ENSG00000005471 and GenBank Acc. No. NM_018849.2 served as genomic and cDNA reference sequences. Mutations found by DNA sequencing were independently confirmed by restriction fragment length polymorphism analysis after digestion of the corresponding PCR product with restriction enzymes. In addition, *ABCB4* was scanned for deletions/duplications by multiplex ligation-dependent probe amplification, using SALSA MLPA KIT P109 *ABCB4* (MRC-Holland, Amsterdam, The Netherlands) according to manufacturer's instructions.

The low expression allele c.1331T>C of *ABCB11*[11] was detected as the presence of a PCR-*Bsu*RI restriction fragment length polymorphism. The variation c.55G>C in *ABCG8* associated with cholesterol gallstones in adults[12] was detected as described by Hubáček *et al*[13]. Pathogenicity of missense variations was predicted *in silico* by SIFT[14], PMut[15], PolyPhen-2[16] and MutationTaster[17].

Statistical analysis

The data are presented as mean and standard deviation,

Table 1 Characteristics of young women with low phospholipid-associated cholelithiasis syndrome and proven *ABCB4* mutations

Patient ID	Year of birth	Age at cholecystectomy (yr)	Age at liver biopsy (yr)	Age at pregnancies (yr)	Age (yr), contraceptive, complication
I	1980	20	19, periportal fibrosis	Nulliparous	18 Minisiston, withdrawn for CIC 22 Mercilon, withdrawn for CIC
II	1978	17	21, periportal fibrosis	Nulliparous	18 Tri-regol, withdrawn for CIC
III	1973	22	22, periportal fibrosis	30, with ICP	20-22 Cilest, withdrawn for CIC
IV	1967	28	31, normal histology	19, without ICP	28 Tri-regol, withdrawn for CIC 39-now Lunafem, tolerated
V	1973	20	20, periportal fibrosis	23, without ICP 32, without ICP	19-?, withdrawn for CIC 24-31 Marvelon, tolerated 32-now Mirena, permanent pruritus, GGT twice normal

Chemical composition of contraceptives: (1) Minisiston (Jenapharm, Jena, Germany): ethinylestradiol 0.030 mg; levonorgestrel 0.125 mg; (2) Mercilon (Organon, Oss, The Netherlands): ethinylestradiol 0.020 mg; desogestrel 0.150 mg; (3) Tri-regol (Chemical Works of Gedeon Richter, Budapest, Hungary): ethinylestradiol 0.030 - 0.040 - 0.030 mg; levonorgestrel 0.050 - 0.075 - 0.125 mg; (4) Cilest (Janssen Pharmaceutica, Beerse, Belgium): ethinylestradiol 0.035 - 0.035 - 0 mg; norgestimate 0.250 - 0.250 - 0 mg; (5) Lunafem (Bayer Schering Pharma, Berlin, Germany): ethinylestradiol 0.020 mg; gestodene 0.075 mg; (6) Marvelon (Organon, Oss, The Netherlands): ethinylestradiol 0.030 mg; desogestrel 0.150 mg; (7) Mirena (Bayer Schering Pharma, Berlin, Germany): levonorgestrel 0.020 mg. Estrogen free intrauterine application. CIC: Contraceptive-induced cholestasis.

or as frequencies when appropriate. We used chi-square testing to check whether genotype frequencies were consistent with Hardy-Weinberg equilibrium. Differences between genotype frequencies were analyzed by two-sided Fisher exact testing, using the approximation of Katz, with the InStat3 program (GraphPad Software, La Jolla, CA).

RESULTS

Pediatric patients with gallstones

In the group of pediatric patients with idiopathic gallstones selected for genetic examination, analysis of protein-coding exons and intron/exon junctions of *ABCB4* identified no obvious pathogenic mutations. In patient 31, a novel heterozygous variation was found (c.2222C>T, leading to predicted conservative amino acid substitution p.Pro741Leu in the extracellular loop between transmembrane domains 7 and 8). The substitution was rated as neutral by all four pathogenicity prediction programs. Another predicted amino acid substitution (p.Gly773Val, localized in transmembrane domain 8 and caused by the novel mutation c.2318G>T) was found in a heterozygous state in patient 32. This conservative substitution was rated as disease-causing by MutationTaster, possibly pathogenic by PolyPhen-2, and neutral by SIFT and PMut. In addition, 6 known coding (5 synonymous) and 6 known non-coding variations were found (Table 2). None of these changes is reportedly associated with hepatobiliary disease, with the possible exception of c.1954A>G (p.Arg652Gly), found previously in a heterozygous state in subjects 4, 8 and 26[10]. However, the c.1954A>G variant was not overrepresented (3/70, allelic frequency 0.043) in our patients as compared with a healthy adult Czech Caucasian population (allelic frequency 0.090, 27 heterozygotes in 150 controls, OR = 0.48, 95%CI: 0.16-1.48, $P = 0.17$).

Families with suspect LPAC

Two of the five probands carried a single heterozygous nonsense mutation, two were heterozygotes for the missense mutation c.523A>G (p.Thr175Ala, rs58238559), and one was a compound heterozygote for the same missense mutation (c.523A>G) and for the frameshift mutation c.1371delG (p.Gln458Argfs*7) (Figure 2). The variation c.523A>G was found on 30% (3/10) of alleles in patients with LPAC, whereas only 2.7% of control alleles from the Czech population carried guanine at the position 523 (8/300, 8 heterozygotes in 150 control individuals, OR = 8.00, CI: 2.20-29.24, $P = 0.012$). While the number of patients was too low to make the result fully convincing, this observation suggests that p.Thr175Ala at least confers susceptibility to hepatobiliary disease. All three null mutations were novel to our best knowledge.

No deletions/duplications in *ABCB4* were detected in index patients by multiplex ligation-dependent probe amplification.

Two probands were homozygous and the other three probands were heterozygous for the low-expression *ABCB11* variant c.1331T>C (p.Val444Ala) (Figure 2). One proband had a c.55G|C genotype while four other probands were homozygous for the wildtype allele c.55G in *ABCG8*. To assess the segregation of the genotype and phenotype in the families of all index patients, first degree relatives were examined. As can be seen from the family trees depicted in Figure 2, the parents in families I, II, IV, and V who carried the same mutation as the probands in a heterozygous state were symptomatic. This indicates that the null mutations in families I - III and even the missense mutation leading to p.Thr175Ala in families IV and V all are likely sufficient in a heterozygous state to promote the LPAC phenotype. In contrast, variations *ABCB11* c.1331T>C and *ABCG8* c.55G>C, found in probands and 11 family members carrying mutations in *ABCB4*, do not seem to affect the penetrance of LPAC (Figure 2).

DISCUSSION

The only possible pathogenic mutation in *ABCB4* found

Table 2 Known variations in *ABCB4*, *ABCB11* and *ABCG8* found in 35 pediatric subjects with idiopathic gallstones

Patient. ID	Variations in the coding sequence of *ABCB4*						*ABCB11* low expression allele	*ABCG8* variation
	c.147C>T Ser49Ser rs8187789	c.175C>T Leu59Leu rs2302387	c.459T>C Phe153Phe rs2230027	c.504C>T Asn168Asn rs1202283	c.711A>T Ile237Ile rs2109505	c.1954A>G Arg652Gly rs2230028	c.1331T>C Val444Ala rs2287622	c.55G>C Asp19His rs11887534
1	CC	CC	TT	TT	AA	AA	CC	GG
2	CC	CC	TT	CT	AA	AA	TC	GG
3	CC	CC	TT	CT	AA	AA	TC	GG
4	CC	CC	TT	CT	AT	AG	TC	GG
5	CC	CC	TT	TT	AA	AA	CC	GG
6	CC	CC	TT	CC	AA	AA	TC	GG
7	CC	CC	TT	CT	AA	AA	TC	GG
8	CC	CC	TT	CC	AA	AG	TC	GG
9	CC	CC	TT	TT	AA	AA	TT	GG
10	CC	CC	TT	TT	AA	AA	CC	GG
11	CC	CC	TT	TT	AA	AA	TC	GG
12	CC	CC	TT	TT	AA	AA	CC	GG
13	CC	CC	TT	CT	AA	AA	TC	GG
14	CC	CC	TT	CC	AA	AA	CC	GG
15	CC	CC	TT	TT	AA	AA	TC	GC
16	CC	CC	TT	CC	AA	AA	TC	GC
17	CC	CC	TT	CC	AA	AA	TC	GG
18	CC	CC	TT	TT	AA	AA	TC	GC
19	CC	CC	TT	CT	AA	AA	TC	GC
20	CC	CC	TT	CT	AA	AA	TT	GG
21	CC	CC	TT	TT	AA	AA	TC	GG
22	CC	CC	TT	CT	AA	AA	TT	GG
23	CC	CC	TT	CT	AA	AA	TT	GG
24	CC	CT	TT	CC	AT	AA	CC	GG
25	CC	CC	TT	CC	AA	AA	TC	GG
26	CT	CT	TC	CC	AA	AG	TC	GG
27	CC	CC	TT	TT	AA	AA	TC	GG
28	CC	CC	TT	TT	AA	AA	TT	GG
29	CC	CC	TT	CT	AA	AA	TT	GG
30	CC	CC	TT	TT	AA	AA	CC	GC
31	CC	CC	TT	CT	AA	AA	TT	GC
32	CC	CC	TT	CT	AA	AA	TT	GG
33	CC	CC	TT	TT	AA	AA	TC	GG
34	CC	CC	TT	CC	AA	AA	TT	GG
35	CC	CC	TT	TT	AA	AA	CC	GG
Allelic frequency of variant alleles in patients with gallstones, HapMap populations and Czech population controls								
Allele	T	T	C	T	T	G	C	G
Gallstone patients	0.014	0.029	0.014	0.571	0.029	0.043	0.471	0.086
HapMap CEU	0	0.112	0[1]	0.664	0.175	0.075	0.408	0.085
HapMap HCB	0	0.167	0[1]	0.344	0.222	0.023	0.333	0.022
HapMap JPT	0	0.273	0[1]	0.442	0.300	0.023	0.261	0.011
HapMap YRI	0.042	0.525	0.11	0	0.362	0.392	0.425	0.042
Czech controls (n = 150)	n.d.	n.d.	n.d.	n.d.	n.d.	0.090	0.400	0.067[2]

[1]Results from corresponding populations studied in Environmental Genome Project (NIEHS ES15478 project). HapMap population data were not available for this variation; [2]Frequency in 285 Czech controls[15]. n.d.: Not done.

in pediatric patients with idiopathic gallstones who met clinical criteria for the diagnosis of LPAC was the variation c.2318G>T (p.Gly773Val) found in a heterozygous state in only one affected subject. The nucleotide change c.1954A>G found in 3 other pediatric gallstone subjects is common in the European, Caucasian, and African general population, but it has also been found in a patient with LPAC and low biliary phospholipid in whom its predicted consequence p.Arg652Gly was hypothesized to be conditionally penetrant, leading to clinical symptoms only under certain circumstances, such as pregnancy, or when combined with another mutation[18]. In contrast, no correlation of the *ABCB4* genotype c.1954A|G with the MDR3 expression level in the liver, as measured by Western blot, was observed in a study by Meier *et al*[11] and the substitution was rated as neutral by all software tools used. Our finding that the genotype c.1954A|G was neither overrepresented nor significantly underrepresented in patients with gallstones may indicate the negligible role of this variation in etiology of pediatric idiopathic gallstones. Similar conclusions could be drawn for both carriers and homozygotes for the low expression variant of the bile salt export pump and for the carriers of the *ABCG8* variation c.55G>C.

Interestingly, the *ABCB4* variation c.523A>G (p.Thr175Ala), found in three index patients with LPAC,

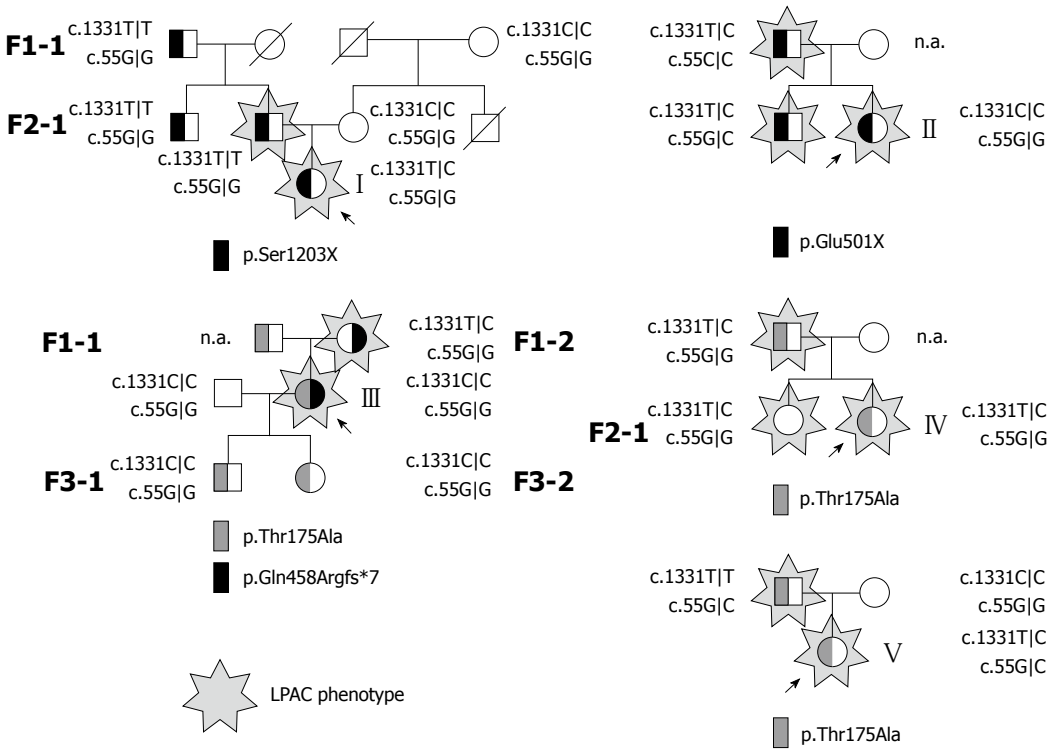

Figure 2 Family trees of the five unrelated female probands with low phospholipid-associated cholelithiasis syndrome and *ABCB4* mutations. Phenotype of low phospholipid-associated cholelithiasis syndrome (LPAC) is indicated with gray asterisks, index patients are marked with arrows. Genotypes for *ABCB11* c.1331T>C and *ABCG8* c.55G>C are shown at the genealogical symbols. n. a. - DNA not available. The father´s brother of the proband Ⅰ (F2-1) aged 53 years had no signs of LPAC. The proband´s grandfather (F1-1) developed gallstones after 40 years of age. Similarly, the father of proband Ⅲ (F1-1) experienced his first attack of biliary pain at the age of 60 years. His DNA sample was not available for analysis; however, he is most likely a heterozygous carrier of c.523A>G (p.Thr175Ala) as depicted. The children (F3-1 and F3-2) of proband Ⅲ are nine year old twins with no signs of LPAC. The DNA of the sister of proband Ⅳ (F2-1), who met the clinical criteria of LPAC, was analyzed for *ABCB4* mutations with negative results.

was not present in any of our patients with pediatric gallstones. The allele c.523G is linked to cholestatic disease[1] although it is also found in healthy Caucasian populations at an allelic frequency of 0.025-0.032[9,19,20]. The threonine residue at position 175 is highly conserved, lying in a Thr-Arg-Leu-Thr cluster required for MDR3 adenosine triphosphatase (ATPase) activity. While the functional consequences of replacement of threonine at position 175 by a neutral amino-acid residue having a hydrophobic side chain were not evaluated in MDR3, they were studied in yeast in the close homologue P-glycoprotein[21], in which the substitution p.Thr169Ile resulted in a complete loss of substrate-induced P-glycoprotein ATPase activity. The substitution p.Thr175Ala, predicted uniformly to impair protein function by SIFT, PMut, PolyPhen-2 and MutationTaster is thus considered a disease-associated mutation[3] with incomplete penetrance.

Neither the 14 confirmed and 1 suspected heterozygous carriers of *ABCB4* mutations investigated in the second part of our study nor the heterozygotes reported previously by others[1,2,4,22-24] developed symptomatic gallstones without progressive familial intrahepatic cholestasis in childhood. This suggests that other pathogeneses of idiopathic gallstones in childhood should be sought. Since we did not assay phospholipid and cholesterol concentrations in bile from our 35 pediatric subjects, we cannot definitively claim that they did not have LPAC; only that, if they had LPAC, it was associated neither with demonstrable *ABCB4* mutation (this study) nor with the studied variations in *ABCB11* and *ABCG8*[10]. We suggest that to carry out *ABCB4* sequencing in pediatric patients with idiopathic cholesterol gallstones who meet only some of the present criteria for assigning the diagnosis of LPAC may be unproductive. We believe that the validity of these criteria for LPAC associated with *ABCB4* mutation should be re-assessed, in pediatric patients at least, and propose that the present criteria at this juncture be considered to apply only to adults aged less than 40 years.

The observations that LPAC syndrome becomes manifest after middle adolescence and that young females heterozygous for pathogenic mutations in *ABCB4* developed CIC and/or manifested previously asymptomatic gallstones during administration of combined oral contraceptives are most likely explained by known changes in biliary lipid composition during the second decade of life. Gallstones hardly ever occur in children, but are frequent in adults; this difference seems to be due to the low concentrations of cholesterol in the bile of children[25]. Children have reduced biliary cholesterol:bile salt excretion ratios[26]. Therefore, even at low rates of phospholipid secretion caused by incomplete MDR3 deficiency, bile is not saturated with cholesterol. The known increase in the biliary cholesterol saturation index in

young adults[26], together with the decreased biliary secretion rate of phosphatidylcholine in carriers of mutations in *ABCB4*, shifts the cholesterol-solubility equilibrium to the borderline. Even the low load of exogenous hormones contained in contraceptives or other hormonally active drugs, which inhibit bile salt secretion[27] and further decrease secretion of phospholipids into bile proportionally to bile salt flow[28], can precipitate cholestasis and promote cholesterol crystallization from supersaturated bile, with formation of intrahepatic sludge and of gallstones.

A practical question can be raised on the safety of contraceptives in women with MDR3 deficiency. Our patients heterozygous for null mutations developed CIC rapidly and consequently contraceptives had to be withdrawn. In contrast, two patients heterozygous for the missense MDR3 variant p.Thr175Ala tolerated long-term administration of oral contraceptives after cholecystectomy without apparent worsening in hepatobiliary disease. Interestingly, patient V reported pruritus and her serum levels of GGT were repeatedly increased twofold when she used estrogen-free intrauterine contraception. We therefore believe that the heterozygous state for missense mutations in *ABCB4* is not an *a priori* contraindication to oral contraception. However, monitoring of clinical status and clinical-laboratory indices of hepatobiliary injury is essential in such cases.

In conclusion, our findings indicate that clinical criteria for LPAC caused by mutations in *ABCB4* cannot be applied to pediatric patients with idiopathic gallstones. Sexual immaturity prevents manifestation of LPAC even in carriers of pathogenic mutations in *ABCB4*. In young females, manifestation of LPAC as intrahepatic cholestasis with elevated serum activity of gamma-glutamyltransferase may be triggered by hormonal contraceptives.

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COMMENTS

Background

Mutations in *ABCB4*, the variation c.55G>C in *ABCG8* and the low expression allele c.1331T>C of *ABCB11* may affect biliary lipid composition and increase saturation of bile with cholesterol. Mutations in *ABCB4* are known to cause low phospholipid-associated cholelithiasis (LPAC) in young adults. The variation c.55G>C in *ABCG8* has been linked with gallstones in adults.

Research frontiers

Since saturation of bile with cholesterol in children is lower than in adults, the authors anticipated a strong contribution of the above listed genetic variations to pediatric idiopathic gallstones and conducted a genetic study in pediatric LPAC-like gallstone patients and in young adults with suspect LPAC who came to medical attention due to contraceptive-induced cholestasis. Whereas young adult females with clinically defined LPAC carried mutations in *ABCB4*, no association with the studied variants was found in pediatric LPAC-like subjects.

Innovations and breakthroughs

Sexual immaturity prevents manifestation of LPAC even in carriers of pathogenic mutations in *ABCB4*.

Applications

Clinical criteria for LPAC caused by mutations in *ABCB4* cannot be applied to pediatric patients with idiopathic gallstones and to carry out *ABCB4* sequencing in pediatric patients with idiopathic cholesterol gallstones may be unproductive. Heterozygous state for some missense mutations in *ABCB4* is not an *a priori* contraindication to oral contraception; however, monitoring of clinical status and clinical/laboratory indices of hepatobiliary injury is essential in such cases.

Terminology

Biliary lipid secretion is mediated by three ABC transporters: *ABCB11* encodes the bile salt export pump, *ABCB4* encodes the canalicular lecithin pump MDR3 (multidrug resistance protein 3) and the genes *ABCG5* and *ABCBG8* encode two proteins named sterolins which form a heterodimeric ABC transporter responsible for biliary secretion of cholesterol and plant sterols.

Peer review

It is an excellent manuscript submitted to reevaluate the criteria for LAPC associated with *ABCB4* mutation and provide the data of both pediatric idiopathic gallstone and young women with LAPC hormonal cholestasis by oral contraceptives. The mechanism of cholelithiasis formation with *ABCB11* and *ABCG8* mutation was also considered and 5 probands with detailed pedigrees were presented. Ethics of the research was given by written informed consent.

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CLINICAL TRIALS STUDY

Prognostic significance of SUVmax and serum carbohydrate antigen 19-9 in pancreatic cancer

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Abstract

AIM: To investigate the prognostic significance of pretreatment standardized maximum uptake value (SUVmax) and serum carbohydrate antigen (CA)19-9 in pancreatic cancer.

METHODS: From January 2007 to October 2011, 80 consecutive patients with pancreatic cancer who received positron emission/computed tomography before any treatment were enrolled in this study. The pretreatment SUVmax and CA19-9 level of the primary pancreatic tumor were obtained and compared with clinicopathological and prognostic factors. Student's t test for unpaired data was used to analyze the differences between two groups. Univariate analysis and Cox proportional hazards regression were used to examine the independent effects of each significant variable. Survival was analyzed by the Kaplan-Meier method.

RESULTS: There was a significant correlation between both the SUVmax and serum CA19-9 of pancreatic cancer and R0 surgical resection (P = 0.043 and P = 0.007). Lymph node metastasis was associated with SUVmax (P = 0.017), but not serum CA19-9 (P = 0.172). On the contrary, the tumor stage was significantly related to serum CA19-9 (P = 0.035), but not SUVmax (P = 0.110). The univariate analysis showed that survival time was significantly related to tumor stage (P < 0.001), lymph node metastasis (P = 0.043), R0 surgical resection (P < 0.001), serum CA19-9 (P = 0.001), SUVmax (P < 0.001) and SUVmax plus CA19-9 (P = 0.002). Multivariate analysis clearly showed that only tumor stage (hazard ratio = 0.452; P = 0.020) was an independent prognostic factor for overall survival in pancreatic cancer. Higher SUVmax or CA19-9 showed worse prognosis. We found that high serum CA19-9 plus SUVmax was the most significant variable.

CONCLUSION: Higher pretreatment SUVmax and serum CA19-9 indicates poor prognosis. SUVmax plus serum CA19-9 is the most significant variable in predicting survival.

Key words: Pancreatic cancer; Maximum standardized uptake value; Carbohydrate antigen 19-9; Prognostic factors

Core tip: We investigated the clinicopathological characteristics and prognostic significance of standardized maximum uptake value (SUVmax) of pancreatic cancer and serum carbohydrate antigen (CA)19-9. Higher SUVmax or CA19-9 showed worse prognosis, and high serum CA19-9 plus SUVmax was the most significant variable in predicting survival.

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INTRODUCTION

Despite advances in diagnostic technology and therapeutic methods, pancreatic cancer is still a medical challenge. Even if early diagnosis and surgical resection are achieved, the average 5-year survival rate remains at approximately 5%[1]

Nowadays, after ultrasonography, contrast-enhanced computed tomography (CT) and enhanced magnetic resonance, fluorodeoxyglucose positron emission tomography (FDG-PET)/CT has assumed an important role in preoperative staging of patients with all kinds of cancers, including lung and breast cancer[2,3]. For any kind of pancreatic cancer, the success of surgical treatment does not depend on the histological type of the tumor but rather on its staging. Accurate preoperative staging is difficult and important for evaluating the disease extension and choosing the most appropriate treatment[4,5]. Some studies have concluded that the efficacy of FDG uptake measured as standardized maximum uptake value (SUVmax) in the primary tumor is predictive of an inferior outcome[6,7]; however, other studies have not agreed[8]. Several studies have shown that FDG uptake possibly plays a role in predicting the prognosis of pancreatic cancer[9,10]. However, the prognostic value of FDG uptake in pancreatic cancer remains uncertain because of the small number of studies reported in the literature.

Carbohydrate antigen (CA)19-9 is the most useful tumor marker for pancreatic cancer. Preoperative serum CA19-9 level has been reported as a useful prognostic marker in pancreatic cancer[11]. Some authors have reported that CA19-9 is an important independent prognostic variable in patients with inoperable pancreatic cancer[12,13].

In this study, we investigated the clinicopathological characteristics and prognostic significance of SUVmax of pancreatic cancer and serum CA19-9.

MATERIALS AND METHODS

Patients

From January 2007 to October 2011, 80 patients with pancreatic cancer were reviewed at Peking Union Medical College Hospital, China. Clinical, histological, and imaging data of patients were collected and stored in a computerized database. Twelve patients had diabetes. Informed consent was obtained and the study design was approved by the Ethics Committee of the hospital.

The inclusion criteria were as follows: (1) all patients were diagnosed with pancreatic cancer by surgical pathology or endoscopic-ultrasound-guided staging pathology; (2) all patients were examined for tumor markers including CA 19-9; (3) pretreatment FDG-PET/CT was performed in all patients; (4) no previous diagnosis of another malignant disease; (5) no prior antitumor treatment, such as chemotherapy or radiotherapy; and (6) lymph node metastasis was confirmed by surgical pathology. The diagnosis and staging of pancreatic cancer was based on the 7th edition Staging Manual of the American Joint Committee on Cancer. Overall survival was defined as the time from pathological diagnosis to death or loss to follow-up. All patients were followed for 7-35 mo.

PET/CT

Patients were asked to fast for at least 6 h before examination and serum glucose level < 160 mg/dL was ensured. Before and after injection, patients were kept comfortable in the prone position. Scanning was initiated 1 h after administration of the tracer. PET/CT data were prospectively evaluated by consensus by two nuclear medicine physicians who were aware of the clinical and imaging results, but blinded to the pathological results. The SUVmax of the primary tumor was measured and calculated by the software according to standard formulas. The SUVmax of the liver and vascular pool were both measured. The same procedure was performed for two adjacent planes and the average of these data was considered.

Serum CA19-9 assay

Serum CA19-9 concentration was measured by an automated, commercially available enzyme immunoassay on an AxSYM analyzer (Abbott Diagnostics Laboratory). A value of 37 U/mL was used as the upper limit of normal.

Statistical analysis

Student's *t* test for unpaired data was used to analyze the differences between two groups. Univariate analysis was performed using the following factors: patient age, tumor stage, tumor histological type, tumor size, blood vessel invasion, lymph node metastasis, distant metastasis, SUVmax, and serum CA19-9. All tests were two-tailed and $P < 0.05$ was considered significant. Survival curves were estimated by the Kaplan-Meier method and examined by the log-rank test. Cox proportional hazards regression was used to examine the independent effects of each significant variable. The statistical software SPSS version 13.0 was used for all analyses.

RESULTS

Only 59 patients underwent R0 surgical resection. The patient characteristics including age, sex, histology, tumor location, tumor pathological stage, metastasis, and treatment methods are summarized in Table 1. The mean serum CA19-9 for all patients was 842.1 U/mL (range: 151.3-2937.0 U/mL). The median serum CA19-9 was 604.5 U/mL. The mean SUVmax for all patients was 6.20 (range: 2.20-16.30). The median SUVmax was 5.35.

The mean SUVmax and serum CA19-9 grouped by stage, lymph node status, pathological differentia-

Table 1 Standardized maximum uptake value or serum carbohydrate antigen 19-9 of pancreatic cancer stratified by stage, differentiation, lymph node and tumor size

	n	SUVmax (*n* = 80)		Serum CA19-9 (*n* = 80)	
		mean ± SE	*P* value	mean ± SE	*P* value
Sex					
Male	48	5.73 ± 0.36	0.091	749.30 ± 72.99	0.102
Female	32	6.91 ± 0.64		981.31 ± 132.74	
Age (yr)					
≤ 60	37	5.74 ± 0.42	0.206	766.43 ± 93.14	0.316
> 60	43	6.60 ± 0.52		907.22 ± 101.44	
Pancreatic location (n)					
Head	62	6.39 ± 0.41	0.293	871.46 ± 84.01	0.437
Body or tail	18	5.53 ± 0.50		740.99 ± 108.47	
Stage					
Ⅰ/Ⅱ	55	5.83 ± 0.43	0.110	729.09 ± 71.31	0.035
Ⅲ/Ⅳ	25	7.01 ± 0.49		1090.74 ± 148.23	
Pathological differentiation					
Well	28	5.46 ± 0.52	0.110	720.83 ± 99.27	0.055
Moderate	18	5.81 ± 0.62		667.87 ± 126.90	
Poor	34	0.70 ± 0.57		1034.22 ± 119.19	
Tumor size					
> 2 cm	56	6.32 ± 0.40	0.584	901.61 ± 87.16	0.193
≤ 2 cm	24	5.91 ± 0.65		703.27 ± 108.27	
Lymph node metastasis					
Yes	34	6.54 ± 0.52	0.017	831.84 ± 90.02	0.172
No	25	4.72 ± 0.50		634.35 ± 112.96	
Distant metastasis					
Yes	8	7.48 ± 0.89	0.210	1363.26 ± 338.95	0.134
No	72	6.06 ± 0.36		784.20 ± 65.13	
R0 surgical resection					
Yes	59	5.79 ± 0.38	0.043	731.19 ± 71.35	0.007
No	21	7.35 ± 0.67		1153.72 ± 156.96	

SUVmax: Standardized maximum uptake value; CA19-9: Carbohydrate antigen 19-9.

tion, distant metastasis, R0 surgical resection, and other clinicopathological variables including sex, age, tumor size and tumor location are shown in Table 2. There was significant correlation between the SUVmax and serum CA19-9 of pancreatic cancer and R0 surgical resection ($P = 0.043$ and $P = 0.007$) (Table 2). Lymph node metastasis was associated with SUVmax ($P = 0.017$), but not serum CA19-9 ($P = 0.172$). On the contrary, tumor stage was significantly related to serum CA19-9 ($P = 0.035$), but not SUVmax ($P = 0.110$). There was no correlation between SUVmax and serum CA19-9 of pancreatic cancer and sex, age, tumor location, tumor size, pathological differentiation, and distant metastasis.

Results of Cox regression analysis are shown in Table 3. The univariate analysis showed that survival time was significantly related to tumor stage ($P < 0.001$), lymph node metastasis ($P = 0.043$), R0 surgical resection ($P < 0.001$), serum CA19-9 ($P = 0.001$), SUVmax ($P < 0.001$) and SUVmax plus CA19-9 ($P = 0.002$). However, age, sex, pathological differentiation, tumor location and tumor size were not significantly related to survival time. Multivariate analysis clearly showed that only tumor stage (HR = 0.452; $P = 0.020$) was an independent prognostic factor for overall survival in pancreatic cancer.

Figure 1 shows the survival curves for all patients who underwent pretreatment CA19-9 and SUVmax analysis of the primary tumor. The SUVmax and CA19-9 cutoff values for the primary tumor as determined by the median value were 5.35 and 604.5 U/mL, respectively. In patients with SUVmax and serum CA19-9 more than cutoff value, survival time was 17.69 ± 1.08 and 17.80 ± 1.08 mo, respectively. In patients with SUVmax and serum CA19-9 less than cutoff value, survival time was significantly longer (23.47 ± 1.19 and 23.33 ± 1.22 mo, respectively). Figure 1C shows the survival time of the patients in the following four groups: high SUVmax plus serum CA19-9; high SUVmax plus low serum CA19-9; low SUVmax plus high serum CA19-9; and low SUVmax plus serum CA19-9. Survival time in the high SUVmax plus serum CA19-9 group was shorter than in the other groups (17.37 ± 1.23 *vs* 22.32 ± 1.08 mo).

DISCUSSION

For prognostic factors for pancreatic cancer, the tumor stage and grade, R0 surgical resection, serum tumor marker levels, size of the primary lesion, and lymph node metastasis have been reported[14,15]. The increased glycolytic activity of the tumor detected by SUVmax may represent tumor growth and biological behavior[16]. FDG-PET possibly plays a role in predicting the prognosis of pancreatic cancer[9]. However, the significance of FDG-PET imaging in the diagnosis, staging, or predicting prognosis of pancreatic cancer has not yet been established.

The metabolic activity measured by FDG-PET, usually through SUVmax, seems to be useful in evaluating

Table 2 Univariate analysis of prognostic factors for overall survival

Variable	*n*	*P* value
Sex		
Male	48	0.119
Female	32	
Age (yr)		
≤ 60	37	0.993
> 60	43	
Pancreatic location (n)		
Head	62	0.378
Body or tail	18	
Stage		
Ⅰ / Ⅱ	55	< 0.001
Ⅲ/Ⅳ	25	
Pathological differentiation		
Well	28	0.697
Moderate	18	
Poor	34	
Tumor size		
> 2cm	56	0.339
≤ 2cm	24	
Lymph node metastasis		
Yes	34	0.043
No	25	
Distant metastasis		
Yes	8	< 0.001
No	72	
Surgical resection		
Yes	59	< 0.001
No	21	
SUVmax		
High	40	< 0.001
Low	40	
CA19-9		
High	40	0.001
Low	40	
SUVmax plus CA19-9		
Both are high	29	0.002
Others	51	

SUVmax: Standardized maximum uptake value; CA19-9: Carbohydrate antigen 19-9.

Table 3 Multivariate analysis of prognostic factors for overall survival

Variable	Hazard ratio (95%CI)	*P* value
Stage	0.452 (0.192-1.064)	0.020
Lymph node metastasis	0.786 (0.418-1.478)	0.455
Surgical resection	11.521 (0.799-166.191)	0.073
SUVmax	0.553 (0.216-1.413)	0.216
CA19-9	0.452 (0.192-1.064)	0.069
SUVmax plus CA19-9	1.558 (0.439-5.525)	0.493

SUVmax: Standardized maximum uptake value; CA19-9: Carbohydrate antigen 19-9.

the prognosis of pancreatic cancer. Most authors consider SUVmax to be an independent prognostic factor that is expressed in tumor growth and biological behavior: higher SUV predicts worse prognosis[17]. In particular, Maemura *et al*[18] have shown that pancreatic cancer with distant metastases has a higher SUVmax than that without metastases. Sperti *et al*[10] have demonstrated that SUV > 4 is associated with shorter survival. Nakata *et al*[19] have shown in patients with unresectable disease that higher SUV is correlated with shorter survival. Tomita *et al*[20] have demonstrated that preoperative SUVmax and serum carcinoembryonic antigen (CEA) level are independent prognostic factors for survival in non-small cell lung cancer, and combined use of the two indicators might be better prognostic factors. Schellenberg *et al*[21] have shown that median survival for patients with a low and high SUVmax value was 15.3 and 9.8 mo, respectively, and patients with SUVmax < 5 had longer progression-free and overall survival than those with SUVmax > 5.

Sperti *et al*[22] have found that patients with preoperative CA19-9 level < 200 U/mL have significantly better prognosis than those with CA19-9 > 200 U/mL. After surgical resection, survival of patients with normal serum CA19-9 is significantly longer than that of patients with persistently elevated CA19-9. It has also been found that serum CA19-9 is an important independent prognostic variable in patients with inoperable pancreatic cancer, and baseline CA19-9 more or less than the median value is an independent prognostic factor for overall survival[13]. Ni *et al*[23] have shown that higher serum CA19-9, CA242 and CEA is related to distant metastasis, advanced stage, and worse prognosis of pancreatic cancer.

In the current study, we found that pretreatment SUVmax and serum CA19-9 in patients with pancreatic cancer were significantly associated with R0 surgical resection. Furthermore, we showed that lymph node metastasis was associated with SUVmax but not serum CA19-9. On the contrary, tumor stage was significantly related to serum CA19-9 but not SUVmax. Moreover, we found that the SUVmax in poorly differentiated cancer was near to 0. Thus, we suggest that SUVmax is associated with pancreatic cancer differentiation. Our univariate analysis demonstrated that survival time was significantly associated with tumor stage, and lymph node metastasis, R0 surgical resection, serum CA19-9, SUVmax. SUVmax plus serum CA19-9 were the main risk factors for prognosis of pancreatic cancer. However, although there was a clear correlation of CA19-9 and SUVmax with survival, multivariate analysis clearly showed that only tumor stage, and not CA19-9 and SUVmax, was an independent prognostic factor for overall survival in pancreatic cancer. We think that these results may have been related to the small number of patients.

Kaplan-Meier analysis revealed that SUVmax and serum CA19-9 were associated with prognosis in pancreatic cancer. The results showed that high SUVmax (≥ 5.35) and serum CA19-9 (≥ 604.5 U/mL) were correlated with worse prognosis. More importantly, we found that SUVmax plus serum CA19-9 was the most significant variable in predicting survival. Thus, we think that the combination of SUVmax plus serum CA19-9 is more meaningful in predicting survival of pancreatic cancer.

Although this study was retrospective, it did shed some light on the usefulness of SUVmax and serum CA19-9 in prognosis of pancreatic cancer. We can draw

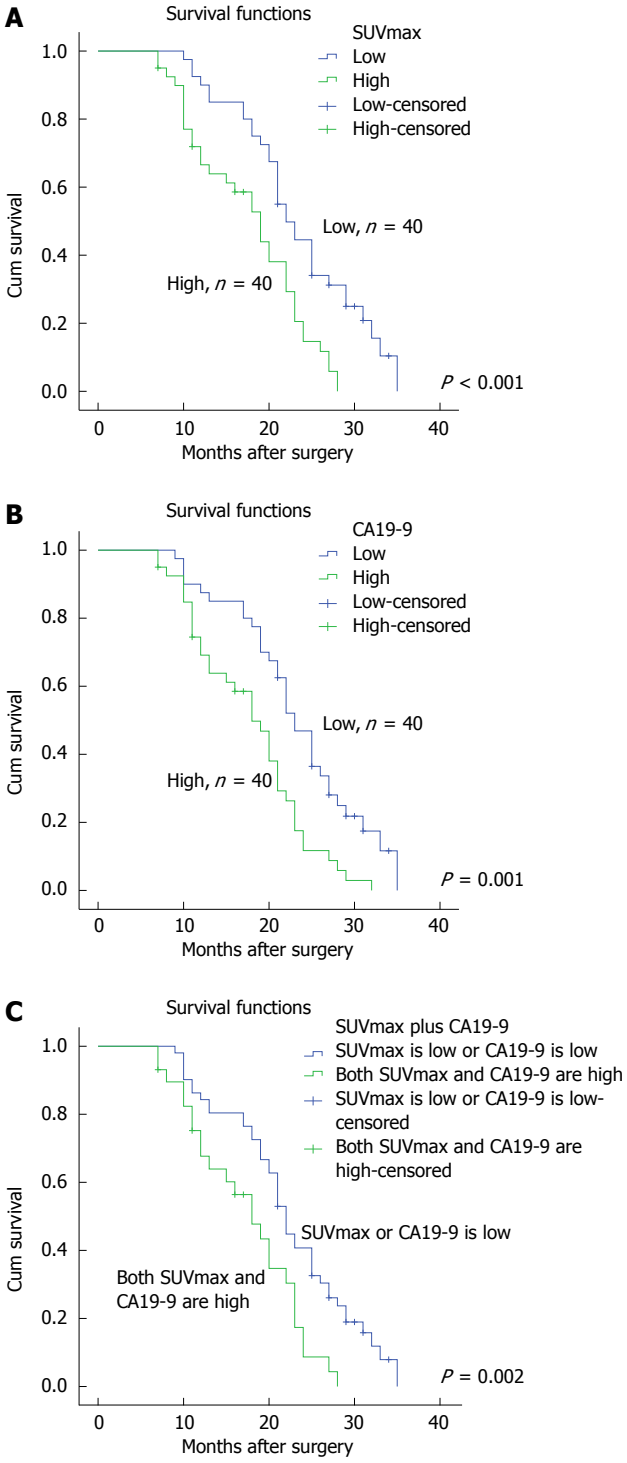

Figure 1 Kaplan-Meier curve for the survival time of patients with pancreatic cancer according to their standardized maximum uptake value or serum carbohydrate antigen 19-9 (*n* = 80). A: Standardized maximum uptake value (SUVmax) and survival; upper line, low SUVmax of primary tumor (< 5.35) (*n* = 40); lower line, high SUVmax of primary tumor (≥ 5.35) (*n* = 40); B: Serum Carbohydrate antigen (CA)19-9 and survival; upper line, low serum CA19-9 (< 604.5 U/mL) (*n* = 40); lower line, high serum CA19-9 (≥ 604.5 U/mL) (*n* = 40); C: SUVmax plus CA19-9 and survival; upper line, low SUVmax (< 5.35) or serum CA19-9 (< 604.5 U/mL) of primary tumor (*n* = 51); lower line high serum SUVmax (≥ 5.35) plus serum CA19-9 (≥ 604.5 U/mL) of primary tumor (*n* = 29).

the following conclusions. Tumor stage is an independent predictor of survival in pancreatic cancer. Higher pretreatment SUVmax and serum CA19-9 level in pancreatic cancer indicate worse prognosis. SUVmax plus serum CA19-9 are significantly associated with poor prognosis. These findings may help to guide the treatment of pancreatic cancer and evaluate its prognosis.

COMMENTS

Background

Despite advances in diagnostic technology and therapeutic methods, pancreatic cancer is still a medical challenge. Several researchers have found that fluorodeoxyglucose (FDG) uptake possibly plays a role in predicting the prognosis of pancreatic cancer. However, the prognostic value of FDG uptake in pancreatic cancer remains uncertain because of the small number of reports in the literature. In this study, the authors investigated the clinicopathological characteristics and prognostic significance of standardized maximum uptake value (SUVmax) of pancreatic cancer and serum carbohydrate antigen (CA)19-9. CA19-9 is the most useful tumor marker for pancreatic cancer.

Research frontiers

The prognostic value of FDG uptake in pancreatic cancer remains uncertain. CA19-9 is the most useful tumor marker for pancreatic cancer. The authors evaluated the significance of FDG-positron emission tomography (PET) imaging and CA19-9 in predicting the prognosis of pancreatic cancer.

Innovations and breakthroughs

Serum CA19-9 is the most useful tumor marker for pancreatic cancer. FDG-PET also possibly plays a role in predicting the prognosis of pancreatic cancer. Most authors think that SUVmax is an independent prognostic factor. Higher SUVmax predicts worse prognosis. However, the significance of FDG-PET imaging in the diagnosis, staging, or prognosis of pancreatic cancer has not yet been established. The significance of combination of SUVmax and CA19-9 has seldom been evaluated for predicting prognosis of pancreatic cancer. In this study, the authors investigated the clinicopathological characteristics and prognostic significance of SUVmax and serum CA 19-9 of pancreatic cancer.

Applications

Their results suggest that higher pretreatment SUVmax and serum CA19-9 level of pancreatic cancer indicate worse prognosis. Moreover, the combination of SUVmax plus serum CA19-9 is significantly associated with poor prognosis. These may help to guide the treatment of pancreatic cancer and evaluate its prognosis.

Terminology

FDG-PET/CT is the fastest growing diagnostic modality in oncology. It is based on the concept that proliferating tumors have increased uptake and metabolism of glucose, and therefore, preferentially take up tracers, such as 18F-FDG, compared with normal tissue. Not only can this form of functional imaging detect malignant disease, irrespective of lesion morphology, it might also convey prognostic information related to the metabolic activity of the cancer.

Peer review

The topic of exploring the relevance of SUVmax and CA19-9 in the prediction of prognosis and overall survival in pancreatic cancer is interesting. It is noted that most papers regarding this issue were from western countries. In the present study, the authors analyzed the data of SUVmax and CA19-9 from Chinese pancreatic cancer patients with follow-up time. Their results provide some useful information for the diagnosis and prognosis of pancreatic cancer. The prognostic value of SUVmax and serum CA19-9 in pancreatic cancer has already been reported before, thus, the emphasis of this article may be put on the combination of these two parameters.

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RETROSPECTIVE STUDY

Late biliary complications in human alveolar echinococcosis are associated with high mortality

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Abstract

AIM: To evaluate the incidence of late biliary complications in non-resectable alveolar echinococcosis (AE) under long-term chemotherapy with benzimidazoles.

METHODS: Retrospective analysis of AE patients with biliary complications occurring more than three years after the diagnosis of AE. We compared characteristics of patients with and without biliary complications, analyzed potential risk factor for biliary complications and performed survival analyses.

RESULTS: Ninety four of 148 patients with AE in Zurich had non-resectable AE requiring long-term benzimidazole chemotherapy, of which 26 (28%) patients developed late biliary complications. These patients had a median age of 55.5 (35.5-65) years at diagnosis of AE and developed biliary complications after 15 (8.25-19) years of chemotherapy. The most common biliary complications during long-term chemotherapy were late-onset cholangitis (n = 14), sclerosing cholangitis-like lesions (n = 8), hepatolithiasis (n = 5), affection of the common bile duct (n = 7) and secondary biliary cirrhosis (n = 7). Thirteen of the 26 patients had undergone surgery (including 12 resections) before chemotherapy. Previous surgery was a risk factor for late biliary complications in linear regression analysis (P = 0.012).

CONCLUSION: Late biliary complications can be observed in nearly one third of patients with non-resectable AE, with previous surgery being a potential risk factor. After the occurrence of late biliary complications, the median survival is only 3 years, suggesting that late biliary complications indicate a poor prognostic outcome.

Key words: Alveolar echinococcosis; Biliary strictures; Biliary cirrhosis; Cholangitis; Cholestatic liver disease; Chronic liver disease; Complications; Echinococcal

cysts; Prognosis

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INTRODUCTION

Human alveolar echinococcosis (AE) is caused by the larval stage of the fox tapeworm, Echinococcus multilocularis. The parasites' dominant definitive host is the red fox, which can be infected in up to 60%[1]. Echinococcus multilocularis is widely distributed throughout the Northern Hemisphere, with a disease-endemic area stretching from North America through central Europe (eastern France, southern Germany, Switzerland and western Austria) to central and east Asia[2,3]. AE remains a rare disease in Western Europe, but not in highly endemic regions such as Sibiria and China, where a human prevalence rate of 3% or higher has been described[4].

In early untreated cohorts, the fatality rate exceeded 90% within 10 years[5]. However, survival rates have improved tremendously within the last three decades due to the increasing success of several treatment options, including surgery as the preferred first line therapy[6], long-term chemotherapy with benzimidazoles (available since 1975[7]), interventional procedures, and in rare cases liver transplantation[8]. As a result, the life expectancy for a middle aged patient with AE has increased by approximately 20 years and is now only slightly reduced (by about 3 years) compared to healthy controls[7]. However, if complete surgical resection of the parasite is not possible, long-term (life-long) chemotherapy with benzimidazoles remains the standard of care[6], and AE continues to be a disease with significant morbidity and mortality.

In unresected AE, biliary complications can influence the course of the disease significantly. The intrahepatic bile ducts or the extrahepatic biliary tree can either be directly infiltrated and destroyed by the growing parasite or compressed due to surrounding growth. Biliary (and other) complications include cholangitis, liver abscess, septic shock, portal hypertension and biliary cirrhosis[9]. Detailed knowledge of biliary complications is crucial for the management of patients with non-resectable AE.

Currently, our knowledge of adverse effects in AE is very limited. There are case series describing biliary complications of cystic hydatid disease (caused by Echinococcus granulosus). In these publications, bile duct obstruction with liver atrophy in the cyst-bearing lobes[10], rupture of hepatic hydatid cysts in the biliary tree with secondary cholangitis[11] or biliary obstruction by daughter cysts with further complications such as portal hypertension, ascites, abscesses and the development of bronchobiliary fistulas have been described[12,13]. However, these data are not directly applicable to alveolar hydatid disease (AE; caused by Echinococcus multilocularis) which is much more frequent in central Europe. We aimed to evaluate late biliary complications in a cohort of patients with non-resectable AE.

MATERIALS AND METHODS

Patients

From the prospective database of the Swiss National Center for Echinococcosis, we retrospectively analysed all available data of AE patients followed at the University Hospital of Zurich with a diagnosis of AE before 2003. The detailed study protocol has been published earlier[14]. The study was performed according to the declaration of Helsinki and the protocol was approved by the hospital ethics committee. All patients provided written informed consent to participate in the study. Patients were examined at yearly intervals, including a physical examination, routine laboratory tests, and additional immunodiagnostic tests, a chest radiograph, ultrasonography, and CT scan of the abdomen according to the study protocol. Further magnetic resonance imaging of the upper abdomen was performed in unclear cases.

The patient selection process as described above is illustrated in Figure 1. All patients in Zurich with a diagnosis of AE between 1967 and 2003 were included (n = 148). Fifty-four patients with successful R0 resection were excluded. A total of 94 patients had non-resectable or recurrent AE and received continuous long-term chemotherapy with benzimidazole carbamates (either mebendazole or albendazole). Of the 94 non-resectable AE patients, 26 patients developed late biliary complications as diagnosed by imaging [endoscopic retrograde cholangiopancreaticography (ERCP), computed tomography scan (CT), magnetic resonance cholangiopancreaticography (MRCP)] and blood tests. Liver biopsies were not routinely performed in these patients to find biliary cirrhosis.

To assess the prognostic value of late hepatobiliary complications, we compared the 26 patients with control patients without any biliary complications. The control group consisted of 32 patients with a first diagnosis of non-resectable AE between 1979 and 2003 and a follow up of at least 3 years.

Definitions

Late biliary complications were defined as biliary disease occurring at least 3 years after initial diagnosis of AE. The following were assessed: (1) Sclerosing cholangitis-like lesions, defined as abnormal ERCP or MRCP findings with features resembling those of primary sclerosing cholangitis such as multilocular annular strictures within the intrahepatic and/or extrahepatic bile ducts with alternating normal or slightly dilated segments; (2)

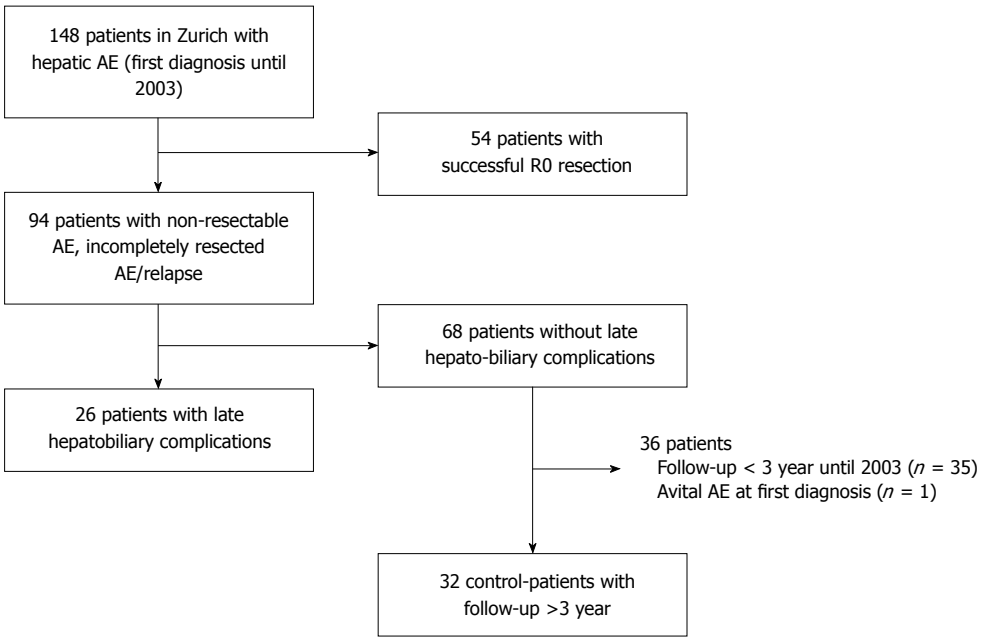

Figure 1 Patient selection process of patients with and a matched control group without biliary complications. Of all patients with hepatic alveolar echinococcosis (AE), patients with successful surgery were excluded. The remaining patients either developed biliary complications or not. From the latter group of patients, individuals with insufficient follow up time or avital AE were excluded; the remaining patients were defined as the control group.

Stenosis of the common bile duct, defined as isolated strictures in the proximal or distal common bile duct as well as strictures in the hilar region or extensions to the extrahepatic left and right duct; (3) One or several episodes of late cholangitis; (4) Hepatolithiasis and choledocholithiasis; (5) Secondary biliary cirrhosis; (6) Postoperative biliary stenosis after hepaticojejunostomy; and (7) Biliary fistulas.

Statistical analysis

Descriptive statistics were calculated using Microsoft Office Excel 2007. Results of numerical data are presented as medians (quartiles). Group comparisons were performed using the Fisher's exact test or the Mann-Whitney-U test of the statistics program GraphPad Prism 5. Furthermore, Pearson's correlation analysis and linear regression analysis were done using SPSS. For the linear regression analysis the potentially confounding factors: gender, age at diagnosis of AE, years of follow-up, years from diagnosis of AE until endpoint (either late biliary complications or end of follow-up), years of albendazole, mebendazole and total benzimidazole treatment as well as previous surgery were considered.

RESULTS

Characteristics of patients with late biliary complications

Late biliary complications developed in 26 patients with non-resectable AE undergoing chemotherapy after a median of 15 (interquartile range, IQR: 8.25-19) years. The 11 male and 15 female patients had a median age of 55.5 (35.5-65) years at diagnosis of AE. Further patient characteristics are provided in Table 1, which summarizes patients with and without late biliary complications. AE was diagnosed between 1967 and 1997. All patients received long-term chemotherapy with benzimidazoles, mostly mebendazole (25/26), rarely albendazole (5/26). Switching from one benzimidazole to the other was necessary in 4/26 patients. Benzimidazoles were given for a median of 13 (IQR: 8.1-20) years.

Late biliary complications

Late biliary complications were grouped according to the presenting clinical syndrome or disease and the underlying pathogenic mechanisms. Table 2 summarizes the clinical syndromes of the 26 patient with late biliary complications and the respective long-term outcome. Symptoms occurred 15 years after diagnosis of AE at a median age of 64.5 (IQR: 54-75) years.

Biliary complications included late-onset cholangitis (n = 14), sclerosing cholangitis-like lesions (n = 8), hepaticolithiasis (n = 5), secondary biliary cirrhosis (n = 7), stenosis of the common bile duct (n = 2), and postoperative stenosis after hepaticojejunostomy (n = 1). In addition, biliary fistulas occurred in 2 patients, including one biliary cutaneous and one bronchobiliary fistula. Altogether, 44 biliary complications in our 26 patients were noted. No obvious associations between different biliary complications could be detected. Eleven patients (42%) required interventions for late biliary complications, a total of 23 procedures were performed. 10 patients (38.5%) needed ERCP for treatment of biliary complications. In 2 patients (7.7%), PTCD was performed. Five patients (19.2%) needed surgery, including 3 hepaticojejunostomies and 2 resections. In 1 patient (3.8%), percutaneous enterostomy was performed.

Table 1 Comparison of patients with and without late biliary complications

	Patients with late biliary complications (n = 26)	Control group without late biliary complications (n = 32)	P^1 value
Male	42.30%	37.50%	NS
Year of AE diagnosis	1967-1997	1979-2003	
Age at AE diagnosis, (yr)	55.5 (35.5-65)	60.0 (44.2-64.6)	NS
Previous liver surgery	13 (50)	5 (15.6)	0.009
Years between AE diagnosis and first biliary complication, (yr)	15.0 (8.3-19.0)	NA	
Age at first biliary complication, (yr)	64.5 (54.0-75.8)	NA	
Deaths during follow-up until 2006	15 (57.7)	5 (15.6)	NA[2]
Liver related	5	1	
Non-liver related	10	4	
Age at death, (yr)	80.0 (67.5-81)	78.5 (65.0-85.1)	NS
Benzimidazole treatment (total) , (yr)	13.0 (8.1-20.0)	7.2 (4.2-16.1)	0.0404
Follow-up (total) , (yr)	23.0 (13.0-25.0)	8.6 (5.1-18.8)	< 0.0001
Follow-up of survivors until study endpoint (end of 2006 or drop-out), (yr)	11	27	
From AE diagnosis	- 24.0 (23.0-29.0)	7.1 (5.0-14.7)	0.0001
From biliary complication	- 9.0 (7.3-11.8)	NA	
Follow-up until death, (yr)	15	5	NS
From AE diagnosis	15.0 (11.0-24.5)	21.2 (9.7-25.1)	
From biliary complication	2.0 (1.5-5.0)	NA	

Data are expressed as absolute numbers (percentage) or median (quartiles). Comparison of patients with and without late biliary complications. [1]For statistical analyses Fisher's exact test was used for gender and outcome (death); for all other comparisons the Mann-Whitney *U* test was employed; [2]Direct comparison is not appropriate at this point since time of follow up is different for both patient groups. After adjustment for this difference in the survival analysis (compare Figure 2), no significant difference remained. AE: Alveolar echinococcosis; NA: Not applicable; NS: No significant.

Table 2 Summary of 44 late biliary symptoms and complications occurring in 26 patients and their respective outcome

Biliary complication	No. of patients	Outcome Number deaths during follow-up	Time to death of non-survivors, (yr)	Length of follow-up after BC, (yr)
Sclerosing cholangitis-like lesions (subtype2)	8 (31)	7/8 (87.5)	3 (1.5-5)	3.75 (1.75-6)
Stenosis of the common bile duct (subtype 4)	7 (27)	4/7 (57)	4 (1.75-6.75)	6 (4-10)
Late cholangitis (subtype 1)	14 (54)	5/14 (35)	2 (2.5-6)	7 (4-10.25)
Hepatolithiasis (subtype 3)	5 (19)	3/5 (60)	2 (2-3)	4 (2-6)
Secondary biliary cirrhosis(subtype 5)	7 (27)	4/7 (57)	3 (2-4.5)	6 (3-7)
Postoperative biliary stenosis after hepaticojejunostomy (subtype 7)	1 (4)	No deaths		11
Biliary fistula (subtype 8)	2 (8)	No deaths		8

Since one patient can have more than one late biliary complication, numbers do not add up to 100%. Data are expressed as absolute numbers (percentage) or median (IQR).

Analysis of potential risk factors for late biliary complications

To identify potential risk factors for late biliary complications, we performed Pearsons' correlation and linear regression analysis of several possible risk factors (Table 3). Age at diagnosis of AE, age at diagnosis of biliary complications and time from diagnosis of AE until endpoint were not associated with an increased risk. However, longer duration of benzimidazole treatment was associated with an increased risk of developing biliary complications, also after adjustment for additional factors in the linear regression analysis. Both years of albendazole and mebendazole treatment correlated with the risk for the development of biliary complication even though directionality was different with albendazole apparently protective and mebendazole increasing the risk. At this point, this different directionality is based on very small subgroups, difficult to explain and other unknown confounding factors could be responsible.

Importantly, previous surgery was a risk factor for biliary complications both in the correlation and regression analysis (P = 0.02 and 0.031, respectively). While 13 of the 26 patients with biliary complications had previously undergone surgery, only 5 out of 32 patients without biliary complications had a history of surgery. However, within the biliary complication group, biliary complications were observed both in patients with (n = 13) and without previous surgery (n = 13). Surgery was performed shortly after the diagnosis of AE in 10 patients or within the first 5 year after diagnosis in 3 patients (after 1, 2 and 5 year). Thus, previous surgery seems to be a risk factor for late biliary complications, but these are nevertheless not simply always consequence of earlier surgical procedures (Table 4).

Survival analysis

A total of 15/26 patients with late biliary complications died until the endpoint of 2006. Five of the 15 deaths

Table 3 Correlation and linear regression analysis for the risk of developing biliary complications

Variable number	Variable	Pearson correlation		Linear regression analysis	
		Pearson correlation coefficients	*P* value for Pearson correlation	Unadjusted regression coefficients	*P* value for regression coefficients
1	Gender	0.049	NS	0.141	NS
2	Age at diagnosis of AE	-0.146	NS	Redundant	-
3	Mebendazole treatment	0.403	0.001	Redundant	-
4	Albendazole treatment	-0.351	0.003	-0.037	0.016
5	Benzimidazole treatment	0.274	0.019	0.030	0.013
6	Whether or not surgery was performed	0.370	0.02	0.354	0.031
7	Age at diagnosis of biliary complication	-0.069	NS	0.001	NS
8	Years from diagnosis of AE until endpoint (either BC or end of follow up)	0.198	NS	-0.024	NS

Pearson correlation coefficients show the interdependence between pairs of variables, positive values indicate correlation and negative values anticorrelation. Regression coefficients from a multivariate linear-regression model are shown in column 5. Redundant variables (Var2 = Var7-Var8 and Var3 = Var5-Var4) do not appear in regression coefficients. R-square of the regression model is: 0.291. *P* values (95%CI) for Pearson correlation and regression coefficients are also indicated.

Table 4 Previous surgery and biliary complications

	Previous surgery (n = 13)	No previous surgery (n = 13)
Late cholangitis	8	6
Sclerosing-cholangitis like lesions	2	6
Hepatolithiasis	3	2
Affection of big bile ducts	3	4
Secondary biliary cirrhosis	4	3
Postoperative biliary stenosis (after hepaticojejunostomy)	1	0
Biliary fistula	2	0

Distribution of various late biliary complications among patients with and without previous surgery. None of these differences reached statistical significance (Fisher's exact test).

were AE induced liver failures. The ten other deaths were caused by infectious complications (n = 3), lethal gastrointestinal bleeding (n = 2), advanced age (n = 2) and cardiovascular diseases (n = 3). Overall, a substantial number of deaths (8/15, 53%) were probably or likely linked to the AE infection (5 liver failures, 1 variceal bleed after secondary biliary cirrhosis and 2 septic shocks in patients with biliary cirrhosis). 7/17 deaths (47%) were not clearly liver-related or due to biliary complications.

Death occurred on average 3.0 (2-5) years after the diagnosis of biliary complications.

The short time interval between the occurrence of late biliary complications and death underlines the prognostic value of such complications. However, survival after first diagnosis of AE was not different in the biliary complications group compared to the control group. Of note, only 4 out of 7 patients with secondary biliary cirrhosis died (1 due to progressive liver disease after terminating treatment, 1 due to gastrointestinal bleeding, 2 because of septic shock). Five of 14 patients with late cholangitis died during follow-up.

Is the occurrence of a late biliary complication a poor prognostic sign?

To assess the prognostic relevance of biliary complications, we compared the 26 patients with late biliary complications to 32 control patients without biliary complications (Table 1). The control group included 27 patients with inoperable AE, 4 patients who had received non-curative surgery and 1 patient with a relapse after curatively intended AE surgery but no postoperative benzimidazole treatment due to non-compliance.

In the group with late biliary complications, substantially more patients died within the follow-up period until 2006 (57.5% *vs* 15.6%). However, the mean and median follow up time in patients with late biliary complications was substantially longer than in control patients, leading to a possible bias. Nevertheless, the range of follow-up in these patient groups was comparable. Moreover, as shown in Figure 2, the diagnosis of a biliary complication seemed to mark a turning point in the natural course of the disease. When we compared the survival of patients immediately after the occurance of biliary complications to the overall survival of patients with and without biliary complications after AE diagnosis a much shorter survival became apparent (Figure 2B). When survival after biliary complications was compared to survival of the control group, this difference reached statistical significance. In contrast, a comparison of the groups with and without biliary complications after diagnosis of AE showed no differences in survival. This is probably due to the much longer mean and median follow-up time in the biliary complication group (Table 1),

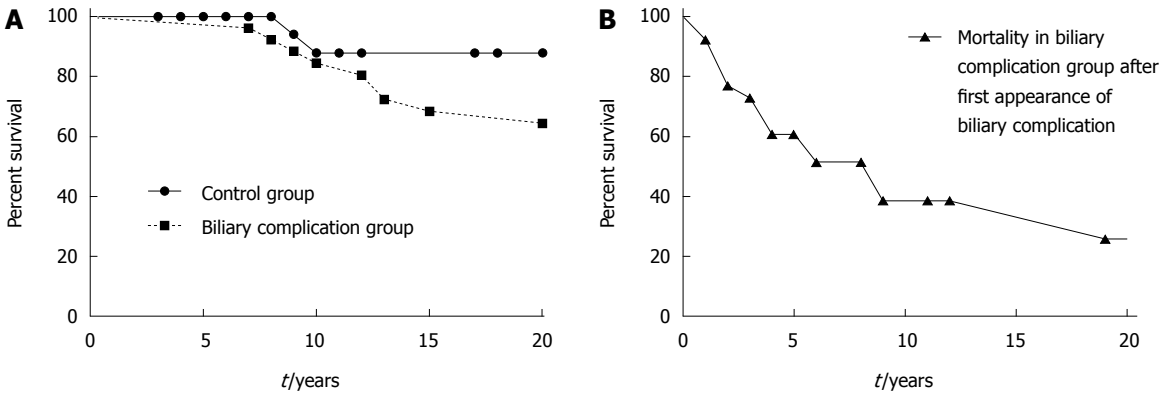

Figure 2 Survival of patients with alveolar echinococcosis with and without late biliary complications. Survival of patients with and without late biliary complications is shown over the course of 2 decades. Survival of the biliary complication group is shown either as time after diagnosis of alveolar echinococcosis (AE) (solid line) or time after diagnosis of the late biliary complication (dotted line). Survival after diagnosis of AE does not differ. In contrast, survival after diagnosis of the late biliary complication is significantly shorter compared to overall survival in the control group ($P < 0.0001$) and in patients with AE after initial diagnosis ($P = 0.0002$), Log-rank (Mantel-Cox) test.

leading to a possible bias. In summary, our data suggest that the occurrence of biliary complications indicates a "turning point" in the natural course of non-resectable AE and implies a poor prognosis after this timepoint.

DISCUSSION

AE is a severe infectious disease with relevant morbidity despite substantial increases in life expectancy over the last three decades[5,7]. Diagnostic and therapeutic improvements include better imaging techniques, improvements in surgical and perioperative strategies and the possibility of indefinite treatment with benzimidazoles in patients with non-resectable AE. Our data from a large national AE cohort demonstrate that biliary complications are a frequent problem in these patients in the course of the disease. Despite prolonged life expectancy, our data show that late biliary complications may predict a poor prognostic outcome. Mortality after the occurrence of biliary complications is substantially increased, with a median and mean survival of only 3 and 3.6 years, respectively.

Literature on biliary complications in non-resectable AE is scarce. There are many case series on biliary complications in "hydatid disease" ("cystic hydatid disease"), due to cystic echinococcosis, which differs from AE ("alveolar hydatid disease") and shows a divergent biological behaviour. Thus, our study fills a gap in the knowledge of the clinical course of AE.

We tried to analyze potential risk factors for late biliary complications in non-resectable AE. In our analysis, length of benzimidazole treatment and previous liver surgery were associated with late biliary complications. The association of benzimidazole treatment and late biliary complications cannot be equated with a causal relationship. However, linear regression analysis showed that surgical interventions are a risk factor for biliary complications, although late biliary complications ocurred both in patients with and without previous surgery. According to the literature, liver surgery can be associated with perioperative complications, and biliary complications after hepatic resections are known to be associated with a high risk of liver failure and operative mortality[15]. Indeed, other case series have shown that there is a learning curve in liver resections (for other indications), accounting for up to 10% of all postoperative biliary complications[16]. In a Turkish study, focussing on hydatid liver surgery (treatment of cystic hydatid disease), biliary leakage occurred in 26% of 54 patients[17]. It should be noted that the frequency of non-curative "debulking" resections in our cohort was high, but most of these operations were performed several decades ago. Today it has become clear that such surgery should be avoided[18], in line with case series reporting postoperative recurrences in 3 of 9 patients[19]. Although length of albendazole treatment was associated with biliary complications in linear regression analysis, this seems difficult to explain. Of note, the albendazole subgroup was very small, including only 5 (19%) patients, of which 4 had previously been treated with mebendazole.

Despite the known beneficial effect of benzimidazoles on longterm survival[5,7], benzimidazoles seem to be unable to completely prevent biliary complications in non-resectable AE. Despite the regression or stabilization of lesions, 28% (26/94) of patients in our study with non-resectable AE developed late cholestatic complications such as cholestatic jaundice or cholangitis 3 to 26 year after the initial diagnosis of AE. In regression analysis, follow-up time was not associated with biliary complications. This suggests that our data are credible despite different mean and median follow-up times in the biliary complications and control group (mean follow up 11 years *vs* 20.3 years, median follow up 8.6 years *vs* 32 years). However, the range of follow-up was not substantially different. This makes it unlikely that differences in follow-up time are distorting the data.

The reasons for late cholestatic complications on benzimidazole treatment are probably multifactorial and not just a consequence of previous surgery, larval proliferation and biliary infiltration. Hypothetical explanations

include malcompliance with benzimidazole treatment, which was not addressed in our study. However, in clinical practice benzimidazole levels are measured regularly (usually 4 h after drug intake) making malcompliance an unlikely cause for most patients. Furthermore, inactive AE cysts may trigger a non-productive immunological reaction, resulting in progressive fibrosis. In another scenario, the altered bile duct anatomy after AE infiltration might promote secondary infections or duodenal reflux causing secondary inflammation and fibrosis. This would explain why benzimidazoles seem to be able to inhibit AE growth, but not always the occurrence of AE complications such as biliary obstructions[19]. In any case, we are unable to determine whether benzimidazoles are only partially effective for the prevention of biliary complications or were started too late in the course of the disease.

Our study has several strengths and limitations. A major strength is the considerable number of affected patients who were evaluated in this nationwide prospective cohort and who followed a rigorous study protocol since 1980. A first limitation is that some of the studied complications occurred many years before the current data analysis, not all details about specific procedures performed and their immediate complications could be retrieved. We therefore focused on the first occurrence of biliary complications and death as major endpoints, for which a robust analysis could be performed despite these limitations. Second, follow-up times in patients with and without late biliary complications were substantially different. However, the range of follow-up was comparable between the two groups, as discussed above. Third, our analysis has to remain descriptive in many aspects and cannot provide clear risk factors for late biliary complications. There are no "parasitic factors" which could be identified as risk factors for late biliary complications. One could speculate that the PNM stage might be crucial, but we did not have access to concise PNM data. Thus, previous surgical interventions stay as major risk factor for late biliary complications.

How should the knowledge of biliary complications and their impact on long term survival change our management of non-resectable AE? It seems crucial that primary care physicians and gastroenterologists caring for such patients pay attention to the relevant risk of late biliary complications under long-term benzimidazole treatment, which is no guarantee for symptom-free long-term survival. After the detection of biliary complications, patients might benefit from advanced treatment options and tertiary care centers should be consulted. Centers will consider the benefits and risks of interventional procedures including ERCP and PTCD in the treatment of biliary complications. Furthermore, early liver transplantation in incurable symptomatic biliary AE has been suggested[20], which has so far only rarely been done due to insufficient available data. Nevertheless, an individual discussion about the risks and potential benefits of liver transplantation with otherwise eligible patients can be considered.

In summary, we have been able to demonstrate in a large cohort of patients with non-resectable AE that biliary complications are a frequent problem despite long-term chemotherapy with benzimidazoles. Late biliary complications occurred in nearly one third of patients, with previous surgery as a main risk factor. The survival in patients after occurrence of late biliary complications was short, with a median and mean survival of only 3 and 3.6 years. These data suggest that the occurrence of biliary complications can cause substantial morbidity and is associated with increased mortality.

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PROSPECTIVE STUDY

Frequency of HER2/neu overexpression in adenocarcinoma of the gastrointestinal system

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Abstract

AIM: To determine the frequency of HER2/neu protein overexpression in gastric (group A), small intestine (group B), and colorectal (group C) adenocarcinoma.

METHODS: A descriptive, cross-sectional study was performed on 50 cases of gastrointestinal adenocarcinoma (stomach, small intestine, and colorectal); 11 from group A, 8 from group B, and 31 from group C. The samples were grossed and processed in the pathology department, and sections were stained with HE (hematoxylin and eosin stain) for histopathological confirmation of malignancy (well-differentiated, moderately-differentiated, and poorly-differentiated). The confirmed samples were processed for immunomarker study of HER2/neu.

RESULTS: HER2/neu protein overexpression was found in 33 (66%) patients overall (P = 0.000). Out of 33 HER2/neu positive subjects, 23 (69.6%) were from group C, while the remaining 10 (30%) were from group A. None of the patients from group B had positive HER2/neu protein overexpression. No protein overexpression or membrane staining in < 10% tumor cells was observed in 17 (34%) patients, which were labeled as score "0" and considered negative for HER2/neu protein overexpression. Faint/weak staining (in ⩾ 10% of tumors cells) were observed in 8 (16%) patients and given the "1+" score. Similarly 13 (26%) patients reported moderate staining (in ⩾ 10% tumor cells) and were thus labeled as "2+", and strong staining (in ⩾ 10% tumors cells), labeled as "3+", was observed in 12 (24%) patients. Out of 50 patients, 26 (52%) were suffering from grade-Ⅱ malignancy, 16 (32%) from grade-Ⅰ, and 8 (16%) from grade-Ⅲ. There was highly significant association between tumor grades and HER2/neu protein overexpression (P = 0.0000).

CONCLUSION: HER2/neu protein is credibly overexpressed in colon and gastric adenocarcinomas in immunohistochemistry. There is significant association between grade of tumor and HER2/neu protein overexpression.

Key words: HER2/neu; Immunohistochemistry; Adenocarcinoma; Gastrointestinal system

Core tip: The role of HER2/neu was seen in 50 cases of gastrointestinal adenocarcinoma by immunohistochemistry. Overall, HER2/neu protein overexpression was found in 33 (66%) patients (P = 0.000), with 23 (69.6%) being colorectal carcinoma while the remaining 10 (30%) were gastric carcinoma. There was highly significant association between tumor grades and HER2/neu protein overexpression. HER2/neu protein was overexpressed in those adenocarcinomas showing a significant association between grade of tumor and HER2/neu protein overexpression. This discovery may improve treatment options for cases of gastric and

colorectal carcinomas.

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INTRODUCTION

HER2/neu is an oncogene located on the chromosome 17q21. Similar to epidermal growth factor receptors HER1, HER3, and HER4, it is a member of the tyrosine kinase receptor family. Activation of HER2/neu leads to initiation of signaling pathways like MAPK/P13K/AKT, essential for cell proliferation and differentiation[1]. Overexpression of HER2/neu has been reported in many epithelial malignancies, including cancers of the lungs[2], prostate[3,4], bladder[5], pancreas[6], and osteosarcoma[7]. The role of HER2/neu directed therapy and its success in breast cancer patients has led to evaluation of protein overexpression, gene amplification, and antitumor activity of Herceptin in multiple tumor types, including colorectal and gastric adenocarcinomas[8,9].

The data available in the literature for HER2/neu positivity rates in gastric cancers vary from about 7%-43%. HER2/neu positive status in gastric cancer also appears to be associated with more aggressive disease, a poorer prognosis, and shorter survival. Recently, the EMEA (European Medicine Agency) has approved trastuzumab in combination with chemotherapy for use in patients with advanced gastric cancer[10-13].

In the West, colorectal cancer is one of the most common malignancies. Survival of patients with colorectal carcinoma has improved due to development of new cytotoxic agents (oxaliplatin and irinotecan) and advanced surgical techniques, but no further treatment options are available when patients become refractory to these modern chemotherapeutic regimens. Recently, however, the effects of these cytotoxic agents have been augmented by the development of monoclonal antibodies against vascular endothelial and epidermal growth factor receptors. Several studies have reported a variety of HER2/neu protein overexpression and gene amplification in colorectal adenocarcinoma. Data available about the prevalence of HER2/neu protein overexpression in colorectal cancer ranges from 0%-83%. However HER2/neu protein overexpression and its relation with clinicopathological features like Dukes classification and survival is conflicting[8,9].

This wide range of HER2/neu protein overexpression in colorectal adenocarcinoma reflects both differences in reagents used and methods, as well as study bias associated with patients selection (*i.e.*, early versus advanced disease).

MATERIALS AND METHODS

The study was carried out at the Department of Pathology of the King Edward Medical University's Mayo Hospital, Lahore and the Nawaz Sharif Social Security Teaching Hospital, Multan Road, Lahore. Informed consent was obtained from patients or their guardians. Patient identities were concealed, and approval from the University Research Ethical Committee was obtained prior to conducting this study.

Study design

This was a descriptive, prospective cross-sectional study on 50 cases of gastrointestinal (GIT) adenocarcinoma (stomach, small intestine, and colorectal) collected from the Pathology Department of the King Edward Medical University's Mayo Hospital, Lahore and the Nawaz Sharif Social Security Teaching Hospital, Multan Road, Lahore.

History and clinical findings of the patients were entered *pro forma*. Patient consent was obtained *via* their own signature, a thumb sign, or by the signature of a guardian. The samples were grossed and processed at the Department of Pathology, King Edward Medical University, Lahore. The sections were stained with HE (hematoxylin and eosin stain) for histopathological confirmation of malignancy. The histological grading of these tumors as well-differentiated (Ⅰ), moderately-differentiated (Ⅱ), and poorly-differentiated (Ⅲ) was done according to the criterion of the American Joint Committee on Cancer[14,15]. The confirmed samples were processed for immunmarker study of HER2/neu. Known positive breast cancer cases were used as positive controls, with the omission of primary antibodies for the negative control. All sections were evaluated by two independent senior pathologists. Tumors with HER2/neu staining showing in ⩾ 10% cancer cells were labeled as positive for HER2/neu protein overexpression. The results were reported as positive (cytoplasmic and membranous) or negative, and scored according to the intensity of the stain (weak, moderate, and severe) as 1+, 2+, or 3+.

Study duration

The study was completed within 6 mo after the approval of final synopsis.

Operational definitions

HER2/neu oncogene, a member of the tyrosine kinase receptor family, is essential for cell proliferation and differentiation, and its overexpression and/or amplification is associated with many types of tumors. Immunohistochemistry was performed by using a Hercep test kit to determine positive protein overexpression of HER2/neu in different grades of stomach, small intestine, and colorectal adenocarcinomas.

Table 1 Positive intensity of HER2/neu staining with respect to study group age

Intensity of HER2/neu staining	Study groups			
	Group A	Group B	Group C	Total
No stain or membrane staining < 10% (0)	1	8	8	17
Weak staining ⩾ 10% (1+)	2	0	6	8
Moderate staining ⩾ 10% (2 +)	6	0	7	13
Strong staining ⩾ 10% (3 +)	2	0	10	12
Total	11	8	31	50
Mean age (yr)	52.81 ± 9.41	50.25 ± 9.41	46.51 ± 15.92	48.50 ± 13.62
Minimum age (yr)	40.00	45.00	17.00	17.00
Maximum age (yr)	70.00	60.00	75.00	75.00

χ^2 = 19.49, *P* value = 0.000 (regarding ratio of positivity among different groups); χ^2 = 23.023, *P* = 0.001 (regarding intensity of positivity among different groups). Group A: Gastric; Group B: Small intestine; Group C: Colorectal; Score 0: Negative HER2/neu protein overexpression; Score 1+, 2+, and 3+: Positive HER2/neu protein overexpression.

Inclusion and exclusion criteria

Fifty fresh cases of stomach, small intestine, and colorectal adenocarcinomas diagnosed *via* histopathology were included in this study. Patients of both sexes and a wide range of ages were taken in this study. Inadequate biopsies, autolysed specimens, and inflammatory lesions were excluded.

Immunohistochemistry scoring

Results were evaluated according to the criteria recommended by the manufacturer by using scores from 0 to 3+. No staining at all or membrane staining in < 10% of tumor cells was given the score "0". Faint staining in ⩾ 10% of tumor cells was given the score "1+", as well as in cells which were only stained in part of the membrane. Weak to moderate staining of the entire membrane in ⩾ 10% of the tumor cells was given the score "2+", and strong staining of the entire membrane in ⩾ 10% tumor cells was given the score "3+". Scores of 0 were labeled as negative tumors, while scores of 1+, 2+, and 3+ were labeled as weak, moderate, and strong positive expression of HER2/neu, respectively[16].

Evaluation of gastric cases was performed according to the modified gastric cancer testing protocol[17]. Accordingly, strong incomplete (basolateral) membranous staining was accepted as positive (3+), and the 10% cut-off for this group in biopsy cases was abolished (Hofmann M *et al*[17] 2008). In biopsy cases, a focus (clone) that is allowed to be scored should have at least 5 stained evaluable cells. Score 0 was considered as negative HER2/neu protein overexpression, while score 1+, 2+, and 3+ were considered as positive HER2/neu protein overexpression in our study.

Ethics

The Institutional Review Board of the King Edward Medical University of Lahore approved the present study.

Statistical analysis

All information related to patients and their ailments was noted in a prescribed *pro forma* method. The data was analyzed by SPSS version 18. The comparison of HER2/neu protein overexpression in different grades was compared by chi-square test. A *P* value less than 0.5 was considered statistically significant.

RESULTS

The mean age of the studied patients was 48.5 ± 13.63 years (range: 17-75). The mean age of subjects in group A was found to be 52.82 ± 9.41 years (range: 40-70). In group B, the mean age observed was 50.25 ± 5.99 years (range: 45-60). The mean age in group C was 46.52 ± 15.92 years (range: 17-75) (Table 1).

In terms of gender, 23 males and 27 females were included in this study, with a male to female ratio of 1:1.13. The highest number of males was observed in group C (12, 52%) followed by group A (9, 39.1%), with the fewest in group B (2, 8.6%). There was a statistically insignificant association between gender and study groups (*P* = 0.078) (Figure 1).

HER2/neu protein overexpression was found in 33 (66%) patients overall. Out of 33 HER2 positive subjects, 23 (69.6%) were from group C, while the remaining 10 (30%) were from group A. None of the patients from group B had positive HER2/neu status. HER2/neu protein overexpression was not found in 17 (34%) patients, which was regarded as negative expression. Among these 17 HER2 negative patients, 8 (47%) were from group C, 1 (5.8%) was from group A, and 8 (47%) were from group B. There was a highly significant association between HER2/neu protein overexpression (positivity) status and the different study groups (*P* = 0.000) (Table 1). No expression or membrane staining (in < 10% of tumors cells) was observed in 17 (34%) of the patients who were scored as "0" and labeled negative. Out of these, 8 (47%) were in group C, 1 (5.8%) was from group A, and 8 (47%) were from group B. Faint/weak staining (in ⩾ 10% of tumor cells) was observed in 8 (16%) of the patients ("1+" score), of which 6 (75%) were from group C and 2 (25%) were from group A. Similarly, 13 (26%) patients reported moderate staining (in ⩾ 10% tumors cells) and were scored "2+"; 7 of these patients were from group C and 6 were from

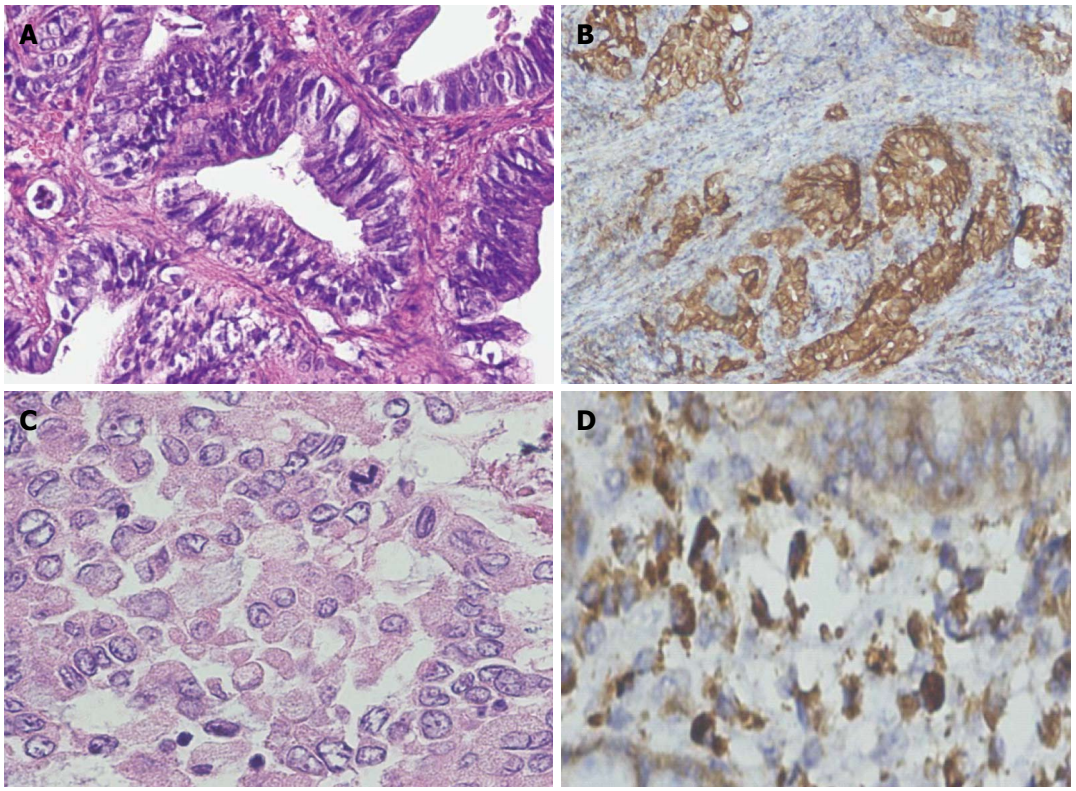

Figure 1 HER2/neu overexpression in gastric carcinoma (HE, x 20). A: Photomicrograph showing well-differentiated non-mucinous colon adenocarcinoma; B: Photomicrograph showing expression of HER2/neu in well-differentiated non-mucinous colon adenocarcinoma; C: Photomicrograph showing the signet ring variant of stomach adenocarcinoma; D: Photomicrograph showing expression of HER2/neu in gastric carcinoma.

Table 2 HER2/neu status and lymph nodes involvement

Lymph nodes	HER2/neu status		Total
	+Ve	-Ve	
Tumor involvement	8	1	9
No tumor involvement	12	3	15
Total	20	4	24

$\chi^2 = 0.320$; $P = 0.572$.

group A. Strong staining (in ⩾ 10% of tumor cells) was scored "3+", and was observed in 12 (24%) patients, of which 10 (83%) were from group C and 2 (16.6%) were from group A. Score "0" was regarded as negative while scores of 1+, 2+, and 3+ were regarded as positive for HER2/neu protein overexpression. Statistically, a highly significant association was seen between intensity of HER2/neu overexpression and the different study groups ($P = 0.000$) (Table 1).

Among 33 HER2/neu positive subjects, 18 (54.5%) were grade-Ⅱ malignancy, 11 (33%) were grade-Ⅰ, while 4 (12%) were grade Ⅲ. Out of 17 HER2/neu negative subjects, 8 (47.05%) were grade-Ⅱ, 5 (29.4%) were grade-Ⅰ, and 4 (23.5%) were grade Ⅲ. There was a highly significant association between tumor grades and HER2/neu overexpression ($P = 0.0000$) (Table 1). Among 9 subjects with lymph nodes metastasis, 8 (88.8%) were HER2/neu positive and 1 (11.1%) was HER2/neu negative. Out of 15 subjects with no lymph nodes metastasis, 12 (80%) were HER2/neu positive and 3 (20%) were HER2/neu negative. There is no significant association between lymph node involvement and HER2/neu overexpression ($P = 0.572$) (Table 2).

Regarding the morphological types of adenocarcinomas positive for HER2/neu, we found 16 (48%) cases of adenocarcinoma (not otherwise specified) positive for HER2/neu overexpression, among which 13 (81.25%) were from group C and 3 (18%) were from group A. Five (15%) cases positive for HER2/neu overexpression were mucinous adenocarcinoma, out of which 4 (80%) were from group C and 1 (20%) was from group A. Five (15%) cases of positive HER2/neu overexpression were of the signet ring variant of adenocarcinoma; 2 (40%) from group C and 3 (60%) from group A. Both cases of adenocarcinoma with neuroendocrine differentiation were from group C and were strongly positive for HER2/neu overexpression (3+). Similarly, both cases of the adenocarcinoma papillary variant from group C were weak to moderately positive for HER2/neu status. All 3 cases of the intestinal variant of adenocarcinoma were from group A and moderately positive for HER2/neu status (2+). A highly significant association was observed between different morphological types and HER2/neu overexpression ($P = 0.0000$) (Table 3).

Tumor staging was not applicable (NA) on biopsy specimens 18 (36%); 6 (33%) were from group C, 7 (38%) from group A, and 5 (27%) from group B. Staging was only done on surgically resected specimens. In

Table 3 HER2/neu status and morphological types

Morphological type grade of tumor	HER2/neu status		
	Positive	Negative	Total
Grade-			
Ⅰ	11	5	16
Ⅱ	18	8	26
Ⅲ	4	4	8
Total[1]	33	17	50
Adenocarcinoma (NOS)	16	13	29
Mucinous adenocarcinoma	5	2	7
Adenocarcinoma (signet ring variant)	5	1	6
Adenocarcinoma (intestinal variant)	3	0	3
Papillary adenocarcinoma	2	0	2
Adenocarcinoma neuroendocrine differentiation	2	0	2
High grade neuroendocrine carcinoma	0	1	1
Total[2]	33	17	50

[1]χ^2 = 22.906; P = 0.0000; [2]χ^2 = 15.062; P = 0.0000.

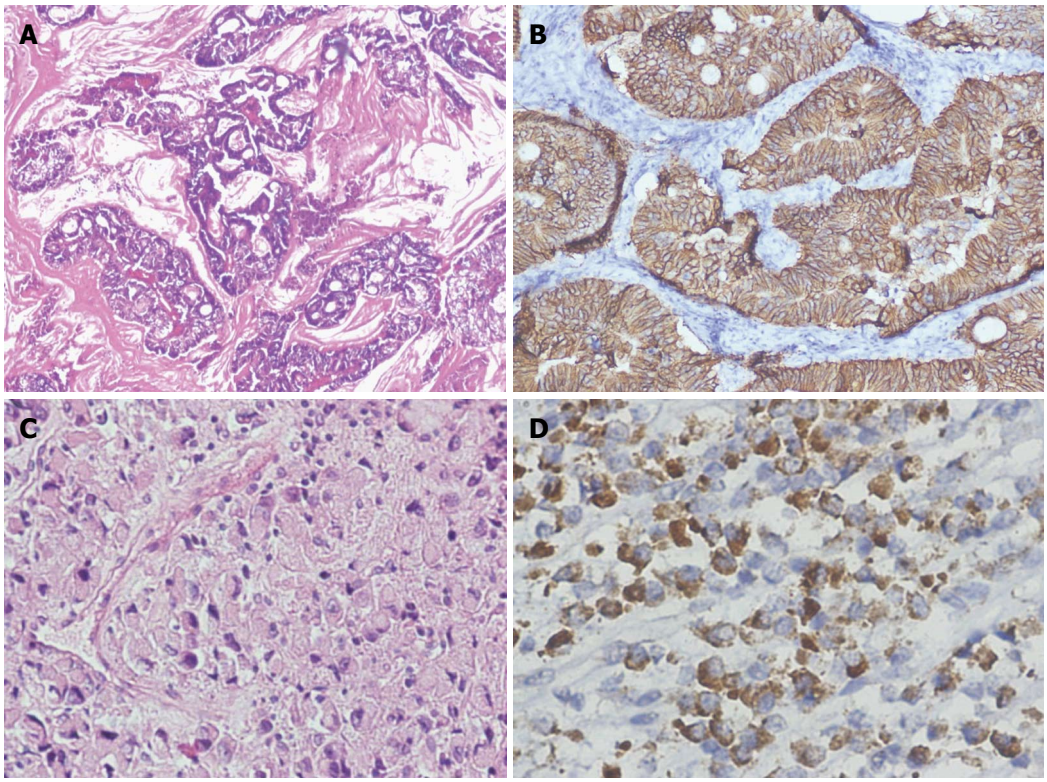

Figure 2 HER2/neu overexpression in colon adenocarcinoma (HE, x 20). A: Photomicrograph showing well-differentiated mucinous colon adenocarcinoma, B: Photomicrograph showing expression of HER2/neu in well-differentiated non-mucinous colon adenocarcinoma; C: Photomicrograph showing the signet ring variant of colon adenocarcinoma; D: Photomicrograph showing expression of HER2/neu in colonic carcinoma.

the tumor stage of modified Dukes, B2 was found to be most frequent [*i.e.*, 19 (38%) group C patients], with C2 being the second most frequently observed tumor stage at 4 (8%), followed by Dukes C1 in 2 (4%). The remaining tumor stages were found in each study group, except for tumor stage-ⅡA in one patient in both group A and B. A highly significant association was found between all tumor stages and study groups (P = 0.000) (Table 3). Expression of HER2/neu is shown in Figures 1 and 2.

DISCUSSION

In our study, a total of 50 subjects were included; 11 from group A, 8 from group B, and 31 from group C. The mean age of overall patients was 48.5 ± 13.63 years (range: 17-75). The mean age in our study shows that presentation is more common in later life, which is an important focus in many studies based on HER2/neu overexpression. A comparative study in 2004 found old-

er age to be statistically correlated with overexpression of HER2/neu in endometrial carcinoma[18]. Additionally, a total of 23 males and 27 females were included in our study, with a male to female ratio of 1:1.13. There was a very slight difference in male to female ratio in our setting, similar to another study in New York (2003), in which the male to female ratio was 1.03:1 (86 males and 83 females)[19]. A statistically insignificant association was found between gender and study groups (P = 0.074) in our setting. This insignificant association was compatible to another study conducted in 2006 by Schuell *et al*[16], which evaluated the frequency of HER2/neu protein overexpression in colorectal cancer. 77 cases of surgically resected malignant colorectal cancer lesions were evaluated *via* a similar mode of immunohistochemistry, as was used in our study. The relationship with gender was also found to be statistically insignificant in that study[16].

The tumor grades in our study varied with respect to the different study groups. Out of 26 (52%) patients who were suffering from Grade Ⅱ malignancy, 20 (76.9%) were from group C, 2 (7.7%) were from group A, and 4 (15.3%) were from group B. The Grade I category was second commonest (16, 32%) and consisted of 10 (62.5%) from group C, 4 (25%) from group A, and 2 (12.5%) from group B. Very few patients (8, 16%) were observed to have a grade Ⅲ tumor. Importantly, the prime focus of our study was to establish the obvious positivity status of HER2/neu protein overexpression by immunohistochemistry among different study groups. In our study, HER2/neu expression was found to be positive in 33 (66%) patients overall, whilst it was negative in 17 (34%) patients. Out of these 33 HER2 positive subjects, 23 (69.7%) were from group C, while the remaining 10 (30.3%) were from group A; none of the patients from group B had a positive HER2 status. A highly significant association was also observed between HER2/neu protein positivity status and the different study groups (P = 0.000). Among 33 HER2/neu positive patients in our study, 18 (54.5%) were of grade Ⅱ malignancy, 11 (33%) were of grade I malignancy, and 4 (12%) were of grade Ⅲ malignancy. A highly significant association between tumor grades and HER2/neu status was seen in our study. A study conducted in Iran in 2006 to evaluate the frequency and staining pattern of HER2/neu protein overexpression in colon carcinoma by immunohistochemistry found HER2/neu positivity to be as high as 59.4% of cases. The association between HER2/neu protein overexpression and tumor grades was also highly significant (P = 0.04) in this study; 66.7% cases were grade I , followed by 36% grade Ⅱ malignancies. In higher grades, HER2/neu staining was decreased[20].

Half *et al*[21] conducted a study to assess HER2 expression in colon cancer. They documented cytoplasmic staining in 63.5% of primary tumors, with strong membranous staining observed in 5% of primary colorectal carcinomas. They also found significant correlation between HER2 positive overexpression (cytoplasmic staining) and tumor differentiation, as is the case in our study. The HER2 positivity in our study (66%) was much greater than that indicated in a study conducted in 2007, in which HER2/neu overexpression and gene amplification was assessed in 137 colorectal patients using immunohistochemistry and FISH. 47.4% subjects were determined to have overexpressed HER2/neu by immunohistochemistry. However, similar to our own study, a significant correlation of malignancy with HER2/neu overexpression was observed[22]. In a study which involved only gastric carcinoma, patients showed the even smaller percentage of only 18.8% patients with HER2 positive status, with a statistically insignificant association contrasting with our results in which a higher frequency, as well as significant association of HER2 positivity, was observed[23]. A Chinese study that included 145 patients with gastric carcinoma showed a total frequency of 32.4% positive HER2/neu protein overexpression, which was statistically significant. In this study, the evaluation of lymph nodes involvement was also assessed and found to be significantly associated with HER2/neu status[24].

Contrary to these results, our study showed an insignificant association of lymph node involvement and HER2/neu status (P = 0.572). Lymph nodes were identified in 24 subjects, of which 9 (37.5%) displayed lymph node metastasis. Among these, 6 (67%) were from group C, with the other 3 (33%) being from group A. Among the 9 subjects with lymph nodes metastasis, 8 (88.8%) were HER2 positive and 1 (11.1%) was HER2 negative. Of the 15 subjects with no lymph nodes metastases, 12 (80%) were HER2 positive and 3 (20%) were HER2 negative. In the above study, only gastric carcinoma patients were studied, and they were found to have significant association with lymph node involvement. In a Chinese study conducted in 2006, a significant association was found between HER2/neu protein overexpression, lymph node metastasis, and clinical stage[25]. Hence, lymph node involvement is generally considered in adenocarcinomas when HER2 protein overexpression is concerned. We were unable to establish this relationship statistically despite having a considerable frequency apparently involved with tumors. General cancer studies that were not GIT-related malignancies, particularly breast cancer, have also indicated possible involvement of HER2/neu in lymph node metastatic tumors[26].

McKay *et al*[27] studied HER2/neu protein overexpression in a large cohort of colorectal tumors and lymph node metastases. HER2/neu was expressed in 81.8% tumors. They did not find any correlation between HER2/neu protein overexpression and lymph node metastasis. The results were very similar to our study, especially with regards to the correlation between HER2/neu protein overexpression and lymph node metastasis.

Another study assessed how the expression of HER2/neu correlates with the stage of disease and survival in colorectal cancer. This study included 201 samples and

found HER2 positivity status to be significantly associated with adenocarcinomas, similar to our study[28]. Ross *et al*[29] studied HER2/neu protein overexpression in gastrointestinal tract tumors. They found a wide range of HER2/neu protein overexpression in esophageal, gastric, and colon carcinomas. They concluded that either HER2/neu protein overexpression or gene amplification is associated with one-fourth of all GIT malignancies, and strategies should thus be designed to employ this marker in therapy selection. Among adenocarcinomas of the gastrointestinal system, colorectal and gastric carcinomas frequently present with protein overexpression of HER2/neu, and are reported to be affected by its presence as well[29].

From our study, it would appear that HER2/neu protein overexpression may play a crucial role in the therapeutic management of GIT adenocarcinomas, particularly in the colorectal region. Further studies are suggested to increase the knowledge in this area, assess other clinical dimensions of HER2/neu involvement, and to provide in depth impact evaluation of cancers. HER2/neu protein is credibly overexpressed in colorectal and gastric adenocarcinomas

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COMMENTS

Background

Colorectal carcinoma and carcinoma of the stomach are the most prevalent cancers worldwide as well as in Pakistan, where it has a poor prognosis. HER2/neu is an epidermal growth factor receptor 2, and its overexpression has been detected in gastric and colorectal carcinomas that are essential for understanding the biological behavior of HER2/neu on these cancers. Chemotherapy with Herceptin (trastuzumab) can improve the prognosis of patients, but a standardized HER2 scoring system is still required. This study on the role of HER2/neu in these malignancies, as well as its clinicopathological attributes, could help patient selection for clinicians performing targeted therapy using anti-HER2/neu drugs like Herceptin.

Research frontiers

Anti-HER2/neu therapy is playing a significant role in breast carcinoma and as a new treatment option for gastrointestinal malignancies in suitable candidates. The Food and Drug Administration has approved the use of Herceptin for the treatment of gastric and colorectal carcinomas. Many researchers have been evaluating HER2/neu positive patients daily *via* the accurate and reliable Immunohistochemistry scoring system for selection of Herceptin targeted therapy.

Innovations and breakthroughs

In Pakistan, there have been limited studies concerning the role of HER2/neu expression in patients with gastrointestinal (GIT) malignancies. The results show HER2/neu protein overexpression in high grade tumors and lymph node metastases, and these cases could represent ideal candidates for Herceptin targeted therapy.

Applications

HER2/neu overexpression is significantly associated with tumor grade. Therefore it could be applied as a reliable immunmarker, with patients overexpressing HER2/neu being potential candidates for new adjuvant monoclonal antibody-based targeted immunotherapy.

Peer review

The study design was valid and the data was sufficient to make a conclusion on the frequency of HER2/neu proteins overexpression in adenocarcinomas of the GIT system.

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PROSPECTIVE STUDY

Determining hepatitis C virus genotype distribution among high-risk groups in Iran using real-time PCR

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Abstract

AIM: To assess hepatitis C virus (HCV) genotype patterns among high-risk Iranian groups, using real-time RT-PCR.

METHODS: In this study, we evaluated the distribution of different HCV genotypes among injection drug users and other high-risk groups over a 4-year period (from 2009 to 2012) using real-time polymerase chain reaction (PCR). Sera from 888 HCV-infected patients residing in southern and southwest Iran were genotyped using real-time PCR with common primers and specific probes. These patients were grouped into distinct exposure categories. Illicit drug users constituted the primary group and were further evaluated for HCV genotype distribution and parameters such as age range.

RESULTS: Of the examined HCV-infected patients, 62% were substance abusers, although the route of transmission could not be determined in approximately 30% of these patients. HCV genotyping revealed that Gt1 was the most prevalent genotype among the drug users as well as among patients with thalassemia, hemophilia, solid organ recipients and those on hemodialysis. Mixed infections were only seen in addict groups, where Gt2 genotype was also found. The highest frequencies in HCV-positive addict patients were observed in the 31-40 age group. Our research also showed that the addiction age has increased, whereas the addiction rate has dropped in this region. Most illicit drug users had more than one risk factor such as tattoo and/or a history of imprisonment.

CONCLUSION: This study revealed that the most common HCV-infection route and HCV-genotype in southern and southwest Iran was illicit drug abuse and Gt1, respectively.

Key words: Hepatitis C virus genotype distribution; Injection drug users; Real-time PCR; Iran

Core tip: The primary treatment method in hepatitis C virus (HCV) infection, determination of evolution pathways, assessment of epidemiological status, and knowledge of HCV genotype distribution among high-risk groups such as addicts are very important. We assessed the different HCV genotypes among illicit drug users and other high-risk groups during a 4-year period from 2009 to 2012 using real-time PCR. We found that the most affected high-risk groups were illicit drug users and specified the respective age distribution and risk factors. An important finding in this research was the genotype pattern shift from 3 to 1, especially among addicts.

Jamalidoust M, Namayandeh M, Asaei S, Aliabadi N, Ziyaeyan M. Determining hepatitis C virus genotype distribution among high-risk groups in Iran using real-time PCR. *World J Gastroenterol* 2014; 20(19): 5897-5902 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5897.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5897

INTRODUCTION

The increasing use of different types of injections, including herbal injections, particularly chemical drugs, in many developed and developing countries, including Iran, and the lack of an effective hepatitis C virus (HCV) vaccine have resulted in HCV infection becoming a major public health concern[1,2]. A lower prevalence of HCV infection has been reported in Iran compared to other parts of the world, particularly the Middle East[1,2]. Given that Injection Drug Users (IDUs) constitute a major group of HCV patients, planning their treatment is essential for community health[3,4].

HCV genotyping is the most significant predictor of treatment duration and evaluation of the course of infection, with different genotypes showing different treatment responses and varying epidemiological as well as virological features[5]. HCV has a single-stranded RNA genome and at least six different genotypes, more than 90 subtypes and many types of quasi-species variants based on scattered and local variations of its genome. This genetic heterogeneity is due to the lack of fidelity of viral RNA-dependent RNA polymerases[6]. HCV-RNA diversity is concentrated in the E1 and E2 glycoprotein-coding regions of the genome, while least heterogeneity is found in the regions encoding core and NS3 proteins, which represent structural and non-structural proteins, respectively. In total, the nucleotide sequences of the different genotypes differ from each other by 30%-35%, with a divergence of 92% in the 5' -UTR regions[1].

The HCV genotype pattern varies among different groups of infected individuals, particularly IDUs, in different areas. For example, the predominant genotypes are Gt3a and Gt4 in European countries and Gt3a in Argentina[1,7-9]. Mahfoud *et al*[10] showed that HCV Gt3 was distributed among Lebanese IDUs at a frequency of 57%. The aim of this study was to assess HCV genotype distribution among drug-addicted HCV-infected patients in Iran and compare this distribution with other high-risk groups of infected patients using real-time PCR, which simultaneously detects target HCV genomes with genotype-specific primer sets and probes.

MATERIALS AND METHODS

From March 2009 to December 2012, a total of 888 HCV-RNA positive patients from southern and southwest Iran were enrolled in this cross-sectional study and underwent HCV genotyping. These patients completed a preliminary assessment, which allowed the identification of individuals with parenteral risk factors. Of these patients, 550 (61.9%) IDUs aged 18-74 years (mean ± SD, 37.89 ± 10.192 years) were identified, including 546 (99.27%) males and 4 (0.72%) females. None of the patients had previously been treated for HCV infection. Ethylenediaminetetraacetic acid (EDTA) plasma was prospectively collected, and aliquots were stored in 1.5 mL vials at -70 ℃. HCV infection was reaffirmed by the detection of antibodies using an immunochromatographic assay (Artron, Burnaby, BC, Canada).

Viral RNA was isolated from 200 μL aliquots of serum samples using the InviTrap® Spin Blood RNA Mini kit (Invitec, Berlin) as per the manufacturer's instructions, and eluted using 50 μL of nuclease-free water. The concentration of the isolated HCV-RNA was determined for the various samples and then genotyped, followed by real-time PCR using commercially available HCV kits (Genome Diagnostics Pvt. Ltd., Hague, Netherland) to determine HCV genotypes Gt1 to Gt4. All tests were carried out in accordance with the manufacturer's instructions. The PCR conditions were as follows: 50 ℃ for 25 min and 95 ℃ for 10 min, followed by 50 cycles of 94 ℃ for 10 s, 55 ℃ for 32 s and 72 ℃ for 25 s. The reactions were performed in a 7500 real-time PCR system (Applied Biosystems, United States).

Statistical analysis

This study was performed using SPSS for Windows systems (Version 16.0, 2007, SPSS Inc, Chicago, IL, United States). Comparison of HCV genotypes, their distribution among different high-risk groups and among different age groups of addicts were analysed by the χ^2 test.

Table 1 Frequency of hepatitis C virus infection among high-risk groups *n* (%)

High-risk group	Frequency	M/F
Addict	550 (62.2)	546/4
Thalassemia	38 (4.3)	17/21
Hemophilia	8 (0.9)	7/1
Kidney graft	4 (0.5)	2/2
Dialysis	6 (0.7)	5/1
Liver graft	3 (0.3)	1/2
Unknown	277 (31.3)	209/68
Total	886 (100.0)	787/99

RESULTS

A total of 888 HCV RNA-positive samples, collected between 2009 and 2012 from patients with chronic HCV infection, were included in this study [787 (83%) men and 99 (11.2%) women, 2 misplaced]. Of these, 738 (83%) HCV-positive serum samples were genotyped successfully using HCV genotype real-time PCR kits from Applied Biosystems. The frequency of HCV infection among various high-risk groups and HCV genotype distribution among the patients are presented in Table 1 and 2.

Table 2 Distribution of hepatitis C virus genotypes among Iranian hepatitis C virus infected patients according to route of infection *n* (%)

High-risk group	Frequency	Undetectable	Gt1	Gt2	Gt3	Gt4	Gt1/2	Gt1/3
Addict	550	67 (12.2)	283 (51.5)	0 (0.0)	192 (34.9)	0 (0.0)	2 (0.4)	6 (1.1)
Thalassemia	38	15 (39.5)	17 (44.7)	0 (0.0)	6 (15.8)	0 (0.0)	0 (0.0)	0 (0.0)
Hemophilia	8	2 (25.0)	5 (62.5)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
Kidney graft	4	1 (25.0)	2 (50)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)
Dialysis	6	1 (16.7)	4 (66.7)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)
Liver graft	3	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.)	0 (0.0)	0 (0.0)	0 (0.0)
Unknown	277	64 (23.1)	140 (50.5)	3 (1.1)	67 (24.2)	3 (1.1)	0 (0.0)	0 (0.0)
Total	886	151 (17.0)	453 (51.1)	3 (0.3)	267 (30.1)	4 (0.5)	2 (0.2)	6 (0.7)

HCV: Hepatitis C virus.

Table 3 Comparison of illicit drug user age-groups in terms of hepatitis C virus genotypes *n* (%)

	Undetectable	Gt1	Gt2	Gt3	Gt4	Gt1/2	Gt1/3	Total
< 20	0 (0.0)	1 (33.3)	0 (0.0)	2 (66.66)	0 (0.0)	0 (0.0)	0 (0.0)	3 (0.57)
21-30	14 (11.02)	59 (46.45)	0 (0.0)	53 (41.73)	0 (0.0)	0 (0.0)	1 (0.78)	127 (24.47)
31-40	23 (10.95)	113 (53.80)	0 (0.0)	69 (32.85)	0 (0.0)	2 (0.95)	3 (1.42)	210 (40.46)
41-50	18 (16.36)	58 (52.72)	0 (0.0)	33 (30)	0 (0.0)	0 (0.0)	1 (0.9)	110 (21.19)
51-60	6 (11.53)	25 (48.07)	0 (0.0)	20 (38.46)	0 (0.0)	0 (0.0)	1 (1.92)	52 (10.01)
> 60	1 (5.88)	10 (58.82)	0 (0.0)	6 (35.29)	0 (0.0)	0 (0.0)	0 (0.0)	17 (3.27)
Total	62 (11.9)	266 (51.25)	0 (0.0)	183 (35.26)	0 (0.0)	2 (0.38)	6 (1.15)	519 (100.0)

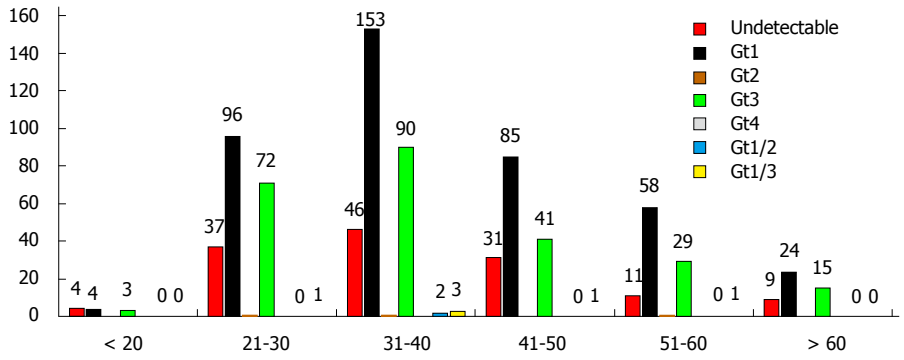

Figure 1 Hepatitis C virus genotype distribution among different addict age groups.

Table 4 Comparison of hepatitis C virus genotypes in the addict and non-addict high-risk groups

Genotype	Non-addict	Addict
Gt1	50.7%	51.5%
Gt2	1.0%	0.0%
Gt3	22.0%	34.9%
Gt4	1.3%	0.0%
Gt1/2	0.0%	0.4%
G1/3	0.0%	1.1%
Unknown	25.0%	12.2%

As shown in Table 1, HCV infection was most prevalent among substance abusers (62.2%), with the highest frequency (> 99%) among male drug users. The high prevalence of HCV infection can also be attributed to unknown factors compromising approximately 31% of cases. The other groups at high-risk for HCV infection included patients with thalassemia, hemophilia and dialysis as well as recipients of solid organs.

Overall, the most prevalent HCV genotypes were Gt1 (51.1%) and Gt3 (30.1%), whereas Gt4 (0.5%) and Gt2 (0.3%) were less prevalent. Mixed HCV genotypes were reported in only 8 addicts and included Gt1/3 and Gt1/2 co-infections with 0.7% and 0.2% prevalence, respectively (Table 2).

Distribution of HCV genotypes among addicts

A past or current history of illicit drug use was the predominant risk factor for HCV infection. A total of 550 addicts [546 (99.27%) men and 4 (0.72%) women] underwent HCV genotyping; 483 (87%) addicts were genotyped, and included 475 (87.8%) patients with a single HCV genotype and 8 (12.2%) patients with a mixed genotype distribution. The most prevalent HCV genotype was Gt1, which was found in 283 addicts (51.5%), followed by Gt3 in 192 (34.9%), Gt1/3 in 6 (1.1%) and Gt1/2 in 2 (0.4%) addicts. Single or mixed genotypes of Gt2 and Gt4 were not detected in any of the addicts.

The prevalence of HCV infection and HCV genotype distribution varied between different age groups; as shown in Table 3 and Figure 1, the highest frequency of HCV-

Table 5 Important risk factors in Iranian illicit drug users *n* (%)

HCV infection	+ Imprisonment and tattoo	+ Imprisonment	+ No other risk factor	Total
Injection Drug Users	70	235	134	439 (80)
Non-Injection Drug Users	63	23	25	111 (20)
Total	133 (24)	265 (48)	159 (28)	550 (100)

positive patients was observed in the 31-40 age group.

DISCUSSION

As the most significant route of HCV infection in both developed and developing countries is addiction and the use of illicit drugs[10,11], the consideration of issues related to HCV infection such as viral load, genotyping, treatment response rate and epidemiological status is very important[2].

HCV genotyping among a defined population serves as a useful tool for determining HCV evolution in different geographical regions and in identifying the respective high-risk groups[12]. Specific HCV genotypes have also been reported as primary tools for determining infection course and assessing the duration of treatment[13]. However, certain investigations have implicated body mass index, IL28 genotype, gamma glutamyl transpeptidase, triglycerides and the level of miR-122 as other predictors of treatment duration[14].

Unfortunately, even in developed countries, only a small proportion of IDUs receive HCV treatment[15], and clinicians are often reluctant to treat them. The reasons for this trend may include high risk of reinfection, concomitant excessive alcohol intake and high rates of concurrent mental health problems[16].

Various studies suggest that the SVR Should you explain what SVR is? rate among IDUs is comparable to that among non-IDUs and that it is not significantly related to IDU status[10,17].

In clinical trials where peg interferon and ribavirin were prescribed, the median SVR rate among IDUs with chronic HCV infection was 54.3% (range, 18.1%-94.1%), comparable to 54%-63% seen in their non-IDU counterparts, whereas it was 68.5% in IDUs with acute HCV infection as opposed to 81.5% among non-IDUs[18,19].

HCV prevalence rate and genotype distribution among Asian and Middle Eastern countries is very diverse. Although Iran is surrounded by countries with a high prevalence of HCV of various genotypes, HCV prevalence in Iran (< 2%) is much lower than that of its neighbouring nations, Pakistan (5.31%) and Egypt (14.7%)[20]. Genotypes 1, 2 and 3 (Gt1-Gt3) have been found to have a global prevalence, while Gt4-Gt6 have a restricted pattern[20]. Gt4 has been chiefly detected in Egypt, some Arab countries such as Saudi Arabia, Syria and United Arab Emirates, and recently, also in certain parts of Europe[21]. Gt5 is restricted to South Africa, and Gt6 is primarily found in Southeast Asian countries such as China, Hong Kong and Taiwan[22,23].

Different studies have shown that Gt4 and Gt1 are the predominant genotypes in Arab and non-Arab countries, respectively, in the Middle East region[21]. A recent study carried out in a neighbouring country, Pakistan, identified Gt3 as the predominant HCV genotype[22,23]. Several investigations have also been conducted in different parts of Iran to determine HCV genotype distribution. The latest studies have indicated that HCV Gt among IDUs in south, southeast and north Iran (Mazandaran province), Gt1 in northeast and Gt3 in Isfahan province in central Iran, are the predominant genotypes[24-29].

The current study was performed to determine the distribution of HCV genotypes based on the route of infection, and revealed a significantly high prevalence (approximately 62%) of HCV infection among drug addicts. Many studies have been carried out in IDUs in different regions of Iran. In general, HCV seropositivity in Iranian addicts is higher than that in the general population (30%-90% *vs* 0.2%-2.0%).

Our data also show that Gt1 is the most common genotype among thalassemia and hemophilia patients, solid organ recipients and hemodialysis patients as well as IDUs. A discrepancy between HCV genotype distribution among the addict and non-addict groups was observed, with Gt3 frequency in IDUs being less than in non-IDUs (Table 4). Among IDUs, Gt2 and Gt4 were rarely seen; the latter was not detected in any IDU patient, while Gt2 was detected in just 2 patients with mixed infections of Gt1/2. Overall, among the 888 patients, mixed HCV genotypes were detected in 8 serum samples from addicts and not in non-addict groups. This could be due to multiple injections in the addict group.

A comparison of these results with our previous study[30-32] revealed an impressive shift in the distribution of HCV genotypes, from the dominant prevalence of HCV Gt3a seen previously[24] to an increased prevalence of Gt1, particularly among IDUs, in the current study.

As shown in Table 3, most of the HCV-infected addicts belonged to the 31-40 age group and accounted for 35% of the infected addicts, followed by the 21-30, 41-50 and 51-60 age groups accounting for 25%, 19% and 12% infected addicts, respectively. These results indicate that unlike the study conducted in northern Iran[25], the addiction age may not have decreased in southern and southwest Iran.

According to Table 5, although all IDUs in this study had an addictive-drug use history of a few months to several years, 72% of them had more than one risk factor. More than 70% of these patients had a history of imprisonment of varying lengths. Tattoos were another crucial risk factor that was seen only in patients who had a history of imprisonment. Drinking alcohol and having multiple sex partners were other risk factors, however,

there was little data on these risk factors.

We found that the genotype distribution also varied with respect to underlying conditions among patients from the same geographical area, and it is noteworthy that the least prevalent unknown genotypes were found among IDUs (Table 4), probably due to high mean quantitative viral load.

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COMMENTS

Background

From 2009 to 2012, hepatitis C virus (HCV) genotype patterns among different high-risk groups were determined using real-time PCR, a very sensitive and fast assay.

Research frontiers

In this research, 888 HCV patients in different high-risk groups underwent genotyping. Genotype 1 was determined to be the predominant genotype in both non-addicts (thalassemia, hemophilia, and hemodialysis patients) and addicts, the most prevalent high-risk group.

Innovations

This is the first report on HCV genotypes among HCV patients with different exposure categories, residing in south and southwest Iran, where genotype 1 was found to be the most frequent genotype. Compared to the results of the authors' previous study, they found that addiction age had increased. An important finding in this research was that the genotype pattern had shifted from 3 to 1, especially among addicts.

Application

This study demonstrated changing HCV genotype patterns in Iran and it can serve as basic research for prospective studies on HCV genome variation. This study could also be helpful for health authorities and decision makers.

Peer review

In the present study, authors examined HCV genotype in 888 Iranian patients by real-time PCR assay and evaluated the relation between infective root and HCV genotype.

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META-ANALYSIS

Histological changes of gastric mucosa after *Helicobacter pylori* eradication: A systematic review and meta-analysis

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Abstract

AIM: To systematically review pathological changes of gastric mucosa in gastric atrophy (GA) and intestinal metaplasia (IM) after *Helicobacter pylori* (*H. pylori*) eradication.

METHODS: A systematic search was made of PubMed, Web of Science, EMBASE, ClinicalTrials.gov, OVID and the Cochran Library databases for articles published before March 2013 pertaining to *H. pylori* and gastric premalignant lesions. Relevant outcomes from articles included in the meta-analysis were combined using Review Manager 5.2 software. A Begg's test was applied to test for publication bias using STATA 11 software. χ^2 and I^2 analyses were used to assess heterogeneity. Analysis of data with no heterogeneity (P > 0.1, I^2 < 25%) was carried out with a fixed effects model, otherwise the causes of heterogeneity were first analyzed and then a random effects model was applied.

RESULTS: The results of the meta-analysis showed that the pooled weighted mean difference (WMD) with 95%CI was 0.23 (0.18-0.29) between eradication and non-eradication of *H. pylori* infection in antral IM with a significant overall effect (Z = 8.19; P <0.00001) and no significant heterogeneity (χ^2 = 27.54, I^2 = 16%). The pooled WMD with 95%CI was -0.01 (-0.04-0.02) for IM in the corpus with no overall effect (Z = 0.66) or heterogeneity (χ^2 = 14.87, I^2 =0%) (fixed effects model). In antral GA, the pooled WMD with 95% CI was 0.25 (0.15-0.35) with a significant overall effect (Z = 4.78; P < 0.00001) and significant heterogeneity (χ^2 = 86.12, I^2 = 71%; P < 0.00001). The pooled WMD with 95% CI for GA of the corpus was 0.14 (0.04-0.24) with a significant overall effect (Z = 2.67; P = 0.008) and significant heterogeneity (χ^2 = 44.79, I^2 = 62%; P = 0.0003) (random effects model).

CONCLUSION: *H. pylori* eradication strongly correlates with improvement in IM in the antrum and GA in the corpus and antrum of the stomach.

Key words: *Helicobacter pylori* eradication; Gastric atrophy; Intestinal metaplasia; Pathological changes; Gastric mucosa; Meta-analysis

Core tip: This study reports the results of a meta-analysis conducted on a large number of articles using an extensive and thorough method. The inclusion of only high-quality relevant articles resulted in the identification of a very strong correlation between the eradication of *Helicobacter pylori* infection and intestinal metaplasia of the antrum, and a strong correlation with gastric atrophy in both the antrum and the corpus of the stomach.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer in the world and the second leading cause of cancer-related deaths, accounting for 10.4%[1]. The incidence and mortality of GC have fallen dramatically over the past 7 decades as a result of improved socioeconomic situations, sanitation, food preservation, as well as a decline in the incidence of *Helicobacter pylori* (*H. pylori*) infection[2-4]. Despite these declines, however, GC cure rates have not changed[4-6]. *H. pylori* infection has been known as a gastric carcinogen for over 10 years[7], and is the main cause of GC[8,9]. Infection triggers a multistep progression from chronic gastritis to gastric atrophy (GA), intestinal metaplasia (IM), dysplasia, and finally invasive cancer. *H. pylori* is a spiral-shaped, microaerophilic, Gram-negative bacterium measuring approximately 3.5 × 0.5 microns that is the cause for the most common chronic bacterial infection in humans, infecting 50% of the world population[10,11].

H. pylori, which causes active chronic gastritis in all infected patients, leads to clinically relevant diseases, such as gastric and duodenal ulcers, mucosa associated lymphoid tissue lymphoma and GC, in 20% of infected carriers[12-17]. Furthermore, meta-analyses have indicated that the infection confers a 2- to 3-fold increased risk of GC development[18,19]. While the course of the infection depends on microbial virulence, host genetic factors and environmental factors, the clinical outcomes are determined by the type and intensity of gastritis, which can be categorized as either a simple benign gastritis, a duodenal ulcer phenotype, or a GC phenotype.

As *H. pylori* infection plays a causal role in the formation of GC, eradication of infection may play a role in GC prevention[15,16]. After *H. pylori* eradication, neutrophils disappear and mononuclear cells slowly return to normal[20]. However, the improvement in gastric mucosal lesions following eradication of *H. pylori* is not entirely clear. While the majority of studies have reported a reversal of atrophy, no reversal of IM has been shown. To further examine and resolve these discrepancies, a systematic review and meta-analysis was conducted to determine if the eradication of *H. pylori* infection eliminates the precancerous lesions of GA and IM.

MATERIALS AND METHODS

Search strategy

A systematic search of PubMed, Web of Science, EMBASE, ClinicalTrials.Gov, OVID and the Cochran Library databases was made to identify relevant review articles, editorials, and original studies published through March 2013 using the following key words: *H. pylori* OR *Helicobacter pylori* (*H. pylori*) OR HP, eradication OR treatment OR cure OR therapy, gastric atrophy OR atrophic OR GA OR intestinal metaplasia, clinical test, English-language. Data were independently extracted from each study by two of the authors working independently and using a predefined form; disagreements were resolved by discussion with a third investigator.

Inclusion and exclusion criteria

Published reports were selected for inclusion in the meta-analysis according to the following criteria: (1) English language publication; (2) prospective and randomized controlled trials on *H. pylori* eradication; (3) studies of adults testing positive for the presence of *H. pylori* prior to treatment and eradication of the infection documented both by histology and carbon (C) 14 urea breath test (UBT) or 13 C-UBT (sensitivity, 100%; specificity, 96%)[21]; (4) *H. pylori* eradication as the only treatment; and (5) gastric histology from at least three pathological specimens per sample processed for hematoxylin-eosin and modified Giemsa staining. Specimens were required to have been taken at baseline and at least 6 mo after treatment, evaluated separately for the antrum and corpus, and scored using the Sydney system[22] or the updated Sydney system[23]. Studies not meeting these criteria, those without data for retrieval, and duplicate publications were excluded from the meta-analysis.

Study quality and data extraction

The quality of included studies was assessed using the Risk of Bias table outlined in the Cochrane Reviewer's Handbook 5.0.1[24]. This method evaluates biases originating from sequence generation (selection bias), allocation sequence concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), and selective outcome reporting (reporting bias). Every facet was judged as either yes, no, or unclear. A judgment of "yes" indicated that the method described was clear and correct, the information was complete, and indicated a low likelihood of bias. A judgment of "no" indicated a high likelihood of bias due to improper use of methods, unused allocation concealment, incomplete information, or selective reporting bias. An "unclear" judgment indicated that an assessment of bias could not be obtained due to insufficient descriptions. Judgments were assigned by two of the authors working independently, and discrepancies were remedied through discussions with a third investigator to obtain a consensus.

The data extracted from each study included the following: general article information (author, publication date, journal name, *etc.*); data to calculate the value of the total effect (treatment number, effective number, *etc.*); clinical heterogeneity of the study (sex, age, concurrent disease, treatment regimen, *etc.*); methodological heterogeneity of the study (design type, randomized, blinded, follow-up, quantity of and processing methods

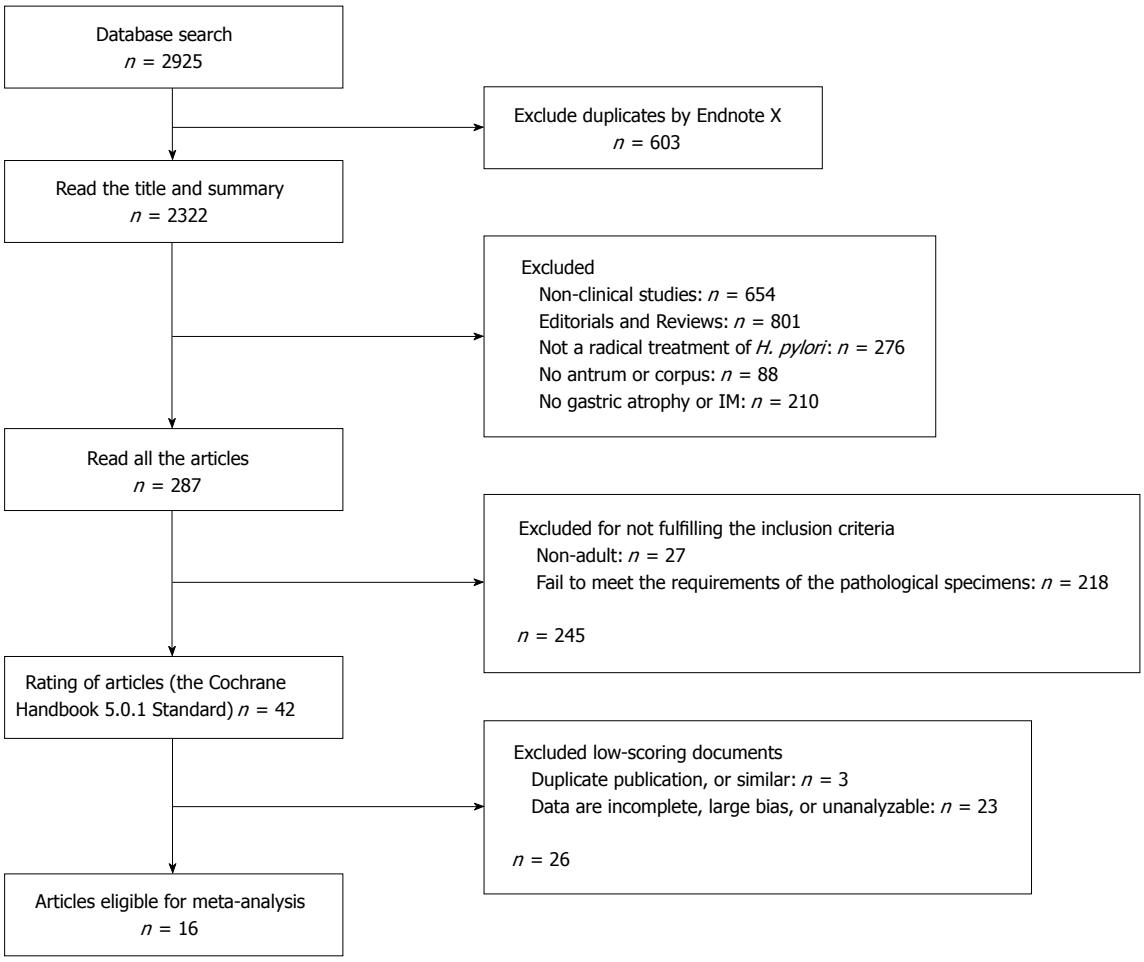


Figure 1 Flow diagram of the selection of included studies. *H. pylori*: *Helicobacter pylori*; IM: Intestinal metaplasia.

for pathological specimens, and methodology for histology scoring). Assessment of the degree of gastritis was performed according to the Sydney system[22] or the updated Sydney system[23]. For each graded variable, the following scores were assigned: 0 for absence and 1, 2 or 3 for mild, moderate or severe presence, respectively. The ultimate histology scores were used to weigh the severity of glandular atrophy or IM graded from 0 (normal) to 3 (markedly abnormal). Studies were reviewed and data extracted by two independent reviewers with knowledge of clinical medicine, epidemiology, and medical statistics, with discrepancies resolved through discussion. This process for data extraction was repeated to ensure accuracy.

Statistical analysis

Agreement on the selection of studies between the two reviewers was evaluated by the κ coefficient. Review Manager 5.2 and Begg's test with STATA 11 were used to perform the meta-analysis to compare continuous variables, such as histological scores before and after *H. pylori* eradication. The inverse variance of the weighted mean difference (WMD) and 95%CIs for gastric mucosal histology scores was estimated for each study. The chi-square test and *P*-value analysis were used to indicate the presence of heterogeneity, and the size of the heterogeneity was tested with I^2. If there was no heterogeneity, a fixed effects model was applied. In cases where heterogeneity was indicated ($P < 0.1$, $I^2 > 25\%$), causes for the heterogeneity were first analyzed; a random effects model was applied when the clinical and methodological heterogeneity could not be identified[25] and subgroup analysis or sensitivity analysis was performed when the clinical or methodological heterogeneity was identified. In the presence of significant statistical heterogeneity, sensitivity analyses were performed to examine sample size, follow-up duration, number of biopsy samples, *etc*. To perform these analyses, meta-analyses were repeated following the exclusion of each individual study one at a time, in order to assess the overall effect of each study on the pooled WMD[26]. Overall effects were considered as statistically significant with a *P*-value < 0.05. Funnel plots were constructed to assess the likelihood of publication bias[27].

RESULTS

Search results

The selection of studies included in the meta-analysis is described in a flow chart shown in Figure 1. The initial search strategy yielded 2925 citations. Of these, 1034

Table 1 Main characteristics of the 16 studies selected for meta-analysis

Ref.	Author, year (country)	Study arms, *n*		Follow-up in year	Medication	Histologic parameters			
		Eradicated	Not eradicated			GA		IM	
						Antrum	Corpus	Antrum	Corpus
[28]	Annibale B, 2000 (Italy)	25	7	0.5	BAM	Yes	Yes	Yes	Yes
		15	15	0.5	BAM				
		15	15	1	BAM				
[29]	Wambura C, 2004 (Japan)	107	118	1	L/A/C	Yes	Yes	Yes	Yes
		107	118	2	L/A/C				
		107	118	3	L/A/C				
[30]	Annibale B, 2002 (Italy)	8	0	0.5-1	BAM	Yes	Yes	Yes	Yes
		32	0	0.5-1	BAM				
[31]	Kamada T, 2005 (Japan)	20	233	1	O/L/A/C	Yes	Yes	Yes	Yes
		1767		1	O/L/A/C				
[32]	Tucci A, 1998 (Italy)	10	0	1	BAM	Yes	No	Yes	No
		8	2 (lost)	1	BAM				
[33]	Sung JJ, 2000 (China)	226	245	1	OAC	Yes	Yes	Yes	Yes
[34]	Ito M, 2002 (Japan)	22	22	5	PPI/A/C	Yes	Yes	Yes	Yes
[35]	Lahner E, 2005 (Italy)	38	36	6.7	B-BTI	Yes	Yes	Yes	Yes
[36]	Toyokawa T, 2009 (Japan)	241	19	5	PPI/A/C	Yes	Yes	Yes	Yes
[37]	Ohkusa T, 2001 (Japan)	115	48	1-1.25	PPI/A/C	Yes	Yes	Yes	Yes
[38]	Iacopini F, 2003 (Italy)	10	0	1	OMC	Yes	No	Yes	No
[39]	Kamada T, 2003 (Japan)	37	8	3	OMC	Yes	No	No	No
[40]	Lu B, 2005 (China)	92	62	3	O/LAC	Yes	No	Yes	No
[41]	Ruiz B, 2001 (Colombia)	29	21	1	BAM	Yes	No	No	No
[42]	Yoshio O, 2004 (Japan)	59	0	1	O/A/C	Yes	Yes	Yes	Yes
[43]	Yamada T, 2003 (Japan)	87	29	0.83-4.17	PPI/A/C	Yes	Yes	Yes	Yes

A: Amoxicillin; B: Bismuth subcitrate; B-BTI: Bismuth-based triple regimen; C: Clarithromycin; GA: Gastric atrophy; IM: Intestinal metaplasia; L: Lansoprazole; M: Metronidazole; O: Omeprazole; PPI: Proton pump inhibitor; BAM: Bismuth subcitrate, Metronidazole and Amoxicillin; OMC: Omeprazole, Metronidazole and Clarithromycin.

were rejected as duplicates or the title suggested that the articles were not appropriate, and a further 1604 were excluded after initial review (editorials, review articles, animal experiments, non-English language, *etc.*). Of the remaining 287 candidate articles, 245 did not fully meet the inclusion criteria and were excluded. A quality assessment of the 42 remaining papers led to elimination of a further 26 articles, leaving 16 studies eligible for the meta-analysis[28-43]. Initial agreement between the reviewers for the selection of relevant articles was high (κ = 0.96).

Characteristics of included studies

The main characteristics of the 16 articles included in the meta-analysis are shown in Table 1. With the exception of one randomized control study[35], all studies were single-center observational studies conducted in different parts of the world, mostly Japan and Italy. All the papers gave data for the four histological parameters evaluated (GA and IM separately for gastric corpus and antrum). *H. pylori* eradication in these studies consisted of a standard therapy with proton pump inhibitors, bismuth-based triple regimens, or dual regimens for 1-2 wk. Two studies enrolled patients with early gastric cancer who underwent endoscopic mucosal resection without recurrence[33,34]. Histological scores were calculated twice in one study, as *H. pylori* eradication occurred at different time points in two different groups[36]. Another study calculated the histological scores of both the lesser and greater parts of the antrum and corpus before and after *H. pylori* eradication[31]. Initial agreement between the reviewers for the data extraction was high (κ = 0.95).

Intestinal metaplasia

Results of the analyses indicated no publication bias for reports on the effects of *H. pylori* eradication on IM in the antrum and corpus. The pooled WMD in the gastric antrum before and after *H. pylori* eradication with 95%CI was 0.23 (0.18-0.29) with a significant overall effect ($P < 0.05$) (Figure 2A). For IM in the corpus, the pooled WMD with 95%CI was -0.01 (-0.04-0.02) with no significant overall effect (Figure 2B). There was no significant heterogeneity among any of these trials, therefore fixed effects models were used.

Gastric atrophy

Results of the analyses indicated no publication bias for reports on the effects of *H. pylori* eradication on GA in the antrum and corpus. The pooled WMD in the gastric antrum before and after *H. pylori* eradication with 95%CI was 0.25 (0.15-0.35) with a significant overall effect ($P < 0.05$) (Figure 3A). For GA in the corpus, the pooled WMD with 95%CI was 0.14 (0.04-0.24) with a significant overall effect ($P < 0.05$) (Figure 3B). There was significant heterogeneity among these trials, therefore random effects models were applied and multiple sensitivity analyses were performed. These analyses showed that the pooled WMD was not influenced by individual trials, thus no studies were excluded from the meta-analysis. These results indicate that the eradication of *H. pylori* aids in the reversal of both GA and IM in the antrum, but only reversal of GA, and not IM, was

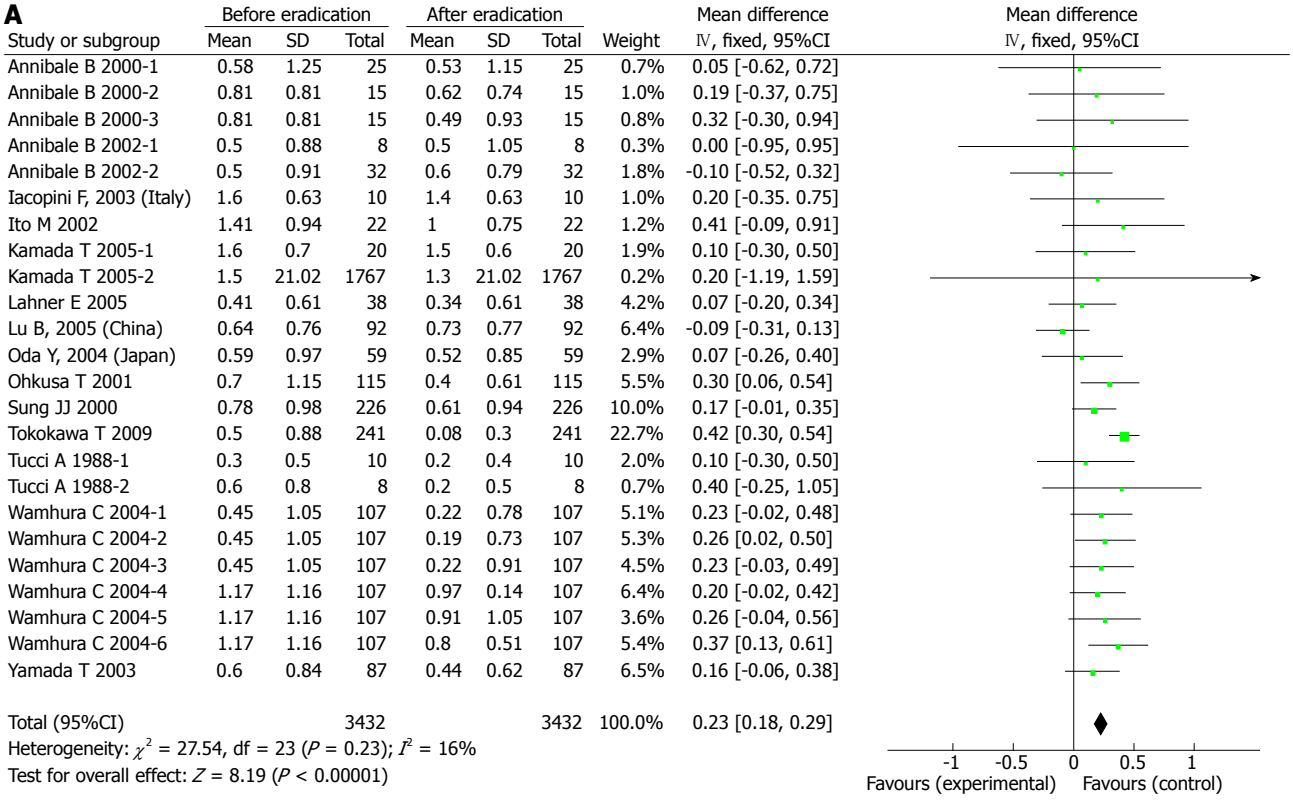


Figure 2 Forest plot comparing intestinal metaplasia in the antrum (A) and the corpus (B).

observed in the corpus.

DISCUSSION

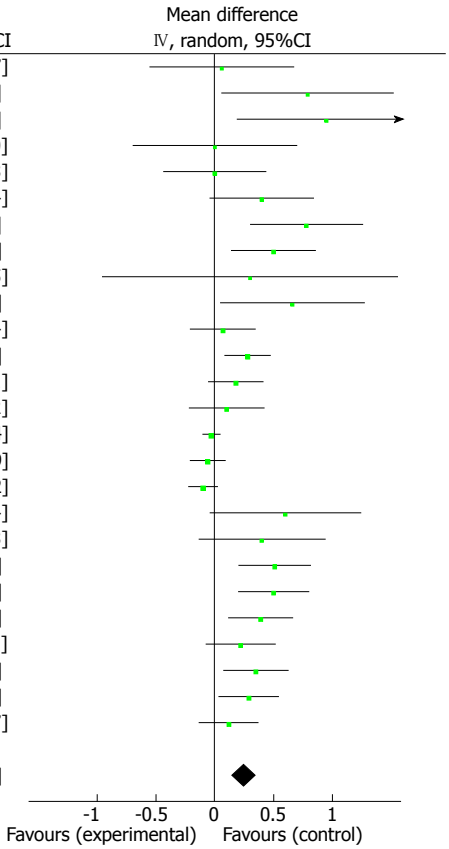
Despite the numerous reports on the improvement of gastric mucosal lesions following *H. pylori* eradication[44-55], some inconsistencies still remain[48,51,56]. Thus, it is still disputed whether the pathology of gastric mucosa, particularly GA and IM, improves after curing of the *H. pylori* infection. In this meta-analysis, data from relevant published studies were pooled with an effort to determine if GA and IM of the stomach are reversible after *H. pylori* eradication, and therefore whether therapeutic intervention is possible, or if efforts should be more appropriately directed at prevention.

The results of this study indicated that *H. pylori* eradi-

A

Study or subgroup	Before eradication Mean	SD	Total	After eradication Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Annibale B 2000-1	0.56	1.2	25	0.5	1	25	2.0%	0.06 [-0.55, 0.67]
Annibale B 2000-2	1.19	1.13	15	0.4	0.9	15	1.5%	0.79 [0.06, 1.52]
Annibale B 2000-3	1.19	1.13	15	0.24	1	15	1.4%	0.95 [0.19, 1.71]
Annibale B 2000-1	0.25	0.71	8	0.25	0.71	8	1.7%	0.00 [-0.70, 0.70]
Annibale B 2000-2	0.6	0.96	32	0.6	0.79	32	3.2%	0.00 [-0.43, 0.43]
Iacopini F, 2003 (Italy)	1.2	0.63	10	0.8	0.32	10	3.1%	0.40 [-0.04, 0.84]
Ito M 2002	2.14	0.81	22	1.36	0.8	22	2.8%	0.78 [0.30, 1.26]
Kamada T 2005-1	1.9	0.7	20	1.4	0.4	20	3.9%	0.50 [0.15, 0.85]
Kamada T 2005-2	1.9	21.02	1767	1.6	16.81	1767	0.6%	0.30 [-0.95, 1.55]
Kamada T, 2003 (Japan)	1.4	1.34	37	0.74	1.34	37	2.0%	0.66 [0.05, 1.27]
Lahner E 2005	0.41	0.61	38	0.34	0.61	38	4.8%	0.07 [-0.20, 0.34]
Lu B, 2005 (China)	1.25	0.44	92	0.97	0.83	92	5.8%	0.28 [0.09, 0.47]
Oda Y, 2004 (Japan)	0.95	0.68	59	0.77	0.6	59	5.3%	0.18 [-0.05, 0.41]
Ohkusa T 2001	0.9	1	115	0.8	1.41	115	4.3%	0.10 [-0.22, 0.42]
Ruiz B, 2001 (Colombia)	0.28	0.16	29	0.31	0.11	29	7.0%	-0.03 [-0.10, 0.04]
Sung JJ 2000	0.64	0.78	226	0.7	0.82	226	6.3%	-0.06 [-0.21, 0.09]
Tokokawa T 2009	2.1	0.7	241	2.2	0.66	241	6.6%	-0.10 [-0.22, 0.02]
Tucci A 1988-1	0.9	0.9	10	0.3	0.5	10	1.9%	0.60 [-0.04, 1.24]
Tucci A 1988-2	0.6	0.7	10	0.2	0.5	10	2.4%	0.40 [-0.13, 0.93]
Wamhura C 2004-1	1.35	1.23	107	0.84	1.02	107	4.4%	0.51 [0.21, 0.81]
Wamhura C 2004-2	1.35	1.23	107	0.85	0.96	107	4.5%	0.50 [0.20, 0.80]
Wamhura C 2004-3	1.35	1.03	107	0.96	0.98	107	4.8%	0.39 [0.12, 0.66]
Wamhura C 2004-4	1.64	1.04	107	1.42	1.14	107	4.6%	0.22 [-0.07, 0.51]
Wamhura C 2004-5	1.64	1.04	107	1.29	0.99	107	4.8%	0.35 [0.08, 0.62]
Wamhura C 2004-6	1.64	0.97	107	1.35	0.91	107	5.0%	0.29 [0.04, 0.54]
Yamada T 2003	1.67	0.99	87	1.55	0.63	87	5.1%	0.12 [-0.13, 0.37]
Total (95%CI)			3500			3500	100.0%	0.25 [0.15, 0.35]

Heterogeneity: Tau^2 = 0.04, χ^2 = 86.12, df = 25 (P < 0.00001); I^2 = 71%
Test for overall effect: Z = 4 (P < 0.00001)



B

Study or subgroup	Before eradication Mean	SD	Total	After eradication Mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Annibale B 2000-1	1.64	1.3	25	1.36	0.9	25	2.2%	0.28 [-0.34, 0.90]
Annibale B 2000-2	1.88	1.74	15	1.3	1.76	15	0.6%	0.58 [-0.67, 1.83]
Annibale B 2000-3	1.88	1.74	15	1.09	1.76	15	0.6%	0.79 [-0.46, 2.04]
Annibale B 2000-1	1.12	0.34	8	0	0	8		Not estimable
Annibale B 2000-2	2.03	0.68	32	1.83	0.85	32	4.7%	0.20 [-0.18, 0.58]
Ito M 2002	2.09	0.7	22	0.9	0.8	22	3.7%	1.19 [0.75, 1.63]
Kamada T 2005-1	2.1	0.6	20	1.6	0.4	20	5.8%	0.50 [0.18, 0.82]
Kamada T 2005-2	1.8	21.02	1767	1.6	16.81	1767	0.6%	0.20 [-1.05, 1.45]
Lahner E 2005	2.16	0.62	38	2.22	0.62	38	6.6%	-0.06 [-0.34, 0.22]
Ohkusa T 2001	0	0.96	115	0.1	0.87	115	7.6%	-0.10 [-0.34, 0.14]
Sung JJ 2000	0.06	0.31	226	0.02	0.18	226	12.7%	0.04 [-0.01, 0.09]
Tokokawa T 2009	0.73	0.76	241	0.53	0.6	241	11.0%	0.20 [0.08, 0.32]
Wamhura C 2004-1	0.9	1.5	107	0.74	1.1	107	5.1%	0.16 [-0.19, 0.51]
Wamhura C 2004-2	0.9	1.5	107	0.82	1	107	5.3%	0.08 [-0.26, 0.42]
Wamhura C 2004-3	0.9	1	107	0.85	0.05	107	9.0%	0.05 [-0.14, 0.24]
Wamhura C 2004-4	1.4	1.2	107	1.45	1.11	107	5.9%	-0.05 [-0.36, 0.26]
Wamhura C 2004-5	1.4	1.2	107	1.33	1.03	107	6.1%	0.07 [-0.23, 0.37]
Wamhura C 2004-6	1.4	1.2	107	1.43	0.67	107	7.0%	-0.03 [-0.29, 0.23]
Yamada T 2003	0.68	1.36	87	0.53	0.71	87	5.6%	0.15 [-0.17, 0.47]
Total (95%CI)			3253			3253	100.0%	0.14 [0.04, 0.24]

Heterogeneity: Tau^2 = 0.02, χ^2 = 44.79, df = 17 (P = 0.0003); I^2 = 62%
Test for overall effect: Z = 2.67 (P = 0.008)

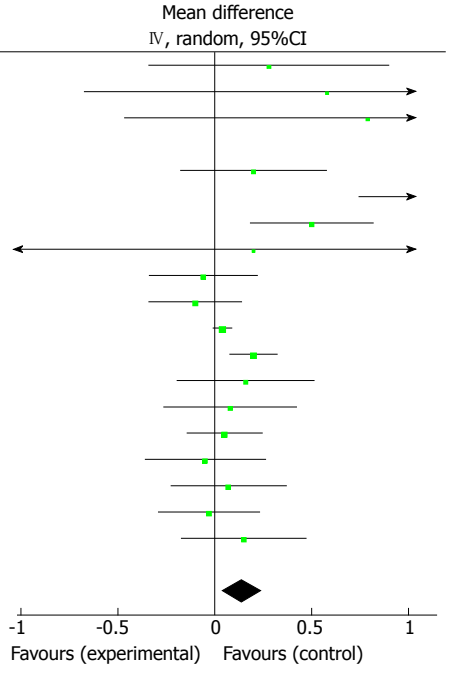


Figure 3 Forest plot comparing gastric atrophy in the antrum (A) and the corpus (B).

cation did indeed have beneficial long-term effects on gastric pathologies, such as halting the progression of pre-neoplastic lesions in the antrum and corpus. More specifically, IM in the antrum and GA in both the antrum and corpus showed regression after eradication of *H. pylori*, although this effect was not seen in IM of the gastric corpus. The interpretation of this finding is not clear, but histological changes occurring after *H. pylori* eradication may play a role[44].

The results reported here differ from similar previously published meta-analyses[45,57]. There are several reasons that may explain this discrepancy. First of all, the previous analyses included a limited number of studies, whereas our analysis included 16 comparatively high-quality scor-

ing studies. Second, the analysis by Rokkas *et al*[45] used the odds ratio as a statistical index, which may not be as precise as WMD for continuous variables. Additionally, there were errors in the analysis reported by Wang *et al*[57], which may have led to an incorrect conclusion.

The results of the current meta-analysis should be considered more reliable as a result of the extensive and thorough measures employed. For articles that merely reported results in chart format, the authors were contacted to obtain the raw data. Failure to obtain the raw data resulted in exclusion of the study to ensure reliability of the included data. Articles reporting varying treatment durations for the same group of patients were included as a separate set of data in the analysis, while taking into account the fact that medications had not been changed during the full course of treatment. Sensitivity analyses were performed to exclude the effects of different treatment courses, resulting in more accurate results[29]. Furthermore, data from articles that segregated results according to outcome were analyzed separately on the basis of the numbers of patients with successful eradication therapy in two groups[28,30-32], and only the patients with successful eradication were included in the analysis. Lastly, random effects models were used, which result in wider confidence intervals and, thus, a more conservative estimate of treatment effects.

The meta-analysis reported here is not without limitations. One inherent weakness involves the methodological flaws of the included studies, dependent on factors such as number of biopsy samples taken, method for histological classification of findings, sample size, and duration of follow-up. To alleviate such influences and unify the method of histological evaluation of biopsy samples, we selected only reports employing the updated Sydney system and had greater than three pathological samples of every specimen that were stained by hematoxylin-eosin methods. Another weakness is the inability to retrieve unpublished studies or published abstracts, due to the absence of a specific searching mechanism. However, we maximized the chances of detecting such studies by going through the references of the selected articles. Furthermore, although we used medical subject heading terms and keywords, some studies may have been missed, particularly studies in which the association of *H. pylori* infection with GA or IM was not the primary research question.

In conclusion, this study illustrates a very strong correlation between the eradication of *H. pylori* infection and improvement in IM in the gastric antrum but not in the corpus, in addition to a strong correlation with GA in both the antrum and the corpus. However, the follow-up periods of the analyzed studies are relatively short compared to the long process of mucosal carcinogenesis. Therefore, more high quality clinical studies with longer follow-up periods are necessary to assess the long-term benefit and whether the eradication of *H. pylori* infection delays disease progression.

COMMENTS

Background

Gastric cancer (GC) is the fourth most common cancer in the world and the second leading cause of cancer-related deaths. Overall GC incidence and mortality have fallen dramatically over the past 7 decades, but despite that decline, the cure rates for GC have not changed. Therapeutic eradication of *Helicobacter pylori* (*H. pylori*) infection is one factor contributing to these declines; however, this association is still debated.

Research frontiers

Although *H. pylori* eradication has been reported to improve gastric mucosal lesions, there are many studies with contradictory results. A clear understating of the role *H. pylori* eradication plays in the incidence and progression of GC will help guide therapies towards effective treatment or prevention.

Innovations and breakthroughs

The results of this meta-analysis indicate that there is a very strong correlation between *H. pylori* infection and improvement in intestinal metaplasia in the antrum, but not the corpus, of the stomach. Furthermore, a strong correlation between *H. pylori* infection and improvement in gastric atrophy in the antrum and corpus was identified.

Applications

The results of this study confirm the association between *H. pylori* eradication and improvements in gastric pathologies. Although additional high quality clinical studies with longer follow-up periods are necessary to assess the long-term benefit of treatments, the findings implicate a viable treatment option for patients with intestinal metaplasia and gastric atrophy.

Terminology

Intestinal metaplasia is the transformation (metaplasia) of epithelium, usually of the stomach or the esophagus, to a type that bears some resemblance to the intestine, as seen in Barrett's esophagus. Chronic *H. pylori* infection in the stomach and gastroesophageal reflux disease are seen as the primary instigators of metaplasia and subsequent adenocarcinoma formation.

Peer review

This article presents a well-designed meta-analysis of high quality studies evaluating the effect of *H. pylori* eradication on intestinal pathologies, namely intestinal metaplasia and gastric atrophy in the antrum and corpus of the stomach. The analyses show a strong correlation with improvement of intestinal metaplasia in the antrum, and gastric atrophy in the antrum and corpus, following eradication of *H. pylori* infection.

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CASE REPORT

TNF inhibitors to treat ulcerative colitis in a metastatic breast cancer patient: A case report and literature review

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Abstract

Adalimumab (ADA) is a tumor necrosis factor (TNF) inhibitor, used for the treatment of inflammatory bowel disease. Previous studies have reported an increased risk of cancer following exposure to TNF inhibitors, but little has been reported for patients with cancer receiving TNF-inhibitor treatment. We present a female patient with metastatic breast cancer and ulcerative colitis (UC) who was treated with ADA. A 54-year-old African American female with a past history of left-sided breast cancer (BC) diagnosed at age 30 was initially treated with left-breast lumpectomy, axillary dissection, followed by chemotherapy and radiation therapy. Years after initial diagnosis, she developed recurrent, bilateral BC and had bilateral mastectomy. Subsequent restaging computed tomography (CT) scan demonstrated distant metastases to the bone and lymph nodes. Three years into her treatment of metastatic breast cancer, she was diagnosed with UC by colonoscopy. Her UC was not controlled for 5 mo with 5-aminosalicylates. Subcutaneous ADA was started and resulted in dramatic improvement of UC. Four months after starting ADA, along with ongoing chemotherapy, restaging CT scan showed resolution of the previously seen metastatic lymph nodes. Bone scan and follow-up positron emission tomography/CT scans performed every 6 mo indicated the stability of healed metastatic bone lesions for the past 3 years on ADA. While TNF-α inhibitors could theoretically promote further metastases in patients with prior cancer, this is the first report of a patient with metastatic breast cancer in whom the cancer has remained stable for 3 years after ADA initiation for UC.

Key words: Tumor necrosis factor inhibitor; Ulcerative colitis; Breast Cancer; Inflammatory Bowel Disease; Adalimumab

Core tip: Tumor necrosis factor (TNF)-α inhibitors are widely-used and effective treatments for many autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease and psoriasis. However, it is believed that TNF-α inhibitors may also place patients at increased risk of cancer occurrence or recurrence. Many studies report increased risk of cancer following exposure to TNF-α inhibitors, but little has been reported for patients with cancer, receiving anti-TNF-α treatment. This is the first case of metastatic breast cancer in long term remission for 3 years in a patient treated with TNF-α inhibitors for ulcerative colitis, suggesting that patients with metastatic cancer could be treated with this class of medications without worsening.

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INTRODUCTION

Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that plays a major role in the pathogenesis of inflammatory bowel disease (IBD)[1]. IBD patients are often administered prolonged treatment with anti-TNF-α agents. Adalimumab (ADA) is a monoclonal antibody that blocks the interaction of TNF-α with its cell surface receptors[2]. In the past, several small open-label trials and case reports had suggested that ADA is an effective therapy for ulcerative colitis (UC)[1,3-5]. Recently, a randomized controlled trial demonstrated that ADA is effective in inducing and maintaining clinical remission in patients with moderate to severe UC, who did not have an adequate response to conventional therapy with steroids[2].

TNF-α acts as an early tumor suppressor, thus drugs blocking TNF-α may possibly increase the risk of initial occurrence of malignancies, especially in those patients with long-term therapy[6-8]. Additionally, blocking TNF-α can also possibly lead to reactivation of latent malignancies[7]. Little has been reported on patients with cancer receiving anti-TNF-α treatment, and the issues concerning the long-term safety of these biologic agents in patients with prior malignancy remain to be clarified. We report the first case of metastatic breast cancer treated with ADA for UC.

CASE REPORT

A 54-year-old African American female was initially diagnosed in 1989 with left sided breast cancer (BC) at the age of 30. She was treated with a left-breast lumpectomy and axillary dissection, followed by four cycles of adjuvant chemotherapy with CMF (cytoxan, methotrexate, 5-fluorouracil). The chemotherapy was discontinued due to side effects. She then received radiotherapy to the left breast, and had no further treatment following that. In 2007, she developed recurrent, bilateral BC. Bilateral mastectomy with right axillary lymph node dissection in 2007 showed metastases in 14 out of 16 right axillary lymph nodes. The tumor was estrogen receptor (ER) negative, progesterone receptor (PR) negative, Her-2/neu (human epidermal growth factor receptor) positive by immunohistochemistry (IHC) with amplified fluorescence *in situ* hybridization. In addition to the axillary nodes that were histologically positive, restaging computed tomography (CT) scan after the surgery showed metastatic disease also in the internal mammary lymph nodes (Figure 1A) and thoracic spine. Biopsies for histologic confirmation of the additional metastatic lesions were not attempted due to high-risk for cancer progression, poor accessibility of the metastases, and compelling imaging. She was started on chemotherapy with vinorelbine and trastuzumab as well as zoledronic acid. Vinorelbine was discontinued after one cycle due to severe myalgias. The patient continued to receive trastuzumab, and zoledronic acid for 11 mo; then, paclitaxel was added at low dose due to the development of right retropectoral lymphadenopathy (Figure 1B). She had stable disease on this regimen for 15 mo, until she developed right supraclavicular lymphadenopathy and further progression of the right retropectoral lymphadenopathy. Also, her tumor marker, carcinogenic embryonic antigen (CEA), rose dramatically at that time and reached a level of 70 ng/mL. This necessitated changing her chemotherapy regimen to gemcitabine and trastuzumab, while continuing zoledronic acid. After 2 mo with this new regimen, she was diagnosed with severe pancolitis, compatible with UC on colonoscopy and biopsies, following an acute episode of diffuse abdominal pain and bloody diarrhea. Gemcitabine was discontinued, but she was continued on trastuzumab and zoledronic acid for an additional 6 mo after the UC diagnosis, when she was found to have cancer progression in the right supraclavicular lymph nodes, and when she was diagnosed with right mandibular osteonecrosis due to zoledronic acid. At that time, zoledronic acid and trastuzumab were discontinued, and the patient was started on capecitabine and lapatinib. She had stable disease on this regimen and she was continued on this regimen for 22 mo and then was continued on lapatinib as a single agent. For UC, she was

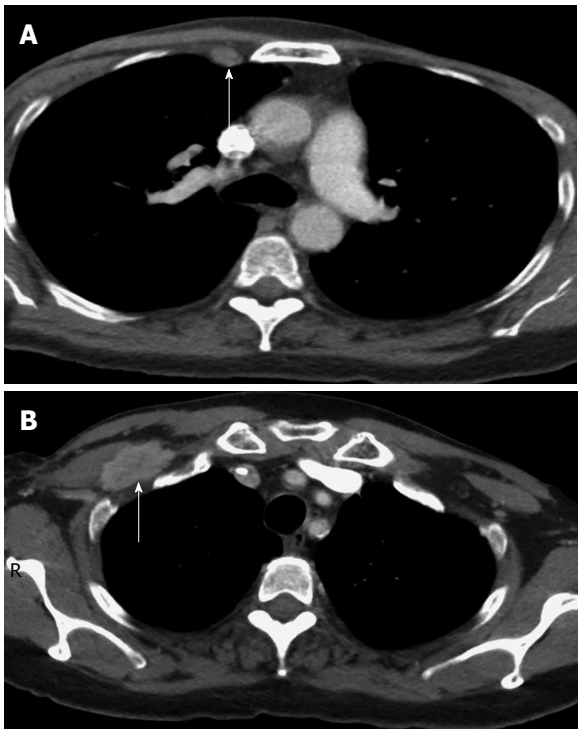

Figure 1 Computed Tomography scan of the chest before initiation of Adalimumab. A: Internal mammary lymph node (arrow); B: Retropectoral lymph node (arrow).

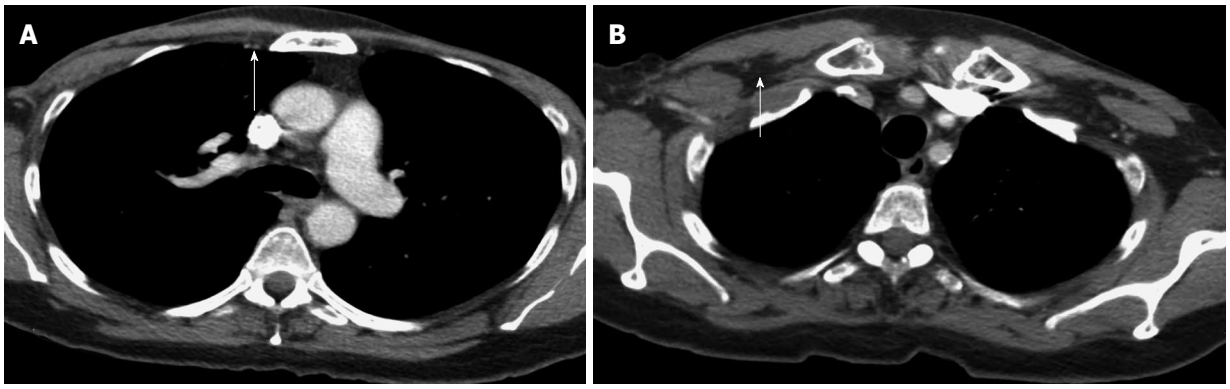

Figure 2 Computed tomography scan of the chest 4 mo after initiation of Adalimumab. A: Resolved internal mammary lymph node (arrow); B: Resolved retropectoral lymph node (arrow).

started on 5- aminosalicylates and prednisone, but her UC was not controlled for 5 mo on this regimen, as the tumor was progressing. Subcutaneous ADA (40 mg every 2 wk) was started and resulted in dramatic improvement of her UC symptoms. Four months after starting ADA along with ongoing chemotherapy with capecitabine and lapatinib, restaging CT scan of the chest, abdomen and pelvis showed the resolution of the previously seen internal mammary lymph nodes (Figure 2A), and the right retropectoral lymph node (Figure 2B) and no evidence of distant metastases. Bone scan and follow-up PET/CT scans performed every 6 mo indicated metabolically inactive lesions at the prior sites of metastatic bone lesions suggesting control of BC for the past 3 years on ADA. She has been clinically asymptomatic and progression free since 2010. Currently, she remains in complete clinical remission on maintenance lapatinib. In 2013, she had a biopsy of her L4 vertebral body to look for histological metastatic disease to the bone; and the pathology was benign. She was genetically tested for BC predisposition and found to have no BRCA1 and 2 mutations by full sequencing of both genes.

DISCUSSION

Many studies have been undertaken to understand whether TNF-α inhibitor therapy increases the rate of malignancies. The hypothetical risk of recurrent malignancy in patients with prior malignancy has previously led researchers to exclude almost all cancer patients from randomized clinical trials of TNF-α inhibitors[9]. TNF-α inhibitor therapy, in general, and ADA in particular, has been associated with an increased risk for malignancy[10]. A meta-analysis of nine randomized controlled trials of anti-TNF-α antibody therapies (infliximab and ADA) versus placebo in patients with rheumatoid arthritis, found a significantly increased risk for malignancies in the TNF-α inhibitor treated patients with a pooled odds ratio of 3.3 (95%CI: 1.2-9.1), compared to placebo-treated patients[11]. Another meta-analysis[12] found a higher malignancy risk in patients treated with etanercept compared to controls, although the relative risk estimate did not achieve statistical significance.

While malignancy risk in general seems increased in patients receiving TNF inhibitors, when the type of malignancies are examined, most common ones encountered are lymphomas and skin cancers. In particular, among IBD patients, a meta-analysis by Siegel *et al*[13] found a significant increase in NHLs. In 26 studies that enrolled a total of 8905 patients with 21178 patient-years of follow-up, 13 cases of NHL were reported (6.1/10000 patient-years) among TNF-α inhibitor treated subjects. This rise in NHLs was also confirmed in another meta-analysis by Wong *et al*[14]. In the literature, there are also several case reports that highlight an increased risk of lymphoma development after initiation of TNF-α inhibitors[15-18]. Among other malignancies in case reports of patients exposed to TNF-α inhibitors are a papillary thyroid carcinoma[19]; a salivary gland tumor[20]; a metastatic invasive ductal carcinoma of the breast and a diffuse B-cell lymphoma (after high-dose of ADA)[21]; malignant melanomas[22,23]; and a pulmonary carcinoid tumor[24].

While a potential role of TNF-α inhibitors in the development of lymphoma and skin cancers is fairly well accepted, the role of TNF-α inhibitors in solid malignancies is not as clear. A possible theory regarding the risk of cancer in patients on TNF-α inhibitors relates to the important role of TNF-α for natural killer/CD8 lymphocyte dependent cell lysis and for modulating adaptive immunity, which is an important component of tumor surveillance[25,26]. Therefore, it is postulated that suppressing TNF-α may enhance proliferation of solid organ malignancies[25,26]. However, in an observational study, Wolfe *et al*[27] analyzed the association between malignancy and biologic therapy in approximately 13000 patients with RA, and reported that there were no increases in the risk of solid tumors. Another observational study by Askling *et al*[28,29] used the Swedish inpatient registry to compare 4160 TNF-α inhibitor treated patients with 53067 other patients in the registry: Cancer risks in the TNF inhibitor treated patients were largely similar to those of other patients with RA.

Data regarding the safety of anti-TNF-α agents prescribed to patients with prior malignancy are available

in only a few studies. One study analyzed data from the British Society of Rheumatology Biologics Register and attempted to assess the potential malignancy risk associated with starting TNF-α inhibitor therapy in patients with RA who had pre-existing malignancies[30]: Analysis of data from approximately 10000 patients, showed an increased incidence rate ratio of 2.5 (95%CI: 1.2-5.8); indicating that patients with a malignancy prior to the initiation of TNF-α inhibitor therapy have a higher risk of another malignancy compared to patients without a previous malignancy. In contrast, a recent study found no significant increase in the risk of tumor recurrence for those patients under treatment with TNF-α inhibitor agents, and no overall increase in the incidence of malignancies in patients exposed or unexposed to TNF-α inhibitor treatment[6]. Additionally, clinical trials of the TNF-α inhibitor etanercept in patients with breast cancer, ovarian cancer and hematological malignancies have resulted in disease stabilization or partial improvement of the cancer[31-33]. Also, infliximab therapy of 41 patients with advanced cancer was well tolerated with no evidence of disease acceleration[34]. But tumor recurrence was reported in two cases of metastatic melanoma and these occurred after initiation of TNF-α inhibitors[35].

In the terms of tumor regression with withdrawal of TNF inhibitors, there are two case reports: One is a case of recurrent Hodgkin's lymphoma, 10 mo after ADA initiation, with spontaneous regression observed after withdrawal of TNF-α inhibition[36]. The other is a case report of a non-small cell lung cancer developing 4 years after TNF-α inhibitors in a patient with Crohn's disease treated initially with infliximab for 2 years and then with ADA. The tumor was found to have TNF receptors type 1 and type 2. After TNF-α inhibitors were withdrawn, the tumor regressed with follow-up chest CT scans 1 year after withdrawal, showing virtually no evidence of the primary lung tumor, nodules, or lymphadenopathy, with complete clinical and radiological remission[37]. Looking at these reports, it is clear that in some patients TNF-α inhibitors may further cancer and in others they do not. Additional studies on tumor characteristics and immune mechanisms at play are needed to determine in which patients TNF-α inhibitors may be harmful.

The case reported here is remarkable because our patient with metastatic BC achieved a complete remission despite initiation of TNF-α inhibitors (which theoretically can worsen malignancies) and she has been progression free for 3 years, along with excellent control of her UC. This case is also interesting because UC could have accounted for the rising CEA observed. Some previous studies suggest that the elevation of CEA titers in some patients can be related to the degree and the extent of active inflammation of the colon and can return to normal with remission[38,39]. In this case, the CEA was difficult to interpret due to the presence of both metastatic BC and active colitis.

In terms of TNF-α therapy, the potential benefits of the latter therapy need to be considered against risks related to the potential recurrence of pre-existing malignancy. If patients have been free of any recurrence of their malignancy for 10 years, there appears to be no evidence for a contraindication to anti-TNF-α therapy[40]. On the basis of all available data, the question of whether anti-TNF-α therapy can be initiated in individuals with previous malignancies less than 10 years in remission is still not solved and requires careful data collection going forward[41]. Our case, illustrates that, it is possible to successfully treat an advanced cancer like metastatic BC and concomitant IBD with TNF-α inhibitor therapy. Further in-depth studies are needed in cancer patients who remain in remission, who are also given TNF-α inhibition to elucidate the role of TNF-α in tumor progression and surveillance.

COMMENTS

Case characteristics

A 54-year-old female with past history of breast cancer presented with tumor recurrence and developed bloody diarrhea three years into the course of treatment for metastatic breast cancer.

Clinical diagnosis

The patient was diagnosed with colitis, while getting treated for metastatic breast cancer.

Differential diagnosis

The differential diagnosis included infectious diarrhea *vs* other causes of colitis.

Laboratory diagnosis

The patient was diagnosed with ulcerative colitis on the basis of colonoscopy and biopsies; and also had a rising carcinogenic embryonic antigen.

Imaging diagnosis

Computed tomography (CT) scan of the chest before treatment of ulcerative colitis showed enlarged internal mammary lymph nodes and a large retropectoral lymph node; but a follow-up CT scan 4 mo after initiation adalimumab therapy showed resolution of the previously demonstrated metastatic lymphadenopathy.

Pathological diagnosis

Bilateral mastectomy with right axillary lymph node dissection showed metastases in 14 out of 16 right axillary lymph nodes. The tumor was estrogen receptor negative, progesterone receptor negative, Her-2/neu (human epidermal growth factor receptor) positive by immunohistochemistry with amplified fluorescence *in situ* hybridization.

Treatment

After the development of ulcerative colitis, the patient was treated with capecitabine and lapatinib for metastatic breast cancer, and 5-ASAs and steroids for ulcerative colitis; and after 5 mo, the ulcerative colitis treatment had to be changed to adalimumab, following which rapid resolution of the patient's colitis symptoms and regression of the metastatic lesions occurred.

Related reports

Tumor necrosis factor (TNF)-α inhibitors such as adalimumab have been reported to increase the risk of occurrence of malignancies but little has been reported for patients with cancer, receiving anti-TNF-α treatment.

Term explanation

IHC is used to characterize various surface and intracellular proteins from cells of all types of tissues ratio between the numbers of HER2 and CEP17 sequences.

Experiences and lessons

Ulcerative colitis was successfully treated in a patient with metastatic breast cancer, using adalimumab, suggesting that metastatic cancer with solid tumors is not an absolute contraindication to TNF inhibitor therapy.

Peer review

The case report is an example of a common scenario in clinical care, when a patient with inflammatory bowel disease and cancer has to be concomitantly treated for both conditions. While the case illustrates it is possible to use TNF inhibitors in this scenario, the follow-up is three years and is relatively short.

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CASE REPORT

Two rare gastric hamartomatous inverted polyp cases suggest the pathogenesis of growth

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Abstract

Gastric hamartomatous inverted polyps (GHIP) are difficult to diagnose accurately because of inversion into the submucosal layer. GHIP are diagnosed using the pathological characteristics of the tumor, including the fibroblast cells, smooth muscle, nerve components, glandular hyperplasia, and cystic gland dilatation. Although Peutz-Jeghers syndrome, juvenile polyposis, and Cowden disease are hereditary, it is rare to encounter 2 cases of monostotic and asymptomatic gastric hamartomas. The pathogeneses of hamartomatous inverted polyps and inverted hyperplastic polyps remain controversial because of the paucity of reported cases. There are 3 hypotheses regarding the pathogenesis of complete gastric inverted polyps. Based on our experience with 2 successive, rare GHIP cases, we affirm the hypothesis that after a hamartomatous change occurs in the submucosal layer, some of these components are exposed to the gastric mucosa and, consequently, form a hypertrophic lesion. In Case 1, our hypothesis explains why a tiny hypertrophic change was first detected on the top of the submucosal tumor using a detailed narrow band imaging-magnified endoscopy. There was no confirmation that the milky white mucous and calcification structures were exuding directly from the biopsy site like Case 1, and in Case 2 the presence of this mucous was indirectly confirmed during an endoscopic submucosal dissection (ESD). Regarding the pathogenesis of GHIP, a submucosal hamartomatous change may occur prior to the growth of hypertrophic portions. An *en bloc* resection using ESD is recommended for treatment.

Key words: Hamartomatous inverted polyps; Hypotheses of pathogenesis; Milky white mucous; Endoscopic submucosal dissection; Pathological findings

Core tip: The pathogeneses of hamartomatous inverted polyps (HIP) and inverted hyperplastic polyps remain controversial. Our experience with 2 rare successive cases of HIP suggests that after a hamartomatous change occurs in the submucosal layer, some of these components are exposed to the gastric mucosa and, consequently, form a hypertrophic lesion. An *en bloc* resection by endoscopic submucosal dissection is recommended for treatment.

Mori H, Kobara H, Tsushimi T, Fujihara S, Nishiyama N, Matsunaga T, Ayaki M, Yachida T, Masaki T. Two rare gastric hamartomatous inverted polyp cases suggest the pathogenesis of growth. *World J Gastroenterol* 2014; 20(19): 5918-5923 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5918.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5918

INTRODUCTION

Gastric hamartomatous polyps (GHP) have been reported as GHIP because of inversion into the submucosal layer of the main body[1]. GHIP located in the submucosal layer and gastric submucosal tumors (SMT) are rarely reported, making an accurate diagnosis difficult[2]. GHIP are diagnosed using the pathological characteristics of the tumor, including the fibroblast cells, smooth muscle proliferation, nerve components, vasoformative tissue, glandular hyperplasia, and cystic gland dilatation[2,3]. GHIP > 20 mm in diameter are recommended to be treated with an *en bloc* resection with laparoscopic wedge resection of the stomach or endoscopic submucosal resection (ESD) rather than a conventional polypectomy, endoscopic mucosal resection (EMR) with piecemeal resection, or subtotal or total gastrectomy because there is a paucity of studies regarding the long term outcomes of GHIP and these polyps occasionally contain paracancerous portions (approximately 20% of GHIP)[2-4]. Furthermore, there are some controversial reports regarding the pathogenesis or natural growth history of GHIP. One hypothesis suggests that the submucosal hamartomatous or heterogenic gastric mucosa components grow up to the mucosal surface; however, another hypothesis suggests that hyperplastic components or the mucosa is inverted into the submucosa[5,6].

Here, we report 2 cases (31-year-old and 65-year-old patients) that will aid in the understanding of the pathogenesis and growth process of GHIP.

CASE REPORT

A 31-year-old woman was found to have a gastric SMT in the great curvature of the upper body of the upper GI tract during her medical exam. She underwent an esophagogastroduodenoscopy (EGD) and endoscopic ultrasound (EUS) (radial scan, 20 MHz). The EGD showed a gastric SMT measuring 30 mm in diameter in the greater curvature of upper body with bridging folds and a tiny reddish spot on the top (Figure 1A). With the exception of the tiny reddish spot, the SMT surface was normal gastric mucosa. Using narrow band imaging-magnified endoscopy (NBI-ME), the tiny reddish area was shown to be dilated with banded marginal crypt epithelium that differed from the surrounding mucosa and consisted of small round pits of normal fundic glands (Figure 1B). A biopsy specimen taken from the tiny reddish spot showed hyperplastic gastric mucosa. Milky white mucous and calcification structures were found to be exuding from the biopsy site (Figure 1C). After draining the mucous, a solid portion with small cystic changes was found inside the SMT wall using forceps (Figure 1D). An EUS (radial scan, 20 MHz) before the biopsy revealed a heterogeneous tumor with a cystic area and calcification spots with acoustic shadow (Figure 2A). After the biopsy and draining of the mucous, we

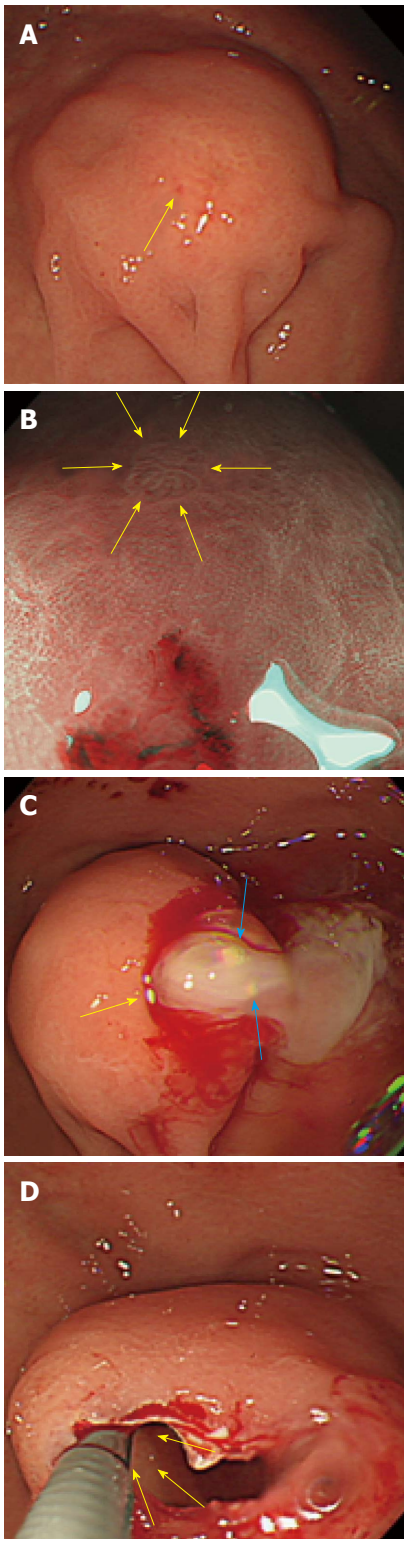

Figure 1 Esophagogastroduodenoscopy showed a submucosal tumor. A: The esophagogastroduodenoscopy (EGD) showed a gastric submucosal tumor (SMT) measuring 30 mm in diameter in the greater curvature of upper body with bridging folds and a tiny reddish spot on top (yellow arrow); B: Using NBI-ME, the tiny area was observed to be dilated with banded marginal crypt epithelium (yellow arrows); C: Milky white mucous and calcification structures (blue arrow) were exuding from the biopsy site (yellow arrow); D: The solid portion inside the wall of the SMT with small cystic changes were exposed using forceps (yellow arrow).

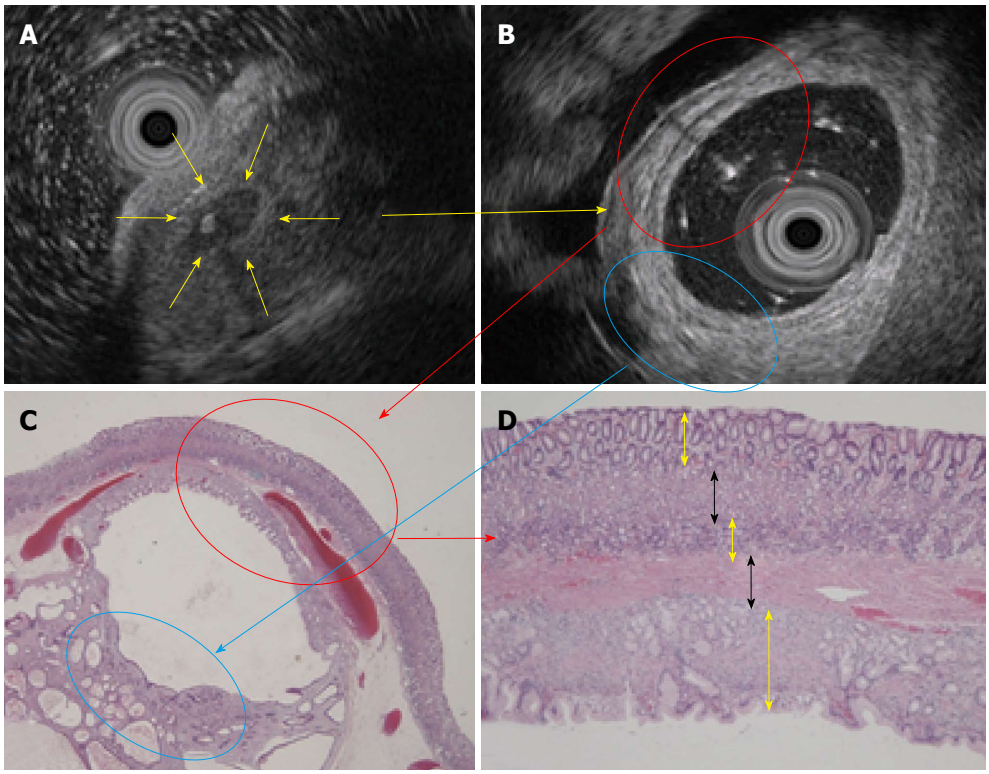

Figure 2 Endoscopic ultrasound before and after the biopsy and histology. A: An EUS before biopsy revealed a heterogeneous tumor with a cystic area and calcification spot (yellow arrow); B: An endoscopic ultrasound (EUS) from inside the submucosal tumor (SMT) filled with distilled water and an inserted EUS probe showed 5 specific layers (red circle); C: An HE stain (× 20) also showed the 5 structural layers. The hyperechoic portion showed proliferation of pseudo-pylorus glands and cystic gland dilatation without cytological atypia (blue circle); D: The 5 layers consisted of a normal mucosal layer (yellow arrow), immature fibroblasts (black arrow), pyloric glands (yellow arrow), muscularis mucosa (black arrow) and another normal mucosal layer (yellow arrow) (HE stain × 100).

conducted an EUS from inside the SMT by filling the tumor cavity with distilled water and inserting the EUS probe (Figure 2B). The EUS from inside the SMT cavity showed 5 specific structural layers (high-low-high-low-high). The EUS findings suggested that the 5 layers (high-low-high-low-high) were mucosa, muscularis mucosa, submucosa, muscularis mucosa, and mucosa, similar to a reverse layer pattern. The patient had no clinical symptoms, no family history, and no laboratory data abnormalities. Based on the EGD and EUS findings, including the heterogeneous echo pattern, small cystic structure, and spotty calcification, we suspected the presence of GHIP and lymphangioma with inflammatory changes. As it was difficult to make a definitive diagnosis and disprove the likelihood of a paracancerous lesion, we believed that an *en bloc* resection of the SMT was necessary to obtain an accurate diagnosis. After obtaining written informed consent from the patient, we resected the SMT by endoscopic submucosal dissection (ESD). A hematoxylin and eosin (HE) stain of the resected SMT is shown in Figure 2C (× 20) and Figure 2D (× 100). Histologically, the 5 structural layers (high-low-high-low-high) shown in the EUS and with the HE stain (× 20, Figure 2B and C red circle) consisted of a normal gastric mucosal layer of columnar epithelium of pyloric glands and fundic glands (high) (Figure 2D, yellow arrow), an immature fibroblast cell rich layer (low) (Figure 2D, black arrow), a pyloric gland rich layer (high) (Figure 2D, yellow arrow), muscularis mucosa (low) (Figure 2D, black arrow), and a normal gastric mucosal layer of columnar epithelium (high) (Figure 2D, yellow arrow) from inside the stomach. As shown in Figure 1C, the milky white mucous and calcification structures were not confirmed. The high echo solid portion of the SMT with tiny low echoic cystic spots (Figure 2B, blue circle) were shown to be the proliferation of pseudo-pylorus glands, cystic glands without cytological atypia, fibroblast cells, smooth muscle proliferation, nerve elements, glandular hyperplasia, and cystic gland dilatation.

A 65-year-old man was admitted to treat a SMT measuring 40 mm in diameter in the greater curvature of the middle body. The size of the SMT had increased from 20 mm in diameter, which had been reported 5 years previously; in addition, on the top of the SMT, there was a reddish and erosive nodular surface measuring 10 mm in diameter that had been previously reported to measure only a few mm in diameter (Figure 3A, B). An EUS (radial scan, 20 MHz) revealed a heterogeneous tumor with small spotty cystic areas, a large anechoic cystic part with papillary structures, and 5 specific structural layers (high-low-high-low-high) as its wall (Figure 3C, D). Considering the EGD and EUS results and the clinical progression of the SMT size, the GHIP may have been paracancerous. Therefore, an *en bloc* resection with ESD was performed to obtain an accurate diagnosis (Figure 4A). During the ESD (as shown in Figure 4B, C), milky white compo-

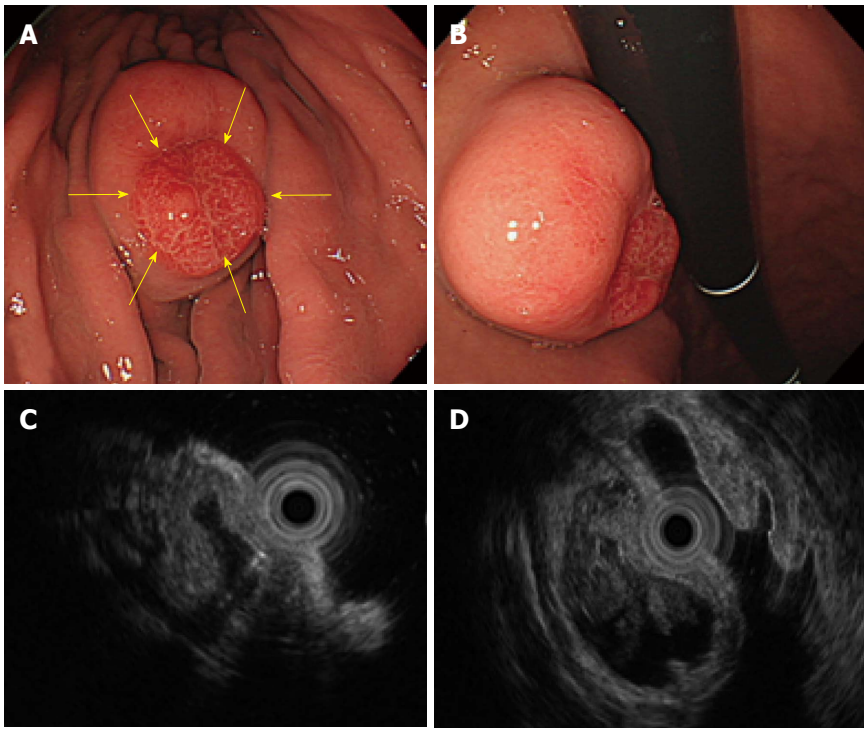

Figure 3 An submucosal tumor measuring 40 mm in diameter was found in the greater curvature of the middle body. A and B: The size of the submucosal tumor (SMT) had increased from the 20 mm in diameter that was reported 5 years before. In addition, a reddish erosive part 10 mm in diameter was found on the top of the SMT (yellow arrows). C and D: An endoscopic ultrasound revealed a heterogeneous tumor with small spotty cystic areas, a large anechoic cystic part, with papillary structures and 5 specific structural layers as its wall.

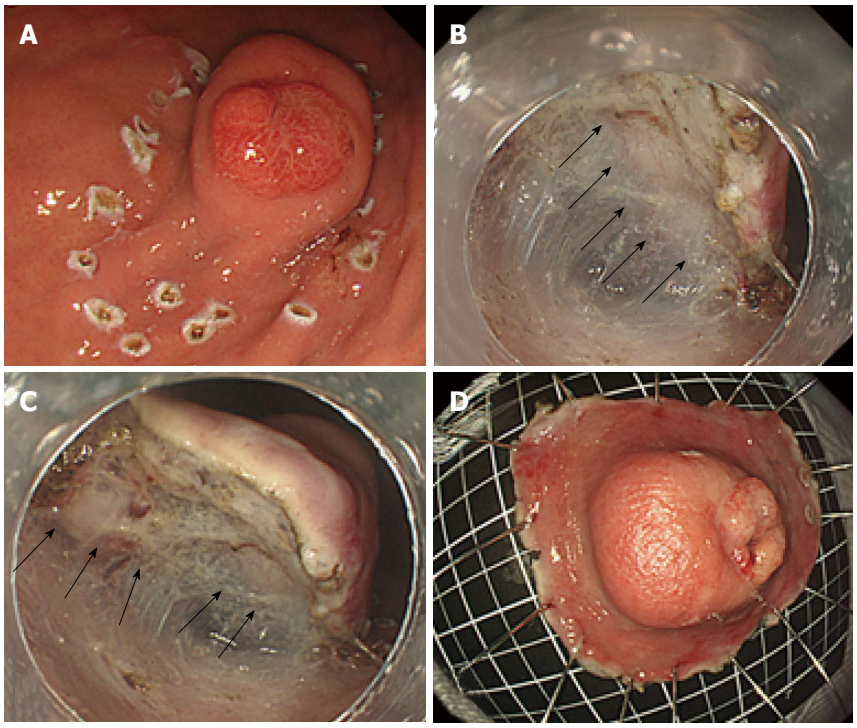

Figure 4 Endoscopic submucosal dissection to resect the submucosal tumor. A: An en bloc resection using endoscopic submucosal dissection (ESD) was performed to obtain an accurate diagnosis; B and C: Similar to Case 1, milky white components were observed during the ESD (black arrows); D: The lesion was completely resected with a sufficient safety margin.

nents similar to those in Case 1 were observed. The lesion was resected as a complete *en bloc* specimen with a sufficient safety margin from the tumor (Figure 4D).

Similar to Case 1, the 5 structural layers (high-low-

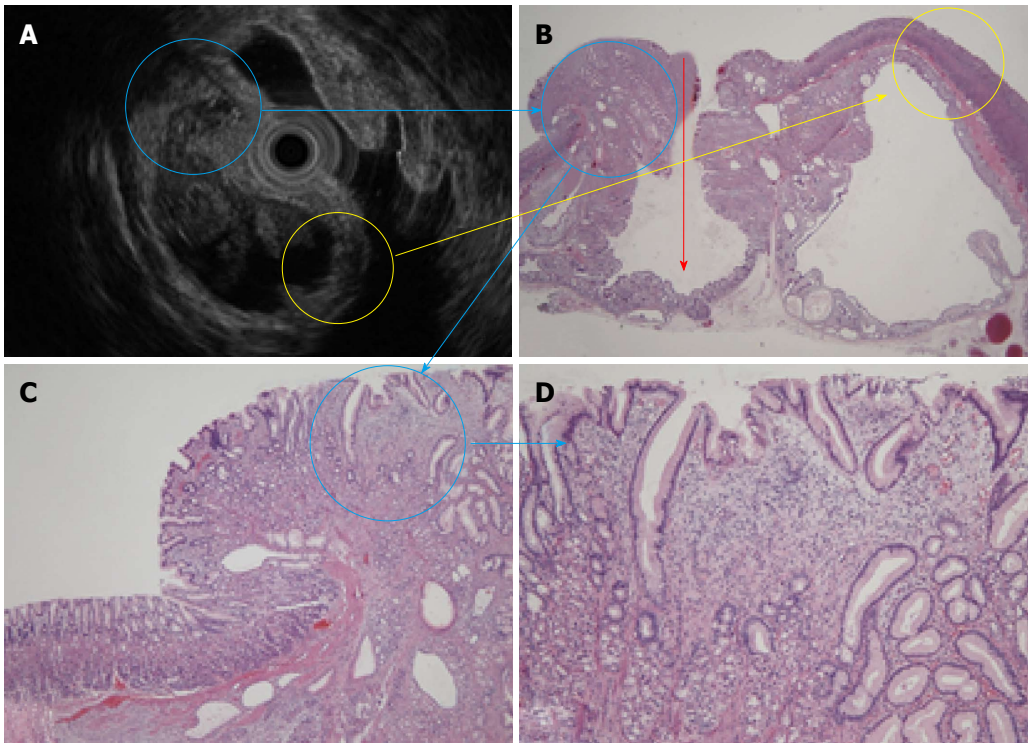

Figure 5 An endoscopic ultrasound and histological comparison. A: An endoscopic ultrasound (EUS) revealed a heterogeneous tumor; B: The 5 layers shown in the EUS and HE stain (× 20) (yellow circle) consisted of a normal mucosa layer, immature fibroblast cells, pyloric glands, muscularis mucosa, and another normal mucosa layer. The surface mucosa was inverted into the submucosal layer (red arrow); C and D: The hyperechoic solid portion had tiny low echoic cystic spots (blue circle) and showed the proliferation of pseudo-pylorus glands, cystic glands, fibroblast cells, smooth muscle, and nerve elements.

high-low-high) shown in the EUS and HE stain (× 20) (Figure 5A, B, yellow circle) consisted of a normal gastric mucosal layer, immature fibroblast cell rich layer, pyloric gland rich layer, muscularis mucosa, and normal gastric mucosa. The high echo solid portion with tiny low echoic cystic spots (Figure 5A, B and C, blue circle) showed proliferation of pseudo-pylorus glands, cystic glands with cytological atypia, fibroblast cells, smooth muscle proliferation, nerve elements, glandular hyperplasia, and cystic gland dilatation (Figure 5D). This SMT was inverted into the submucosal layer, as highlighted by the red bold arrow (Figure 5B).

DISCUSSION

GHPs are pathologically defined by cystic dilated hypertrophic pseudo-pylorus gland proliferation and smooth muscle located in the submucosal layer with branching from the proliferation of smooth muscle bundles[7]. Although Peutz-Jeghers syndrome, juvenile polyposis and Cowden disease are hereditary, it is rare to encounter 2 cases of monostotic and asymptomatic gastric hamartomas[8-12]. GHIP is difficult to diagnose accurately without endoscopic resection and pathological investigation because of its inverted grown into the submucosal layer and the paucity of case reports[2]. However, GHIP may be identified by the exuding of milky mucous, calcifications from the biopsy site, and specific EUS findings. There have been reports that GHIP was diagnosed by EUS examination and the observation of hyper echoic lesions, including small cystic hypo echoic spots (a heterogeneous tumor), in the 3rd layer (submucosal layer)[3]. In Case 1, an EUS examination from inside the tumor cavity was performed by inserting an EUS probe after cutting a portion of the tumor surface; forceps were used to obtain additional detailed information about the wall structure (high-low-high-low-high). A previous Japanese case report mentioned that EUS-fine needle aspiration was useful for diagnosing GHIP with 82% sensitivity, 100% specificity, and an overall 92% success rate. In addition, a differential diagnosis of GHIP and an aberrant pancreas with cystic pancreatic ducts was reported to be particularly difficult[2]. Pathological findings of inverted ectopic gastric pseudopyloric glands in the submucosal layer is vital for diagnosing GHIP; therefore, an *en bloc* resection using an ESD method was a viable treatment option in our 2 reported cases.

The pathogeneses of hamartomatous inverted polyps (HIP) and inverted hyperplastic polyps (IHP) remain controversial because of the paucity of documented cases[13]. A previous report suggested that GHIP should be classified as IHP because the rupture, thinning, and frailty of muscularis mucosae is caused by repeated erosions reaching the muscularis mucosae and gastric glands that are inverted into the submucosal layer, and the obstruction of a drain outlet for produced mucous leads to cystic dilatation of ectopic gastric glands[3]. There are currently 3 hypotheses regarding the pathogenesis of complete

gastric inverted polyps. One hypothesis contends that after a hyperplastic change occurs on the gastric mucosa surface, these components invert into the submucosal layer, and the submucosal glandular or cystic elements transform into an SMT-like tumor. Another hypothesis speculates that after a hamartomatous change occurs in the submucosal layer, some of these components are exposed to the gastric mucosa, thereby forming a hypertrophic lesion. A third hypothesis argues that a hyperplastic polyp is incidentally formed on the top of mucosa from the original submucosal hamartomatous change. Based on our experience with 2 rare cases of GHIP, we affirm that the second hypothesis is likely the basis for the GHIP pathogenesis. Case 1 supports this hypothesis because a tiny hypertrophic change was first detected on the top of the SMT using detailed NBI-ME. We found that a portion of the submucosal hamartomatous components were exposed to the surface of the SMT, with a tiny hypertrophic change. In Case 2, the SMT grew to 20 mm in diameter in the greater curvature of the middle body after it had been originally detected 5 years prior; in addition, a tiny reddish and erosive nodular spot in the hypertrophic mucosa on top of the SMT had grown to 10 mm in diameter. These data confirm the chronological changes in the hypertrophic mucosa on top of the SMT.

No report has confirmed the milky white mucous and calcification structures exuding from the biopsy site directly and confirmed indirectly during the ESD[14]. Although GHPs are benign tumors, approximately 20% coexist with paracancerous or cancerous areas[15]; therefore, it is important to diagnosis these polyps, and the recommended treatment is an *en bloc* resection using an ESD method. There was an atypical portion with high density nuclei in Case 2; thus, it was suggested that *en bloc* resection using an ESD method was pathologically more important than a piecemeal resection with EMR.

In conclusion, as submucosal hamartomatous changes of GHIP might occur prior to the development of secondary hypertrophic portions from our experiences of two rare cases, more cases are needed to be stored to evaluate the pathogenesis of GHIP. For treatment, an *en bloc* resection using an ESD method is recommended.

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CASE REPORT

Transmesosigmoid hernia: Case report and review of literature

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Abstract

Transmesosigmoid hernia has previously been considered as a rare condition. The clinical symptoms can be nonspecific. Here, we report a case of acute intestinal obstruction because of transmesosigmoid hernia. In addition, after a comprehensive review of PubMed and China National Knowledge Infrastructure, we present a review of 22 cases of transmesosigmoid hernia. We summarize several valuable clinical features that help early recognition of transmesosigmoid hernia. As a result of easy strangulation, in patients without a history of surgery or abdominal inflammation who present with symptoms of progressive or persistent small bowel obstruction (SBO), surgeons should consider the possibility of transmesosigmoid hernia. In addition, based on our data, in patients with SBO because of transmesosigmoid hernia, the defect is usually 2-5 cm in diameter. Furthermore, because of the high risk of strangulation with transmesosigmoid hernia, it is mandatory to reassess the condition timely and periodically when patients receive conservative treatment.

Key words: Transmesosigmoid hernia; Acute intestinal obstruction; Internal hernia

Core tip: Transmesosigmoid hernia is a rare condition. We report a case of transmesosigmoid hernia. In addition, we carried out a comprehensive literature analysis and summarized the important clinical traits. Our data indicated for the first time that small bowel obstruction due to transmesosigmoid hernia can easily lead to strangulation, with the defect being 2-5 cm in diameter. It is necessary to reassess the condition timely and periodically if patients receive conservative treatment.

Li B, Assaf A, Gong YG, Feng LZ, Zheng XY, Wu CN. Transmesosigmoid hernia: Case report and review of literature. *World J Gastroenterol* 2014; 20(19): 5924-5929 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5924.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5924

INTRODUCTION

Internal hernia is the protrusion of a viscus through a mesenteric or peritoneal aperture. It causes up to 5.8% of cases of small bowel obstruction (SBO)[1,2]. Sigmoid-related hernias are especially rare, and account for 6% of internal hernias[3]. There are three main types of sigmoid-related hernias[4,5]. One is the intersigmoid type, which develops when herniated bowel protrudes into the inter-

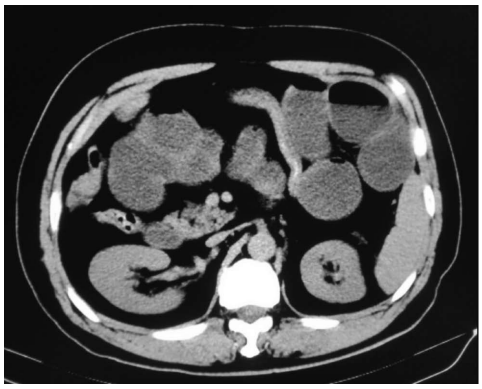

Figure 1 Abdominal computed tomography showed dilated loops of small bowel with multiple air fluids levels and collapsed distal small bowel, consistent with small bowel obstruction.

sigmoid fossa. This is actually a normal congenital variant, and the hernias are often easily reducible. Whether there is a true aperture of the intersigmoid fossa is still debatable. The other two types are transmesosigmoid hernia and intersigmoid hernia. The difference between them is that the former type involves a complete defect involving both layers of the sigmoid mesocolon, while the latter has an incomplete defect involving only one of the layers (usually the left leaf). Due to the lack of herniatic sac, transmesosigmoid hernia is assumed to progress rapidly, with a higher incidence of strangulation. The clinical presentation is often featureless until frank obstruction or strangulation occurs. Additionally, imaging changes can also be nonspecific, such as abdominal X-ray, contrast series, or computed tomography (CT)[6]. Without a heightened awareness and understanding of this hernia, it can always be misdiagnosed, with subsequent significant morbidity and mortality. To the best of our knowledge, only a few cases have been reported regarding this topic. We present a case and review of the literature to give an up-to-date perspective on transmesosigmoid hernia.

CASE REPORT

A 59-year-old man came to the emergency department complaining of acute severe colicky abdominal pain, with no flatus and feces passed for five consecutive days. This patient had a history of an episode of painful abdomen lasting for 1 d 2 years previously. When he was 29 years old, he underwent surgery for inguinal hernia repair (total extraperitoneal repair). He had no history of abdominal trauma. On examination, his temperature was normal, pulse rate was 90 beats/min, and blood pressure was 125/92 mmHg. Heart and lungs were clinically normal. Observation of the abdomen showed a surgical scar about 5 cm in length in the right lower quadrant. There was mild tenderness in the lower abdominal region with hyperactive bowel sounds. He was negative for intestinal peristalsis, rebound tenderness, hepatosplenomegaly, or shifting dullness. The external hernia orifices were normal bilaterally. Rectal examination was inconclusive. Blood counts revealed a white blood cell count of 68000/L 68000 with neutrophil leukocytosis of 71.1%. Metabolic panel and liver function tests were within normal limits. Abdominal CT showed dilated loops of the small bowel with multiple air fluid levels and collapsed distal small bowel (Figure 1). Preoperative provisional diagnosis was acute intestinal obstruction/adhesive ileus. Emergency exploratory laparotomy was carried out. We found a mechanical SBO due to an incarcerated internal hernia. Proximal ileum was dilated while the distal ileum was collapsed. A loop of ileum about 15 cm had herniated through a congenital defect in the sigmoid mesocolon (Figure 2A). The herniated loop and the other parts of the ileum were viable, with no gangrene, but there was a constriction ring over the bowel wall (Figure 2B). The herniated loop was reduced successfully and the defect was approximated. No bowel resection was done. No

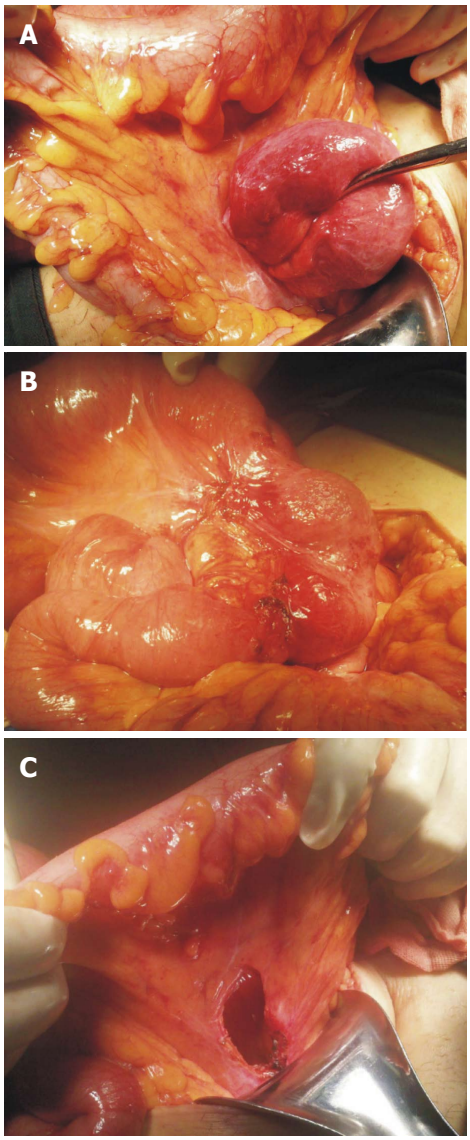

Figure 2 Sigmoid mesocolic hernia during laparotomy, with 15-cm loop of ileum herniated into the hiatus of the sigmoid mesocolon (A), herniated loop showing no gangrene, but the presence of a constriction ring (B), orifice about 2.5 cm in diameter (C).

Table 1 Case reports of transmesosigmoid hernia

Ref.	Age/sex	History of surgery or inflammation	Time from onset to operation	Acute bowel obstruction	Chronic abdominal pain	Peritonitis	Sepsis	Ascites (hemorrhagic or turbid)	Imaging before operation	Diagnosed preoperatively	Diameter of defect (cm)	Bowel resection (cm)	Exploratory laparotomy
Papanikolaou *et al*[12]	3/F	N	12 h	Y	Y	Y	Y	Y	U/S	SBO	5	60	Y
Collins *et al*[13]	60/M	N	48 h	Y	N	N	N	N	CT	SBO	4	0	Y
Van der Mieren *et al*[14]	Postpartum woman	N	Early	Y	N	N	N	N	CT	SBO	-	0	Y
Yang *et al*[15]	66/M	N	48 h	Y	N	N	N	N	CT	SBO	-	0	Y
Yu *et al*[16]	81/F	N	12 h	Y	1 time of SBO	Y	N	Y	CT	SBO	5	5	Y
Sasaki *et al*[17]	63/M	Appendicitis	-	Y	N	Y	N	Y	CT	SBO	3.5	30	Y
Johnson *et al*[18]	20/F, 14 wk gestation	N	1 wk	Obstruction and vaginal bleeding	N	Y	N	Y	AXR	SBO	5	160	Y
Benson *et al*[19]	42/F	N	1 wk	Y	N	N	N	N	AXR	SBO	2	0	Y
[1]Li *et al*	59/M	N	5 d	Y	Y	N	N	N	CT	SBO	2.5	0	Y
Yao *et al*[20]	60/M	N	7 h	Y	N	Y	N	Y	U/S	SBO	4	30	Y
Guo *et al*[21]	39/M	N	9 h	Y	N	Y	Y	Y	CT	SBO	4	65	Y
Yang *et al*[22]	62/M	N	9 h	Y	N	Y	N	Y	CT	SBO	5	20	Y
	48/M	N	24 h	Y	N	Y	Y	Y	CT	Appendicitis, SBO	4	30	Y
Bao *et al*[23]	62/F	N	37 h	Y	N	Y	N	Y	CT	SBO	3	12	Y
Zhou *et al*[24]	57/M	N	48 h	Y	N	Y	N	Y	CT	SBO	3	20	Y
Yang *et al*[15]	50/M	N	48 h	Y	N	Y	N	Y	CT	SBO	2	0	Y
He *et al*[25]	55/M	N	72 h	Y	N	Y	N	Y	AXR	SBO	3	100	Y
Luo *et al*[26]	69/F	Hysterectomy	24 h	Y	N	Y	N	Y	AXR	Appendicitis, SBO	3	40	Y
Zhang *et al*[27]	51/M	N	37 h	Y	N	Y	N	Y	AXR	SBO	3	40	Y
Mo *et al*[28]	59/M	N	10 d	Y	Y	Y	N	Y	AXR	SBO	3	40	Y
Li *et al*[29]	69/F	N	6 h	Y	N	Y	Y	Y	AXR	SBO	4	30	Y
Li *et al*[30]	62/F	hysterectomy	4 d	Y	N	Y	N	Y	AXR	SBO	2	30	Y
Average	53.04	13.6%	-	100%	18.2%	77.3%	18.2%	77.3%	-	9%	3.39	44.5	100%

[1]Present case report, laparoscopy converted to laparotomy; U/S: Ultrasound. CT: Computed tomography; SBO: Small bowel obstruction; AXR: Abdominal X-ray.

hernia sac was present. The hernia orifice measured 2.5 cm in diameter and consisted of two leaves of the sigmoid mesentery (Figure 2C). The operation was successful, lasting about 40 min, with minimal blood loss. The patient started to take liquids orally at 72 h after surgery. The suture was removed at day 7 after surgery. The patient recovered well with no complications at 6 wk follow-up.

Literature review

The studies were identified by searching the PubMed database using the terms (transmesosigmoid hernia) OR (sigmoid mesocolon hernia) or (mesosigmoid hernia) with no time limitation. Five reports with no abstract available were excluded[7-11]. We also searched the China National Knowledge Infrastructure database from January 1989 to December 2013 with the terms transmesosigmoid hernia. For each eligible report, the information was extracted as shown in Table 1, including the first author with year of publication, age and sex of the patients, history of surgery, symptoms, preoperative diagnosis, and some characteristics about the sigmoid mesocolon defect and herniation.

We found 27 reports, and five with no English abstract available were excluded. Twenty-two reports of surgically proven transmesosigmoid hernias were identified; eight in English and 14 in Chinese. Among these, there were 13 men (59.1%) and 9 women (40.9%; M: F =1.44:1), with a mean age of 53.1 (SD = 17.55, range 3-81 years) (Table 1). All the patients presented with acute intestinal obstruction. The associated obstructive symptoms included abdominal pain, nausea, vomiting, constipation, and obstipation. There were two patients with a history of hysterectomy and one with previous appendicitis. Three patients had chronic intermittent abdominal pain. However, this abdominal pain had no relation to the history of surgery or appendicitis. The interval between the development of symptoms and onset of surgery ranged from several hours to 10 d (median 1.5 d, mean 2.9 d). There were nine patients (40.9%) who received conservative treatment first, converting to surgery because of worsening conditions. Physical examination revealed that 18 (77.3%) patients had signs of peritonitis, including acute abdominal pain and tenderness, rebound, and abdominal guarding. All these patients had hemorrhagic or turbid ascites during laparotomy. Four patients developed sepsis (18.2%). Preoperatively, 12 (50%) patients underwent CT scanning of the abdomen; eight with abdominal X-ray (AXR) and two with ultrasound examination. The classical finding of CT scanning was dilated loops of small bowel and collapsed distal small bowel. AXR showed multiple, dilated small-intestinal loops. Ultrasound examination of the abdomen revealed dilated fluid-filled loops with free intraperitoneal liquid, suggesting bowel obstruction. Two patients were misdiagnosed with appendicitis. All the others were diagnosed with SBO of unknown etiology. Not a single case was considered as internal hernia preoperatively.

For management, all patients received exploratory laparotomy. One case received laparoscopy first, and then converted to laparotomy. The average diameter of the mesosigmoid orifice in the 20 available cases was 3.39 cm (SD = 0.99, range 2-5 cm). Sixteen patients had gangrenous bowel and underwent bowel resection. Sixteen (72.7%) patients had gangrenous bowel and underwent bowel resection. The average length of bowel resection was 44.5 cm (SD = 38.31, range 5-160 cm). The longest was in a gestational woman who had SBO symptoms with vaginal bleeding for 1 wk and had intestinal resection of about 160 cm.

DISCUSSION

Transmesosigmoid hernia is rare and characterized by the small bowel herniating through a complete defect involving both layers of the sigmoid mesocolon. To date, only a few cases of transmesosigmoid hernia have been reported. The general opinion is that the clinical presentation is featureless and the imaging tests nonspecific. Here, we summarize several valuable clinical features to help early recognition of transmesosigmoid hernia.

According to the current study, transmesosigmoid hernia may be underdiagnosed. Our data showed that the male to female ratio is 1.44:1, with an average age of 53.1 years. The interval between the development of symptoms and onset of surgery is 1.5 d (median). We suggest that, in patients without a history of abdominal surgery or inflammation, who present with progressive or persistent SBO, internal herniation or transmesosigmoid hernia should be considered as one of the differential diagnoses.

Generally, abdominal CT scan is important for SBO, which provides information of obstructive location, content, and the possible underlying causes, such as malignancy, strictures, and congenital atresia and stenosis within the bowel wall, and other intraluminal problems such as intussusception, gallstone ileus, feces or meconium, or Bezoar[1]. There are some traits that are especially meaningful for transmesosigmoid hernia: (1) CT scan showing dilated loops of small bowel with collapsed distal segments suggests the possibility of internal hernia; and (2) CT scan revealing anteromedial displacement of the sigmoid colon due to entrapped bowel loops behind the left posterior or lateral aspect of the sigmoid colon[5]. Therefore, for patients with SBO, after comprehensive history taking and physical examination, abdominal CT scan is important for early acute diagnosis.

In transmesosigmoid hernia, the herniated loop is easily strangulated, causing necrosis. According to our data, 72.7% patients had bowel necrosis and underwent bowel resection, with the average length of resection being 44.5 cm. Among the patients, 77.3% had peritonism and 18.2% developed systemic compromise (sepsis). Although a conservative approach was attempted in some patients, all eventually underwent exploratory laparotomy. These data are meaningful. On the one hand, during conservative treatment, it is mandatory to reassess timely and periodically the patient's condition. Any persistence or progression of the clinical symptoms and signs should lead to urgent surgical exploration. On the other hand, SBO due to transmesosigmoid hernia has a high incidence of strangulation, which rapidly progresses to gangrene. In contrast, for SBO secondary to postoperative adhesion, a substantial percentage of selected patients can be treated successfully using with a conservative approach[31]. If strangulation occurs, the risk of complications is high, including fluid leakage, bleeding, and stenosis in the anastomosis.

Based on our data, strangulation of the herniated loops in the transmesosigmoid defect is closely related to the size of the opening. The average diameter of the defect in transmesosigmoid hernia is about 3.39 cm (range 2-5 cm). Two cases with no data about the size of the orifice had no bowel necrosis[14,15]. It is reasonable to infer that when the orifice diameter is > 5 cm, the bowel may move in and out spontaneously; when it is < 2 cm, it may cause partial herniation of the bowel wall only. For these two conditions, spontaneous restoration occurs, and the symptoms of SBO are mild and vague. When the defect is 2-5 cm, spontaneous restoration is unlikely and the risk

of strangulation is high. In addition, the symptoms of ileus based on transmesosigmoid hernia can always recur because of the congenital defect, even though there is transient subsidence after conservative treatment in a few cases. Therefore, this can explain why all patients in our literature series received laparotomy. To the best of our knowledge, this is a major novel feature of our study.

We suggest that surgeons should consider the possibility of transmesosigmoid hernia in patients without a history of surgery or abdominal inflammation, who present with progressive or persistent symptoms of SBO. We considered the common causes such as postoperative adhesions, abdominal wall hernia, and tumor. After ruling out these conditions, we should maintain a high index of suspicion of transmesosigmoid hernia. We suggest that the orifice diameter because of transmesosigmoid hernia causing SBO is usually 2-5 cm. Timely and periodically re-evaluating the condition of patients who receive conservative treatment is mandatory so as to enable early recognition of bowel ischemia. For treatment, laparotomy is useful and there are no special considerations for SBO due to transmesosigmoid hernia.

COMMENTS

Case characteristics

A 59-year-old man presented with acute severe colicky abdominal pain, with no flatus and feces passed for five consecutive days.

Clinical diagnosis

Transmesosigmoid hernia with small bowel obstruction (SBO).

Differential diagnosis

Postoperative adhesions, abdominal wall hernia, tumor.

Laboratory diagnosis

White blood cell count of 68000/L 68000 with neutrophil leukocytosis of 71.1%; metabolic panel and liver function tests were within normal limits.

Imaging diagnosis

Abdominal computed tomography showed dilated loops of small bowel with multiple air fluids levels and collapsed distal small bowel, consistent with SBO.

Treatment

The patient underwent emergency exploratory laparotomy. The herniated loop was reduced successfully and the defect was approximated. No bowel resection was done.

Related reports

Twenty-two reports of patients with surgically proven transmesosigmoid hernias were identified; 8 in English and 14 in Chinese.

Term explanation

Transmesosigmoid hernia involves a complete defect involving both layers of the sigmoid mesocolon. As a result of the lack of herniatic sac, transmesosigmoid hernia is assumed to progress rapidly, with a high incidence of strangulation.

Experiences and lessons

This case report not only represents the process of diagnosis and treatment of a patient with transmesosigmoid hernia, but also summarizes the important clinical traits that SBO due to transmesosigmoid hernia can easily become strangulated, with the defect being 2-5 cm in diameter.

Peer review

This article reports a case of transmesosigmoid hernia (a rare internal hernia), carries out a comprehensive literature analysis, and summarizes the important clinical traits.

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CASE REPORT

Colonic metastasis after resection of primary squamous cell carcinoma of the lung: A case report and literature review

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Author contributions: Lou HZ has made substantial contributions to conception and design and was writing the manuscript; Wang CH made substantial contributions to acquisition, analysis and interpretation of the data; Pan HM was revising it critically for important intellectual content and authorized the publication of the article; Pan Q has made substantial contributions to conception and design and has been involved in drafting the manuscript; Wang J provided the pathological data and critically review the manuscript; all authors read and approved the final manuscript.
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Abstract

Lung cancer is a common malignancy in the world; however symptomatic colonic metastasis from primary lung cancer is rare. A 64-year-old man was originally found poorly differentiated squamous cell carcinoma of right lung and received right lower lobectomy and lymph node dissection. Three years later, the patient presented to our emergency room with the symptom of upper abdominal pain and weight loss. Abdominal palpation and computed tomography scan of the abdomen revealed a large mass measuring 7.6 cm × 8.5 cm in the ascending colon. Colonoscopy and biopsy revealed poorly differentiated squamous cell carcinoma with similar morphological pattern to that of the previous lung cancer. Chemotherapy was given and the patient died 5 mo later. Lung cancer metastatic to the colon confers a poor prognosis: overall survival ranged from 5 wk to 1 year, with a median survival of 3 mo after the diagnosis of the colonic metastasis.

Key words: Lung neoplasm; Colonic metastasis; Squamous cell carcinoma; Chemotherapy

Core tip: Lung cancer with colonic metastasis is a rare condition, accounting for only 0.5% of lung cancer cases. Symptomatic colonic metastases are often emergent and colonoscopy with biopsy can make further diagnosis. Herein we report a case of patient with upper abdominal pain and weight loss after lung cancer resection. Subsequent colonoscopy and pathology confirmed poorly-differrentiated squamous cell carcinoma due to colonic metastasis of lung cancer. The patient improved after receiving chemotherapy but died from rectal bleeding. We report the case for its rarity and emphasize disease management after prompt clinical and pathological analyses.

Lou HZ, Wang CH, Pan HM, Pan Q, Wang J. Colonic metastasis after resection of primary squamous cell carcinoma of the lung: A case report and literature review. *World J Gastroenterol* 2014; 20(19): 5930-5934 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i19/5930.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i19.5930

INTRODUCTION

Lung cancer is a common malignancy in the world; how-

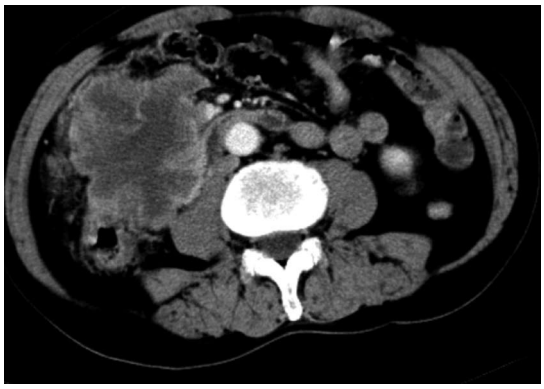

Figure 1 Abdominal computed tomography which revealed a large soft tissue mass of 7.6 cm × 8.5 cm in size in the ascending colon.

ever symptomatic colonic metastasis from primary lung cancer is rare. Clinically, patients may present with symptoms of abdominal pain, obstruction, bowel perforation, and lower gastrointestinal bleeding. In this report, we describe a rare case of symptomatic colonic metastasis from squamous cell carcinoma of the lung and with literature review.

CASE REPORT

A 64-year-old man originally presented to local hospital for left chest pain in May 2009. At that time, he underwent a contrast-enhanced computed tomography (CT) scan of the chest,which showed a mass in the right lower lobe of lung. Flexible bronchoscopy identified focal areas of thickening in the bronchus of right lower lobe. Histopathologic examination revealed a poorly differentiated squamous cell carcinoma (SCC) of the lung. Then he underwent right lower lobectomy and lymph node dissection.The final pathologic diagnosis of the tumor was stage Ⅱ poorly differentiated SCC. Margins were negative and no involved lymph nodes. The patient refused any adjuvant chemotherapy or radiation therapy after surgery.

In February 2012, nearly three years after his initial lung cancer diagnosis, the patient presented to our emergency room with the symptom of upper abdominal pain and weight loss. On physical examination at admission, the patient was fully alert and oriented, afebrile. His vital parameters were normal. There was slight pallor, no peripheral lymphadenopathy or pedal edema. Abdominal palpation revealed a large mass in the right upper quadrant. The mass was tender on palpation, but there was no rebound tenderness or guarding. Rest of the systemic examination was unremarkable. On laboratory tests,hemoglobin was 10.6 g/dL, blood count showed slight leukocytosis with neutrophils elevated. Liver and renal functions were normal.Fecal occult blood test was positive.

CT scan of the abdomen revealed a large mass measuring 7.6 cm × 8.5 cm in the ascending colon with heterogeneous enhancement (Figure 1). Subsequent colonoscopy disclosed a large ulcerated lesion in the ascending colon, and biopsy revealed poorly differentiated SCC with similar morphological pattern to that of the previous lung cancer. Furthermore, immune histochemical results of the tissue specimen were positive for CK5/6 and p63, but negative for CDX2 and CK20 (Figure 2). Upon review by our hospital's tumor board, it was concluded that these results were consistent with primary lung cancer. Then chemotherapy with intravenous cisplatin and oral S-1 every 5 wk was initiated and a total of 2 cycles were given. The patient improved remarkably after 2 cycles of chemotherapy. CT scan of the abdomen showed significant reduction in the abdominal mass. Just before the 3rd cycle, the patient developed rectal bleeding with bright red blood in stool. He was treated with somatostatin and hemostatic drugs and symptoms relieved soon. But three weeks later the patient refused further chemotherapy and was discharged from the hospital. The patient died 5 mo after diagnosis.

DISCUSSION

Lung cancer is one of the most common primary malignancies, and nearly 50% have distal metastasis at the time of diagnosis[1,2]. The preferential sites of distal metastasisare the liver, adrenal gland, bone, and brain[3,4]. Gastrointestinal metastasis of primary lung cancer is considered to be rare. The overall incidence of primary lung cancer metastatic to gastrointestinal tract is about 4.7%-14.0% at autopsy[1,5]. Symptomatic gastrointestinal metastasis of lung cancer is 1.77% (6/339) as reported by Yang *et al*[6]. Lung cancer metastatic to the colon are very rare, accounting for only 0.5%of lung cancer cases[5].

Gastrointestinal metastases from lung cancer are often asymptomatic. Symptomatic colonic metastasis is very rare and only a few cases were reported in the English literature. After review of the literature, a total of only 12 such cases were identified since 1970 (Table 1)[2-4,6-13]. In these patients, the mean age was 64.4 years and all patients were male. Abdominal pain and anemia are the most common symptoms[1,10]. Other symptoms like intestinal obstruction, lower GI bleeding, bowel perforation, or GI fistula may occur[2,10].These findings can occur synchronously or before the diagnosis of lung cancer, but more frequently after the diagnosis of primary lung cancer[6].

Symptomatic colonic metastases are often emergent and intended to undergo emergent computed tomography .Colonoscopy with biopsy can make further diagnosis. PET-FDG has become useful in patients with potentially resectable non-small cell lung cancer[14,15]. In patients with clinical stage Ⅲ disease, PET scanning willdetect extrathoracic metastases in approximately 25% of patients[16,17]. Recently, case of colonic metastasis from lung cancer has been assessed by PET-CT imaging[11]. PET-CT scanning may reveal a higher incidence of colonic metastases than previously suspected. However, the role of PET-CT in the diagnosis of gastrointestinal metastasis from lung cancer is still unclear because of

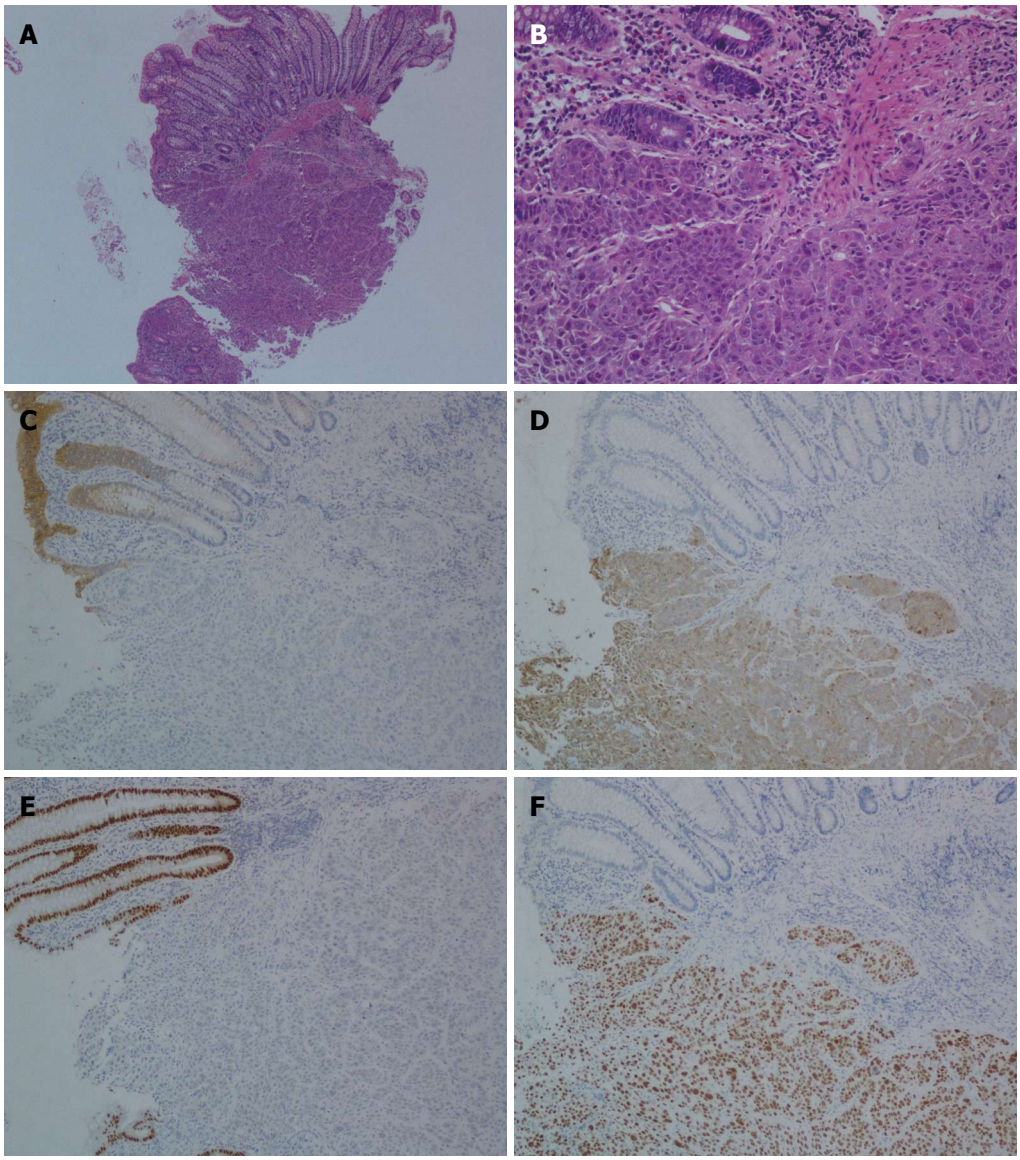

Figure 2 Histological findings. A: Poorly-differentiated squamous cell carcinoma metastatic to the descending colon [hematoxylin eosin (HE) × 40]; B: Cancercells infiltrating mucosa of the descending colon (HE × 200); C: Negative immunohistochemical staining for CK 20 (× 100); D: Positive immunohistochemical staining for CK 5/6 (× 100); E: Negative immunohistochemical staining for CDX-2 (× 100); F: Positive immunohistochemical staining for p63 (× 100).

few clinical data.

The histological type of lung cancer that causes gastrointestinal metastasis varies according to different series[6].The most common type was squamous cell carcinoma which accounting for colonic metastasis in more than 50% of cases in our review. But the study of Stenbygaard *et al*[18] showed adenocarcinoma was more prominent. The pathologic results of colonic metastasise usually consistent with the primary lung cancer. These findings can be confirmed by immunohistochemistry. Lung carcinomas usually show positive staining forCK5/6 or p63 and negative staining for CK20 and CDX-2, whereas colon adenocarcinoma is typicallyCK5/6 or p63 negative and CDX-2 or CK20 positive[19]. CDX-2 is a highly specific and sensitive marker for gastrointestinal adenocarcinoma andcan be used to differentiate from metastasis of lung cancer[20].

Secondary colonic involvement from lung cancer suggests widespread dissemination and is associated with a poor prognosis. According to our review, after the detection of colonic metastasis secondary to primary lung cancer, survival times ranged from 5 wk to 1 year (Table 1), with most patients dying within 6 mo. Treatment of colonic metastasis depends on the extent of the disease and the nature of the initial presentation. The most important point is which lesion should be treated first-the colonic metastasis or the primary lung cancer? In case of a complicated colonic lesion (obstruction, bleeding or perforation), proper surgery for colonic metastasis provides excellent palliation, increases the quality of life and shortens the time of hospitalization[9,13].Chemotherapy is useful in selected patients. In our case sufficient palliation was achieved with chemotherapy. This patient survived for a further 5 mo after diagnosis.

Table 1 Clinical characteristics of published colonic metastasis from primary lung cancer

Case	Year	Age	Sex	Site of metastasis	Pathology type of lung cancer	Location of lung cancer	Interval time to diagnosis	Survival
1	1978	52	Male	Sigmoid colon	Poorly differentiated squamous cell carcinoma	Left upper lobe	Same time	> 1yr
2	1978	57	Male	Sigmoid colon	Squamous cell carcinoma	Left lower lobe	2 yr later	1 yr
3	1980	63	Male	Sigmoid colon	Large cell carcinoma	Left upper lobe	1 mo	NA
4	1988	69	Male	Ascending colon	Small cell carcinoma	Right medial lobe	Same time	2 mo
5	1998	69	Male	Sigmoid colon	Squamous cell carcinoma	Right main bronchus	9 mo	2 mo
6	2001	68	Male	Sigmoid colon	Squamous cell carcinoma	Right lower lobe	10 wk	6 mo
7	2002	73	Male	Entire colon	Large cell carcinoma	Right lower lobe	Same time	3 mo
8	2006	60	Male	Ascending colon	Squamous cell carcinoma	Left upper lobe	Same time	NA
9	2006	57	Male	Caecum	Small cell carcinoma	Left upper lobe	645 d	70 d
10	2008	74	Male	Descending colon	Squamous cell carcinoma	Right upper lobe	Same time	23 wk
11	2008	59	Male	Caecum	Small cell carcinoma	Left hilar mass	Same time	NA
12	2010	53	Male	Descending colon	Adenocarcinoma	Left lower lobe	Same time	6 mo
32	2012	67	Male	Descending colon	Squamous cell carcinoma	Right lower lobe	32 mo	5 mo

NA: Not available.

Although lung cancer metastatic to colon is rare, it should be included in the differential diagnosis for any patient with colonic tumor.Accurate differentiation is necessary because treatment differs significantly for patients with colonic metastasis of lung cancer, as compared with patients with primary colon cancer. Lung cancer metastasis to the colon confers a poor prognosis and early diagnosis can prevent further complications.

COMMENTS

Case characteristics

The patient presented with the symptom of upper abdominal pain and weight loss.

Clinical diagnosis

Clinical diagnosis was colonic metastasis of primary squamous cell carcinoma of lung.

Differential diagnosis

Differential diagnosis of primary colon carcinoma, malignant lymphoma, Crohn disease and diverticulosis should be considered.

Laboratory diagnosis

On laboratory tests, hemoglobin was 10.6 g/dL, blood count showed slight leukocytosis with neutrophils elevated.

Imaging diagnosis

Computed tomography scan revealed a large mass in the ascending colon with heterogeneous enhancement and colonoscopy disclosed a large ulcerated lesion in the ascending colon.

Pathological diagnosis

Pathology with Hematoxylin and eosin stain and immunohistochemistry revealed poorly differentiated squamous cell carcinoma.

Treatment

Chemotherapy with intravenous cisplatin and oral S-1 every 5 wk was initiated and the patient improved remarkably after 2 cycles of chemotherapy.

Related reports

Most patients presented with symptoms like intestinal obstruction, lower glycemic index or glycaemic index (GI) bleeding, bowel perforation, or GI fistula and pathology with immunohistochemistry can confirm the diagnosis.

Experiences and lessons

Although lung cancer metastatic to colon is rare, it should be included in the differential diagnosis for any patient with colonic tumor. Accurate diagnosis and chemotherapy with cisplatin and S-1 may help to manage the condition but the prognosis is still very poor.

Peer review

In this manuscript "Colonic metastasis after resection of primary squamous cell carcinoma of the lung: a case report and literature review", Lou *et al* reported a case of symptomatic colonic metastasis from squamous cell carcinoma of the lung.

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Acknowledgments

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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