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EDITORIAL

Intestinal M cells: The fallible sentinels?

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Abstract

The gastrointestinal tract represents the largest mucosal membrane surface in the human body. The immune system in the gut is the first line of host defense against mucosal microbial pathogens and it plays a crucial role in maintaining mucosal homeostasis. Membranous or microfold cells, commonly referred to as microfold cells, are specialized epithelial cells of the gut-associated lymphoid tissues (GALT) and they play a sentinel role for the intestinal immune system by delivering luminal antigens through the follicle-associated epithelium to the underlying immune cells. M cells sample and uptake antigens at their apical membrane, encase them in vesicles to transport them to the basolateral membrane of M cells, and from there deliver antigens to the nearby lymphocytes. On the flip side, some intestinal pathogens exploit M cells as their portal of entry to invade the host and cause infections. In this article, we briefly review our current knowledge on the morphology, development, and function of M cells, with an emphasis on their dual role in the pathogenesis of gut infection and in the development of host mucosal immunity.

Key words: M cell; Gastrointestinal; Development; Pathogenesis; Mucosal immunity

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INTRODUCTION

The gastrointestinal (GI) tract, in addition to its role as an organ for nutrient absorption, represents a key interface between the host and its external environment. Since the GI tract has the highest recorded bacterial cell density of any microbial ecosystem[1], it is not surprising that the GI immune system is both extensive and complex. The GI tract contains more antibody-producing cells than in the spleen and lymph nodes combined, and it contributes the majority of the body's immunoglobulin production in the form of IgA secreted into the intestinal lumen[2,3]. The GI mucosa, due to its large surface area (200 times greater than the skin)[4], requires consistent monitoring for potentially harmful agents (such as pathogens) while discriminating these from harmless food and non-pathogenic antigens. Gut-associated lymphoid tissue (GALT), consisting of Peyer's patches (PP), appendix, and other lymphoid aggregates in the large intestine, plays crucial roles in the maintenance of homeostasis in the GI system. The membranous or microfold cell (M cell) in the Peyer's patches is one of the primary cell types responsible for the capability of the intestinal immune system to mount both immunological and mucosal tolerogenic responses to foreign antigens.

This review will briefly summarize the current knowledge on intestinal M cells, with the emphasis of its potential role in GI infection and immunity. However, it is worth noting that M cells are also present in other mucosa-associated lymphoid tissues (MALT), such as the bronchus-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT)[5].

MORPHOLOGY AND FUNCTIONS OF THE M CELL

M cells are specialized epithelial cells forming part of the follicle-associated epithelium (FAE) which overlies the PP and other lymphoid aggregates. The most striking feature of the human M cell under light or electron microscopy is the absence of surface microvilli which are characteristic of the intestinal epithelial cells. Instead, the apical membrane of the M cell has a microfold (or membranous) topography (Figure 1)[6-8], and hence the name M cell. Like other epithelial cells, M cells form tight junctions to maintain a barrier function, albeit with different structural features and adhesion protein expression[9]. The basolateral membrane of M cells is invaginated, and forms many "pockets", which harbor infiltrating lymphocytes[10]. The formation of these "pockets" greatly reduces the intracellular distance that antigens have to travel and allows M cells to rapidly transport (within 10 to 15 min) antigenic materials to the basolateral membrane[11,12].

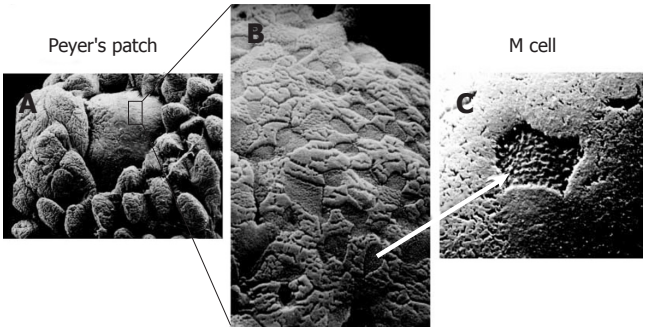

Figure 1 Ultrastructure of the Peyer's patches and FAE (Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology[32], copyright 2003). **A**: At low magnification, the dome shape of the Peyer's patch protrudes between villi into the lumen of the intestine; **B**: At higher magnification, M cells can be seen as epithelial cells with surface microfolds rather than the microvilli that are seen on the surrounding conventional enterocytes; **C**: Antigen is taken up preferentially through M cells.

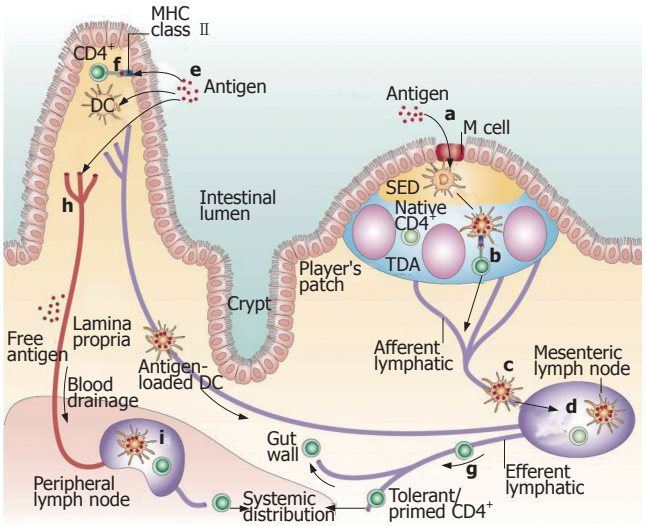

Figure 2 Antigen uptake and recognition by $CD4^+$ T cells in the intestine. Antigen may enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (**a**), and after transfer to local dendritic cells (DCs), might then be presented directly to T cells in the Peyer's patch (**b**). Alternatively, antigen or antigen-loaded DCs from the Peyer's patch may gain access to draining lymph (**c**), with subsequent T-cell recognition in the mesenteric lymph nodes (MLNs) (**d**). A similar process of antigen or antigen-presenting cell (APC) dissemination to MLNs may occur if antigen enters through the epithelium covering the villus lamina propria (**e**), but in this case, there is the further possibility that MHC class II^+ enterocytes may act as local APCs (**f**). In all cases, the antigen-responsive $CD4^+$ T cells acquire expression of the $\alpha4\beta7$ integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (**g**) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propria. T cells which have recognized antigen first in the MLN may also disseminate from the bloodstream throughout the peripheral immune system. Antigen may also gain direct access to the bloodstream from the gut (**h**) and interact with T cells in peripheral lymphoid tissues (**i**). (Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology[32], copyright 2003).

The morphology of M cells varies greatly amongst different animal species, and within anatomic sites of a species. For instance, the microfold structure is present only in human M cells[7], and human M cells lack microvilli. In contrast, the microvilli are present on the surface of murine M cells, but these are short and irregular[13] in contrast to the microvilli on the M cells of rabbit caecal lymphoid patches which are longer than the neighboring enterocytes[14]. The M cells express a wide range of carbohydrate markers with diverse glycoconjugate profiles[15], which perhaps allows M cells to interact with a broad range of microbes[16,17]. For example, while Ulex europaeus agglutinin-1 (UEA-1), an α-L-fucose residue-specific lectin which selectively labels fucose, recognizes M cells and goblet cells overlying the mouse PP[18,19], it fails to react with M cells on the mouse caecum or colon[15,20]. Conversely, UEA-1 does not bind to M cells of rabbit PP but reacts with those in the caecal lymphoid patches[21]. As a result, studies of rabbit M cells have frequently used vimentin, instead of UEA-1, as histochemical markers[22-25]. On the other hand, human M cells are generally negative for specific lectin binding[26], but are positive for the sialyl Lewis A antigen[27]. M cells in rats, guinea pigs and cats share similar lectin-binding patterns to enterocytes, although the cytokeratins 8 and 18 are over-expressed in M cells of rats and pigs, respectively, compared to neighboring enterocytes[28,29]. Because of these variations and diversity in the morphology and lectin-binding patterns, multiple confirmatory characteristics are usually required for the positive identification and characterization of M cells. Although glycosylation patterns and lectin-binding properties remain commonly used identifiers of M cells due to their relative ease of analyses, electron microscopy currently remains the most definitive method for M cell identification[15,20,30,31].

One of the major functions of M cells is believed to be the uptake and transport of antigens from the gut lumen to the underlying mucosal immune system (Figure 2)[32]. The apical membrane of M cells is specialized for the uptake and transport of antigens, featuring a reduced glycocalyx[33], and a general lack of membrane hydrolytic enzymes[34]. Additionally, the dramatic reduction of lysosomes may allow M cells to transport microorganisms into the lymphoid follicles without altering their antigenic properties[35]. M cells have been shown to be able to transport proteins[36,37], bacteria[31,38,39], viruses[40] and non-infectious particles[41,42] from the apical membrane to the basolateral surface. Bacteria and large particle transport is accomplished by phagocytosis, accompanied by apical membrane ruffling and actin cytosketeton rearrangements[38,43]. Under electron microscopy observation, M cells appear to reach out and engulf these large particles. Viral, and small adherent particles are endocytosed in clathrin-coated vesicles[41], while non-adherent antigens undergo fluid phase pinocytosis[6,11]. However, the role of M cells in antigen processing and presentation *per se* remains unclear. Although there have been several reports of M cells expressing major histocompatibility complex (MHC) class Ⅱ molecules[44-46], these findings could not be confirmed by others[32,47,48]. However, M cells do express cathepsin E, which is typically expressed on antigen-presenting cells[49], and M cells can also produce the pro-inflammatory cytokine interleukin 1 (IL-1)[50]. In addition, M cells are the main producers of CC chemokine ligand (CCL) 9 and CCL20 in the FAE[51], and also produce CXC chemokine ligand (CXCL) 16[52].

DEVELOPMENT OF M CELLS

During embryonic and postnatal development, each crypt in the intestine is a clonal unit[53,54] whose cells differentiate into multiple types as they migrate. Cells on the villous side of the crypt differentiate into absorptive enterocytes, goblet cells and enteroendocrine cells. The cells on the FAE side of the crypt acquire the phenotype of absorptive enterocytes, M cells and, rarely, goblet cells[12,55,56]. Within this framework, two hypotheses have been proposed for the development of M cells. The first hypothesis suggests that M cells originate from a distinct cell lineage following an independent differentiation program. Evidence supporting this hypothesis includes that M cell development in the PP is restricted to specialized dome-associated crypts, and that both M cell precursors and their developmental intermediates have been identified within these dome-associated crypts[34,57] with early commitment of M cells observed in the mid-crypt area of caecum, appendix and PP[58,59]. In addition, the arrangement of some M cells as radial strips on the FAE dome with a single, predominating glycosylation pattern also implies M cell commitment occurs in the dome-associated crypts[14,60,61]. However, even in this example, the M cell glycosylation pattern is heterogenous.

The second hypothesis postulates that M cells develop from FAE enterocytes either as a developmental/transient stage of enterocytes, or in response to local signal stimulations (such as contact with lymphocytes and chemokines/cytokines). Indeed, Caco-2 cells, a human intestinal adenocarcinoma cell line, differentiate into an M cell-like morphology and phenotype after *in vitro* co-culture with murine PP-derived lymphocytes[39] or human B lymphoma cell lines. Moreover, intravenous injection of PP lymphocytes or normal bone marrow transplantation into severe combined immunodeficient (SCID) mice correlates with the development of M cells in the FAE[62,63]. The hypothesis that M cells are derived from enterocytes[64] is also supported by ultrastructural studies of chicken caecal tonsils[65], and by cell division and apoptosis studies in mouse PP[66] and in rabbit ileal PP[61]. Furthermore, a possible intermediate M cell/enterocyte cell type has been recently identified in upper regions of the dome in pigs[59].

Kerneis and Pringault have merged these diverse observations together into a single postulation that intestinal cell differentiation is largely determined by the crypt stem cells[67]. However, with the proper stimuli, alternate differentiation pathways could be followed (which they term "intestinal cell plasticity")[67]. In the case of M cells, enterocytes (perhaps immature) may convert into an M cell phenotype[67].

Although it is generally recognized that the mucosal lymphoid cells induce the development of the overlying specialized FAE, and that cell-to-cell contact and/or soluble factors provide important signals for the development of M cells[12], the events and signaling pathways directly involved in M cell differentiation and development remain poorly understood. The tumor necrosis factor (TNF) family of cytokines, particularly lymphotoxin (LT)-α, LT-β, and TNF-α produced by B cells, appear to play crucial roles in the development of Peyer's patches and FAE[68,69]. Their involvement in the development of M cells *per se* is, however, less clear. In the absence of LT-α and LT-β, the specialized areas of PP anlagen in the embryonic intestine are not formed[70-72]. Also, LT-β receptor-knockout mice lack PP[69]. However, mice whose B cells do not express LT-β do have normal FAE and M cells, although with smaller PP. Recombinase-activating gene (RAG)-1 -/- knockout mice, which lack mature B and T lymphocytes, have small PP-like aggregates having a normal M cell density[73]. When the LT-β receptor signaling is blocked by the antagonist lymphotoxin-β receptor-immunoglobulin G fusion protein in RAG-/- mice, the percentage of M cells in the PP-like aggregates decreases, suggesting that the LT-β signaling is essential for the differentiation and development of M cells, but LT-β signaling molecules could be supplied by other cell types in the absence of mature B and T lymphocytes[73]. On the other hand, mice having defective CD40 or IL-4 signaling, defective B-cell proliferation, or deficient in signal transducer and activator of transcription 6 (STAT6) have normal FAE and M cells[18,74]. Furthermore, although Toll-like receptors (TLRs) are expressed by the M cells[75-78], and exposure to bacteria can trigger TLR signaling resulting in induction of M cell proliferation[79] and up-regulation of transcytosis[80], TLR signaling does not appear to be essential for the development of M cells since MyD88-knockout, TLR-2 or TLR-4-knockout mice have normal M cell populations[18,79,81].

The notch signaling system is a recently characterized, highly conserved mechanism which regulates the differentiation, proliferation and apoptotic events at all stages of cell development, including the differentiation and renewal of intestinal epithelial cells and other types of intestinal cells, such as goblet cells, enteroendocrine cells, and Paneth cells[18,82,83]. Therefore, notch and notch ligands may play an important role in M cell developmental signaling. Indeed, the expression of Jagged-1 mRNA, a notch ligand, is increased in the *in vitro* M cell system compared to the parental epithelial cell line[84]. A subset of cells of the FAE in mice with a mutated Delta-3 gene, a notch ligand, showed abnormal apical membranes (dubbed as 'C cells'), and it has been suggested that these cells are precursor M cells[18]. This 'C cell' morphology has also been observed in normal mice[85].

DISTRIBUTION OF M CELLS IN THE GI TRACT

In the human GI system, M cells are mainly found on the FAE overlying the dome structure of Peyer's patches in the small intestine[85,86]. The FAE, aside from having M cells, is distinguished by a reduced number of goblet cells and enterocytes[87,88]. Beneath the FAE lies the sub-epithelial dome (SED), a diffuse region of dendritic cells (DCs), naive B cells, $CD4^+$ and $CD8^+$ T cells, and macrophages[55]. Particles transported by the M cells from the lumen can be captured in SED by immature DCs[89], which then migrate to B-cell follicles and parafollicular T-cell zones and become mature DCs[90].

However, M cells are also present over lymphoid

follicles in the colon and rectum[91]. These follicles in the colonic crypts have a specialized epithelium with a greater proportion of goblet cells than PP, but fewer than the surrounding colonic regions. Similar to PP M cells, colonic M cells have transport vesicles, a thin glycocalyx, and a basolateral invagination containing pockets of lymphocytes[13].

The percentage of M cells comprising FAE varies substantially among host species and their anatomical locations, ranging from 5% to 10% in the human and murine PP[92] to about 50% in the rabbit and human caecal lymphoid aggregates[27,93].

M CELLS AS SENTINELS OF THE GI IMMUNE SYSTEM

M cells and GI microbial infections

The accessibility of M cells on the mucosal surface and their ability to transcytose particulate material make the M cells an ideal entry point for potential pathogens. Indeed, it has been demonstrated that M cells can transport a diverse array of mucosal microorganisms across the intestinal epithelial barrier, including bacteria (*Vibrio cholerae*[94], *Campylobacter jejuni*[95], *Mycobacterium tuberculosis*[13], *Shigella spp.*[96,97], *Salmonella spp.*[98,99], *Escherichia coli*[100,101], *Yersinia spp.*[102]), viruses (MMTV virus[74,103], polioviruses[104], reoviruses[105-107], prions[108] and HIV[40,109]) and parasites (Cryptosporidium[110]). In fact, many pathogens exploit the M cells as a conduit to invade the host and establish an infection. In this regard, enteropathogens, such as *Salmonella typhimurium*, *S. typhi*, *Shigella sp.* and *Yersinia spp.,* are capable of directly invading and destroying M cells and spreading the infection to neighboring enterocytes. For example, *S. typhimurium* initially invades the M cells[98,99], and induces a spotty and diffuse infection pattern with small groups of infected M cells[111]. Experimental infection in calves have shown that *S. typhimurium* is ingested by M cells within 5 min of contact[99,112]. The process ends with the exfoliation of majority of the infected M cells within 30 min, and cell death within an hour. This disruption of M cells allows the pathogen access to the neighboring enterocytes, and results in the sloughing off of the FAE[113,114]. Although these results have not been directly confirmed in humans, ulcerations are nevertheless present in regions corresponding to PP in cases of typhoid infection[115]. Similarly, free HIV particles use both, M cells and DCs, as conduits to infect local $CD4^+$ T cells[40,109,116]. In addition, it has been shown that the success of host adaptation of Salmonella in pigs is closely associated with the increased number of pathogens per M cell, as compared to the parental strain[117].

Other enteropathogens, such as Shigella species, are capable of attaching and adhering to M cells, but do not necessarily induce any cytotoxicity to the infected cells[97,118,119]. Instead, it induces membrane ruffling[96], and the afflicted M cells increase in size, rather than proliferating, to accommodate increased numbers of mononuclear cells in their basolateral pocket[96].

The interaction between intestinal pathogens and M cells are likely influenced and controlled by factors deriving from both the pathogen and the host. In this regard, the long polar fimbria (LPF) produced by the lpf operon and Salmonella pathogenicity island-1 (SPI-1) encoding the type Ⅲ secretory system play important roles in selective adherence of Salmonella to M cells[120,121]. LpfC or SPI-1 mutants of Salmonella show reduced colonization, decreased virulence, are not cytotoxic to M cells and are not disruptive to the FAE[99,122]. Transformation of the lpf operon into non-piliated *E coli* increased their uptake in PP[120]. Similarly, the uptake of Yersinia and Shigella by M cells is mediated by invasin or mechanisms encoded by a 30-kb virulence plasmid, respectively[102]. The presence or absence of these M cell-targeting gene products in pathogens might explain the differences seen amongst different strains of the pathogen in their attachment to M cells. For example, the rabbit diarrheagenic *E. coli* (RDEC)-1 strain is selective for adherence to M cells[100,101], whereas enterohemorrhagic *E. coli* (EHEC), such as strain O157: H7, has been found to attach to the FAE of human PP[123]. On the other hand, enteropathogenic *E. coli* (EPEC) is not transcytosed by the M cells and remains in the gut lumen.

It appears that most of the bacterial genes and their products identified to date for their invasive role represent the primary, but not the exclusive, mechanism for the entry of pathogens in M cells[124]. In Salmonella cases, some Salmonella serotypes, which are M cell selecting, lack the lpf operon, and others with the lpf operon do not target M cells[120,125]. Similarly, invasin-deficient Y. pseudotuberculosis mutants have delayed uptake of 3 to 5 d *in vivo,* but are nonetheless found in the spleen and liver at the same time and produce the same LD50 values, as the wild-type strain[126]. Perhaps, M cells can also recognize other Yersinia adhesins, such as pH 6 antigen and the plasmid encoded YadA, but with less affinity than for invasin[127]. In addition, the expression of the lpf operon has been found to cycle between 'off' and 'on', being referred to as phase variation[128,129]. It is probably an adaptation to avoid host defence. In this regard, cultivation in Lauria Bertani (LB) broth appears to increase the proportion of *S. typhimurium* in the lpf operon 'on'phase[128].

It is now recognized that the entry of M cells by intestinal pathogens is also mediated by a number of surface adhesion molecules, particularly those within the integrin family, of the host cells. In this regard, enteropathogenic Y. pseudotuberculosis can attach and invade murine M cells *via* β1-integrins expressed by the apical M cell membranes[130-132]. Studies have postulated that β1-integrins were the receptor for Yersinia invasin protein[131]. *In vitro* studies have demonstrated that α2β1 integrins are the exclusive heterodimer form found on the M cell apical membrane[133], although others have found that this heterodimer does not normally interact with invasin[134]. Other studies have shown that inhibition of α5β1 integrin expression on the apical membranes of Caco-2 cells and M cells *in vitro* abolished the abilities of these cells to transport microbes[77,135]. In addition, lymphotropic (X4) HIV transport by M cells is CXC4 receptor-mediated and is lactosyl cerebroside-dependent *in vitro*[109]. Finally, the variation of M cell glycocalyx has led to speculation about its role in pathogen tissue tropism[19,136].

Target M cells for mucosal immunization

Just as pathogens can exploit M cells as the portal of entry for infection, biomedical researchers have, for many years, investigated the potential of using M cell-specific mechanisms for drug or vaccine delivery to the mucosal immune system[137-140]. Compared to parenteral routes, mucosal administration of drugs and vaccines is relatively simple, safe and inexpensive[141]. An additional benefit of mucosal immunization is its capability of priming and inducing both systemic and mucosal immune responses in the host[142,143]. Mucosal vaccination is necessary for protection against mucosal pathogens because parenteral immunization is generally ineffective for the development of protective mucosal immunity[144], and optimal vaccination strategies for many pathogens may require both mucosal and systemic delivery components[145].

Successful mucosal vaccines must circumvent the same barriers that mucosal pathogens have, i.e. mucus, proteases, nucleases, secreted antibodies, and the epithelial glycocalyx. Mucosal pathogens themselves have so far been the most effective models exploited for mucosal vaccination. The advantages of attenuated, live vaccines include their ability to activate multiple, innate immune responses. Currently, most effective oral vaccines are live attenuated poliovirus and live attenuated S. typhi, both of which exhibit selective binding to M cells and exploit M cell transport to the mucosal lymphoid tissue[99,104]. For this reason, other recombinant bacteria, including attenuated *Lactococcus spp.*, Listeria monocytogenes, and *Yersinia spp.*, have been constructed as delivery vectors for heterologous antigens[146-148].

M cells actively transport microparticles up to 1 μm in size, and those that are adherent to M cells are effectively transcytosed[12,33,149]. Thus, formulations that are multimeric or particulate and adhere to the mucosal surfaces, especially if there are some M cell specificities, seem most effective[145] while soluble, non-adherent antigens are frequently poorly internalized and hence generally induce weak immune responses or even immune tolerance[150]. The packaging of drugs and antigen microparticles on polystyrene or latex mircospheres protects them from degradation within the GI tract as well as allows them to be transcytosed by M cells[42,151-155]. Chitosan microparticles have shown promise both for oral vaccines and intranasal application[156-158]. Others have examined the potential of using copolymeric microparticles[159], proteosomes[160], liposomes[161], virus-like particles[162,163], and viral vectors, such as poliovirus and adenovirus[164,165]. However, these formulations can also bind to enterocytes[161], and are readily taken up by mucosal DCs. In addition, small vesicles derived from outer membrane components of bacteria[160,166] are interesting because of their uptake by M cells and DCs and their potential to induce an innate immune response through the activation of TLR pathways. Unfortunately, their tendency to become trapped in mucus necessitates large doses[165]. There are also regulatory concerns regarding the use of live, attenuated vectors for vaccine delivery, especially for use in immunocompromised population and the risk of reversion of the attenuated strain to full virulence.

Several recent studies have elegantly demonstrated the feasibility to specifically exploit M cells for mucosal vaccine development[167,168]. Manocha *et al*[167] have shown that HIV peptide bearing microparticles targeted to M cells, using UEA-1 lectins, are more immunogenic when administered mucosally than systemically. Wang *et al*[169] have used the adhesin protein sigma-1 from the enteric pathogen reovirus, which infects PP M cells, to direct DNA vaccines to the mucosal immune system[168]. Three expression plasmids encoding the genes for HIV gp160, cytoplasmic gp140, and secreted gp140 were conjugated to sigma-1 with poly-L-lysine and individually tested in mice. Intranasal immunization of mice showed specific, long-term CTL responses to gp160[168]. Upon challenge using a standard HIV surrogate test, these mice showed significant antiviral protection.

However, the relative importance of M cells in the induction of protective immunity by mucosal immunization remains unknown. For example, the antigen-specific immune responses as measured by IgG production is not substantially altered in the absence of PP[31]. Although M cells are capable of uptaking and transporting antigens, their role in antigen processing and presentation is less well characterized. In addition, M cells consist of only a small percentage of intestinal epithelial cells, raising the question of their overall efficiency in antigen uptake in the GI system. Moreover, there are redundancies at multiple levels of the mucosal immune system to ensure its continuing functionality. In this regard, intestinal DCs can migrate between mucosal epithelial cells, and directly sample the luminal antigens by forming transepithelial dendrites[170,171]. Other cell types, such as villous enterocytes, also express MHC class Ⅱ molecules and are capable of sampling and presenting intestinal antigens[172,173]. The difficulty in determining the precise role of M cells in the induction of mucosal immune responses is further confounded by the lack of availability of animal models which are completely and specifically deficient in M cells, making studies of intestinal antigen sampling by alternate cell types impossible.

INTESTINAL VILLOUS M CELLS

Although M cells were initially believed to be exclusively located within the FAE region in the GI tract, this notion has been challenged by the recent identification and characterization of the intestinal villous M cells[31]. Intestinal villous M cells share all the known features of traditional M cells, but are independent of PP and not associated with the FAE. Instead, intestinal villous M cells lie on the intestinal villi either as small dense clusters (50 to 60 per animal) or diffusely. Intestinal villous M cells are more common in the terminal ileum than in other areas of the small intestine. Although the role and potential significance of these M cells remain to be elucidated, evidence to date indicates that they are functionally analogous to the PP M cells[31] and may compensate for PP M cell functions. Indeed, GALT-deficient mice produce antigen-specific IgG comparable with that produced in wild-type animals upon non-invasive bacterial challenge, and the population of UEA-1+ cells increased, perhaps the result of villous M

cells developing from epithelial cells upon exposure to foreign antigens or pathogens, such as *S. typhimurium*[31].

PERSPECTIVES

More than three decades have passed since the first description of the M cell as the antigen shuttle for the mucosal immune system[6,7]. Current knowledge has highlighted the dynamic and complex role that M cells play in entry/invasion of pathogens, in antigen sampling, and in facilitating eliciting of immunity to GI infections. The advent of new technologies, such as confocal laser scanning microscopy and the intracellular visualization by use of fluorescence techniques, have supplemented the initial static electron microscopy studies in the characterization of M cells. The ability to cultivate M cells *in vitro* has complemented the *in vivo* models, and makes the molecular analysis of M cell functionality possible. The host-pathogen interactions have shown the varying strategies of the pathogen in exploiting M cells as conduits to initiate an infection, while at the same time evading or circumventing host immune surveillances. However, much work remains to be done to clarify the cellular and molecular mechanisms of the attachment to and the uptake of pathogens by M cells, and the interaction between the M cell and the pathogen, particularly the downstream events are evoked by the M cell antigen transport. Also, how does this transport lead to both mucosal and systemic immune responses? The presence of functional redundancies in the mucosal immune system and the lack of suitable animal models have further hindered the clarification of the precise role of M cells in the induction of mucosal immune responses and the rationale of targeting M cells for mucosal immunization. Further understanding and characterization of the mechanisms involved in the interaction between M cells and microorganisms, in the development and activation of M cells, and in the development of novel M cell targeting approaches will be needed for the development of a new generation of mucosal vaccines. In this regard, the recent identification of intestinal villous M cells and the rapid progress in our understanding of the role of TLR in the regulation of bacterial antigen uptake by M cells are likely to accelerate the development of M cell-based mucosal vaccines.

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TOPIC HIGHLIGHT

Wei Tang, MD, *Series Editor*

Microarray-based analysis for hepatocellular carcinoma: From gene expression profiling to new challenges

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Abstract

Accumulation of mutations and alterations in the expression of various genes result in carcinogenesis, and the development of microarray technology has enabled us to identify the comprehensive gene expression alterations in oncogenesis. Many studies have applied this technology for hepatocellular carcinoma (HCC), and identified a number of candidate genes useful as biomarkers in cancer staging, prediction of recurrence and prognosis, and treatment selection. Some of these target molecules have been used to develop new serum diagnostic markers and therapeutic targets against HCC to benefit patients. Previously, we compared gene expression profiling data with classification based on clinicopathological features, such as hepatitis viral infection or liver cancer progression. The next era of gene expression analysis will require systematic integration of expression profiles with other types of biological information, such as genomic locus, gene function, and sequence information. We have reported integration between expression profiles and locus information, which is effective in detecting structural genomic abnormalities, such as chromosomal gains and losses, in which we showed that gene expression profiles are subject to chromosomal bias. Furthermore, array-based comparative genomic hybridization analysis and allelic dosage analysis using genotyping arrays for HCC were also reviewed, with comparison of conventional methods.

Key words: Liver cancer; Microarray

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INTRODUCTION

Cancer is a genetic disease of somatic cells arising from accumulation of genetic changes, and abnormalities of suppressor genes, *TP53*[1], *RB*[2], and *IGF2R*[3], and oncogenes, *c-myc*[4], *CCND1*[5], *CTNNB1*[6], and *c-Met*[7], have been reported in hepatocarcinogenesis. On the other hand, activation of the matrix metalloproteinase (*MMP*) family[8], angiopoietin[9], and vascular endothelial growth factor (*VEGF*)[10], and inactivation of *E-cadherin*[11] have been demonstrated to play pivotal roles in invasion and metastasis of liver cancer. Considering the complexity of carcinogenesis, many other genes may be involved in the initiation and progression of cancer, and comprehensive expression analysis using microarray technology has great potential for the discovery of new genes involved in carcinogenesis.

cDNA microarray analysis for hepatocellular carcinoma (HCC) was first reported by Lau *et al*[12], using approximately 4000 known human genes in 10 pairs of HCC and non-tumorous tissues, which was followed by many subsequent studies[13-16]. Thereafter, using HCC samples, clustering analysis based on clinicopathological features, including viral infection, tumor differentiation grade, and vessel invasion, were reported[17,18]. Identification of novel candidate genes for biomarkers and discovery of therapeutic targets are helpful for improvement of clinical diagnosis and treatment[19,20]. Tailor-made therapy becomes possible if predictor genes can anticipate therapeutic responses and prognosis precisely[21,22]. Furthermore, bioinformatics technology has enabled the integration of expression profiles with various types of gene information, such as gene ontology, function, and locus[23,24].

Comparative genomic hybridization (CGH) is a molecular cytogenetic analysis of screening neoplasms for genetic changes, and has been used extensively to localize regions of oncogenes and tumor suppressor genes in various types of cancer[25]. Recently, array-based CGH (aCGH) using genomic DNA or cDNA clones has been developed with much higher resolution than conventional

CGH, and accurate identification of genes with DNA copy number changes in carcinogenesis is now possible[26,27].

In addition to aCGH, we and others have developed novel algorithms for global and high-resolution analysis of copy number changes using single nucleotide polymorphism (SNP) arrays[28-31], which were originally designed for high-throughput SNP analysis[32,33]. In comparison to aCGH, the newly developed Genome Imbalance Map (GIM) algorithm has advantages for detecting not only copy number changes but also allelic imbalance, including loss of heterozygosity (LOH) and uniparental disomy (UPD)[28].

In this article, we review the outcomes of microarray analysis for HCC through a literature search of published reports, *i.e.*, clustering analysis based on clinicopathological features, identification of candidate genes for therapy and diagnosis, selection of predictor genes for tailor-made therapy, and integration of microarray data with other types of gene information. Furthermore, we discuss the chromosomal bias of gene expression and pitfalls of gene clustering.

IDENTIFICATION OF ALTERED GENE EXPRESSION IN HEPATOCELLULAR CARCINOMA

Gene expression profile analysis has made it possible to identify novel genes with altered expression that have not been reported in liver cancer. For example, aberrations of *MARKL1* and *MARK3*[34], *VANGL1*[35], *PEG10*[36], *BMAL2*[37], *DDEFL1*[38], *RhoC*[39], *GEP*[40], *HLA-DR*[41], *Claudin10*[42], and *Ephrin A1*[43] were demonstrated by microarray analysis.

Through comprehensive expression analysis in HCC, we also identified up-regulated genes, *GPC3*[20], *ROBO1*[19], and *SP-5*[44]. *GPC3* is a member of the heparan sulfate proteoglycans and binds to the cell membrane *via* glycosyl phosphatidylinositol anchors. We demonstrated that *GPC3* works as a co-receptor, modulating signaling pathways of growth factors, such as FGF2 and BMP-7, and contributes to hepatocarcinogenesis[20]. Up-regulated genes in HCC, compared to the background hepatocytes, are candidate diagnostic markers, and we confirmed that monoclonal antibodies against *GPC3* generated in our laboratory recognize the *GPC3* molecule in HCC, and demonstrated the feasibility of *GPC3* as a marker for HCC in routine histological examination[45]. Furthermore, this molecule is secreted due to the signal peptide in its N-terminus, and we succeeded in measuring serum *GPC3* levels by enzyme-linked immunosorbent assay in HCC patients[46].

In addition to the availability of the neural cell adhesion molecule *ROBO1* as a diagnostic serum marker for HCC, this molecule is also a clinical gene therapy target as a newly generated anti-*ROBO1* monoclonal antibody induced complement-dependent cytotoxicity in the *ROBO1*-expressing liver cancer cell line, PLC/PRF/5[19].

Abnormalities in β-catenin are observed in about 40% of liver cancers, and several genes, including *c-myc*[47], *CCND1*[48], *MDR1*[49], *WISP1*[50], *L1*[51], *GPR49*[52], and *DKK1*[53], have been identified as downstream targets in the Wnt signaling pathway, which may be important for understanding the role of Wnt signaling in carcinogenesis because these genes are involved in cell proliferation, differentiation, and migration. As new downstream target genes of the Wnt signaling pathway, we observed up-regulation of *SP-5*[44] in liver cancer with Wnt signaling abnormality and showed that this molecule is a direct target for β-catenin/TCF4 complex. Especially, transfer of *SP-5* into MCF-7 cells, in which *SP-5* protein is not detectable, resulted in significant growth promotion, and we, therefore, concluded that *SP-5* plays a pivotal role in the progression of liver cancer.

FROM GENERAL CLUSTERING TO PREDICTION OF PROGNOSIS AND TREATMENT EFFECT BASED ON GENE EXPRESSION PROFILING

Most cases of HCC originate from chronic liver disease caused by hepatitis viral infection, including hepatitis B virus (HBV) and hepatitis C virus (HCV), exposure to aflatoxin B1 in mold, and alcohol abuse. After a long period of inflammation of the liver, early-stage HCC, which is small with indistinct margins and consists of well-differentiated cancerous tissues, may occur and develop to less well-differentiated HCC. Through the progression of liver cancer with dedifferentiation, cancer cells may metastasize to the lungs, adrenal glands, bone, and other segments of the liver parenchyma. Many researchers have attempted to determine the associations between gene expression profiles and such clinicopathological features.

Viral hepatitis is one of the most important epidemiological factors, and Okabe *et al*[17] classified 20 HCC specimens into HB- and HC-based liver cancers by two-way clustering after data mining and selected genes that were differentially expressed between the two viral-based HCC, and this was followed by studies by other groups[18]. Takemoto *et al*[54] reported that sex affects not only the incidence of HCC, but also the outcome after treatment, and performed clustering analysis based on gender. After chronic infection forces hepatocytes to regenerate with consequently excessive replication, regenerative nodules appear as precancerous lesions. Genes responsible for hepatocarcinogenesis may be identified through comparison of expression profiles between regenerative nodules and early liver cancer[55-57]. On the other hand, we and other groups have highlighted HCC with nodule-in-nodule appearance to investigate gene alterations in the course of liver cancer progression[58,59].

Intrahepatic recurrence is one of the main causes of poor prognosis in HCC, and Iizuka *et al*[21] reported a gene set consisting of 12 genes that can predict intrahepatic recurrence within 1 year after curative surgery using a supervised learning method to construct a predictive system. On the other hand, Cheung *et al*[60] identified 90 genes that are correlated with intrahepatic metastasis and may provide clues to identify patients with increased risk of developing metastasis.

Patients with advanced HCC with tumor thrombi in the major portal branches are no longer candidates for surgical

resection, and combination chemotherapy with intra-arterial 5-fluorouracil (5-FU) and subcutaneous interferon-alpha (IFN-α) is one of a few effective chemotherapeutic regimens for such advanced HCC. Kurokawa *et al*[22] selected 63 genes capable of predicting chemotherapeutic responses to 5-FU and IFN-α combination therapy using PCR-based array, which will lead to tailor-made medicine for advanced HCC.

KARYOTYPING ANALYSIS OF HEPATOCELLULAR CARCINOMA USING ARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION AND SINGLE NUCLEOTIDE POLYMORPHISM ARRAY

In addition to expression profiling analysis, genome dosage analysis using aCGH for HCC has been reported[26,27]. Takeo *et al*[61] analyzed 20 HCC samples by genomic DNA microarray analysis using an array containing 57 oncogene spots and emphasized the utility of microarray technology compared to conventional CGH. Katoh *et al*[62] investigated the significance of correlations of frequent chromosomal aberrations with various clinicopathological features, and demonstrated that chromosomal loss on 17p13.3 and gain on 8q11 were independent prognostic indicators by multivariate analysis. Furthermore, Patil *et al*[63] correlated gene expression with aCGH data and identified high-level expression of *JAB*1 on 8q, which was shown to have a potential role in the development of HCC by functional analysis.

GIM is a novel algorithm for detecting copy number changes at both gene and allele levels using SNP arrays[28]. We applied GIM to 36 HCC samples, and analyzed copy number alterations and allelic imbalance accurately in the liver cancer genome in a single experiment[64]. That is, in addition to the gains of 1q, 5p, 5q, 6p, 7q, 8q, 17q, and 20q, and LOH of 1p, 4q, 6q, 8p, 10q, 13q, 16p, 16q, and 17p, which were significantly associated with HCC, we identified UPD and UPT on 13 regions, suggesting that genome dosage analysis misses many LOH regions with normal copy number. For example, on 6q24-25, which contains imprinting gene clusters and UPD regions in our data, we observed reduced levels of *PLAGL*1 expression due to loss of the unmethylated allele. Thus, high-resolution GIM analysis can accurately determine the localizations of genomic regions with allelic imbalance, and when integrated with epigenetic information, a mechanistic basis for inactivation of a tumor suppressor gene in HCC was elucidated.

SYSTEMATIC INTEGRATION OF EXPRESSION PROFILES WITH OTHER TYPES OF GENE INFORMATION

As described above, strong correlations between expression profiles and various HCC classifications, including hepatitis viral infection, tumor differentiation grade, and prognosis, have been reported. The next era of gene expression analysis will involve systematic integration of expression profiles and other types of gene information. Patil *et al*[24] analyzed gene ontology categories for the 703 over-expressed genes selected by microarray analysis and concluded that metabolism, cell cycle, growth, and proliferation may be involved in HCC development.

We have integrated gene expression data and gene locus information, and the regions in which the numbers of up-regulated and down-regulated genes were significantly concentrated were mapped on the chromosomal region[65]. This method for detection of regions of mRNA expression imbalance is called Expression Imbalance Map (EIM), and we applied EIM analysis to gene expression data from 31 HCC tissues[23]. Our data revealed that expression gains of 1q21-23, 8q13-21, 12q23-24, 17q12-21, 17q25, and 20q11 and losses of 4q13, 8p12-21, 13q14, and 17p13 were significantly associated with HCC, which is consistent with previous reports using CGH in liver cancer. Furthermore, more poorly differentiated liver cancer contains more chromosomal alterations, which are accumulated in a stepwise manner in the course of HCC progression. Taken together, we demonstrated that gene expression profiles are subject to chromosomal bias in EIM analysis[23].

If not only gene expression but also cytogenetic data can be obtained from the same sample, integration of expression profile with chromosomal loci will enable comparison of gene expression with gene dosage. Furge *et al*[66] obtained regional expression biases (REBs) from a multiple span moving binomial test and demonstrated that REBs overlapped genetic abnormalities identified using aCGH in HCC. Comparing the expression intensity with the genome dosage obtained from GIM directly, we also confirmed that alterations in mRNA expression level reflect gain or loss of genomic copy number, and substantiated our assertion in the previous report using EIM[23,64].

CONCLUSIONS

Candidate genes for gene therapy and diagnostic markers are selected through microarray analysis and shown to be available for clinical application. In addition, clustering analysis based on clinicopathological features is performed. However, bioinformatics technology indicates that gene expression profile is subject to chromosomal bias, *i.e.*, clustering analysis involves the risk of being affected by gene structural abnormalities, such as genomic gains and losses.

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TOPIC HIGHLIGHT

Wei Tang, MD, *Series Editor*

Spleen-preserving distal pancreatectomy with conservation of the splenic artery and vein

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Abstract

Preservation of the spleen at distal pancreatectomy has recently attracted considerable attention. Since our first successful trial, spleen-preserving distal pancreatectomy with conservation of the splenic artery and vein for tumors of the pancreas and chronic pancreatitis has been performed more frequently. The technique for spleen-preserving distal pancreatectomy with conservation of the splenic artery and vein are outlined. The splenic vein is identified behind the pancreas and within the thin connective tissue membrane. The connective tissue membrane is cut longitudinally above the splenic vein. An important issue is to remove the splenic vein from the body of the pancreas toward the spleen, since a different approach may be very difficult. The pancreas is preferably removed from the splenic artery toward the head of the pancreas itself. This procedure is much easier than removing the pancreas from the vein side. One patient had undergone distal gastrectomy for duodenal ulcer, with reconstruction by Billroth Ⅱ tehcnique. If distal pancreatectomy with splenectomy had been performed for the lesion of the distal pancreas at the time, the residual stomach would also have to be resected. The potential damage done to the patient by reconstruction of the gastrointestinal tract in combination with distal pancreatectomy and splenectomy would have been much greater than with distal pancreatectomy only with preservation of the spleen and residual stomach. Benign lesions as well as low-grade malignancy of the body and tail of the pancreas may be a possible indication for this procedure.

INTRODUCTION

For tumors or low-grade malignancies of the body and tail of the pancreas, or for chronic pancreatitis, spleen-preserving distal pancreatectomy with conservation of the splenic artery and vein is performed[1]. This method is already being used worldwide for the treatment of chronic pancreatitis[2,3]. We were the first in the world to perform this procedure for chronic pancreatitis (Figure 1)[1,4]. The patient was a 22-year-old man with familial chronic pancreatitis. Because the patient was very young and had many protein plugs in the dilated ductule of the tail of the pancreas, we decided to perform a Puestow's procedure with removal of the tail of the pancreas. The postoperative course was uneventful. The splenic function of this young man has been preserved in the all of his life.

The relevance of preserving the spleen has been shown in Europe, the United States and Japan. Splenic loss causes either changes in the peripheral blood count, infection or sepsis[5-7]. Some authors have also stressed the potential immuno suppression related to splenectomy[8].

SURGICAL ANATOMY

In order to perform the procedure, adequate anatomical knowledge of the pancreas and supplying vessels, especially the fusion fascia of Toldt and fusion fascia of Treitz, is necessary[9].

Figure 2 displays the fusion fascia of the head of the pancreas in an autopsy case[9]. The fascia is composed of

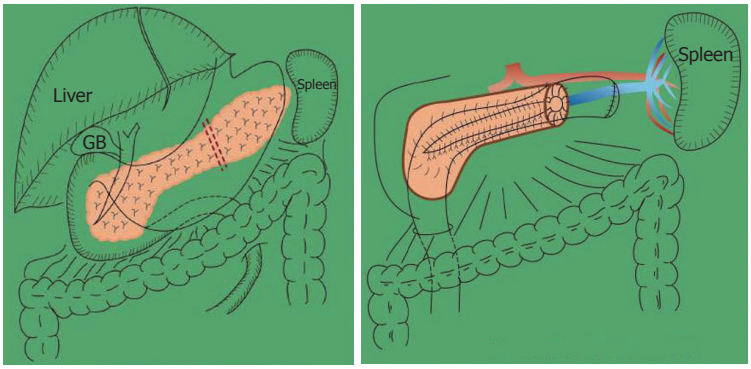

Figure 1 Spleen-preserving distal pancreatectomy with conservation of the splenic artery and vein for chronic pancreatitis. A Puestow's procedure with removal of the tail of the pancreas.

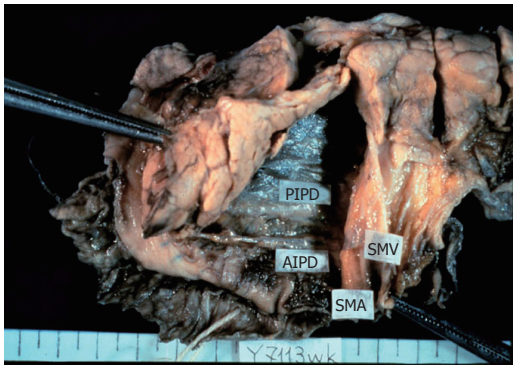

Figure 2 The fusion fascia of the head of the pancreas in an autopsy case.

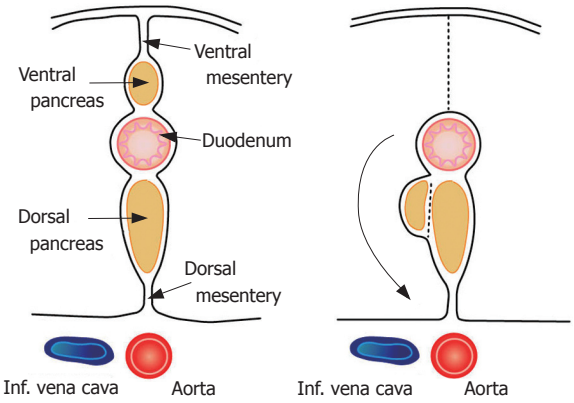

Figure 3 During development, the pancreas is divided into two buds representing the ventral and dorsal anlagen. The ventral anlage of the pancreas then moves around the duodenum until it comes in contact with the dorsal bud.

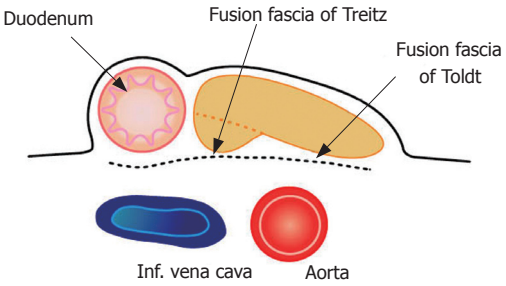

Figure 4 The membrane of the ventral bud and that of the inferior vena cava and abdominal aorta become fused. The fusion fascia of the head of the pancreas is known as the "fusion fascia of Treitz" and that of the body and tail of the pancreas is called the "fusion fascia of Toldt".

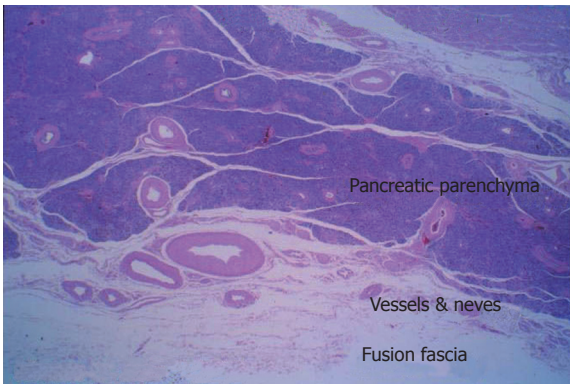

Figure 5 Histological findings of the "fusion fascia" of the pancreas.

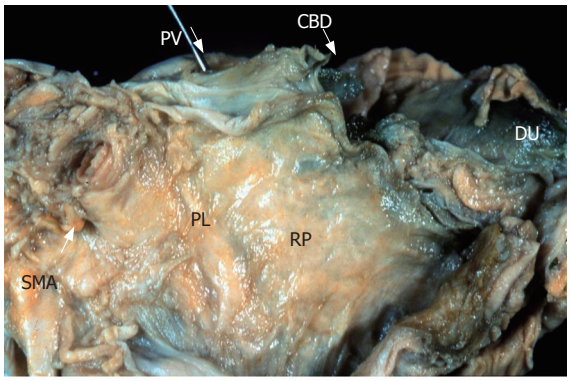

Figure 6 A posterior view of the head of the pancreas in an autopsy case. The posterior surface of the pancreas is covered with fusion fascia of Treitz.

loose connective tissue. By Kocher's maneuver, the fascia adheres to the pancreatic parenchymal side, but not to the vena cava side. All the relevant pancreatico-duodenal arcades of arteries, veins, and nerves are situated on this membrane.

The composition of this membrane has been previously described. During early development, the pancreas is divided into two buds representing the ventral and dorsal anlagen. The ventral anlage of the pancreas then moves around the duodenum until it comes in contact with the dorsal bud (Figure 3).

The membrane of the ventral bud and that of the inferior vena cava and abdominal aorta become fused, and this is reflected by the term "fusion fascia" (Figure 4). The fusion fascia of the head of the pancreas is known as the "fusion fascia of Treitz", while the one of the body and tail of the pancreas is called the "fusion fascia of Toldt".

Histological findings of the "fusion fascia" of the pancreas are shown in the Figure 5. The fusion fascia is composed of loose connective tissue. While it appears to be fragile to the naked eye during an operation, it is actually quite resistant. A large number of arteries,

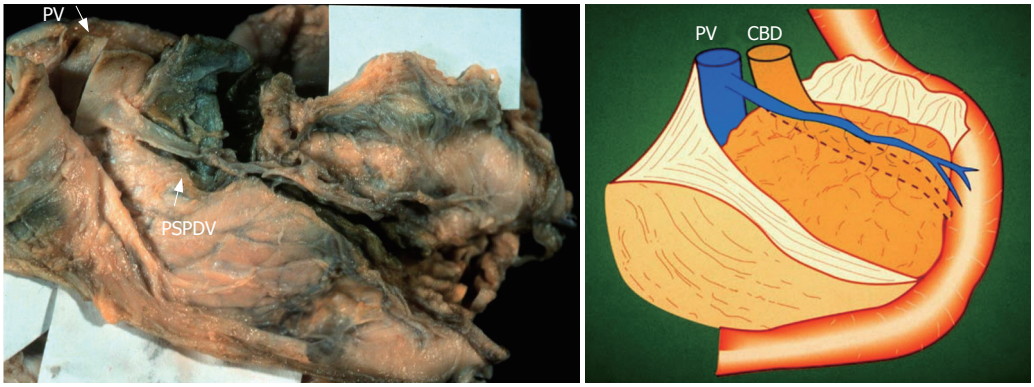

Figure 7 When the fusion fascia of Treitz is ablated from the parenchyma of the pancreas, an important pancreatoduodenal vessel, for example the PSPDV, is revealed. A posterior view of the head of the pancreas after ablation of the fusion fascia of Treitz.

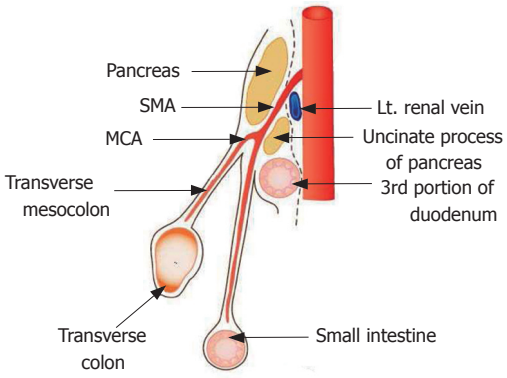

Figure 8 Sagittal section through the neck of the pancreas. The fusion fascia is indicated by the dotted line.

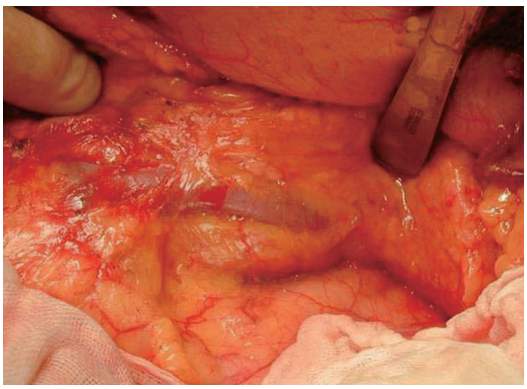

Figure 9 When the pancreas was ablated from the retroperitoneum, the pancreas and splenic vein are covered with the fusion fascia of Toldt.

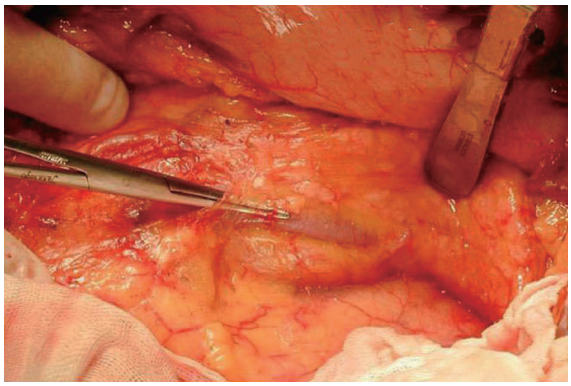

Figure 10 The fusion fascia of Toldt is longitudinally cut above the splenic vein.

arterioles, veins and nerves exist between the fascia and pancreatic parenchyma.

Figure 6 displays shows a posterior view of the head of the pancreas in an autopsy case. This is the surface that is ablated by Kocher mobilization. The posterior surface of the pancreas is covered with fusion fascia of Treitz.

The cut end of the portal vein and the superior mesenteric artery shown at the top of the figure are also covered with fusion fascia and exist on the abdominal side.

Thus, the extrapancreatic nerve plexuses and the portal vein, superior mesenteric artery and parenchyma of the pancreatic head are situated in the same area, and are surrounded by the fusion fascia of Treitz[10].

A similar anatomical configuration is displayed in the body and tail of the pancreas. The splenic vein and artery and parenchyma of the pancreas are situated in the same area, and are surrounded by the fusion fascia of Toldt.

When the fusion fascia of Treitz is ablated from the parenchyma of the pancreas, an important pancreatoduodenal vessel, the PSPDV, is revealed as it is shown in Figure 7.

A Sagittal section as it can be seen through the neck of the pancreas is shown in Figure 8. The fusion fascia is indicated by the dotted line. The superior mesenteric artery (SMA) penetrates the fusion fascia, just after its origin from the abdominal aorta.

SURGICAL TECHNIQUES BASED ON THE ANATOMY

When the pancreas is ablated from the retro-peritoneum, the pancreas and splenic vein are covered with the fusion fascia of Toldt (Figure 9). In the first step of this operation, the fascia of Toldt is cut to reveal the splenic vein (Figure 10). X-ray ductography of the resected specimen of IPMN in the body of the pancreas is shown in the lower part of the Figure 10.

The branches of the splenic vein should be gently ligated and divided (Figure 11). An important technique is to remove the splenic vein from the body of the pancreas toward the spleen. It is very difficult to remove the splenic vein in the other direction because (1) it is difficult to discriminate the distal end of the pancreas from the fatty

Table 1 The spleen preserving procedure for benign lesions and IPMN in eighteen cases

Case No.	Age, Gendar	Diagnosis	Location	Size (cm)	Pathological findings
1	54, F	Endocrine tumor	Body	1.0	Glucagonoma
2	51, F	Cystic tumor	Body	1.2	Mucinous cystadenoma
3	54, F	Insulinoma	Tail	1.5	Insulinoma
4	54, F	Cystic tumor	Tail	2.0	Serous cystadenoma
5	22, M	Chr. pancreatitis	Total	–	Chronic pancreatitis
6	26, M	Cystic tumor	Tail	2.0	Edidermoid cyst
7	42, M	Cystic tumor	Tail	3.0	Edidermoid cyst
8	66, M	IPMN	Body	3.0	Pseudocyst, dysplasia
9	73, M	IPMN	Body & tail	3.0	In situ carcinoma
10	54, M	IPMN	Tail	3.0	Hyperplasia and adenoma
11	78, M	IPMN	Body & tail	7.5 × 5.5	Benign-Borderline
12	79, M	IPMN	Body & tail	2.7	Adenoma
13	70, M	IPMN	Body	2.5 × 1.8	*In situ* carcinoma
14	46, F	IPMN	Body	2.5	*In situ* carcinoma
15	60, F	IPMN	Body & tail	2.5	*In situ* carcinoma
16	57, M	IPMN	Body	3.5	Adenoma
17	61, M	IPMN	Body	4.0	Adenoma
18	70, M	Endocrine tumor	Tail	0.5	Endocrine tumor

Mean age 56.5; M:F = 12:6; IPMN 9; Cystic tumor 4, Endocrine tumor 3, Chr. pancreatitis 2.

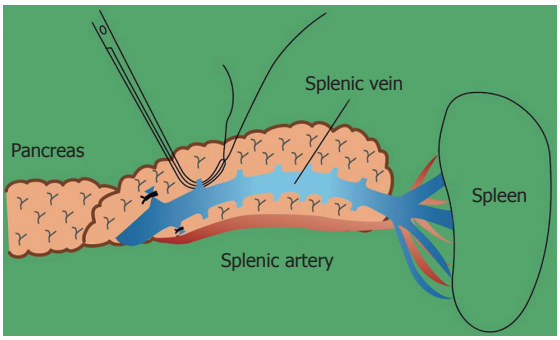

Figure 11 The branches from the splenic vein on both sides should be carefully ligated and divided.

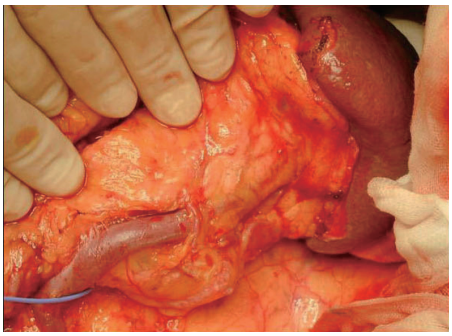

Figure 12 The splenic vein in the body of the pancreas is removed from the pancreatic body.

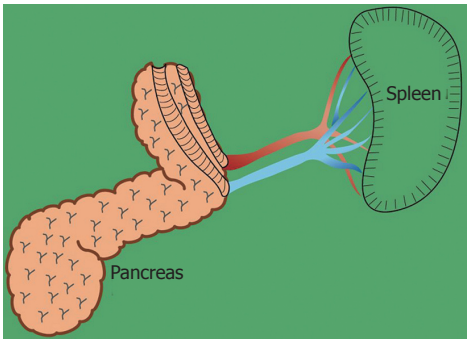

Figure 13 The splenic artery is removed from the spleen toward the head of the pancreas. Then, the distal pancreas can be removed.

tissue in the hilum of the spleen, and (2) in this area the splenic artery and vein are already divided into small vessels that can be easily injured.

The splenic vein in the body of the pancreas is removed from the pancreatic body (Figure 12).

The splenic artery is removed from the spleen toward the head of the pancreas (Figure 13). Then, the distal pancreas can be confidently removed.

Wesafely performed this procedure for benign lesions and Intraductal papillary-mucinous neoplasm (IPMN) in eighteen cases (Table 1). This procedure was also performed for IPMN in 10 cases in the last five years[11,12]. All of the operation were successfully performed. No procedure related or disease recurrence casualties were reported.

The mostly feared potential complication was postoperative bleeding from the splenic vein caused by digestion of the ablated wall of the vein by pancreatic juice originating from the end of the pancreas[1]. Another feared complication was tortion of the splenic vessels. However, these complications did not arise in any case.

IMPORTANT AND INTERESTING CASES

Case No. 1

A 79 year-old patient with simultaneous gastric carcinoma and IPMN in the body and tail of the pancreas:

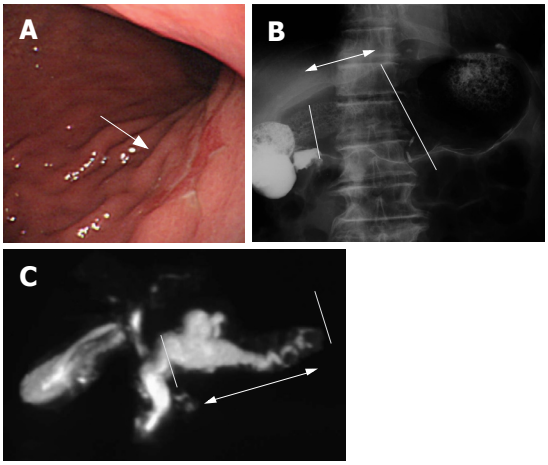

Figure 14 Case No.1: A 79 yr-old patient with simultaneous gastric carcinoma and IPMN in the body and tail of the pancreas. Endoscopic examination and MRCP. **A**: Stomach: Early carcinoma; **B**: Pylorus-preservingdistal gastrectomy; **C**: Spleen-preserving distal pancreatectomy.

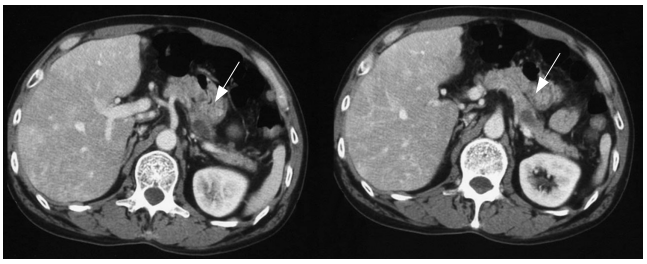

Figure 16 Case No. 2: A 54-yr-old patient with a diagnosis of IPMN in the body of the pancreas. CT scan. The cystic dilatation of the branch duct with protrusive lesions in the body of the pancreas. The main pancreatic duct is not dilated.

Endoscopic examination showed early gastric carcinoma of the body of the stomach (Figure 14). MRCP revealed the extremely dilated main pancreatic duct, as well as cystic dilatation of the branch duct in the body of the pancreas. In combination with other findings, we made a diagnosis of main and branch duct IPMN .

At surgery , the pancreas was not hard and tumor did not seem to invade the parenchyma of the pancreas by both inspection and (Figure 15) intraoperative ultrasonography. No apparent lymphnode metastasis around the pancreas was detected. MRI did not show any malignant findings preoperatively. Therefore, we decided to do pylorus-preserving distal gastrectomy and spleen preserving distal pancreatectomy. Re-construction of stomach was done by end-to-end gastro-gastrostomy.

Figure 15 show the intra-operative view of the pancreas, and egg-like appearance of the main pancreatic duct in the tail of the pancreas by intra-operative pancreatoscope in this case. Histological investigation revealed carcinoma *in situ* in the main pancreatic duct in the body, and adenoma in the branch duct of the body of the pancreas.

The middle part of the stomach was segmentaly resected and distal pancreas was also resected with conservation of the splenic artery and vein and the spleen. The gastro-gastro-anastomosis was done after this

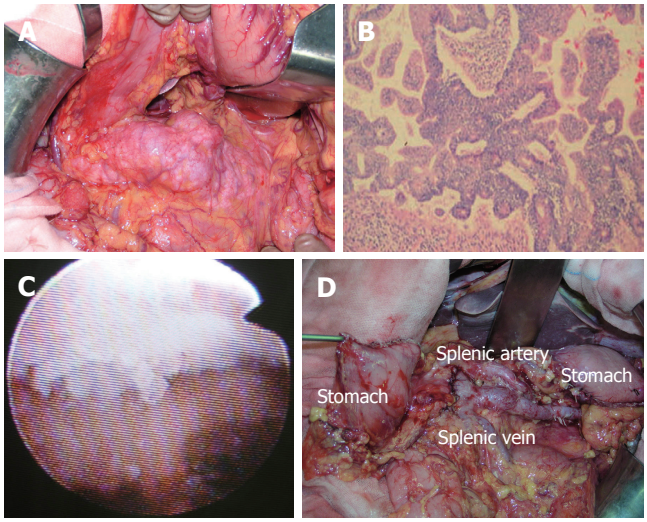

Figure 15 Case No.1 The operative and histological findings, and egg like appearance by intraoperative pancreatoscopy. **A**: IPMN of the pancreas; **B**: Body: Carcinoma, noninvasive; **C**: Body: Egg like appearance; **D**: After stomach resection and spleen-preserving DP.

operation. This patient is still alive with no recurrence of carcinoma in the last three years.

If distal pancreatectomy with splenectomy had been performed for the lesions of the distal pancreas at this operation, the residual stomach should also have been resected because of no blood supply from the short gastric arteries originated from the splenic artery. That is, total gastrectomy with esophago-jejunostomy should have been performed.

However, total gastrectomy would have been excess surgery for early gastric carcinoma of this patient. In addition, the damage done to the patient by reconstruction of the gastrointestinal tract in combination with distal pancreatectomy and splenectomy would have been much greater than with only distal pancreatectomy with preservation of the spleen and residual stomach with end to end anastomosis.

Case No. 2

A 54-year-old patient with a diagnosis of IPMN in the body of the pancreas (13): CT scan revealed two cystic lesions in the body of the pancreas (Figure 16). Endoscopic examination revealed the slight dilatation of the papilla of Vater. Endoscopic ultrasound revealed the cystic dilatation of the branch duct with protrusive lesions in the body of the pancreas (Figure 17). In combination with other findings, we made a diagnosis of branch type of IPMN in this case.

Thirty-two years before the operation, the patient had undergone distal gastrectomy for duodenal ulcer, with reconstruction by the Billroth Ⅱ method. If distal pancreatectomy with splenectomy had been performed for the lesion of the distal pancreas at this operation, the residual stomach should also have been resected because of no blood supply from the short gastric arteries originated from the splenic artery. Furthermore, the adhesion on the left side of the intraabdominal cavity should have been ablated for use of the jejunum for

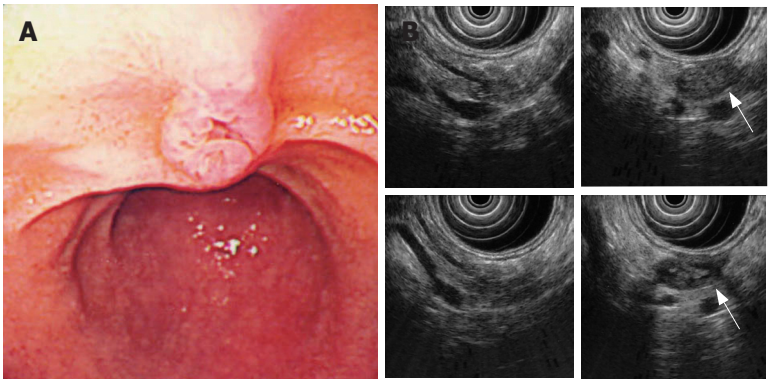

Figure 17 Endoscopic examination revealed the slight dilatation of the papilla of Vater. Endoscopic ultrasound revealed the cystic dilatation of the branch duct with protrusive lesions in the body of the pancreas. **A**: Slight dilatation of the orifice of the papilla of Vater; **B**: The cystic dilatation of the branch duct with protrusive lesions in the body of the pancreas. The main pancreatic duct is not dilated.

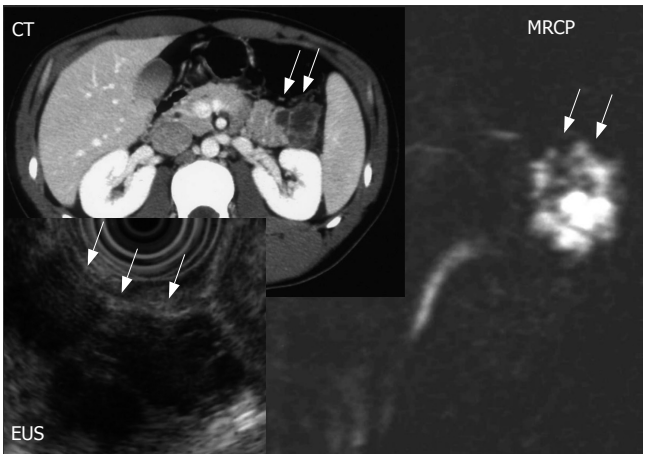

Figure 18 Case No. 3. A 25 yr old male. The imaging procedures such as abdominal CT scan, EUS and MRCP suggested a branch type of IPMN.

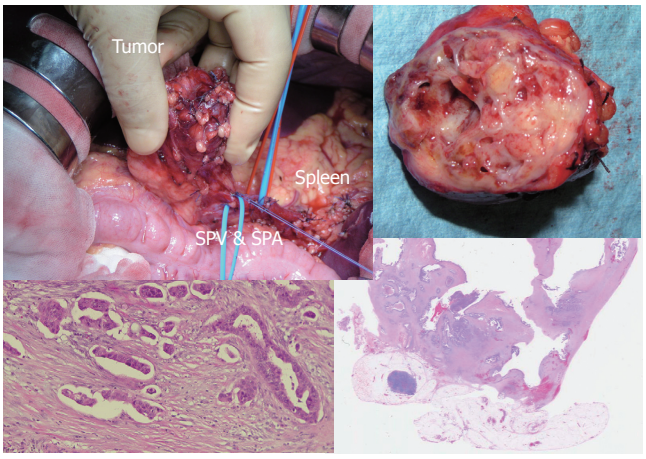

Figure 19 The operative and histological findings, and cut surface of the tumor.

esophago-jejunostomy. The damage done to the patient by reconstruction of the gastrointestinal tract in combination with distal pancreatectomy and splenectomy would have been much greater than with only distal pancreatectomy with preservation of the spleen and residual stomach.

In this case, although the preoperative diagnosis of the cystic lesion was a branch-type IPMN, this was histologically revealed to be a pseudocyst. Dysplasia was found in the branch duct epithelium of the tail of the pancreas.

Even with advances in imaging techniques, diagnosis of a cystic lesion of the pancreas is still very difficult. Ordinary distal pancreatectomy with splenectomy would have been over surgery in this case, and this should be avoided and could be avoided by the spleen preserving method.

Case No. 3

A 25 year old male: The imaging procedures such as abdominal CT scan, EUS and MRCP suggested a branch type of IPMN (Figure 18). The presence of invasion was unclear both preoperatively and intraoperatively. The patient was young that we decided to perform spleen preserving distal pancreatectomy.

Figure 19 top-left shows the distal part of the pancreas with tumors. The spleen, splenic artery and vein were preserved. However, intraoperative histologic examination revealed that the tumor was malignant with invasive parts. Therefore, we converted the procedure to the distal pancreatectomy with splenectomy in combination with radical lymphnode dissection. This is a very rare case of carcinoma derived from IPMN, developed in a very young man.

In conclusion, spleen-preserving distal pancreastectomy with preservation of the splenic vessels can be performed easily and safely. Our experience suggests that this procedure should be conducted for IPMN of the distal pancreas as well as chronic pancreatitis.

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TOPIC HIGHLIGHT

Wei Tang, MD, *Series Editor*

Multi-disciplinary treatment for cholangiocellular carcinoma

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Abstract

Cholangiocarcinoma (CC) is rare malignant tumors composed of cells that resemble those of the biliary tract. It is notoriously difficult to diagnose, and is associated with a high mortality. Traditionally, CC is divided into intrahepatic and extraheaptic disease according to its location within the biliary tree. Intrahepatic cholangiocellular carcinoma (IH-CCC) or peripheral cholangiocellular carcinoma (CCC) appears within the second bifurcation of hepatic bile duct, and is the second most common primary liver cancer following hepatocellular carcinoma (HCC), IH-CCC or peripheral CCC often presents with advanced clinical features, and the cause for this cancer rise is still unclear. MRI, CT and PET provide useful diagnostic information in those patients. Surgical resection is the only chance for cure, with results depending on selected patients and careful surgical technique. Liver transplantation could offer long-term survival in selected patients when combined with chemotherapy. Chemotherapy, radiation therapy or combination therapies remain as the only treatment for inoperable patients. However, these are uniformly ineffective in patients' survival.

Key words: Cholangiocellular carcinoma; Surgical resection; Liver transplantation; Chemotherapy; Radiation

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INTRODUCTION

Cholangiocellular carcinoma (CCC) is the primary cancer of the bile duct and is a frequent tumor of the biliary tree with estimated incidence of 0.01%-0.8 %[1,2]. Histologically, most CCCs are well differentiated adenocarcinomas, and classification based on location classically has been applied[3,4]. Intrahepatic CCC (IH-CCC) or peripheral CCC appears within the second bifurcation of hepatic bile duct, and is the second most common primary liver cancer following hepatocellular carcinoma (HCC). IH-CCC or peripheral CCC often presents with advanced clinical features, and the cause for this cancer is still unclear. The Liver Cancer Study Group of Japan has proposed a classification based on macroscopic features; CCC can be described as mass-forming, periductal infiltrating, intraductal growth, or as a mixed mass-forming, and periductal[5,6]. Tumors located in the left and right hepatic ducts and hilar cholangiocarcinoma were sometime classified as intrahepatic cholangiocarcinoma because these tumors can be very similar in condition to the periductal infiltrating type of CCC. However, hilar chlangiocarcinoma was eliminated from IH-CCC category. Periductal infiltrating type of IH-CCC and hilar cholangiocarcinoma demonstrate similar condition clinically. However, each substance has different epidemiological and pathological findings. Preoperative imagings, such as MRI, MRCP, CT, and FDG-PET, provide useful diagnostic informations in those patients.

Although surgical resection is the only chance for cure with result depending on exceptional surgical technique and patient selection, many patients, at the time of diagnosis, have IH-CCCs that are unresectable because of associated advanced liver disease or lesion involving the portal structure or main hepatic artery[7-9]. For these patients, liver transplantation provides the opportunity for better survival[10-14]. In case of inoperable patients, chemotherapy, radiation therapy or combination therapies remain as the only effective treatment[15,16].

EPIDEMIOLOGY

Worldwide, cholangiocarcinoma (IH-CCC and extra-hepatic cholangiocarcinoma) accounts for 3% of all gastrointestinal cancer and is the second most common primary malignancy[17]. Especially, IH-CCC accounts for approximately 10% to 20% of all primary liver cancer[18]. The incidence rates of IH-CCC are extensive, varying among different parts of the world. The incidence of IH-CCC appears to be higher among men in different areas of the world. The incidence ratios of male to female vary between 1.3 among white Americans to 3.3 among the French. In general, male preponderance is less pronounced

in IH-CCC than in HCC. In Japan, IH-CCC accounts for about 5%-10% of all primary liver cancer[5], prevalence is slightly higher compared to that of Western countries and the United States. Furthermore, in most countries, the percentage of increase in IH-CCC mortality is higher than that of HCC. The 5-year patient survival of IH-CCC is still very low, and nearly unchanged over past 20 years.

RISK FACTORS

Several risk factors have been associated with the development of IH-CCC; however, the cause is still unknown for most IH-CCC cases. The association between IH-CCC and chronic biliary tract inflammation, such as primary sclerosing cholangitis (PSC), liver fluke infestation, or hepatolithiasis, is well recognized. Especially, PSC is a definite risk factor for IH-CCC[19,20], and the risk for developing IH-CCC after the diagnosis of cholestatic liver disease is 1.5% per year[21]. Approximately 30% of the patients with PSC who are likely to develop IH-CCC will be diagnosed with malignancy of the bile duct within two years after diagnosis of the PSC[20,21]. Bile stenosis and recurrence of biliary inflammation might predispose individuals with these conditions to cancer.

TREATMENT

Treatment options for IH-CCC are determined by the local extent of the cancer, vascular invasion, presence or absence of metastasis, basic liver function, and available local expertise.

Surgical resection

Careful preoperative staging is required to determine the applicability of surgery with curative margin. Several factors influence the potency of surgery, and need to be carefully considered. These include the location and the extent of the tumor, and the patient's conditions, such as liver cirrhosis, viral infection of the liver, cardiopulmonary diseases, jaundice, biliary tract infection, and ascites. Other factors, such as the patient's performance status and nutritional condition, also require careful consideration. Routine imagings, such as ultrasonography, abdominal and chest computed tomography, and cholangiography [either magnetic resonance cholangio-pancreatogram (MRCP), percutaneous transhepatic cholangiography (PTC) or endoscopic retrograde cholangiogram (ERC)], are useful for evaluating metastasis, tumor location, and extent of tumor. Angiography or 3-dimensional vascular imagings are helpful for evaluating vascular invasion.

Although surgical complete resection remains the only curative treatment strategy for IH-CCC, most patients present with advanced disease and cannot be applicable for surgical management. The selection of performable curative surgical resection depends on location of the tumors. Surgery for IH-CCC is similar to that of other liver malignancies, and includes hepatic lobectomy, segementectomy or subsegmentectomy with or without resection of the common bile duct. For hilar lesions, preoperative evaluation of tumor location and involvement of intra- or extra-hepatic biliary tree are extremely important for obtaining tumor-free margin. If the patient requires extended hepatic resection, preoperative portal vein embolization (PE) is effective for inducing lobar hypertrophy, and decreasing risk of operative modality and mortality, such as remnant liver failure[22]. In case with involvement of distal common bile duct, pancreato-duodenectomy is performed additionally to obtain negative margin.

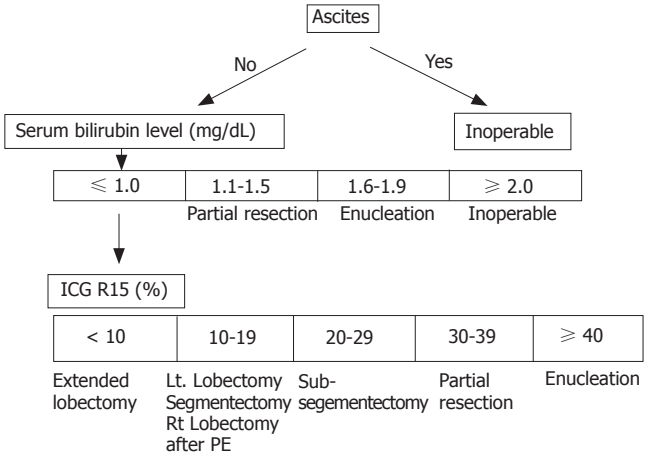
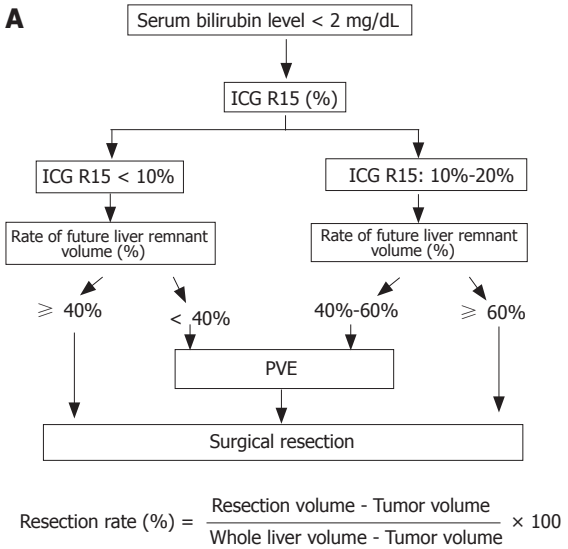


Figure 1 Makuuchi craiteria.

Some centers determined surgical resection and procedure according to Makuuchi criteria (Figure 1)[23]. If patients need extended hepatic resection, surgical strategy is indicated for patients with the total bilirubin level < 2 mg/dL prior to surgery. Indocyanine green (ICG) retention rate at 15 min is useful for evaluating liver function and determining surgical intervention. PE is employed for patients with normal liver function (ICG 15 ≤ 10%) when the future remnant liver volume is estimated to be less than 40% of the calculated total liver volume. For patients with mild liver dysfunction (10% < ICG 15 < 20%), PE is indicated when the remnant liver volume is estimated to be 40%-60% of the total liver volume (Figure 2A). In jaundiced patients, the intervention was performed after the serum total bilirubin level had decreased to less than 5 mg/dL. After total bilirubin level was decreased to less than 2 mg/dL, we calculated the patient's ICG level, and surgery was performed (Figure 2B)[24-26].

In recent years, the outcomes after surgical resection have improved compared with the past results, though further advancements are necessary. Curative resection is associated with 5-year survival rate of up to 22%-36% for IH-CCC[27]. These low survival rates are considered to be due to the difficulty in identifying the extent of disease intra-operatively. Tumor-free surgical margin is the best predictor of patient survival. Bilobar distribution, lymph node involvement, vascular invasion and distant metastases were found to be poor predictors of survival rate of IH-CCC patients. In periductal infiltration type of IH-CCC, curative resection with lymph node dissection improved survival in patients with no more than two positive lymph nodes[28]. Shimada *et al*[29] reported that lymph node dissection did not appear to improve patient survival.



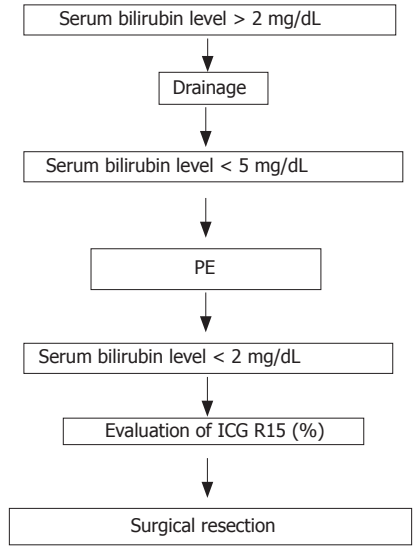


Figure 2 Surgical management of IH-CCC.

Aggressive surgical approach with adjuvant chemotherapy may be effective in obtaining better patient survival rate. However, neoadjuvant chemotherapy with several modalities, including radiation, failed to demonstrate clear benefit[30].

Transplantation

The initial experience with liver transplantation (LT) for IH-CCC was unsatisfactory. Recurrence of IH-CCC was common and 5-year patient survival rate was only 5%-15%[12-14]. Most liver transplant centers consider IH-CCC as a contra-indication for LT[11-14]. Therefore, it is interesting to report that a selected group of patients who underwent LT and had negative surgical margin and no lymph nodes metastasis had long-term survival[14]. Prolonged disease-free survival was reported following LT for IH-CCC that combined preoperative radiation therapy and chemotherapy, and pre-transplantation exploratory laparotomy. These data support liver transplantation as an option for carefully selected patients with unresectable IH-CCC, but this should be offered only in the context of the clinical trial. It is also worth noting that these data have been originated from a single center with specialized interest in this disease; thus, general application of this experience remains to be validated.

Adjuvant therapy

Adjuvant chemotherapy or radiation therapy has been discouraging, and has not been convincingly shown to prolong survival and reduce tumor recurrence. It is worth noting that most studies of adjuvant therapy for IH-CCC patients have been small retrospective studies, and these are insufficient to reach statistically significant conclusions. Furthermore, the efficacy of adjuvant chemotherapy alone remains controversial, as it has been associated with improved survival in some studies, whereas no benefit in others[13,31,32].

Chemotherapy

The role of systemic chemotherapy in the unresectable IH-CCC is undefined. While no single agent or combination therapy has achieved significant response rates, most promising approaches involve the use of single agent gemcitabine (GEM). Combination regimens of GEM with agents, such as 5-FU, docetaxel, oxaliplatin, cisplatin, and capecitabine, have been limited by toxicity without good effective response for unresectable IH-CCC patients[33]. The median survivals of 9.30 (range: 6.43-12.17) mo, 14 mo, and 11 mo were obtained by using the combination chemotherapy of gemcitabine with cisplatin[34], gemcitabine with capecitabine[35], and gemcitabine with docetaxel[36], respectively.

Radiation therapy

External beam radiation therapy and chemotherapy have been administrated as adjuvant treatment to surgical resection. In the cases of complete resection, radiation did not improve survival[16]. Palliative radiation therapy might be suitable for patients with unresectable periductal infiltrating or intraductal growing types of CCC, with locally advanced lesion without distant metastasis. Palliative radiation therapy contributes to biliary decompression, and is pain-free. However, outcome of radiation therapy was also poor, with the patients' survival of 3-6 mo, and without proven survival benefit over surgical resection[37].

OUR EXPERIENCE

From April 2000 to April 2006, a total of 756 cases with primary liver malignancies were treated at our department, among them 23 cases had IH-CCC. Data were collected retrospectively from all patient records and our database. When patients were not considered as candidates for surgical resection, chemotherapy or chemo-radiation therapies were indicated. The protocol administered consisted of GEM 400 mg/m^2 with radiation therapy dose of 50 Gy, or GEM 800 mg/m^2 alone. Patient follow-up was until the end of June 2006.

Statistical analysis

Overall survival rate was calculated using Kaplan-Meier method. The log-rank test was used to compare survival. *P*

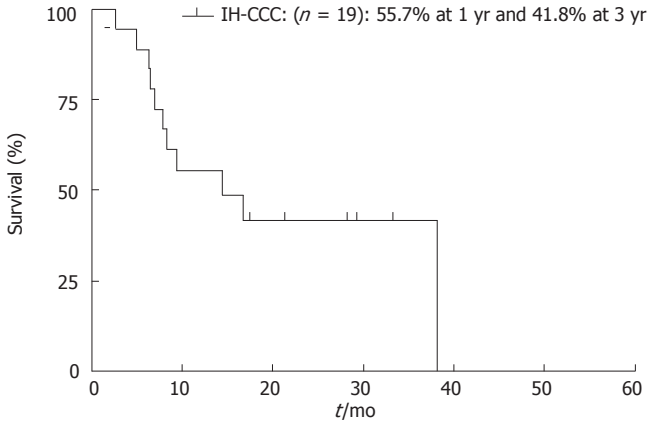


Figure 3 Actuarial patients survival calculated by using Kaplan-Meier methods at 1, 3 and 5 years after surgery for IH-CCC.

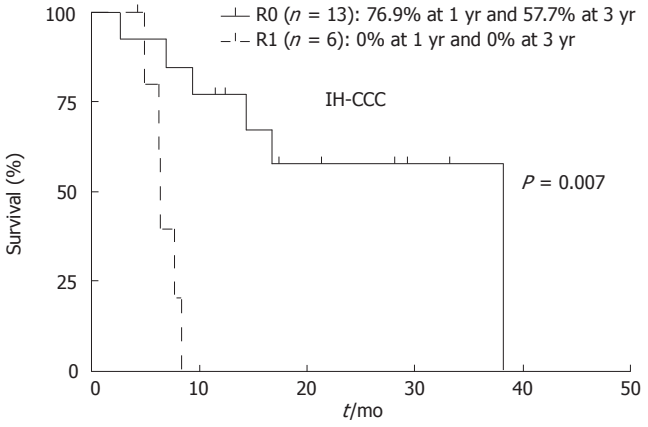


Figure 4 Actuarial patients survival calculated by using Kaplan-Meier methods at 1, 3 and 5 years after R0 *versus* R1 resection for IH-CCC.

value less than 0.05 was considered statistically significant.

Result

A total of 23 cases were found to have IH-CCC; of them, 19 cases (12 men and 7 women) were resectable, while 4 cases (2 men and 2 women) were unresectable. The mean age of patients was 62.1 ± 10.9 years. Out of 19 resectable cases, 14 had periductal infiltrating or intraductal growth-type tumor. Six cases underwent extended right lobectomy with the common bile duct resection (CBD-R) after PE. Three cases underwent extended left lobectomy with CBD-R, 2 cases underwent left lobectomy and posterior segmentectomy, respectively, and one underwent left trisegmentectomy with CBD-R following PE, and partial resection and extended lateral segementectomy. Eight of 19 patients were still alive at the end of the study. The actuarial patient survival at 1 and 3 years after resection was 55.7% and 41.8%, respectively (Figure 3). Fourteen of 19 patients had recurrence of CCC, most common recurrence site was remnant liver (10 of the 14 patients). Five patients had GEM treatment after recurrence; however, GEM did not have a significant effect on patient survival after recurrence (GEM: 11.4 ± 4.1 mo *vs* non-GEM: 15.3 ± 13.7 mo, $P = 0.56$). Thirteen of 19 (64.2%) cases received R0 resection by histological findings. Patient survival at 1 and 3 years for R0 cases were 76.9% and 57.7%, respectively. R0 resection had significant benefit on patients' survival rate compared to R1 (Figure 4, $P = 0.0007$). GEM was administered to all four unresecteable cases for palliative treatment; however, mean survival time was 3.1 mo after treatment.

In our study, the survival rate for the resected IH-CCC patients was still poor, which is similar with the previous reports. Thus, based on our data, it is considered that the effectiveness of neo-adjuvant chemotherapy and palliative GEM treatment is still unclear.

SUMMARY

In view of increasing incidence of CCC, we need better methods of early detection, as well as new, effective treatment to improve the survival of the patients with this difficult disease. Surgical resection can be potentially curative, but many patients present at an advanced stage when resection might not be feasible. Chemotherapy and radiation are uniformly ineffective in prolonging survival.

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Current surgical treatment for bile duct cancer

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Abstract

Since extrahepatic bile duct cancer is difficult to diagnose and to cure, a safe and radical surgical strategy is needed. In this review, the modes of infiltration and spread of extrahepatic bile duct cancer and surgical strategy are discussed. Extended hemihepatectomy, with or without pancreatoduodenectomy (PD), plus extrahepatic bile duct resection and regional lymphadenectomy has recently been recognized as the standard curative treatment for hilar bile duct cancer. On the other hand, PD is the choice of treatment for middle and distal bile duct cancer. Major hepatectomy concomitant with PD (hepatopancreatoduodenectomy) has been applied to selected patients with widespread tumors. Preoperative biliary drainage (BD) followed by portal vein embolization (PVE) enables major hepatectomy in patients with hilar bile duct cancer without mortality. BD should be performed considering the surgical procedure, especially, in patients with separated intrahepatic bile ducts caused by hilar bile duct cancer. Right or left trisectoriectomy are indicated according to the tumor spread and biliary anatomy. As a result, extended radical resection offers a chance for cure of hilar bile duct cancer with improved resectability, curability, and a 5-year survival rate of 40%. A 5-year survival rate has ranged from 24% to 39% after PD for middle and distal bile duct cancer.

Key words: Extrahepatic bile duct cancer; Cholangiocarcinoma; Biliary drainage; Portal vein embolization; Extended hemihepatectomy; Pancreatoduodenectomy; Hepatopancreatoduodenectomy; Right trisectionectomy; Left trisectoriectomy

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INTRODUCTION

Extrahepatic bile duct cancer is difficult to diagnose in the early stage. Most patients with bile duct cancer have developed jaundice at presentation, and the tumor stage is already advanced. The anatomical relationships between hepatic hilar structures and modes of tumor extension are complicated, and this has resulted in low resectability and curability, high morbidity and mortality, and poor long-term survival rate[1-9]. Because of the advances of clinical imaging, such as magnetic resonance cholangiography (MRC) and helical computed tomography (CT)[10,11], the results of bile duct cancer diagnosis have improved, and the number of candidates for surgical resection has increased.

Radical resection is still the only a chance for cure, and various types of surgical procedure have been applied[1-3,6,12-14]. Extended hemihepatectomy has recently been recognized as the standard curative treatment for hilar bile duct cancer and has acceptable mortality[15-19]. Pancreatoduodenectomy (PD), on the other hand, is the choice of treatment for middle and distal bile duct cancer[20-24], and major hepatectomy concomitant with PD (HPD) has been applied to selected patients with widespread tumors[17,18,25-27]. However, these extensive radical procedures are not always safe, because there are risks of postoperative liver failure and pancreatic leakage. Radical resection with a safe and beneficial strategy is needed in the treatment of bile duct cancer. Proper choice of surgical procedure according to the modes and patterns of tumor infiltration is important for curative resection. Preoperative treatments, including biliary drainage (BD) and portal vein embolization (PVE), are necessary before major hepatectomy. In this article we review the current treatment of extrahepatic bile duct cancer and elucidate safe and beneficial surgical treatments and the surgeon's role in treatment.

CLASSIFICATION OF BILE DUCT CANCER

Cholangiocarcinoma is defined as any tumor arising from the ductal epithelium of the biliary tree and is classified as intrahepatic or extrahepatic according to its location[28,29]. Intrahepatic cholangiocarcinoma is usually treated as a hepatic tumor, because it requires hepatic resection alone.

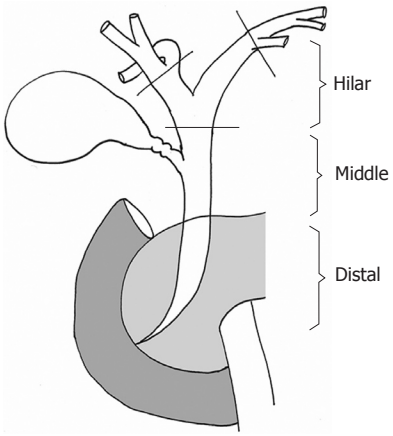

Figure 1 Schema of extrahepatic bile duct.

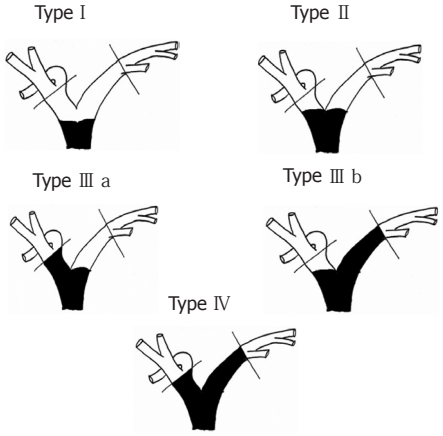

Figure 2 Bismuth-Corlette classification of hilar bile duct cancer[9].

In this article we have focused on the surgical treatment of extrahepatic bile duct cancer, which is classified according to the primary site into hilar, middle, and distal bile duct cancer[30] (Figure 1). The longitudinal extent of bile duct cancers that involve the bifurcation of the hepatic duct is classified according to a modified Bismuth-Corlette classification[2,9] (Figure 2).

MODE OF INFILTRATION AND SPREAD OF BILE DUCT CANCER

Longitudinal tumor spread along the biliary tree

Infiltration by bile duct cancer includes both longitudinal extension and vertical invasion. Microscopic extension of bile duct cancer beyond the border visualized by cholangiography or the margin observed macroscopically is often detected, and is diagnosed as a microscopically positive margin (R1). Longitudinal extension consists of superficial and submucosal infiltration and includes sometimes direct, lymphatic, or perineural invasion[31-36]. Sakamoto *et al*[35] pointed out a correlation between the gross type of the tumor and the pattern of infiltration beyond the macroscopic margin at the proximal border. The submucosal spread was predominant in infiltrating type, and the mean length was 6 mm. The superficial extension was predominant in papillary and nodular types, and the mean length was 20 mm. Ebata *et al*[36] investigated 80 resected specimens with microscopically positive margin and observed invasive (submucosal) extent within 10 mm in all the cases, and non-invasive (superficial) spread within 20 mm in 90% of the cases. Considering these reports, a macroscopic surgical margin over 10 mm is preferable in the invasive type and over 20 mm in the papillary and nodular types. Intraoperative pathologic examination of frozen sections is also advocated to confirm the margin of the cut end of the bile duct. When the margin is positive for cancer, additional resection is necessary to obtain R0 resection whenever possible.

Vertical invasion of the bile duct

Hilar bile duct cancer vertically infiltrates into the hepatoduodenal ligament, in which the hepatic artery and the portal vein are located adjacent to the bile duct. Skeltonization of vessels in the hepatoduodenal ligament and clearance of all perivascular connective tissue is standard surgical procedure[15,37,38]. Hilar bile duct cancer easily invades the right hepatic artery, which usually lies behind the common bile duct, and involves the portal vein. If the tumor shows the signs of invasion of the perivascular connective tissue, en bloc resection of the right hepatic artery is advocated to obtain a negative surgical margin; therefore, right hemihepatectomy is recommended. If tumor invasion of the portal vein is suspected based on the preoperative imaging or the intraoperative findings, combined resection of the portal vein and reconstruction are the treatment of choice to obtain a negative radical margin[39-41].

Need for hepatectomy and caudate lobectomy

When the hilar bile duct cancer has infiltrated the hepatic duct confluence, 3-dimensional knowledge of the hepatic hilum is required to determine the extent of the tumor because of the complicated anatomical relationships. Hilar bile duct cancer spreads not only to the right and left bile ducts but in a craniad and dorsal direction along the thin bile ducts. There are two key points to achieving radical resection of hilar bile duct carcinoma. The first is removal of the liver parenchyma adjacent to the hepatic hilum together with the hilar plate, and extended right or left hepatectomy is preferable for this purpose to remove the infiltration of cancer to the right and left bile ducts. The second key point is caudate lobectomy and resection of the inferior area of segment Ⅳ to extirpate cancer infiltration to the thinner bile ducts near the hilum.

The necessity of resecting the caudate lobe has been described by Japanese authors[12,13,42-44]. Nimura *et al*[43] firstly described caudate lobectomy for hilar bile duct cancer. The clinical efficacy of caudate lobe resection was first described by Sugiura *et al*[13]. The 5-year survival rate in their retrospective study was 46% with caudate lobectomy and 12% without caudate lobectomy.

Cholangiography of a resected specimen obtained by extended left hemihepatectomy with caudate resection showed that the caudate branches of the bile originated from the confluence of the hepatic ducts and that hilar bile duct cancer invaded these branches (Figure 3).

DIAGNOSIS

Resectability was assessed and the type of surgical treatment was selected according to the location and extent of the tumor as determined by ultrasonography, helical-CT, direct cholangiography, and MRCP[10]. Patients with para-aortic lymph node metastasis or other distant metastases were not included as candidates for resectional surgery. Direct cholangiography *via* the percutaneous or endoscopic route provides useful findings, but cholangitis may occur after repeated cholangiography in patients whose right and left hepatic ducts are separated by hilar bile duct cancer because the bile is contaminated with bacteria three days after biliary drainage and undrained bile ducts are infected as a result of reflux of the contaminated bile. Direct cholangiography should be restricted to the time of the first puncture and the evening of the day before surgery if the bile ducts are separated[18]. MRCP provides information regarding the entire biliary tree and eliminates the need for direct cholangiography of the whole biliary tree.

Helical contrast CT angiography as well as conventional angiography reveals not only tumor invasion of the vessels in the hepatoduodenal ligament but also vascular anomalies, especially of the hepatic arteries, which are sometimes a determinant of the operative procedure. Aberrant right hepatic arteries originating from the root of the superior mesenteric artery and aberrant left hepatic arteries originating from the left gastric artery are invaded late by bile duct cancer. Preserving the aberrant arteries increases the probability of curative resection by extended right or left hemihepatectomy.

It is sometimes difficult to make a definite diagnosis of bile duct cancer. Bile cytology or brushing biopsy is the choice of diagnosis, but benign lesions such as primary sclerosing cholangitis, cannot be denied in the treatment of bile duct cancer. The incidence of benign lesion in patients with hilar obstruction ranges from 8% to 15%[45,46]. Surgeons should consider the possibility of benign lesions when treating bile duct cancer. Radical surgery of a lesion suspected of being a malignant neoplasm is justified under the condition of low mortality rate even at the risk of benign diseases.

PREOPERATIVE TREATMENT

Biliary drainage

The aim of preoperative BD is to improve liver function and reduce morbidity and mortality after radical surgery with major hepatectomy, i.e. resection of more than three segment of the liver[47-51]. However, instead of elucidating the benefits of preoperative BD previous reports have emphasized the adverse effects of BD, i.e. infectious complications due to bile contamination and tract seeding[47-58]. Sewnath *et al*[56] reviewed and summarized randomized controlled and comparative cohort studies, comparing surgery plus preoperative BD with surgery without preoperative BD, and they performed a meta-analysis of the efficacy of preoperative BD. The results however, failed to show an effect of preoperative BD on the surgery. However, the following problems determine the pros and cons of preoperative BD based on the previous reports[18,59]. First, the rate of radical resection or major hepatectomy was too low to examine the effect of BD on surgery in those studies. The rate of resection in randomized controlled studies is only 15% on average and the other procedures were palliative treatments. The average resectability rate was 90% in the comparative cohort studies, but pancreatoduodenectomy composed nearly 90% of resected cases and hemihepatectomy only 2%. To perform major hepatectomy in patients with jaundice for hilar bile duct cancer is most likely to develop postoperative liver failure. Second, in the previous studies, recovery of hepatic function was insufficient, since BD was only performed for 10-14 d and the surgical interventions were carried out at a total bilirubin value of about 10 mg/dL[48,50]. By contrast, the studies from Japan insisted that radical surgery be performed after complete recovery from jaundice, i.e., a total bilirubin decrease to under 2.0 mg/dL[15,16,18]. It takes 4-6 wk for liver function to recover after BD for jaundice[47,60,61]. Finally, most tube-related trouble is due to poor technical skill, such as drainage tube failure and cholangitis caused by unnecessary cholangiography. Slipping of the drainage tube out of the bile duct can be prevented by careful management of the BD tube and by using balloon catheters or pig-tail catheters.

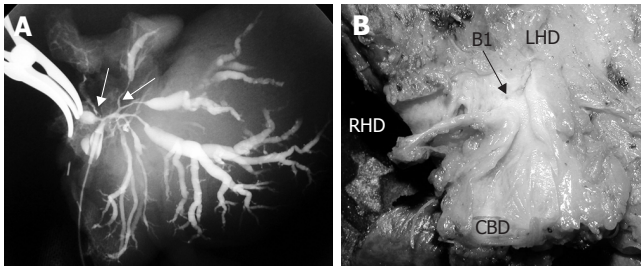

Figure 3 Tumor infiltration of the roots of the caudate branches; **A**: Cholangiography of a resected specimen. Hilar bile duct cancer has infiltrated the root of the caudate branches (B1) arising from the confluence of the hepatic duct (arrows); **B**: Gross appearance of the resected tumor. The caudate branch opens into the hilar neoplasm.

Since suppurative cholangitis is not a rare complication after BD, many reports have pointed out that bile contamination increases postoperative infectious complications and mortality, and that preoperative BD should be avoided. Cholangitis is caused by pushing the contaminated bile into the undrained area during cholangiography or by occlusion of the drainage tube. The most important means of preventing cholangitis is not to perform cholangiography after PTBD in patients whose right and left bile ducts are separated by tumor infiltration. Cholangiography should be limited to the time of PTBD and the afternoon of the day before the operation, because bile juice becomes contaminated by bacteria within 3 d after PTBD.

Once cholangitis has developed regardless of the cause, the treatment of choice is BD by either the percutaneous transhepatic or endoscopic retrograde route. Criticism of preoperative BD due to the risk of cholangitis is nonsense, because doctors claim such opinion indicate that they cannot treat the patients who have cholangitis not by

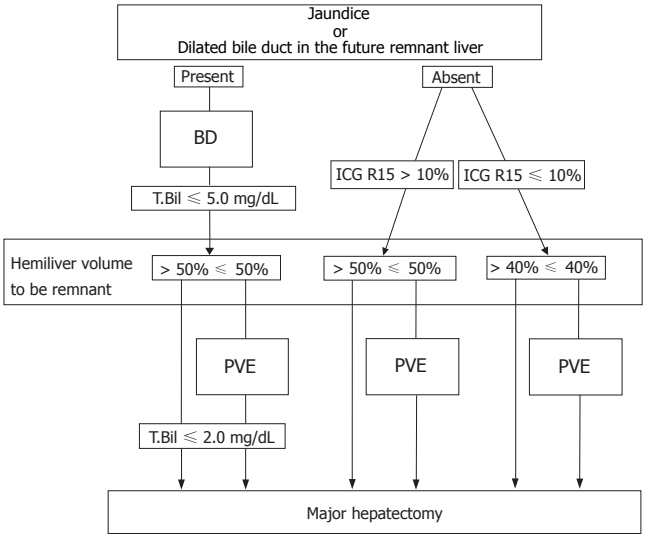

Figure 4 Flowchart of preoperative treatment. If a patient had jaundice or there were dilated bile ducts in the future remnant liver, biliary drainage (BD) was performed. Surgical interventions were scheduled after sufficient recovery of hepatic function. Portal vein embolization (PVE) was carried out to avoid postoperative liver failure, depending on the liver function and the liver volume to be resected.

BD. Bile from the BD tube should be cultured, and the sensitivity of isolates to antibiotics should be checked routinely. During the operation, a solution of antibiotic to which the culture are sensitive should be scattered into the abdominal cavity before closing the wound.

The route of BD is either percutaneous transhepatic or endoscopic retrograde. The only drawback of the percutaneous transhepatic route is catheter tract seeding (described below in detail). The endoscopic route, on the other hand, is closely associated with retrograde infection, and thus it is not advocated for hilar obstruction. The biliary stent tube should be replaced every two weeks to avoid tube occlusion by debris in patients in whom an endoscopic retrograde BD (ERBD) tube has been inserted[62].

Points of preoperative BD procedure are listed below. The following should be taken into consideration: (1) Externally drained bile juice should be taken orally[63], (2) Radical operation should be performed after sufficient recovery of liver function[15,16,18], (3) BD should be performed if hepatectomy is scheduled[64], (4) ERBD stent tube should be replaced every two weeks, (5) Bile culture should be performed routinely, and isolates should be tested for the antibiotic sensitivity[58].

For hilar obstruction with interruption of communication between the right and the left hepatic duct, the following should be taken into consideration (1) PTBD of the future remnant liver alone is the first choice[17,18,65,66], (2) The endoscopic route is not advocated[53,57,67,68], (3) To avoid cholangitis, preoperative cholangiography should be performed only when the tube is inserted and on the evening before the operation[18].

Catheter tract seeding is a problem related to PTBD, and its incidence has been reported to be 5%-10%[18,69]. Multiple BD and PTCS may increase the risk of seeding, but the risk can be decreased by the draining only the future remnant liver and using the minimum number of BD tubes. Tract seeding did not affect outcome because the primary tumors were already far-advanced and most patients had multiple recurrences. Sakata *et al*[69] reported that resection of an isolated metastasis along the catheter tract may prolong survival. Catheter tract seeding is not a rare complication in the treatment of the malignant biliary obstruction, but its incidence is acceptable.

Portal vein embolization

In 1982, Makuuchi *et al*[70,71] were the first to apply PVE to prevent liver failure after extended hemihepatectomy in patients with hilar cholangiocarcinoma. Major hepatectomy results in massive reduction of the liver volume and a increase in portal pressure. If PVE is performed preoperatively, the portal hypertension can be tolerated, and the rate of liver volume loss decreases due to the atrophy-hypertrophy complex theory. Kubota *et al*[72] showed indication criteria of PVE according to the volume of liver to be resected, which was calculated from CT, and ICG R15 value. Several recent reports have confirmed the safety of major hepatectomy following PVE for patients with hilar bile duct cancer and obstructive jaundice[16-18,38,73]. The rate of liver volume to be preserved increased by 8%-12% two weeks after PVE[18,74,75]. In Western countries, evaluations of liver volume after PVE have been made after 4-6 wk. The wait between PVE and operation is shorter in the reports from Japan, but was adequate to perform hepatic resection safely.

Safe strategy for major hepatectomy in patients with hilar bile duct cancer

Seyama *et al*[18] described the safe strategy for hilar bile duct cancer that includes BD and PVE. A flow chart for preoperative treatment is shown in Figure 4. If a patient showed evidence of jaundice or there were dilated bile ducts in the future remnant liver, BD was performed, in principle only in the future remnant liver. Whether PVE was indicated depended on liver function and the volume of the future remnant liver, which was calculated by CT volumetry. In patients with normal liver function, i.e. an ICG R15 value under 10%, PVE was indicated when the remnant hemiliver volume was less than 40%. In patients with jaundice or with an ICG R15 value over 10%, PVE was indicated if the remnant hemiliver volume was less than 50%. Since the standard operative procedure for hilar bile duct cancer is an extended hemihepatectomy, including the whole S1, the remaining hemiliver volume should have a margin from the safety zone. After re-evaluation of the liver volume to be resected, hepatectomy was performed if the patient fulfilled the criteria. Figure 5 showed the intraoperative findings after BD of the future remnant liver followed by PVE of the right portal vein. The right liver was markedly atrophic, and the BD tube was inserted into the bile duct in segment 3, which drained only the future remnant left liver. In this patient, extended right hemihepatectomy was carried out.

SURGICAL TREATMENT FOR BILE DUCT CANCER

In view of their modes and patterns of infiltration, the best treatment for hilar bile duct cancer is extrahepatic bile duct

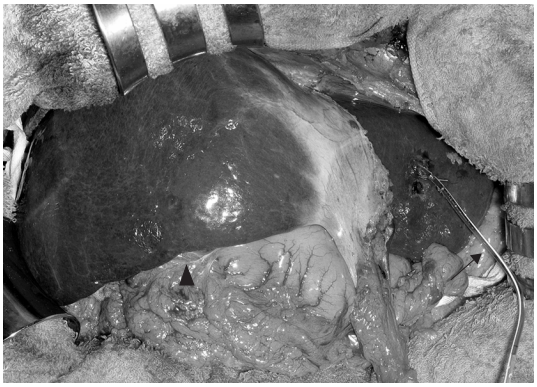

Figure 5 Intraoperative view at laparotomy after biliary drainage and portal vein embolization. A percutaneous transhepatic biliary drainage tube (arrow) has been inserted into the bile duct of segment 3. The right liver is markedly atrophic, and there is a clear line of demarcation between the right and left liver.

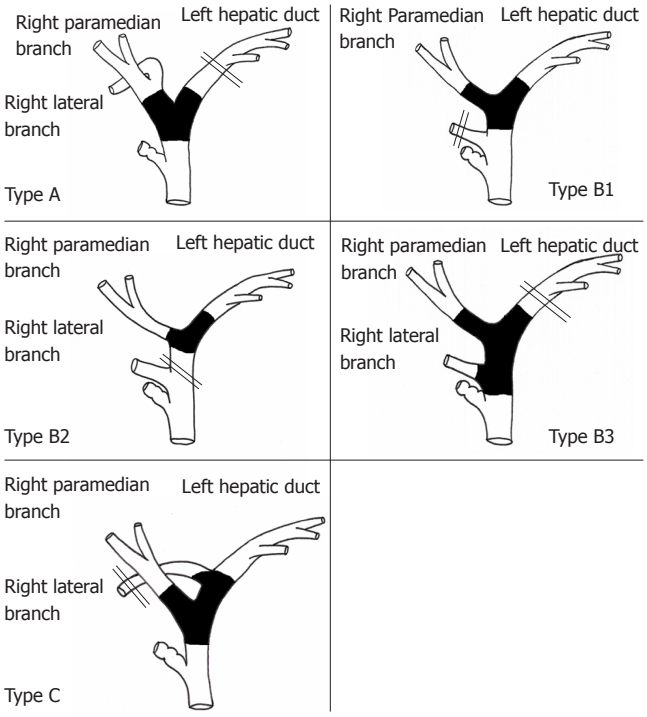

Figure 6 Indications for left trisectoriectomy according to tumor location and the bile duct variations in the anatomy of the right lateral branch of the bile duct. Double lines indicate the scheduled cut end of the bile duct. Type A, ordinary bifurcation; extended right hepatectomy is the choice of treatment. Type B, the right lateral branch originates from the common hepatic duct; left trisectoriectomy, preserving the right lateral branch, is a useful alternative in Types B1 and B2. Type C, the right lateral branch originates from the left hepatic duct; left trisectoriectomy may provide a longer bile duct margin.

resection plus hepatectomy and regional lymphadenectomy, and the best treatment for middle and distal bile duct cancer is pancreatoduodenectomy. Lymph node dissection includes the nodes within the hepatoduodenal ligament, behind the pancreatic head, and along the common hepatic artery. Some authors have reported extended lymphadenectomy including para-aortic lymph nodes, but the incidence of para-aortic lymph node metastasis by bile duct cancer is lower than by pancreatic cancer. Since there is no evidence of a survival benefit[76,77], extended lymph node dissection is not justified.

Extended hemihepatectomy for hilar bile duct carcinoma

Because of its radicality and simplicity, extended hemihepatectomy is recognized as the standard surgical procedure for hilar bile duct cancer[15,17,18,27,38,73]. Extended right hemihepatectomy consists of resection of the right liver, the inferior part of Couinaud's segment Ⅳ, and the entire caudate lobe. Extended left hemihepatectomy consists of resection of the left liver, the hilar part of the anterior segment, and most of the caudate lobe. Even in patients with Bismuth type Ⅰ or type Ⅱ, extended hemihepatectomy is needed to achieve curative resection according to the mode of tumor extension[17,18,73]. If the tumor is predominantly on the right side or centrally located, extended right hepatectomy is selected. Kawasaki *et al*[17] explained why right hepatectomy is more likely to be associated with a negative margin than left hepatectomy based on the anatomic considerations, i.e. length of the extrahepatic part of each hepatic duct, location of the common hepatic duct in the hepatoduodenal ligament, facility for systematic caudate lobectomy, and ease of portal vein reconstruction. Extended left hemihepatectomy is indicated for left-side-dominant tumor. If the tumor has spread diffusely into the intrapancreatic bile duct, pancreatoduodenectomy is performed simultaneously[27] (HPD, described later).

Right or left trisectoriectomy for hilar bile duct carcinoma

Right or left trisectoriectomy is one of the most extensive resections because of the massive loss of volume of the hepatic parenchyma[78-80]. Hilar bile duct cancer widely invades the hepatic hilum (Bismuth type Ⅳ), resulting in jaundice, and sometimes requires trisectoriectomy for curative resection. Right or left trisectoriectomy is advantageous in terms of obtaining a cancer-free margin in the hepatic ducts. A negative hepatic margin was obtained in 75% of cases treated by left trisectoriectomy and 87.5% of cases treated by right trisectoriectomy, both rates are higher than obtained by extended hemihepatectomy[79,80]. Variation in the anatomy of the right lateral branch should be taken into consideration to obtain adequate surgical margin (Figure 6). Type A is an ordinary bifurcation, and extended right hepatectomy is the choice of treatment. If the right lateral branch originates directly from the common hepatic duct (Figure 6 Type B1-2), left trisectoriectomy, which preserves the right lateral branch, is a useful alternative. When the right lateral branch originates from the left hepatic duct, left trisectoriectomy may provide a longer bile duct margin than other hepatic resection (Figure 6 Type C). Since the aberrant right hepatic artery originates from the SMA, which runs behind the portal vein and the right-dorsal aspect of the hepatoduodenal ligament, is hardly invaded by bile duct cancer; left trisectoriectomy, which preserves the right lateral branch of the right hepatic artery, is a useful alternative for advanced hilar cholangiocarcinoma. However, the operative risk of trisectoriectomy is greater than that of extended hemihepatectomy, and preoperative BD of the future remnant liver and PVE are indispensable. Nagino *et al*[80] reported a mortality rate of 7.1% after right

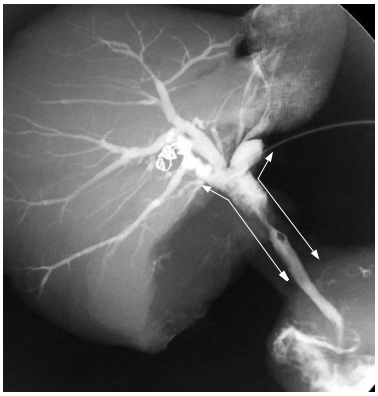

Figure 7 Cholangiography of the resected specimen by HPD. The tumor is located in the common hepatic duct (Bismuth-Corllete type 2), and microscopic examination showed superficial spread to the right and left hepatic duct and the intrapancreatic part of the common bile duct (arrows).

trisectoriectomy despite preoperative PVE. Shimada *et al*[79] reported 12 consecutive cases of left trisectoriectomy for hilar cholangiocarcinoma with no mortality after BD followed by PVE. Proper patient selection and adequate preoperative treatment are required when radical high-risk operations, such as trisectoriectomy, are preformed for hilar bile duct cancer.

Limited resections for hilar bile duct cancer

Parenchyma preserving hepatectomy is a less invasive procedure for hilar bile duct cancer than major hepatectomy. The drawback of the procedure is high incidence of positive margins because of tumor spread along the bile duct, although various type of hepatic resection have been used; segmental, S4 + S1, S4a + S5 + S1, S1, and extended hilar resection[14,81,82]. Other problems include the complexity of the surgical procedure, which takes a great deal of time, intraoperative bleeding, and the difficulty of biliary reconstruction. Consequently, parenchyma preserving hepatectomy for hilar bile duct cancer is the useful alternative in patients in poor general condition or with high-risk factors.

Bile duct resection without hepatectomy is the most minimally invasive, least radical surgical treatment. It is indicated only when the primary tumor is located in the middle of the common hepatic duct and there is no invasion or spread. It is also an option for high-risk patients.

Surgery for middle and distal bile duct cancer

Pancreatoduodenectomy is the treatment of choice for middle and lower bile duct cancer. The pylorus preserving Whipple procedure (PpPD) is commonly performed, and the short- and long-term results of PpPD are comparable to those of standard PD[83-85]. When a middle bile duct cancer arises midway along the extrahepatic duct, the decision has to be made as to whether PD or extended hemihepatectomy is more appropriate according to the tumor location and extension.

Indications for hepatopancreatoduodenectomy

Hepatopancreatoduodenectomy (HPD) has been used to treat biliary malignancies and is a useful alternative for the treatment of bile duct cancer. The clinical indications are longitudinal tumor spread from the hepatic duct to the intrapancreatic bile duct or massive lymph node metastases along the bile duct and behind the pancreatic head. Massive lymph node metastases are not a good indication because the presence of lymph node metastases is itself a negative prognostic factor. However, postoperative hospital mortality has been very high. Nimura *et al*[26] firstly presented a considerable number of patients who had undergone HPD, hospital mortality was as high as 35% and 5-year survival was only 6%. The causes of postoperative mortality after HPD are postoperative liver failure and leakage of pancreatojejunostomy resulting in bleeding from the major arteries. BD followed by PVE is indispensable to prevent postoperative liver failure[17,18,27]. A two-stage operation, in which reconstruction of the pancreatic duct is the second step, has been reported to reduce the risk of leakage of the pancreatic juice[86-88]. Miyagawa *et al*[27] firstly reported twelve consecutive cases of HPD without mortality, applying BD followed by PVE and staged reconstruction of the pancreatic duct.

Figure 7 is a cholangiography of a resected specimen obtained by HPD. The common hepatic duct was obstructed (Bismuth type 2), and the tumor had superficially spread to the right and left hepatic duct and the intrapancreatic part of the common bile duct. This case was considered to be a good candidate for HPD, and all the margins were negative in spite of the wide tumor spread. Pathological examination revealed that the depth of invasion was to the subserosa and that there were five lymph node metastases, all close to the bile duct. The postoperative course after HPD was uneventful, and there was no liver failure. The patient is alive without tumor recurrence as of 10 mo after surgery.

Combined vascular resection and reconstruction

The value of portal vein resection and reconstruction in the treatment of bile duct cancer is still controversial. Previously, invasion of a major vessel meant an unresectable tumor. Recent reports have shown improved results when portal vein resection and reconstruction are performed together with major hepatectomy[39-41,89]. Neuhaus *et al*[66] reported a 5-year survival rate of 65% after curative resection of hilar bile duct cancer with portal vein resection and reconstruction. Although their case series excluded the 60-d deaths (mortality rate of 17%) and non-curative resections, the results indicated that portal vein reconstruction concomitant with hepatectomy improves the probability of long-term survival. Interestingly, macroscopic portal vein invasion was a significant prognostic factor, but microscopic invasion was not[40,66]. When there is severe adhesion between the tumor and portal vein, combined resection and reconstruction is needed to obtain a negative surgical margin. Kondo *et al*[89] advocated portal vein resection and reconstruction prior to hepatic dissection during extended right hepatectomy, and the procedure enables no-touch resection. On the other hand, portal vein reconstruction increases the risk of major hepatectomy. The reported mortality of portal vein reconstruction for hilar bile duct cancer is 10%-20%, but recent studies have shown that PVE improves mortality[17,18,73]. PVE is recommended to increase the safety of major hepatectomy with portal vein reconstruction.

Table 1 Review of the literature on major hepatectomy for hilar bile duct carcinoma

Author	Year	Period	Total resection	Rate of R0 (%)	Major Hx	MH/ resection (%)	BD	PVE	Liver failure (%)	Mortality	5-yr survival rate (%)
Miyagawa[16]	1995	1989-1994	37		33	89	Done	Done	0.0	0.0	NA
Pichlmayr[92]	1996	1975-1993	125	73	92	74	No	No	10.5	12.7	26.3[1]
Klempnauer[93]	1997	1971-1995	151	78	111	74	No	No	3.4	11.7	32.0[1]
Nagino[82]	1998	1977-1996	138	78	109	79	Done	Done[4]	8.0	9.7	25.8[2]
Burke[94]	1998	1991-1997	30	83	22	73	No	No	NA	6.6	45.0
Ogura[95]	1998	1976-1995	66	55	31	47	Done	No	15.9	2.2	NA
Kosuge[38]	1999	1980-1997	65	52	52	80	Done	Done[4]	10.8	9.2	34.8
Neuhaus[66]	1999	1988-1998	95	61	66	69	Done	(TAE)	NA	9.0	22.0[3]
Launois[96]	1999	1968-1993	40	80	22	55	No	No	NA (9)	12.5	12.5
Miyazaki[97]	1999	1981-1998	93	70	66	71	Done	Partially	29.0	12.0	36.0
Gerhards[98]	2000	1983-1998	112	14	32	29	Done	No	12.0	25.0	NA
Todoroki[99]	2000	1976-1998	98	14	32	33	Done	No	8.4	5.0	28.0
Jarnagin[100]	2001	1991-2000	80	78	62	78	Not routine	No	3.2	11.0	39.0
Seyama[18]	2003	1989-2001	67	64	58	87	Done	Done	0.0	0.0	40.0
Kawasaki[17]	2003	1991-2000	79	68	69	87	Done	Done	0.0	1.3	40.0
Kondo[73]	2004	1999-2002	40	95	26	65	Done	Done	0.0	0.0	40 (3-yr)
Rea[101]	2004	1979-1997	NA	80	46	NA	Done	No	11.0	9.0	26.0
Hemming[19]	2005	1997-2004	53	80	52	98	Done	Done	3.7	9.0	35.0

Hx: hepatectomy; NA: not available; R0: microscopic negative margin; BD: biliary drainage; PVE: portal vein embolization. 1, these two reports were from the same center; 2, curative Hx 97 cases; 3, total 95 cases; 4, PVE was started in the middle of the study periods.

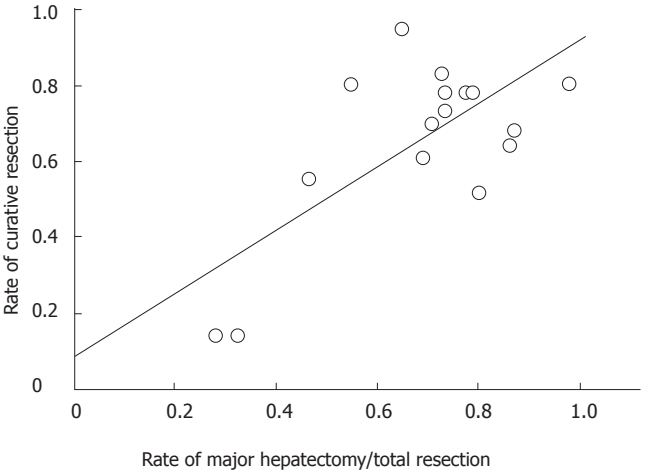

Figure 8 Graph of data from a literature review of the relationship between curative resection and major hepatectomy rate. The studies are listed shown in Table 1. Curative resection is defined as a microscopic ally negative margin. Linear approximation showed a significant correlation between the rate of major hepatectomy and curative resection (P = 0.0027, R = 0.7).

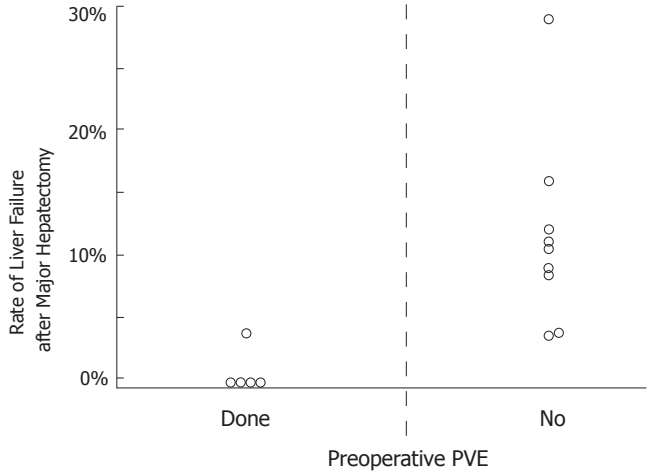

Figure 9 Graph of data from a literature review of the relationship between the preoperative portal vein embolization (PVE) and the rate of postoperative liver failure after major hepatectomy for hilar bile duct cancer. PVE significantly reduced the incidence of postoperative liver failure (P = 0.0113).

The clinical significance of combined resection and reconstruction of the hepatic artery of the remnant liver has not been resolved[17,18,39-41,90,91]. Reconstruction of the hepatic artery has been performed less frequently than reconstruction of the portal vein, because the conditions that require combined resection of the hepatic artery of the remnant liver often mean a locally far advanced tumor. Although there have been recent reports of major hepatectomy with reconstruction of the hepatic artery being performed safely with acceptable mortality rates (0-8%)[90,91], since it is more dangerous than major hepatectomy without reconstruction[41], at present reconstruction of the hepatic artery together with major hepatectomy is advocated if the tumor can be resected without residual tumor at any surgical margin.

SHORT-AND LONG-TERM OUTCOME

As mentioned above, major hepatectomy is the most suitable surgical procedure for removing hilar bile duct cancer with curative intent, and it is the standard procedure. Table 1 shows the results of major hepatectomy for hilar bile duct cancer over the last 10 years based on the review of the literature[16-19,38,66,73,82,92-101]. Hepatectomy improved the resectability and curability of the surgical resection for hilar bile duct cancer. Launois *et al*[96] reviewed selected studies and pointed out the positive correlation between the resectability rate and hepatectomy. The rate of major hepatectomy and curability are plotted in Figure 8 and show a significant positive correlation (P = 0.0027, R = 0.7). Recent reports have shown that the 5-year survival rate for hilar bile duct cancer treated by major hepatectomy has improved to 40%.

A high rate of postoperative liver failure and high mortality rate have been mentioned as the drawbacks of major hepatectomy for hilar bile duct cancer despite its radicality. Table 1 shows that BD alone does not reduce the rate of postoperative liver failure, and that major hepatectomy can be carried out without liver failure or mortality by using preoperative PVE together with BD. Rates of postoperative liver failure of 10%-20% had have been reported without PVE, and PVE has significantly reduced the rate of postoperative liver failure to nearly zero percent (Figure 9, $P = 0.0113$). The low mortality rate shows that PVE not only decreased the occurrence of postoperative liver failure but provided a hepatic functional reserve.

Curative resection (R0), lymph node metastasis negative, tumor size smaller than 2 cm, well differentiated histology, and earlier tumor stage have been reported as favorable prognostic factors after resection for hilar bile duct cancer[17-19,38,66,98,100,102]. Combined vascular resection was not a prognostic factor, if curative resection was achieved[39-41,89]. Concomitant PD with hepatectomy did not significantly influence patient survival[17,18]. Therefore, extended radical resection for local advanced bile duct cancer has been justified only under the acceptable low mortality rate. On the other hand, patients with lymph node metastasis have had significantly poorer long-term results, and long-term survival has rarely been expected even when extended lymph node dissection was performed[34].

The reported five-year survival rates after PD for middle and distal bile duct cancer have ranged from 24% to 39%[20-22,24,37,103,104]. Curative resection, lymph node metastasis negative, intraoperative transfusion negative, well differentiated histology, and location (middle or distal) have been pointed out as a favorable prognostic factors for the treatment of middle and distal bile duct cancer. Sakamoto *et al*[24] emphasized the prognostic significance of the radical margin rather than hepatic margin in the treatment of middle and distal bile duct cancer. They insisted that significance of purchase to the negative hepatic margin is dependent on whether radical margin is negative for the tumor. Survival of patients with middle and distal bile duct cancer and lymph node metastasis is also poor, and Yeo *et al*[77] showed that radical extended lymph adenectomy provided no survival benefit in a randomized controlled trial. Nor have any studies shown a difference in survival between standard PD and PpPD[83-85].

CONCLUSION

Hemihepatectomy with or without pancreatoduodenectomy, plus extrahepatic bile duct resection and regional lymphadenectomy can be performed safely and offer a chance for cure of hilar bile duct cancer if preoperative BD followed by PVE is properly indicated. Major hepatectomy with PD and/or vascular reconstruction can be performed without mortality and results in improved resectability, curability, and a 5-year survival rate of 40%. PD is a standard procedure for middle and lower bile duct cancer, and the 5-year survival rate is nearly 30% with acceptable mortality rate. Radical resection with negative margins and no mortality is the goal of surgeons. Survival of patients with lymph node metastasis is still poor despite extended lymph node dissection, and to improve it is a future issue.

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TOPIC HIGHLIGHT

Wei Tang, MD, *Series Editor*

Artificial and bioartificial liver support: A review of perfusion treatment for hepatic failure patients

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Abstract

Liver transplantation and blood purification therapy, including plasmapheresis, hemodiafiltration, and bioartificial liver support, are the available treatments for patients with severe hepatic failure. Bioartificial liver support, in which living liver tissue is used to support hepatic function, has been anticipated as an effective treatment for hepatic failure. The two mainstream systems developed for bioartificial liver support are extracorporeal whole liver perfusion (ECLP) and bioreactor systems. Comparing various types of bioartificial liver in view of function, safety, and operability, we concluded that the best efficacy can be provided by the ECLP system. Moreover, in our subsequent experiments comparing ECLP and apheresis therapy, ECLP offers more ammonia metabolism than HD and HF. In addition, ECLP can compensate amino acid imbalance and can secret bile. A controversial point with ECLP is the procedure is labor intensive, resulting in high costs. However, ECLP has the potential to reduce elevated serum ammonia levels of hepatic coma patients in a short duration. When these problems are solved, bioartificial liver support, especially ECLP, can be adopted as an option in ordinary clinical therapy to treat patients with hepatic failure.

Key words: Bioartificial liver support; Blood purification therapy; Extracorporeal liver perfusion; Bioreactor; Transgenic pig

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INTRODUCTION

Organ replacement by artificial organ technologies has progressed remarkably in the past decades. The temporary replacement of heart and lung functions has permitted major improvements in cardiac surgery, and renal dialysis has revolutionized the prognosis in acute and chronic renal failure. However, the clinical treatment of fatal hepatic failure with living liver support systems has been far from satisfactory.

The liver is the chief metabolic and synthetic organ and it carries out more than 500 different functions that are difficult to replace by substitute methods. The destruction of homeostasis that is caused by hepatic failure results in a life crisis. This has promoted much research into living liver tissue support to perform various hepatic functions, however, the development of bioartificial liver support is remarkably delayed compared to the development of artificial kidney and artificial heart.

Liver transplantation and blood purification therapy, including plasmapheresis, hemodiafiltration, and bioartificial liver support, are the available treatments for patients with severe hepatic failure. Plasmapheresis involves separating and replacing the plasma of patients in hepatic failure and this treatment has confirmed beneficial effects for patients. Its major drawback lies in the cost of economic and medical resources because it requires a large volume of fresh frozen plasma. Bioartificial liver support, in which living liver tissue supports hepatic function, is anticipated to be an effective treatment for hepatic failure. This review focuses on the development of artificial and bioartificial liver support and evaluates the most desirable strategy for blood purification therapy for hepatic failure patients.

ARTIFICIAL LIVER SUPPORT

Table 1 shows the development of blood purification therapy for hepatic failure. The first extracorporeal perfusion treatments for hepatic failure were applications of artificial hepatic function devices. During the 1950s, hemodialysis was introduced as an optimal treatment for renal failure by removing uremic substances based on the theory of diffusion; that is, the solute substances move according to a concentration incline, and this treatment has prevailed in daily clinical medicine as a reliable therapy for renal failure patients. In 1958, hemodialysis was carried out on patients with hepatic failure in an attempt to remove the toxins thought to cause hepatic encephalopathy. Killey

et al[1] reported an uncontrolled study of five patients with chronic hepatic failure, four of whom showed improvement in their metabolic encephalopathy, although long term survival was not achieved.

For more aggressive removal of protein bound molecules, two forms of mechanical liver support were developed: hemoperfusion and plasmaperfusion. One method involved an adsorbent device able to substitute some hepatic functions by removing toxic substances from the blood. In 1958, Schechter *et al*[2] introduced direct extracorporeal hemoperfusion over an ion-exchange resin matrices column (Dowex 50-X8), which achieved removal of blood ammonia and reversal of coma in 20% of patients. Also in 1958, Lee and Tink *et al*[3] reported the successful treatment of a patient in hepatic coma by exchange transfusions with fresh blood. This trial was based on the concept that the detoxification, regulation, and synthetic functions of the liver can be realized by exchanging the blood volume of a patient in hepatic failure. Neurologic improvements and survival were repeatedly suggested, but this conception was replaced later by the effective method of plasma exchange.

Considerable experience has been gained using activated charcoal as an adsorbent of possible toxins in the range of 500 to 5000 daltons in whole blood or plasma in patients in hepatic failure[4,5]. In 1965, Yatzidis *et al*[4] developed an activated charcoal column for removing serum bilirubin, which is still used for patients suffering from hyperbilirubinemia. Its initial primary side effects were platelet loss and anaphylaxy shock, which have been controlled by plasma separation or prostaglandin administration.

One of the most straightforward approaches to biological liver support is cross-circulation, which was reported in 1967 by Burnell *et al*[6]. In this technique, which is performed under general anesthesia, the circulation of patients with hepatic failure is directly connected to that of healthy human donors. One of three patients were reported to be fully recovered, but the donors suffered from severe adverse reactions during the procedure. It was demonstrated that liver support could[1,2]be provided with an external liver, but the donor suffered serious toxic side effects from the hepatic failure blood, resulting in cessation of further trials.

Plasma exchange, or plasmapheresis, was introduced by Sabin *et al*[7] in 1968. This plasma separation technique, using either a centrifuge or a membrane, separated plasma that was then discarded and replaced by an equivalent volume of fresh plasma. Yamazaki *et al*[8] developed a combination technique of plasmapheresis and hemodialysis that proved effective for reversing hepatic coma and improving coagulation. A major problem with this method was the need for a large volume of normal plasma as a substitute.

The kidney produces urine mainly by filtration in glomeruli. A high quality membrane was needed to modify the dialyser to be equivalent to glomeruli. During the latter 1960s, Henderson *et al*[9] created a novel approach to the hemofiltration method by using a polysulfone membrane.

Considering cellulosic membranes that are permeable to small water-soluble molecules, in 1976, Opolon *et al*[10] used a polyacrylonitril membrane to improve the diffusive transfer of substances up to 15000 daltons by removing medium-weight solutes and small peptides. In a clinical study of 24 patients with acute fulminant viral hepatitis, hemodialysis with this membrane achieved a coma reversal rate of 54%, but the survival times were not improved. An important conclusion from these experiments was that substances of less than 15000 daltons in molecular weight were associated with metabolic encephalopathy. Also in 1976, Knell and Dukes *et al*[11] introduced reciprocal dialysis in which a dialysis fluid with an amino acid concentration identical to that of normal plasma was used to correct increased aromatic amino acids and decreased branched-chain amino acids.

Hemodialysis is effective in removing small sized molecules that are under 5000 in molecular weight, and hemofiltration is especially effective in removing larger sized molecules of 5000 to 10000 in molecular weight. Thus, a combination therapy of hemodialysis and hemofiltration has the potential to be an ideal therapy for severe renal failure and hepatic failure. In 1977, Ota *et al*[12] developed the hemodiafiltration (HDF) method, in which a great deal of water, as much as 10l, is removed and a physiological solution is supplied to adjust for the quantity of water removal. In this method, a dialyser consisting of a hollow fiber membrane with a larger pore size than is used in ordinary hemodialysis, enables removing larger molecules.

Table 1 Development of blood purification therapyin the treatment of liver failure

1958	Killey	Treatment of a hepatic coma patient using hemodialysis
1958	Schechter	Treatment of a hyperammonemia patient using an ion-exchange column
1958	Lee, Tink	Exchange transfusion
1958	Hori	Cross-hemodialysis using living dogs
1965	Yatzidis	Bilirubin adsorbent using activated charcoal
1965	Eisemann	Initial clinical use of ECLP using a resected porcine liver
1967	Burnell	Cross-hemodialysis between a patient in hepatic coma and a healthy donor
1968	Sabin	Plasma exchange (Plasmapheresis)
1970	Abouna	Clinical use of ECLP using a xenogeneic liver
1976	Opolon	Treatment of a patient with fulminant hepatitis using a dialyzer made of a polyacrilonitril membrane
1976	Knell, Dukes	Control of amino acid imbalance using BCAA solution
1978	Yamazaki	Incorporated system of plasmapheresis and hemodialysis
1980	Brunner	Bioreactor immobilized with hepatic enzymes
1982	Ozawa	Cross-hemodialysis using porcine and baboon livers
1985	Teraoka	XDHP and CPP using porcine liver
1987	Matsumura	Perfusion system of suspended rabbit hepatocytes
1988	Marguilis	Perfusion system of suspended porcine hepatocytes
1992	Yoshiba	Incorporated system of plasmapheresis and hemodiafiltration
1993	Demetriou	Bioreactor system of immobilized porcine hepatocytes
1994	Gerlach	Bioreactor system of immobilized hepatocytes
2000	Stange	Molecular adsorbent recirculating system (MARS)

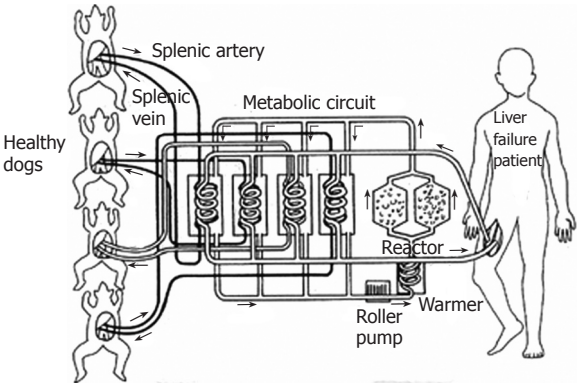

Figure 1 Cross dialysis treatment using four living dogs (Hori *et al* 1958).

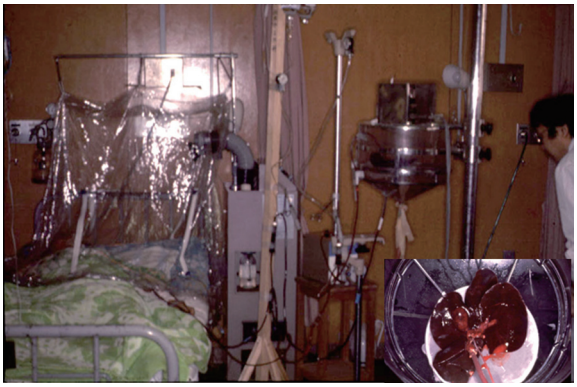

Figure 2 ECLP applied for acute phosphorus intoxication patient (Kawamura *et al* 1974).

In an attempt to provide more specific detoxification therapy, immobilized enzyme systems were developed during the 1980s. In these systems, blood is perfused over liver enzymes that are either linked to an insoluble substrate or encapsulated in artificial cells[13,14]. It is desirable to control hyperammoniemia with a single column containing an immobilized enzyme, but these columns are costly and it is difficult to activate enzymes and keep them activated on request.

During the early 1990s, Yoshiba *et al*[15] consecutively treated 27 patients who had fulminant hepatitis by using plasma exchange in combination with continuous hemodiafiltration, using a high performance membrane (polymethylmetacrylate). Treatment was considered successful for 15 of 27 treated patients, who survived after a mean 16.1 sessions over a mean 19.3 d. The effectiveness of this method (55.6% survival) was attributed to its early application.

Stange and Mitzner *et al*[16] introduced a new dialysis method using their originally developed molecular adsorbent re-circulating system, and applied it to 26 patients with hepatorenal syndrome. They reported a significant decrease in serum bilirubin and creatinine levels in the treated groups, which enabled this procedure to be one of the most important options for treating hepatic failure.

BIOARTIFICIAL LIVER SUPPORT

The liver has more than 500 different functions, which are difficult to replace by one or a few substitute methods, and this has provoked much research into living liver tissue supporting hepatic functions. In 1958, Hori *et al*[17] conducted a primary trial of perfusion treatment using xenogeneic livers and a cross-hemodialysis method consisting of the blood circuits of a patient with cirrhosis and four living dogs. The circuits were separated by a semipermiable membrane, through which low and middle molecular weight waste products from the patient passed into the animals' circuit to be metabolized by canine livers and absorbed into an ion-exchange resin matrix column (Figure 1). This treatment was applied to four patients with cirrhosis, one of whom recovered temporarily from hepatic coma after a remarkable decrease in serum ammonia levels.

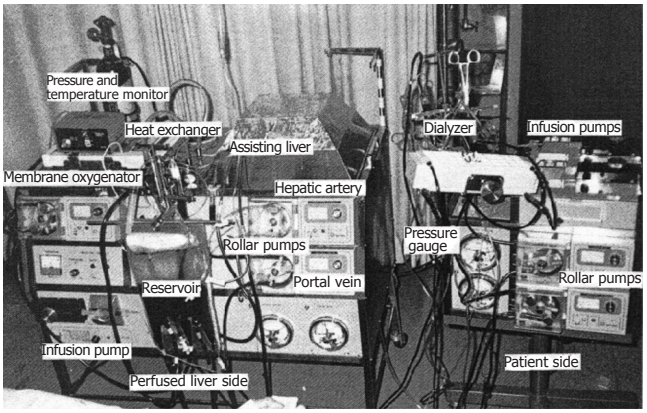

Figure 3 ECLP using baboon or porcine livers (Ozawa *et al* 1982).

After an initial clinical study by Eisemann *et al*[18] in 1965 using whole porcine liver for direct hemoperfusion, many research groups conducted clinical trials of extracorporeal liver perfusion (ECLP). Abouna *et al*[19] clarified that humans have immune responses to pig proteins and that anaphylactic reactions to liver from another species sometimes occurred.

In 1974 in Japan, aggressive clinical trials of ECLP were also performed. Kawamura *et al*[20] clinically applied ECLP using porcine liver for a hepatic coma patient suffering from acute phosphorus intoxication (Figure 2). Direct hemoperfusion was performed, however, the clinical status was so severe that the patient could not be saved. During the early 1980s, Ozawa *et al*[21] developed a cross hemodialysis method using resected porcine or baboon livers, which achieved an overall survival rate of 27% in 13 patients with severe hepatic failure (Figure 3). In 1985, Teraoka *et al*[22] used resected whole porcine liver to treat hepatic failure, with direct hemoperfusion for one patient and cross plasma perfusion for two patients (Figure 4).

In the 1990s, a renewed interest, based on experimental studies, led Fox *et al*[23] to use allogenic ECLP in the management of three patients with fulminant hepatic failure. They used human cadaveric livers, which improved the perfusion time to 72 h, resulting in two patients being successfully bridged to liver transplantation. Other trials were

performed by Chiari[24], and Neuhaus[25] using porcine livers.

New trials of ECLP are being carried out using transgenic porcine livers. Levi *et al*[26] reported the first two cases of ECLP using transgenic porcine livers to overcome a potential immunologic barrier (hCD55/hCD59) and both were successfully bridged to liver transplantation. Xu performed a clinical trial of extracorporeal perfusion treatment using transgenic whole porcine liver in two patients, and reported no symptoms of porcine endogenous retrovirus (PERV) infection[27].

Another bioartificial liver support approach is the hybrid bioartificial liver system in which hepatocytes isolated with chelating solution and collagenase solution are cultured in artificial devices. In 1987, Matsumura *et al*[28] reported the first clinical use of a suspension-cultured hepatocyte system using a hemodialysis chamber in which about 100 g of cryopreserved rabbit hepatocytes (about 1×10^{10} cells) were seeded. A patient in hepatic failure underwent repetitive dialysis using these hepatocyte suspensions, which resulted in a 68% decrease in total serum bilirubin and improved mental status. However, no further clinical use of this system has been reported subsequently.

Hepatocytes are anchorage-dependent cells that must adhere to survive. Thus, hybrid bioartificial liver systems, incorporated with bioreactors immobilized with hepatocytes or a bioreactor system, have been developed and tested in primary clinical trials during the past 10 years. Demetriou *et al*[29] developed a hollow-fiber bioreactor system within which porcine hepatocytes are seeded in the extrafiber space. This system has been used in the treatment of about 200 patients with hepatic failure but there seems to be equivalent hepatocyte suspension as with other treatments with a doubtful number of intact cells. Gerlach *et al*[30] introduced another interesting bioreactor design, which is a three-dimensional woven capillary network bioreactor containing hepatocytes immobilized in the extrafiber spaces. This system was applied in nine patients using porcine hepatocytes, and in eight patients using human hepatocytes obtained from a cadaveric donor liver that was unsuitable for transplantation because of severe injury. In Italy, Flendrig and Van de Kerkhove *et al*[31] developed a bioreactor using polyester non-woven fabric. They reported promising results for its clinical use to treat hepatic coma in seven patients using porcine hepatocytes with perfusion periods of 8 to 35 h. Patzer *et al*[32] investigated the efficacy of hemoperfusion compared with plasma perfusion by using a hollow-fiber bioreactor, which is currently utilized for clinical use with porcine hepatocytes.

In Japan, many research groups are engaged in developing bioreactor systems. Uchino *et al*[33] developed a pioneering system, composed of 200 collagen-coated glass plates bearing monolayer cultured canine hepatocytes stacked in an acrylic resin box. The bioreactor design appeared to be efficient, but its labor-intensive assembly prevented its widespread use.

OUR DEVELOPMENTS

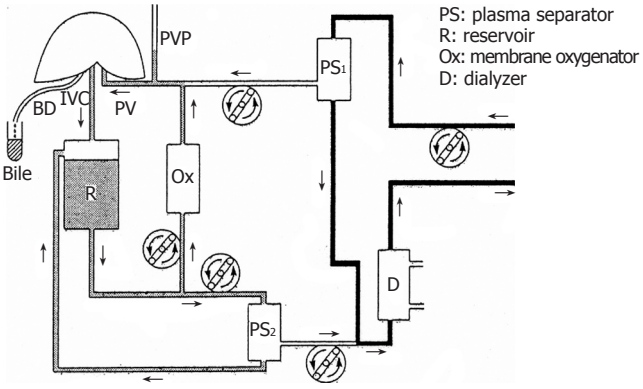

Figure 4 ECLP using porcine livers (Teraoka *et al* 1985).

Our group has developed various types of the hepatic functioning units; i.e., ECLP or bioreactor systems, for bioartificial liver systems. We devised a bioreactor filled with hepatocytes that were immobilized on a collagen-coated non-woven polyester fabric that can accommodate $2\text{-}3 \times 10^{10}$ viable hepatocytes per unit and allows direct contact between hepatocytes and perfused blood[34]. We also developed an original accommodation device of whole liver for extracorporeal perfusion in which the liver is placed in a physiological state that is supported by a special tube inserted into the inferior vena cava with pulsatory blood flow perfused by an artificial heart pump to dilate peripheral vessels and supply oxygenated whole blood to the peripheral liver tissue[35].

In bioartificial liver support in which xenogeneic liver tissue is used, it is necessary to establish a method for perfusing xenogeneic whole blood through a hepatic functioning unit without hyperacute rejection occurring. Therefore, we devised a method of xenogeneic direct hemoperfusion consisting of a leukocyte adsorbent column, an immunoglobulin adsorbent column, and a hepatic functioning unit[36]. Furthermore, we developed a method of cross plasma perfusion in which plasma exchange is carried out between the blood circuit of the patient's side and that of the hepatic functioning unit through which immunologically free whole human blood is perfused in order to achieve epidemic safety for the patient. Comparing various types of bioartificial liver in our *ex vivo* perfusion experiments, we concluded that the best efficacy can be performed by the ECLP system in view of function, safety, and operability[37].

Furthermore, we performed perfusion experiments to compare efficacy between ECLP and apheresis therapy as hemodialysis (HD) and hemofiltration (HF). A Sangen strain pig was surgically induced to hepatic failure by side-to-side portocaval shunting and ligation of the entire hepatoduodenal ligament in the porta hepatis. HD was performed with a dialysate pump at 300-350 mL/min. HF was performed by a post-dilution method with a filtration pump at 2 L/h, a supplement pump at 0.5-1 L/h, and a dialysate pump at 1-1.5 L/h. A renal preservation device (RM3: Waters medical systems; Rochester, USA) was employed for ECLP in direct hemoperfusion (Figure 5). Perfusion treatment was initiated at 3 h after

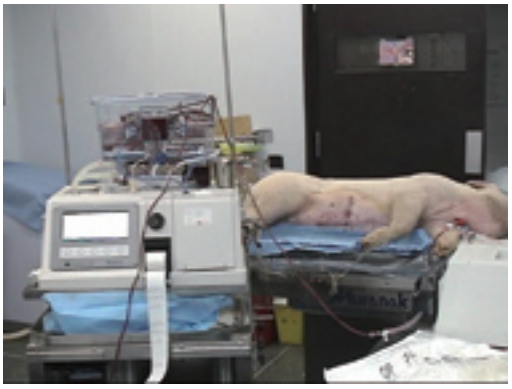

Figure 5 *Ex vivo* direct hemoperfusion experiment of ECLP (Naruse *et al*).

completion of the surgical induction of hepatic failure and continued for 3 h. As a result, in the hepatic failure control group with no treatment, serum ammonia levels increased continuously. In the HF group, it also increased continuously although the rate increase was restricted. In the HD group, it increased or gradually decreased depending on the particular case, and the average rate was almost zero. In the ECLP group, it decreased significantly, exhibiting the most superior efficacy of all the groups, and any kind of apheresis was apparently inferior to ECLP (Figure 6A). Regarding Fischer's ratio, in the Control, HF, and HD groups, it decreased continuously, although the decreasing rate was restricted in the apheresis group probably due to dilution. In the ECLP group, it increased constantly. This result shows that ECLP containing living liver can compensate for an amino acid imbalance, but apheresis therapy using an artificial membrane cannot control the amino acid balance (Figure 6B). Furthermore, ECLP secreted bile approximately 10 mL/h during the perfusion treatment. In summary, ECLP has a higher metabolic function with ammonia than HD and HF, and ECLP can compensate amino acid imbalance and secret bile, while HD and HF cannot. ECLP has the potential to reduce elevated serum ammonia levels of hepatic coma patients in a short duration and can be one option to treat hepatic failure patients in ordinary clinical therapy.

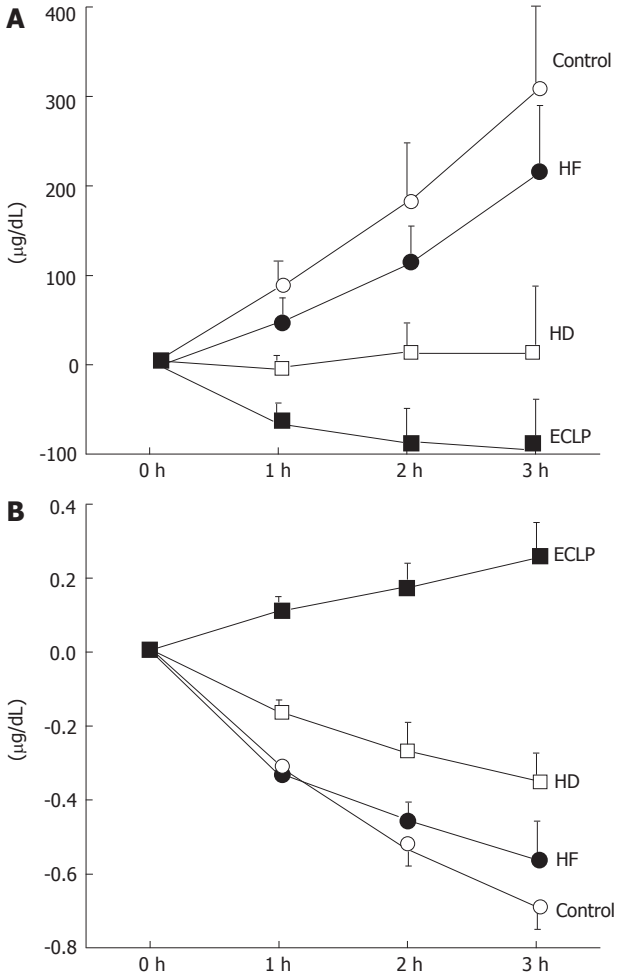

Figure 6 Comparison of efficacy between ECLP and apheresis therapy. (**A**): Ammonia; (**B**) Fischer's ratio. ○: Control group; ●: Hemofiltration (HF) group; □: hemodialysis (HD) group; ■: ECLP group.

PERSPECTIVES

Many excellent systems of bioartificial liver support have been developed, in which ECLP is likely to become a most important procedure. On the other hand, xenogeneic protein influx into the patient during on-line perfusion in bioartificial liver support is an ongoing problem. Moreover, the social antagonism for zoonosis has consistently been raised as a controversial subject surrounding the use of xenogeneic organs for treating humans. However, zoonosis does not seem to be an essential problem except for an individual's sense against treatment using animal tissue. Rather, the essential problem of bioartificial liver support is the high requirement of labor, resulting in high working costs. When these problems are solved, bioartificial liver support, especially ECLP, will greatly contribute to the treatment of patients suffering from severe hepatic failure, resulting in elevated human medicine and welfare.

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LIVER CANCER

Rising costs and hospital admissions for hepatocellular carcinoma in Portugal (1993-2005)

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Abstract

AIM: To determine, for hepatocellular carcinoma (HCC), the patient demographic profile and costs of their admissions to the hospitals of the Portuguese National Health System from 1993 to 2005.

METHODS: The National Registry (ICD-9CM, International Classification of Diseases, 155.0) provided data from the 97 Hospitals in Portugal.

RESULTS: We studied 7932 admissions that progressively rose from 292 in 1993 to 834 in 2005, having a male predominance of 78% (6130/7932). The global rate of hospital admissions for HCC rose from $3.1/10^5$ in 1993 to $8.3/10^5$ in 2005. The average length of stay decreased from 17.5 ± 17.9 d in 1993 to 9.3 ± 10.4 d in 2005, $P < 0.001$. The average hospital mortality for HCC remained high over these years, 22.3% in 1993 and 26.7% in 2005. Nationally, hospital costs (in Euros - €) rose in all variables studied: overall costs from €533000 in 1993, to €4629000 in 2005, cost per day of stay from €105 in 1993, to €597 in 2005, average cost of each admission from €1828 in 1993, to €5550 in 2005. In 2005, 1.8% (15/834) of hospital admissions for HCC were related to liver transplant, and responsible for a cost of about €1.5 million, corresponding to one third of the overall costs for HCC admissions in that same year.

CONCLUSION: From 1993 to 2005 hospital admissions in Portugal for HCC tripled. Overall costs for these admissions increased 9 times, with all variables related to cost analysis rising accordingly. Liver transplant, indicated in a small group of patients, showed a disproportionate increase in costs.

Key words: Hepatocellular carcinoma; Hospitalization; Length of stay; Disease-related group cost analysis; Hospital mortality

Marinho RT, Giria J, Moura MC. Rising costs and hospital admissions for hepatocellular carcinoma in Portugal (1993-2005). *World J Gastroenterol* 2007; 13(10): 1522-1527

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INTRODUCTION

Hepatocellular carcinoma (HCC) is now a major public health problem. It is one of the most frequently occurring malignant tumors throughout the world, being the third cause of death from cancer in men.

The mortality of HCC is particularly elevated in Africa and Asia[1,2]. In the last years there has been an increasing mortality due to HCC in Western Europe,[3,4] the United States of America[5] and Japan[6,7].

In the great majority of cases, HCC appears in patients who have liver cirrhosis, especially in those regions of relatively low incidence as Western Europe. Liver cirrhosis is a chronic disease carrying a high oncogenic risk, with a rate of evolution to HCC at an annual incidence of 1%-4%[8]. HCC has a poor prognosis but the three main situations that cause it are amenable to prevention, i.e. hepatitis B[9,10] and C[11,12] infections, and excessive alcohol consumption[13]. Increasing rates of hepatitis C virus (HCV) infection could explain a proportion of the reported increase in HCC incidence during the last decade[14]. Liver transplant in selected cases is an effective option for patients with HCC. The percentage of HCC cases considered for liver transplant, for both on the waiting list and those already transplanted, has also been increasing, resulting in mounting costs to the health system for the management of these patients[15].

Databases of hospital admissions, obtained from patient classification systems that are based on principal diagnosis of discharge episode, may be used to measure the morbidity of the inpatient population and the demand that certain diseases make on health systems[16]. We used the Diagnosis-Related Groups (DRG)[17] database to classify inpatients according to epidemiological variables of sex, age, principal and associated diagnoses, technical procedures carried out for diagnosis, mortality, and location of hospital admission[18]. In addition, this

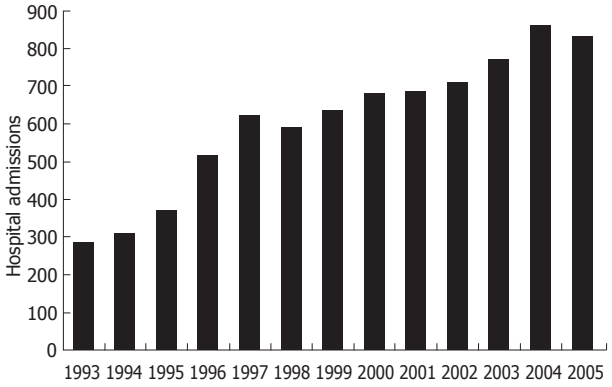

Figure 1 Admissions in the 97 Portuguese hospitals belonging to National Health System (1993-2005).

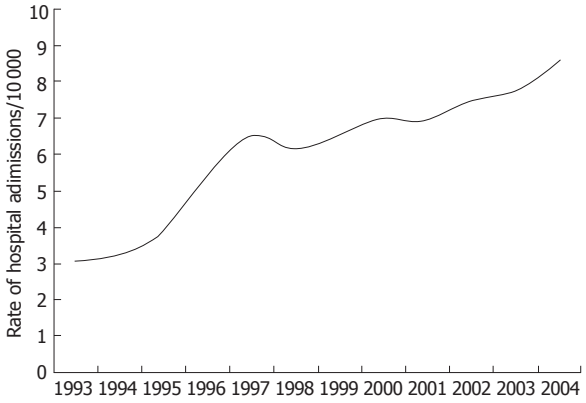

Figure 3 Hospital admissions per 100 000 inhabitants.

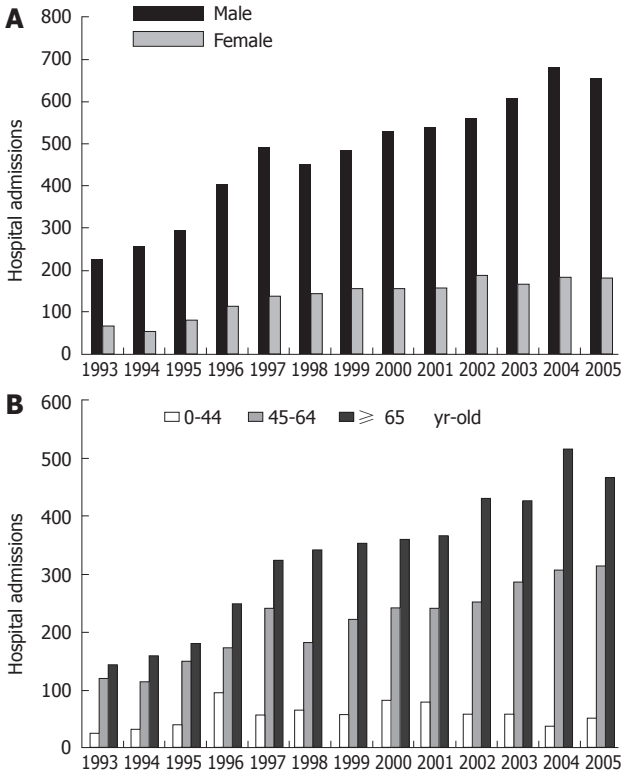

Figure 2 **A**: Distribution of the hospital admissions according to gender; **B**: Distribution of the hospital admissions according to age.

database allows for the tracking of variables of resource consumption i.e. length of stay, costs per day according to the type of episode, and costs of episode of hospital admission. Economic analysis of chronic diseases is a prerequisite for planning a proper distribution of health care resources.

Using such data, the aim of this work was to determine the profile of patients with HCC, and the costs of their hospital admissions during the period between 1993 and 2005 to the 97 public hospitals of the National Health System of Portugal, serving now a population of about 10 million inhabitants.

MATERIALS AND METHODS

We studied the demographic data and resource consumption of hospital admissions for HCC to the 97 hospitals of the National Health System in Portugal during the period of 1993 to 2005.

The study is descriptive, based on the DRG database. We used the International Classification of Diseases, Ninth Revision, Clinical Modification, (ICD-9-CM), primary liver tumor (155.0), and evaluated the following variables: gender, age, mortality, length of stay, overall costs, costs per day and per episode of hospital admission, and costs related to liver transplant in HCC.

The population of Portugal and number of deaths for HCC were obtained from the "Instituto Nacional de Estatística (INE)".

Specific rates for 100 000 were calculated by age group and gender.

RESULTS

In the period 1993-2005, 7932 hospital admissions for HCC were registered in Portugal. A progressive 2.9 fold increase was verified in the period studied: 292 hospital admissions in 1993, 635 in 1999 and 834 in 2005 (Figure 1).

Gender distribution data showed a 77.6% (6155/7932) predominance of male patients, at a ratio of 3.4:1 in 1993, and 3.6:1 in 2005 (Figure 2A). This percentage has remained stable over the years: 77.1% in 1993, and 78.2% in 2005. The evolution of episodes of hospital admission per 100 000 inhabitants similarly showed a progressive increase from 3.1×10^5 (men 4.9, women 1.4) in 1993, to 8.6×10^5 in 2005 (13.4 in men and 3.5 in women) (Figure 3).

The average age for hospital admission was 62.0 ± 16.4 years in 1993, and 63.9 ± 13.3 in 2005. Most hospital admissions occurred in patients older than 64 years, increasing from 49.3% (144/292) in 1993 to 55.8% (466/834) in 2005, P = NS. Findings for all age groups showed that 54.5% (4319/7932) were older than 64 years, 36.0% (2853/7932) were between 45-64 years and only 9.6% (760/7932) were between 0-44 years. The rate of hospital admission per 100 000 in the age groups of 0-44, 45-64 and over 64-years was respectively 0.5, 5.5 and 10.4 in 1993 and 0.9, 12.5 and 26.7 in 2005 (Figure 2B).

The average length of stay for HCC was higher than the national average (9.0 d in 1993 and 7.7 d in 2005) but it has shown a decrease from 17.5 d in 1993 to 9.3 d in 2005, $P < 0.05$ (Figure 4A). Hospital mortality for HCC

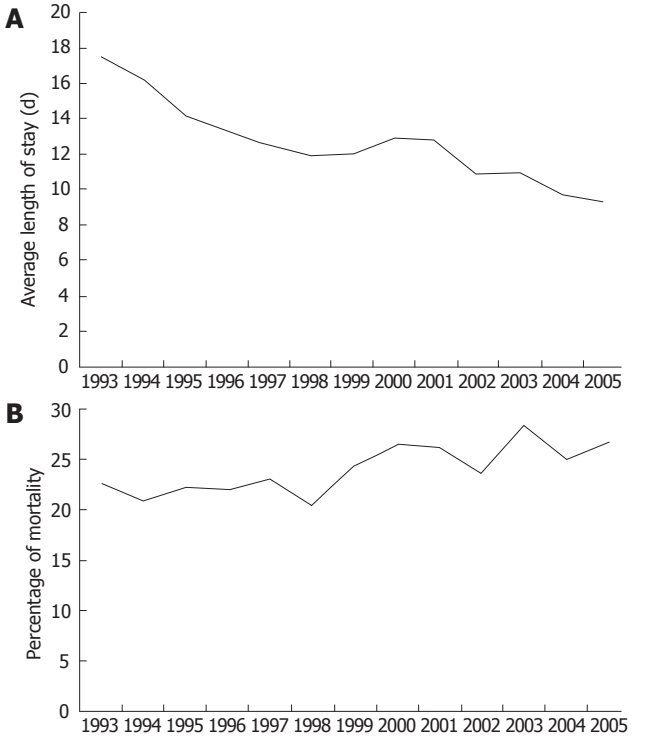

Figure 4 **A**: Evolution of the average length of stay for HCC in Portuguese hospitals; **B**: Evolution of the lethality of the hospital admissions for HCC.

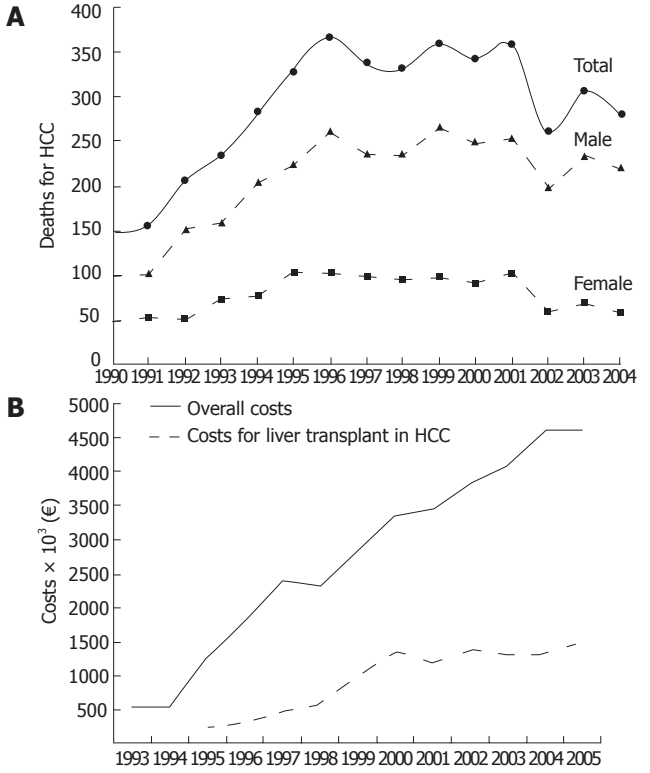

Figure 5 **A**: Deaths for HCC in Portugal (National Institute of Statistics); **B**: Global costs for hospital admissions for HCC.

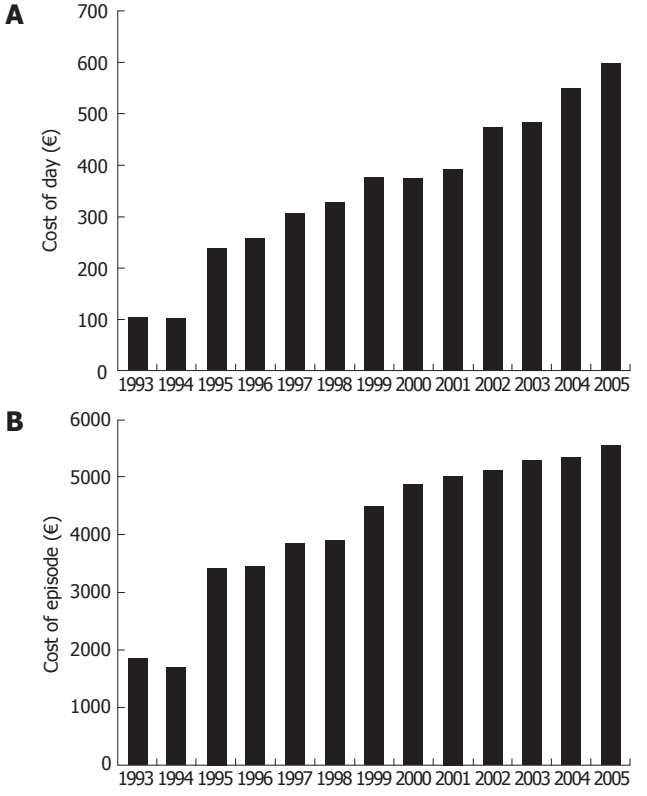

Figure 6 **A**: Cost of day of hospital admission; **B**: Cost of episode of hospital admission.

remained very high, 22.6% in 1993 and 26.7% in 2005 (Figure 4B). As would be expected, the mortality was higher in older patients, 12.5% in the group 0-44 years-old, 27.3% in 45-64 years, and 28.3% in patients older than 64 years. In addition, the figures given by the National Institute of Statistics for inpatient and outpatient mortality for HCC in Portugal showed an increase, from 149 deaths in 1990, to 280 in 2004 (Figure 5A). In determining the costs for hospital admissions for HCC in the Portuguese Health System, we found a significant increase for all economic variables studied. The overall costs of hospital admissions increased 8.7 times, from €533 000 in 1993, to €4 629 000 in 2005 (Figure 5B). In the same period the average cost of each day of hospital admission 5.7 times from €105 in 1993, to €597 in 2005 (Figure 6A). The average cost of each stay in hospital for HCC increased 3 times from €1828 in 1993, to €5550 in 2005 (Figure 6B)

Liver transplants in Portugal began in 1992. Of all liver transplants performed between 1992 and 2005, 7.2% (117/1619) were liver transplants for HCC. But, this percentage almost tripled from 3.3% in 1993 (1/30) to 9.0% in 2005 (15/166), P = NS. Today, costs for hospital admissions for patients transplanted with the indication of HCC are 32.5% of the total costs for all hospital admissions for HCC (Figure 5A).

These means, in effect, that the costs for 15 patients with HCC transplanted in Portugal in 2005 represent one third of the costs for all 834 hospital admissions for HCC in that same year.

DISCUSSION

The number of patients diagnosed with HCC has been increasing in Japan[19], the United States[20,21], and Europe[22]. HCC particularly affects patients who have liver cirrhosis. The characteristics of liver cirrhosis that lead to HCC,

namely its frequent complications and instability, are associated with multiple episodes of hospital admissions[23], mainly in the phase of decompensation (ascites, jaundice, spontaneous peritonitis and encephalopathy).

Our work revealed a 2.9 fold increase in hospital admissions for HCC in Portugal over the twelve years studied (1993-2005), reaching 834 hospital admissions in 2005 ($8.6/10^5$). As some patients are admitted to hospital several times each year, these figures do not correspond to the real incidence of HCC. A recent study in United States has documented a similar increase in the hospital admissions for HCC[24].

Male gender has also been documented as a risk factor for HCC, and the male/female ratio is usually 2 to 3:1[25]. Our data is in accord with this ratio: 78% of hospital admissions were for male patients, and in spite of the increase in numbers for both genders, the male/female ratio was 3.5:1 in the period studied.

HCC has been found to be more frequent in individuals in their sixties[26]. The average age of patients admitted to hospitals in Portugal for HCC was 61.2 ± 16.7 years in 1993, and 63.9 ± 13.5 years-old in 2005. More than half of hospital admissions now occur in patients older than 64, increasing from 49.3% (144/292) in 1993, to 55.8% (466/834) in 2005, P = NS. When we analyzed the rate of hospital admissions per 100.000 inhabitants, the higher rates were found to be more elevated in patients older than 64 ($26.7/10^5$ in 2005). This rate is higher than the group 45 to 64-years-old ($12.5/10^5$). The rarity in younger ages is evident: in the group 0-44 years-old was $0.9/10^5$ in 2005. The rate of hospital admissions increased in all groups of patients, but more in the older ones: 1.8 times more ($0.5/10^5$ in 1999 to $0.9/10^5$ in 2005), 2.3 times more ($5.5/10^5$ to $12.5/10^5$) and 2.6 times more ($10.4/10^5$ to $26.7/10^5$) respectively, in patient groups 0-44, 45-64 and over 64-year-old in 1993 and in 2005. Several studies, notably in Scotland, Denmark, France, and Japan have found the same tendency, corroborating our findings that HCC occurs mainly in patients older than 65 years-old[27,28].

The demographic data and rate of mortality (Ministry of Health 2003) by Chronic Liver Disease and Cirrhosis (ICD-9-CM:347) are similar to that for HCC admissions. In effect, of the 1599 deaths registered in Portugal for cirrhosis, 73.2% (1172/1599) were male, and the age group with the most number of deaths was that over 65 years-old, having 40% of the total (638/1599). A higher rate of mortality was verified in persons between 65 and 74 years old with $39.5/10^5$ (men $66.9/10^5$ and women $17.4/10^5$), compared to the national rate of $15.3/10^5$[29].

In addition, the data provided by the National Registry of Deaths, which included mortality both for inpatients and outpatients, showed that deaths due to HCC (ICD 9:155.0 ICD 10:C220) almost doubled, from 149 in 1990 to 280 in 2004.

One of the most important indexes of health resource consumption, the average length of stay[30], was found to be higher in HCC patients when compared to the national average of 7.7 d; albeit, there was a tendency towards decrease from 17.5 d in 1993 to 9.3 d in 2005, $P < 0.05$, a trend that can be explained by the improvement of medical management for these groups of patients.

HCC is now the third cause of death by malignant tumors in males throughout the world[31]. Hepatocellular carcinoma is a disease with high mortality; one quarter of patients admitted to hospitals in Portugal for HCC died.

The overall mortality during hospitalization did not change over time, 26.7% in 2005 closely following the percentage of twelve years ago of 22.6% (1993). In addition, considering the figures of national mortality for HCC, the increase is more pronounced in the male sex, representing a 2.2 fold increase between 1990-2004, compared to females at 1.2 times. The same has been found by other authors[32]. The rate of mortality adjusted for age, $3.7/10^5$ in men and $0.7/10^5$ in women, being less than that generally found in other Mediterranean countries ($5\text{-}12/10^5$ in Spain, France, Italy)[3], suggests that the real number, in Portugal because of insufficient diagnosis, might be higher.

The reasons for the increase in these figures are not well understood, but several hypotheses can be put forth:[33] the increase in diagnostic yield, the increase of liver cirrhosis associated with hepatitis C infection resulting from intravenous drug addiction and blood transfusions in the sixties[34], the improvement of average survival in patients with liver cirrhosis due to better management[35].

A significant increase in the costs of hospital admissions for HCC was verified for all variables: over-all costs, cost per episode, and costs per day of hospital admission. The over-all costs increased 8.7 times since 1993 (€533 000), reaching the figure of €4 629 000 for the year 2005. In the twelve years studied, the average cost per day increased 5.7 times, from €105 to €597, and the average cost per episode increased 3.0 times, from €1828 to €5551.

It is important to emphasize that liver transplantation in HCC, indicated in around 7% of cases only, is the kind of therapy most effective in the long term[36,37], but carries a very high cost. The relative percentage of costs for transplantation in HCC in relation to the overall costs of hospital admission for HCC in Portugal has been increasing significantly, from 14.3% in 1993 for one transplant, to 32.5% (€1 505 669) in 2005 for 15 liver transplants. Put in another way, in 2005, 1.8% (15/834) of hospital admissions for HCC, i.e. related with liver transplant, were responsible for a cost of about €1.5 million, corresponding to one third of the over-all costs all hospital admissions for HCC in that same year.

These data suggest that patients with HCC must be strictly selected for liver transplant[38] because the cost of a liver transplant for HCC appears to exceed in effect the lower long-term survival rate when compared with other indications[39] deemed significant to a health system.

In conclusion, HCC, from the point of view of hospital admissions, promises to significantly impact the Portuguese Public Health System Hospitals, due to the steady increase in the number of hospital admissions, 2.9 times over the period 1993 to 2005. The mortality for HCC in hospitals has been steadily rising, affecting a quarter of hospital admissions; thus HCC is a disease

that is consuming a growing share of resources. The overall costs for HCC increased dramatically, 8.7 times in the 12 years studied, 5.7 times for the average length of hospital stay, and 3 times for the cost of each day. HCC is indicated for liver transplant in a small number of cases only, remains a therapeutic option with high relative costs for the health care system. So, liver transplant for HCC, albeit potentially curative in selective cases, must be strictly indicated.

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COLORECTAL CANCER

Stool-based DNA testing, a new noninvasive method for colorectal cancer screening, the first report from Iran

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Abstract

AIM: To detect tumor-associated DNA changes in stool samples among Iranian patients with colorectal cancer (CRC) compared to healthy individuals using BAT-26, *p*16 hypermethylation and long DNA markers.

METHODS: Stool DNA was isolated from 45 subjects including 25 CRC patients and 20 healthy individuals using a new, fast and easy extraction method. Long DNA associated with tumor was detected using polymerase chain reaction method. Microsatellite studies were performed utilizing denaturating polyacrylamide gel to determine the instability of BAT-26. Methylation status of *p*16 promoter was analyzed using methylation-specific PCR (MSP).

RESULTS: The results showed a significant difference in existence of long DNA (16 in patients *vs* 1 in controls, $P < 0.001$) and *p*16 (5 in patients *vs* none in controls, $P = 0.043$) in the stool samples of two groups. Long DNA was detected in 64% of CRC patients; whereas just one of the healthy individuals was positive for Long DNA. *p*16 methylation was found in 20% of patients and in none of healthy individuals. Instability of BAT-26 was not detected in any of stool samples.

CONCLUSION: We could detect colorectal cancer related genetic alterations by analyzing stool DNA with a sensitivity of 64% and 20% and a specificity of 95% and 100% for Long DNA and *p*16 respectively. A non-invasive molecular stool-based DNA testing can provide a screening strategy in high-risk individuals. However, additional testing on more samples is necessary from Iranian subjects to determine the exact specificity and sensitivity of these markers.

Key words: Stool DNA; Colorectal cancer; Cancer screening; Long DNA; BAT-26; *p*16

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INTRODUCTION

Colorectal cancer is one of the most common forms of cancer in the world and is curable if diagnosed at an early stage[1]. Extensive research over the past 15 years has shown that a specific series of genetic changes drives the neoplastic transformation of normal colonic epithelium to benign adenomas and subsequently to malignant adenocarcinomas[2]. Colon cancers arise from at least three different genetic pathways: chromosomal instability, microsatellite instability, and CpG island methylation. Chromosomal instability accounts for about 85% of sporadic colorectal cancers. Microsatellite instabilities that are replication errors (RERs) caused by germline or somatic mutations of mismatch gene, are involved in the development of some colorectal cancers[3]. Loss of function of any of mismatch repair genes may lead to a failure in repair mutations and development of cancers[4,5]. One microsatellite, BAT-26, a single locus of 26 consecutive adenine nucleotides is strongly associated with failure of a mismatch gene. Thus, testing for mutations in BAT-26 is almost as effective as screening most microsatellite loci[6,7]. Several studies showed the relationship of this marker and colorectal cancers[8,9]. It is implicated in about 20% of right-sided colorectal cancers

while in only 1% to 2% of left-sided colon cancers.

One other pathway known to be involved in the pathogenesis of colorectal cancer is the methylation of the CpG islands located within the promoter regions of genes regulating cell proliferation, apoptosis, and DNA repair. The detection of hypermethylated fecal DNA has been reported by others in a few studies[10,11]. Methylation often affects multiple genes. It also occurs as an age-related phenomenon in morphologically normal mucosa. *p*16, a tumor suppressor gene silenced by hypermethylation of its promoter in early stages of cancer, provide a valuable approach to screening for early lesions[12].

The discovery of these genetic and epigenetic alterations has raised the possibility of detecting colorectal cancer through examination of the stool DNA as a healthy adult excretes approximately 10^{10} epithelial cells every day[13]. A large number of tumor cells will renew and exfoliate into the intestinal cavity of colonic cancer patients daily. A certain amount of DNA can maintain its stability due to the resistance of intestinal tumor cells to various degradation enzymes or due to the impairment of apoptotic mechanism of tumor cells. Therefore, molecular examination of the genetic composition of the colonic mucosal cells, which are exfoliated into the stool, brought new options for colorectal cancer screening. Sidransky *et al*[14], for the first time, detected the K-ras gene mutations in the fecal samples from early intestinal cancer patients. Since then, several studies have shown that it is possible to detect mutations of these genes in stool samples from patients with colorectal cancer. Stool-based DNA testing has gradually become a screening method for colorectal cancer[15-17].

The amount of human DNA in feces may be increased in individuals with colorectal cancer. Kelaassen *et al*[18] demonstrated increased amounts of human DNA in the feces of patients with colorectal tumors compared with healthy persons. Boynton *et al*[19] showed that the majority of DNAs isolated from the stool of patients with colorectal tumors were of high molecular weight, in contrast to the fragmented apoptotic DNAs found in stools from colonoscopy-negative patients. They proposed that the increased concentrations of human DNA could be explained by decreased apoptosis of bowel cells and/or increased shedding of cancer cells into the colonic lumen. There is evidence that transformed colonic mucosa cells have dysfunctional apoptotic mechanisms[20] and thus may shed cells that have not undergone apoptosis. Because one of the characteristics of apoptosis can be the cleavage of DNA into 180 to 210 bp fragments[21], dysfunction of apoptotic mechanisms will lead to presence of high-molecular-weight fragments (more than 1 Kb) of DNAs, which are named as Long DNA. Therefore, long DNA becomes a valuable marker in stool based DNA testing.

Newer assays examining more than one mutation are significantly more sensitive. They include more than 20 mutations on APC, *p*53 and k-ras, microsatellite analysis for BAT-26 and Long DNA and methylation markers[22,23]. In this study, we have established a stool-based DNA assay to detect Long DNA and BAT-26 markers and *p*16 methylation in patients with colorectal cancer in Iran.

MATERIALS AND METHODS

Sample collection

Human stool samples were collected from 45 individuals including 20 healthy colonoscopic volunteers and 25 patients with colorectal cancer without any dietary restrictions or antibiotic treatment. About 5 g stool was collected from each individual. All the samples were collected in dry clean plastic containers. Informed consent was obtained from every subject prior to the study. Stools were collected prior to any preparation for colonoscopy or 4-5 d following this procedure. Tumor characteristics such as location, size, histological features, stage and, in addition, age, sex and fecal occult blood test (FOBT) results were considered. The stool specimens were stored at -20℃ immediately after collection, to avoid potential enzymatic degradation of nucleic acids, and then transferred to a -70℃ refrigerator within 24 h until use. The information of 21 patients is shown in Table 1.

DNA extraction

For DNA extraction, 1 g of stool, frozen at -70℃, was diluted in 10 mL of lysis buffer (0.5 mol/L Tris-HCl, 20 mmol/L EDTA, 10 mmol/L NaCl, 0.1% SDS, pH = 9.0) (TEN-9) in a 50 mL tube. After vortexing for 5 min, samples were homogenized by shaking for 10 min. A second dilution (1/2) step was performed with 10 mL lysis buffer and homogenized for 5 min. Particulate materials were removed by centrifugation at 4500 r/min for 10 min. The supernatant was transferred to a new tube, approximately 15 mL DNA was precipitated by addition of 7.5 mL ammonium acetate 7.5 mol/L (half the sample volume) and 30 mL of ice-cold ethanol 95%-100% (twice the sample volume). Incubation at -20℃ for 30-45 min rendered a better precipitation. DNA was collected following centrifugation at 4500 r/min for 15 min at RT. In this step, precipitated DNA was not colorless and contained the bile salts. The DNA pellet was re-suspended in 750 μL of TE (pH = 8) and incubated at 65℃ for 15 min. Then DNA was purified using the conventional single step phenol/chloroform protocol. Phenol would eliminate the colored impurities. After isopropanol precipitation, colorless DNA pellet was collected and dissolved in 300 μL of TE buffer following an overnight incubation at 37℃.

Long DNA analysis

A 1476 bp fragment including exons 6 to 9 of p53 gene was used for long DNA analysis. Primers were previously described by Beroud *et al*[24] (Forward: 5' GCCTCTGATTCCTCACTGAT 3' and reverse: 5' AAGACTTAGTACCTGAAGGGT 3'). The PCR reaction mixture consisted of 1 × CinnaGen PCR buffer, 500 nmol/L of each PCR primer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs and 1 U of Taq DNA polymerase (CinnaGen, Tehran, Iran). Five hundred ng DNA diluted in 200 μL TE (pH = 8.0) was used in a reaction volume of 20 μL. PCR conditions were as follows: 3 min at 95℃ followed by 35 cycles of 40 s at 95℃, 120 s at 58℃ and 120 s at 72℃, and 5 min at 72℃ as final extension, with maximum heating and cooling settings in the Techne

Table 1 Demographic and clinicopathological characteristics of patients

Patients	Sex	Age (yr)	Tumor type	Tumor size (cm)	Tumor location	Stage	FOBT	Long DNA	Instability BAT-26	*p16*
1	M	37	A.C	10	AC	B2	+4	+	-	M
2	M	52	A.C	5	C	B2	+4	+	-	U
3	F	64	A.C	15	C	C2	+4	+	-	U
4	M	62	A.C	2	R	B1	+1	+	-	U
5	F	34	A.C	-	Sp	B2	-	+	-	U
6	M	50	A.C	10	Hp	B2	+4	-	-	U
7	M	52	A.C	7	R	C2	-	+	-	U
8	M	70	A.C	3	AC	B2	+2	-	-	U
9	M	83	A.C	1	R	-	+2	-	-	U
10	F	50	A.C	1.5	Sp	B2	+4	+	-	U
11	M	64	A.C	3	R	C2	+3	+	-	M
12	M	83	A.C	4.5	R	-	+4	+	-	M
13	M	72	A.C	8	C	B2	+4	+	-	U
14	M	64	A.C	3	R	C2	+2	+	-	M
15	F	73	A.C	6	-	B1	+4	+	-	U
16	M	43	A.C	7	AC	B1	-	+	-	U
17	F	67	A.C	3.5	AC	B2	+4	-	-	U
18	F	53	A.C	8	Sp	C2	+4	+	-	M
19	M	49	A.C	3	C	B2	+4	-	-	U
20	F	70	A.C	5	R	B2	+3	-	-	U
21	M	51	A.C	6	R	B2	+4	-	-	U

R: Rectum; C: Cecum; AC: Ascending colon; Sp: Splenic flexure; Hp: Hepatic flexure; A.C: Adenocarcinoma; FOBT: Fecal occult blood test. M: Methylated; U: Unmethylated.

Thermal Cycler (Techgene, Techne, UK).

Five microliters of amplified products were electrophoresed on 1.7% agarose gel, and stained with ethidium bromide. The presence of the 1500 bp band was considered as Long DNA. DNA extracted from blood was used as the positive control. We also used amplification of a short fragment (138 bp), including exon 9 of *p*53 gene, to evaluate the extraction method.

Microsatellite studies

For microsatellite analysis, BAT-26 was used as the microsatellite target. The purified stool DNA samples were subjected to PCR amplification of the BAT-26 sequence in 20 μL reaction mixture containing 1 μL (about 200 ng) of purified DNA (or diluted DNA), 1 × PCR buffer (Cinnagen), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 10 pmol BAT-26 sequence-specific primers, and 1 U of Taq DNA polymerase (Cinnagen). The primers have been described by Devouassoux-Shisheboran *et al*[25] (Forward: 5' TGACTACTTTTGACTTCAGCC 3'and reverse: 5' AACCATTCAACATTTTTTAACCC 3'). Amplifications were conducted in a Techgene thermocycler. After an initial denaturation at 94℃ for 5 min, PCR amplification was performed for 35 cycles, each consisting of 45 s at 94℃, 2 min at 54℃, and 2 min at 72℃, followed by a final extension of 30 min at 72℃. Appropriate amount of PCR-products was mixed with a formamide loading buffer and denaturated by boiling for 5 min at 95℃. The mixture was then loaded onto 6% polyacrylamide gel containing 7 mol/L urea.

Following the denaturating gel electrophoresis at 60 W (50 mA; 1200 V) for 60 min in 1 × TBE (89 mmol/L Tris; 2 mmol/L EDTA; 89 mmol/L Boric acid), the gels were stained with silver nitrate as described by Creste *et al*[26]. BAT26-associated instability was identified on the basis of comparison between electrophoretic patterns of tumor and their corresponding blood samples. Increased number of bands in tumor BAT-26 PCR products as compared to blood samples indicated microsatellite instability.

p16 methylation analysis

Stool DNA (2 μg) was treated with sodium bisulfite as previously described[12,27]. Modified DNA was purified using a Promega Wizard DNA Clean-Up System, according to the manufacturer's instructions and then was stored at -20℃ until it was used for PCR. Modified DNA was amplified using primers specific for methylated and unmethylated *p*16 sequences as previously described[12]. DyNAzyme Ⅱ Hot start Taq (Finnzymes, Finland) was used as DNA polymerase. PCR conditions were as follows: 10 min at 95℃ followed by 45 cycles of 45 s at 95℃, 45 s at 60℃ and 60 s at 72℃; and 5 min at 72℃ as final extension, with maximum heating and cooling settings in the Techne Thermal Cycler (Techgene, Techne, UK). Five microliters of amplified products were electrophoresed on 3% agarose gel, and stained with ethidium bromide. The presence of a 150 bp band was considered for methylated and a 151 bp band for unmethylated product. DNA extracted from blood and treated with CpG methylase (M.Sss1, Newengland BioLabs) was used as the positive methylated control.

Statistical analysis

The relationship between Long DNA, *p*16 methylation and BAT-26 instability and clinicopathological parameters, as listed in Table 1, was analyzed using SPSS version 11.5 and the *P* value was calculated using Chi-square and Fisher exact tests to find the significant relationships.

RESULTS

*p*53 exon 9, representing the short fragment (138 bp), was amplifiable in 24 out of 25 patient samples and 18 out of 20 control samples. It was revealed that the extraction

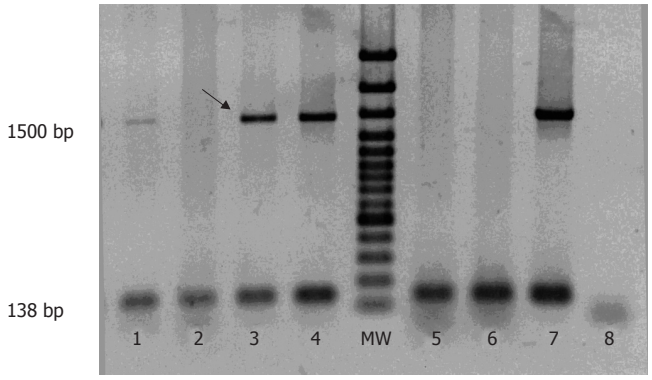

Figure 1 Long DNA analysis in patients with colon cancer in comparison with healthy individuals. 1, 2, 3, 4: Stool DNA of patients with colorectal cancer; MW: Ladder 100 bp; 5, 6: Stool DNA of healthy individuals; 7: Blood DNA as control; 8: Negative control. The arrow indicates Long DNA. Long DNA detected in 3 out of 4 patients in the picture. The 1.7% agarose gel plus ethidium bromide was used for electrophoresis at 120 volt.

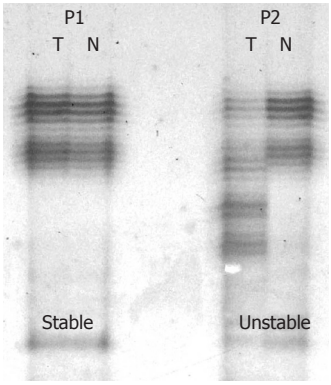

Figure 3 Microsatellite analysis of BAT-26 in two patients with colon cancer. BAT-26 instability detected in patient 2. P1: patient with stable BAT-26, P2: Patient with instable BAT-26, T: Tumor tissue, N: Normal margin. The gel was electrophoresed at 60 W (50 mA; 1200 V) for 60 min using 1 × TBE.

protocol produced enough amplifiable DNAs in most cases suitable for standard PCR amplifications. Long DNA (a nearly 1476 bp band) was detectable in 16 out of 25 colorectal cancer patients and only in 1 out of 20 healthy individuals. There was a significant difference of this marker in the stool samples of two groups ($P < 0.001$). The sensitivity for this marker was 64% and the specificity was 95%. Results of representative CRC patients and healthy individuals are shown in Figure 1. Methylation of *p*16 promoter was detected in 5 out of 25 patients compared to none in healthy controls ($P = 0.043$). The sensitivity for this marker was 20% and the specificity was 100%. Methylation analysis results are shown in Figure 2. Interestingly, all the hypermethylated *p*16 samples were also positive for Long DNA. Instability of BAT-26 was not found in any of colorectal cancer patients using stool DNA; whereas we could detect instability for BAT-26 using tumor tissue samples of the patients after surgery. The instability of BAT-26 was detected in two out of 25 patients (8%) using the tumor tissue. The results are shown in Figure 3.

Four samples were removed from statistical studies, one sample due to not producing the small fragment band (138 bp). It means the extraction protocol has not produced sufficient DNA for amplification. Three other samples were removed due to incomplete profile and lack of clinicopathological parameters. The data for the

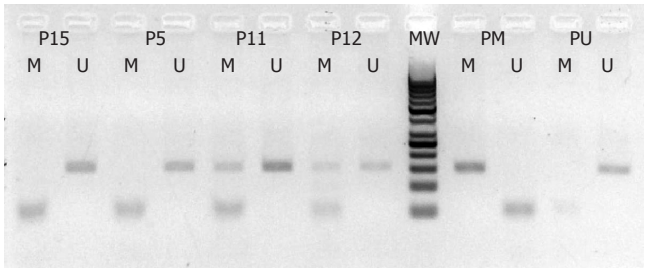

Figure 2 Methylation analysis of p16 among four patients. P15: patient 15; P5: patient 5; P11: patient 11; P12: patient 12; MW: Ladder 50 bp; PM: Methylated control; PU: Unmethylated control; M: Methylated PCR; U: unmethylated PCR. Methylated p16 detected in patients 11 and 12.

remaining 21 cases are presented in Table 1. The mean age of the patients was 59.2 years. Sixty six percent of patients were male and 34% were female. The most common tumor site was rectum (40%); other sites were cecum (20%), splenic flexture of colon (15%), ascending colon (20%) and hepatic flexture (5%), respectively. All the tumors were invasive adenocarcinoma. The tumor stages were reported as 15.8% B1, 57.8% B2, and 26.4% C2 in patients with available clinicopathological data. Tumor sizes were less than 3 cm in 35%, 3-6 cm in 30% and more than 6 cm in 35%. Although it seemed that there might be some relationship between the presence of Long DNA and *p*16 methylation in stool samples and clinicopathological parameters while reviewing Table 1, comprehensive statistical studies, using Chi-square and Fisher exact tests revealed that there was no statistically significant relationship.

DISCUSSION

Many investigators studied different genetic alternations in stool-DNA of CRC patients and determined their sensitivities and specificities as a diagnostic tool. Ahlquist *et al*[15] analyzed stool samples in a blinded fashion from 22 patients with colorectal cancer, 11 patients with adenomas at least 1 cm in size, and 28 patients with endoscopically healthy colons. The assay targeted point mutations at any of 15 sites on K-ras, *p*53, APC, and the microsatellite instability marker BAT-26 and Long DNA. The sensitivity was 91% for cancer and 82% for adenomas 1 cm or larger; the specificity was 93%. They could detect Long DNA in 14 out of 20 cancers (70%) and 6 out of 11 adenomas (54.5%). However, Long DNA and BAT-26 were not detected in any of normal patients. Syngal *et al*[17] used a fecal-based assay to detect 23 DNA markers, including 21 point mutations in K-ras, APC, and p53; the microsatellite instability marker BAT-26; and Long DNA. The sensitivity was 68% for invasive colorectal carcinoma, 40% for adenomas with high-grade dysplasia, and 20% for adenomas with low-grade dysplasia. Overall, the sensitivity of multitarget DNA stool assays ranged from 68% to 91% for colorectal cancer and from 40% to 82% for advanced adenomas. The specificity of the assays was approximately 95%[28,29]. Methylation of *p*16 in stool samples of CRC patients has been reported[10]. Although it was detected in smaller percentages of patients compared to our study, it

still remains unconclusive for its sensitivity and specificity in a stool-based DNA testing.

We could detect 64% of colon cancer cases using three genetic markers, 64% were positive for Long DNA, 20% for methylation of *p*16 and none for instable BAT-26. However, additional testing of more samples is necessary from Iranian subjects to determine the exact specificity and sensitivity of these markers. We could detect the instability for BAT-26 in 2 out of 25 patients (8%) using tumor tissues after surgery, whereas we detected no instability when DNAs extracted from stool were applied for microsatellite studies. This may suggest that the proportion of unstable DNA to total extracted DNA was very low. It seems further procedures are required to increase the unstable DNA following our extraction protocol. Oligo capture was recommended by previous researchers to solve this problem[15].

There are still many obstacles for stool-based DNA testing to become a worldwide screening method. Stool-based DNA testing is noninvasive, and it is more sensitive than fecal occult blood testing. Only a single stool sample is needed, and the patient and physician do not need to handle it as much. The test does not require diet or medication restrictions, it evaluates the whole colon and rectum, and it is now generally available. However, it is expensive, it is less sensitive than colonoscopy, and if the stool-based test is positive, colonoscopy is still necessary. The positive predictive value is low, and there is uncertainty regarding how to manage patients with a positive test and a healthy colonoscopic test. It is unclear whether screening for extracolonic malignancies will prove to be an advantage of stool-based DNA testing.

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COLORECTAL CANCER

Rosiglitazone enhances fluorouracil-induced apoptosis of HT-29 cells by activating peroxisome proliferator-activated receptor γ

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Abstract

AIM: To examine whether and how rosiglitazone enhances apoptosis induced by fluorouracil in human colon cancer (HT-29) cells.

METHODS: Human colon cancer HT-29 cells were cultured *in vitro* and treated with fluorouracil and/or rosiglitazone. Proliferation and growth of HT-29 cells were evaluated by MTT assay and trypan blue exclusion methods, respectively. The apoptosis of HT-29 cells was determined by acridine orange/ethidium bromide staining and flow cytometry using PI fluorescence staining. The expressions of peroxisome proliferator-activated receptor γ (PPARγ), Bcl-2 and Bax in HT-29 cells were analyzed by Western blot.

RESULTS: Although rosiglitazone at the concentration below 30 μmol/L for 72 h exerted almost no inhibitory effect on proliferation and growth of HT-29 cells, it could significantly enhance fluorouracil-induced HT-29 cell proliferation and growth inhibition. Furthermore, 10 μmol/L rosilitazone did not induce apoptosis of HT-29 cells but dramatically enhanced fluorouracil-induced apoptosis of HT-29 cells. However, rosiglitazone did not improve apoptosis induced by fluorouracil in HT-29 cells pretreated with GW9662, a PPARγ antagonist. Meanwhile, the expression of Bax and PPARγ was up-regulated, while the expression of Bcl-2 was down regulated in HT-29 cells treated with rosiglitazone in a time-dependent manner. However, the effect of rosiglitazone on Bcl-2 and Bax was blocked or diminished in the presence of GW9662.

CONCLUSION: Rosiglitazone enhances fluorouracil-induced apoptosis of HT-29 cells by activating PPARγ.

INTRODUCTION

Colon cancer is a leading cause of cancer-related death in developed countries[1]. Fluorouracil (5-Fu) is one of the most widely used chemotherapeutic drugs in the treatment of advanced colorectal cancer patients[2]. However, the patient response to this single anticancer agent is 10%-30%[2]. Several mechanisms are responsible for resistance of tumor cells to fluorouracil. On the other hand, the dose increments of systemic administration of 5-Fu would generate unacceptable levels of toxicity to the normal cells of bone marrow and gastrointestinal tract[3]. Therefore, many attempts have been made to enhance its therapeutic effectiveness and reduce its toxicity[4,5]. As we known, the common strategies are to develop and validate new chemopreventive and therapeutic approaches to colon cancer by making use of chemosensitizers or combination of drugs.

It was recently reported that rosiglitazone (Ros), a well-established oral antidiabetic agent, can protect against myelotoxicity produced by fluorouracil[6]. Sik Lee also reported that rosiglitazone attenuates cisplatin-induced renal damage[7].

Rosiglitazone is a member of the thiaolidinediones (TZDs) and a synthetic ligand of the peroxisome proliferator-activated receptor γ (PPARγ)[8]. Members of thiazolidenediones such as troglitazone and ciglitazone exhibit anti-tumor effects on various types of cancer cells, including colon cancer cells expressing high levels of PPARγ[9]. However, low bioavailability of rosiglitazone[10] limits its application in clinical cancer therapy. We thus investigated the effect of rosiglitazone in combination with fluorouracil on human colon cancer cells.

PPARγ has been implicated in metabolic diseases[11,12]

and is associated with cell proliferation, differentiation and apoptosis[13]. However, the role of PPARγ in fluorouracil-induced apoptosis of HT-29 cells is unknown.

It was reported that ciglitizone induces significant apoptosis of HT-29 cells and reduces Bcl-2 expression by activating PPARγ[14]. On the other hand, Bcl-2 exerts its functions by heterodimerizing with Bax, a protein that accelerates apoptosis. Deficient expression of Bax is also associated with apoptosis resistance. We thus analyzed the effect of rosiglitazone on the expression of Bax and Bcl-2 in HT-29 cells to understand the underlying mechanisms of 5-Fu-induced apoptosis.

In the present study, we investigated whether and how rosiglitazone enhances fluorouracil-induced apoptosis of HT-29 cells. The results demonstrate that rosiglitazone at low concentration has no inhibitory effect on HT-29 cell growth and proliferation, but enhances apoptosis of HT-29 cells induced by fluorouracil. The mechanism of rosiglitazone underlying the improvement of fluorouracil-induced apoptosis may be associated with Bax and Bcl-2 depending on PPARγ.

MATERIALS AND METHODS

Reagents

Propidium iodide (PI), acridine orange (AO), ethidium bromide (EB) were purchased from Sigma Chemical Company (St. Louis, MO, USA). RPMI-1640 medium and newborn calf serum were supplied by Giboco BRL (Grand Island, NY, USA). Methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were bought from Sigma. Polyclonal antibodies against PPARγ, Bcl-2 and Bax were purchased from Santa Cruz Biotech Co. Horseradish peroxidase-conjugated goat antimouse IgG and goat antirabbit IgG were purchased from Santa Cruz biotechnology, Inc.

Cell lines and cell culture

Human colon cancer HT-29 cells were obtained from the China Center for Type Culture Collection (Wuhan, China). HT-29 cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum, 80 U/mL penicillin and 100 U/mL streptomycin in humidified atmosphere (90% relative humidity) with 5% CO_2 at 37℃. The culture media were changed every two days.

MTT assay for proliferation

HT-29 cells were plated onto 96-well plates at approximately 1.0×10^4 cells per well and incubated for 12 h. The cells were treated with rosiglitazone or fluorouracil or both at various concentrations for 72 h. Then 20 μL of 5 g/mL MTT in phosphate-buffered saline (PBS) was added. The plates were incubated for 4 h and formosan was dissolved in 100 μL DMSO. The absorbance at 570 nm was recorded using an enzyme-linked immunosorbent assay reader. The proliferation inhibition rate (IR) was calculated according to the following formula: IR% = [1-absorbance of drug treatment group/absorbance of vehicle control group] × 100%[15]. The IR was analyzed using Calcusyn program to determine the IC_{50} of each drug. The combination index (CI)-isobologram by Chou and Talalay[16] was used to analyze the drug combination: $CI = IC_{50(AB)}/(IC_{50(A)} + IC_{50(B)})$ (A, B represent different drugs).

CI > 1, CI = 1 and CI < 1 indicate antagonism, additive effect, or synergism, respectively.

Cell growth assessment by trypan blue exclusion method

HT-29 cells were plated onto 24-well plates at approximately 1.0×10^4 cells per well and incubated for 12 h. The cells were treated with rosiglitazone or fluorouracil or both at various concentrations. On d 1, 2, 3, 4 and 5, the cells were harvested by trypsinization and counted under microscope after trypan blue staining. Three independent experiments were carried out based on the following formula: cell viability% = number of cells in drug treatment group/ number of cells in control group × 100%[17]. Population doubling time was calculated as follows: $TD = t^{\log 2/\log N_t - \log N_0}$, where TD is population doubling time, t is cell culture time, N_0 and N_t are the number of cells at initiation and t time, respectively).

Cell morphological observation by AO/EB staining

Cells were treated with rosiglitazone or fluorouracil or both for 72 h, then harvested with 0.25% trypsin and resuspended in PBS. After staining for 10 min with 10 μL of 100 mg/mL acridine orange/ethidium bromide (AO/EB) mixture, cells were visualized immediately under a fluorescence microscope (TE2000-S, Nikon, USA)[18].

Apoptosis assay by FCM using PI staining

Cells were treated with rosiglitazone or fluorouracil or both for 72 h, then harvested with 0.25% trypsin and washed with PBS. Cells at a density of 1×10^6 were fixed in 70% ice-cold ethos/PBS and stored at 4℃ overnight, then washed with PBS and incubated in PI solution (69 mom PI, 388 mom sodium citrate, 100 go/mL Raze A) for 15 min at 37℃. Cells were immediately analyzed with a FAC scan flow cytometer (Becton Dichinson, San Jose, USA)[17].

Western blot analysis of PPARγ, NF-KB, Bcl-2 and Bax expression

Cells were lysed in a lysis buffer containing 0.1 mol/L Nacl, 0.01 mol/L Tris-Cl, 0.001 mol/L EDTA, 1 μmol/L aprotinin, and 100 μmol/L phenylmethylsulfonyl fluoride (PMSF) at 4℃ with sonication. The lysates were centrifuged at 15000 × *g* for 15 min and the concentration of protein was determined with a bicinchoninic acid protein assay kit (Pierce Chemicals), using bovine serum albumin as a standard. Loading buffer (42 mmol/L Tris-Cl, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol and 0.02% bromophenol blue) was then added to each lysate, which was subsequently boiled for 5 min and electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred onto a polyvinylidene difluoride membrane (PVDF), and incubated separately with antibodies against PPARγ, Bcl-2, Bax and β-actin, and then labled with horseradish peroxidase-conjugated secondary antibodies. The reactions were visualized using an enhanced chemiluminescence reagent (Santa Cruz). The results were approved by

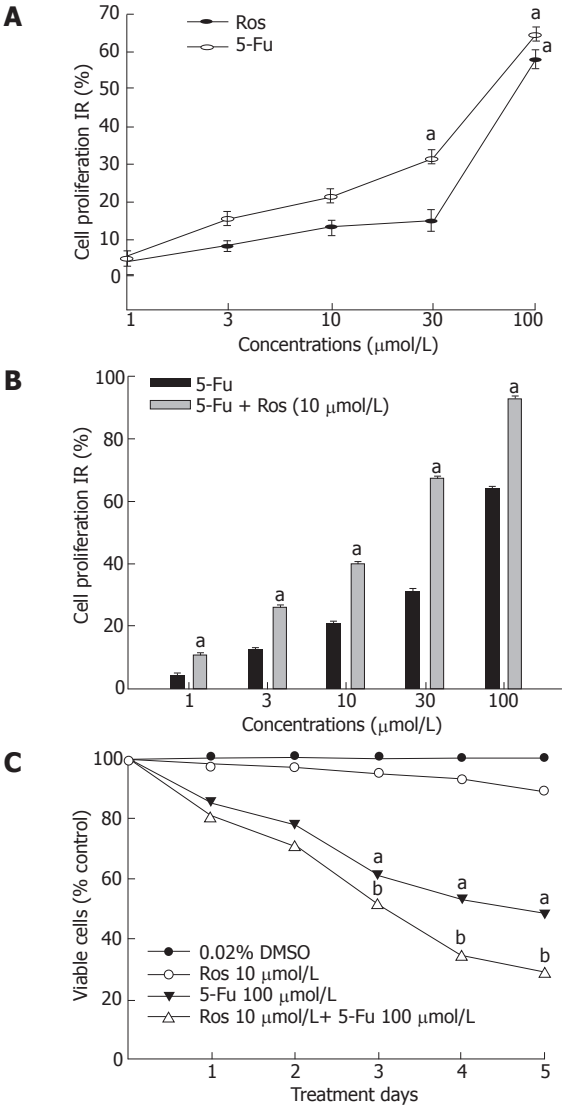

Figure 1 Effect of rosiglitazone and/or 5-Fu on proliferation and growth of HT-29 cells exposed to rosiglitazone or 5-Fu (**A**), 5-Fu with or without 10 μmol/L rosiglitazone (**B**), and treated with rosiglitazone and 5-Fu (**C**) at the indicated concentration for 72 h. [a]$P < 0.05$ *vs* control group, [b]$P < 0.01$ *vs* rosiglitazone group.

repeating the reaction 3 times using different samples[19].

Statistical analysis

Data were expressed as mean ± SD. ANOVA was used to assess the statistical significance of differences. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of rosiglitazone on fluorouracil-induced proliferation and growth inhibition of HT-29 cells

To examine the effect of rosiglitazone on fluorouracil-induced proliferation inhibition of HT-29 cells, the proliferation inhibition rate of HT-29 cells treated with fluorouracil in the presence or absence of rosiglitazone was calculated by MTT method. Rosiglitazone exerted almost no inhibitory effect at the concentration below 30 μmol/L (IR < 20%) for 72 h on HT-29 cells. The IR value for fluorouracil at 30 μmol/L and 100 μmol/L was 30.20% and 64.9%, respectively (Figure 1A). Since the IR value for rosiglitazone at 10 μmol/L was 12.01%, we co-administered 10 μmol/L rosiglitazone with 3, 10, 30, 100 μmol/L of fluorouracil respectively to HT-29 cells. As shown in Figure 1B, fluorouracil inhibited proliferation of HT-29 cells in a dose-dependent manner and rosiglitazone significantly enhanced the proliferation inhibition of HT-29 cells induced by fluorouracil ($P < 0.05$).

The IC_{50} of rosiglitazone, fluorouracil or both was 140.4 ± 21.23 μmol/L, 56.9 ± 6.21 μmol/L, 10.5 ± 0.14 μmol/L respectively and the CI value for rosiglitazone and fluorouracil was 0.257, indicating the synergistic effect of combined drugs.

Trypan blue exclusion assay showed that rosiglitazone potently enhanced the susceptibility of HT-29 cells to fluorouracil. Although 10 μmol/L rosiglitazone was not cytotoxic to HT-29 cells, it could dramatically enhance growth inhibition of HT-29 cells stimulated by 100 μmol/L fluorouracil (Figure 1C). When treated with 100 μmol/L 5-Fu, the doubling time of HT-29 cells was 2.5 d, whereas it was 3.4 d in the presence of 10 μmol/L rosiglitazone.

Effect of rosiglitazone on fluorouracil-induced apoptosis of HT-29 cells by AO/EB staining

Apoptotic cells were detected by morphological observation using AO/EB staining. As shown in Figure 2A, the normal cells (Figure 2A.a) and cells treated with 10 μmol/L rosiglitazone (Figure 2A.b) exhibited uniformly dispersed chromatin and intact cell membrane. Typical morphological changes were found in apoptotic HT-29 cells exposed to 100 μmol/L fluorouracil for 72 h, including apoptotic nuclear condensation (Figure 2A.c). However, the number of cells with nuclear condensation was significantly increased in cells cotreated with 100 μmol/L fluorouracil and 10 μmol/L rosiglitazone for 72 h (Figure 2A.d), revealing that rosiglitazone could enhance fluorouracil-induced apoptosis.

Effect of rosiglitazone on fluorouracil–induced apoptosis of HT-29 cells by FCM using PI staining

To quantify and assess the apoptotic rate of HT-29 cells induced by rosiglitazone in combination with fluorouracil, the proportion of cells that had a DNA content of less than 2N was analyzed by FCM using PI staining (Figure 2B). The apoptosis rate for HT-29 cells treated with 10 μmol/L rosiglitazone for 72 h was 2.1% ± 0.26, which was similar to that for the untreated control group (1.8% ± 0.21). In the presence of 10 μmol/L rosiglitazone, the apoptotic rate for HT-29 cells induced by 10, 30, 100 μmol/L fluorouracil for 72 h was increased from 20.7% ± 0.46%, 23.7% ± 0.43%, and 30.3% ± 0.97 to 28.1% ± 0.70%, 32.7% ± 0.45%, and 40.3% ± 0.73% respectively, indicating that rosiglitazone dramatically promoted apoptosis of HT-29 cells induced by fluorouracil.

Effect of PPARγ antagonist on fluorouracil–induced apoptosis of HT-29 cells induced by rosigliatzone

To confirm that rosiglitazone enhances fluorouracil-induced apoptosis of HT-29 cells depending on PPARγ, the effect of GW9662 on fluorouracil–induced apoptosis

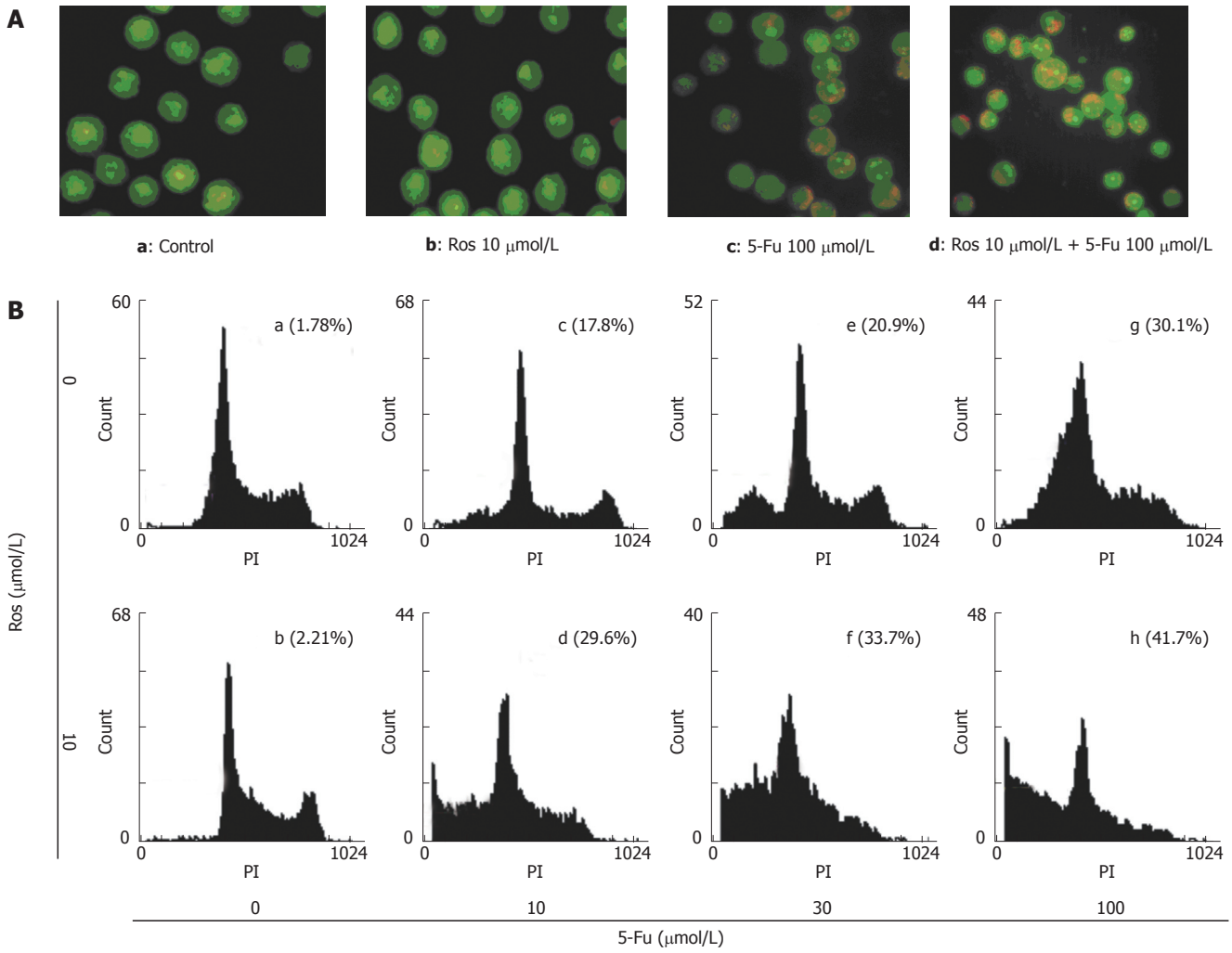

Figure 2 Apoptosis of HT-29 cells induced by AO/EB staining (**A**) and flow cytometrical analysis (**B**). HT-29 cells were treated with rosigliatzone and/or 5-Fu at the indicated concentration for 72 h, then harvested and stained with AO/EB. Results are representative of three experiments.

induced by rosigliatzone was investigated. As shown in Figure 3A , the apoptosis rate of HT-29 cells pretreated with GW9662 30 min before exposed to rosiglitazone and 5-Fu was 33.1% ± 0.81%, lower than that of HT-29 cells not pretreated with GW9662 (40.3% ± 0.73%).

Effect of rosiglitazone on expression of PPARγ, Bax, Bcl-2 in HT-29 cells

As shown in Figure 3B, the expression of PPARγ and Bax increased in a time-dependent manner, while the expression of Bcl-2 decreased in a time-dependent manner in HT-29 cells treated with 10 μmol/L rosiglitazone for 0, 4, 8, 12 h, respectively.

Effect of PPARγ antagonist on Bax and Bcl-2 expression induced by rosigliatzone

To confirm the relationship between the expressions of Bcl-2/Bax and PPARγ in HT-29 cells induced by rosiglitazone, HT-29 cells were pretreated with GW9662, a PPARγ antagonist, 30 min before treatment with 10 μmol/L or 30 μmol/L rosiglitazone for 12 h. We found that the expression of Bcl-2 and Bax in HT-29 cells induced by rosiglitazone was blocked by GW9662 (Figure 3C).

DISCUSSION

A previous study suggested that rosiglitazone inhibits proliferation of the human adrenocortical cancer cell line H295R in a dose-dependent manner with the maximal effect (about 50% inhibition) obtained at 20 μmol/L[20]. Another study also demonstrated that rosiglitazone only at high concentration (> 10 μmol/L) inhibits growth and viability of cancer cells[21]. However, the plasma concentration of rosiglitazone in typical diabetes patients is 1.67 μmol/L[14]. Thus rosiglitazone should not be used as a single anticancer agent.

In this study, rosiglitazone at a low concentration (< 30 μmol/L) did not inhibit HT-29 cell growth *in vitro*. Importantly 10 μmol/L rosiglitazone promoted fluorouracil-induced proliferation and growth suppression of HT-29 cells. The mechanism may be associated with the low concentration of rosiglitazone promoting fluorouracil-induced apoptosis. When a combination of 10 μmol/L rosiglitazone with various concentrations of 5-Fu was used, the apoptotic rate of HT-29 cells improved compared with 5-Fu alone.

Although rosiglitazone is the most potent and selective synthetic ligand of PPARγ, it suppresses cancer cell

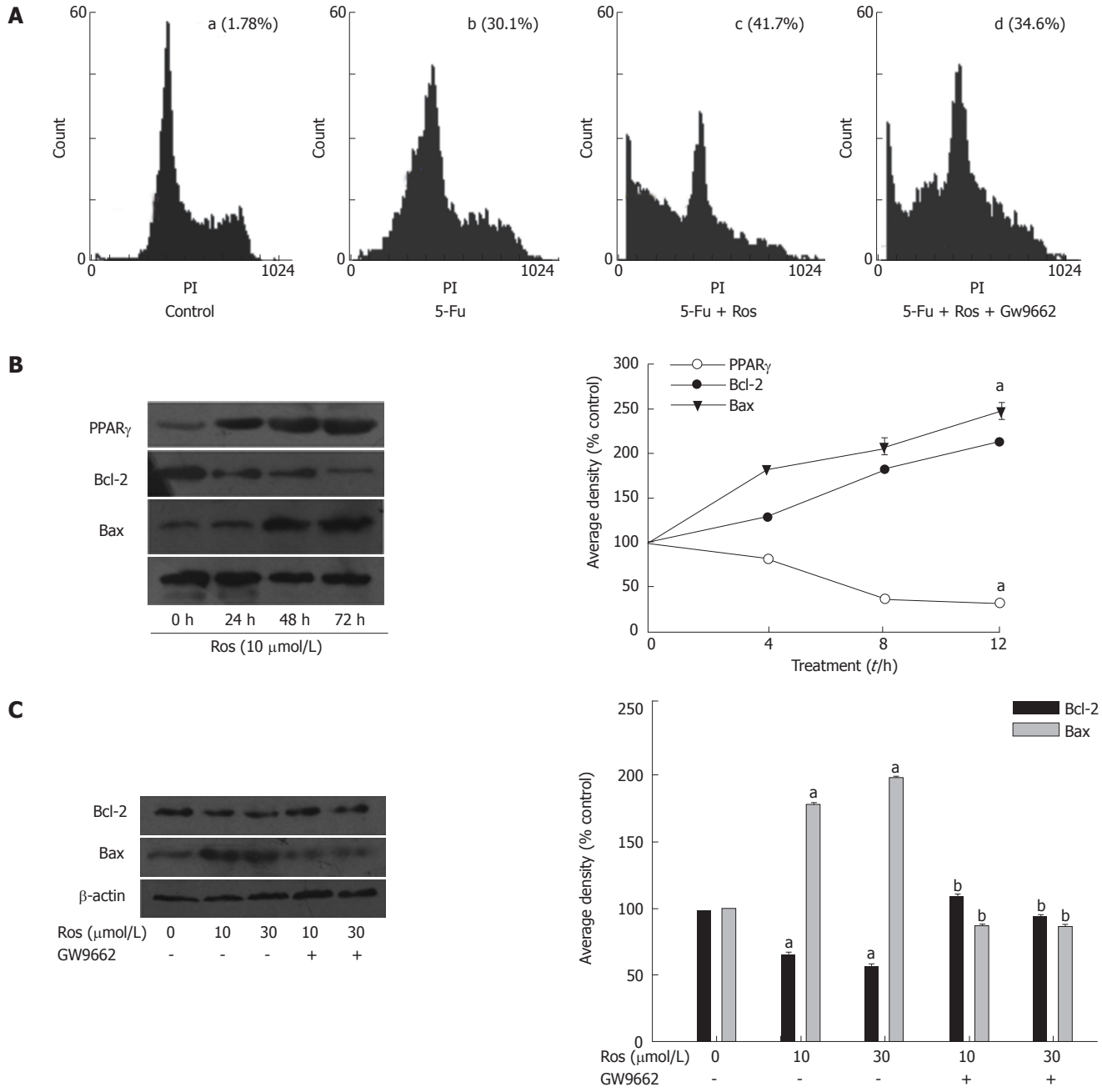

Figure 3 Effect of rosiglitazone on 5-Fu induced apoptosis (**A**), time-dependent expression of PPARγ, Bax and Bcl-2 (**B**), and PPARγ-dependent expression of Bax, Bcl-2 and β-actin (**C**). $^{a}P < 0.05$, $^{b}P < 0.01$.

growth through PPARγ-dependent and independent[22] signal path ways, because different cellular models may be, at least in part, responsible for the discrepancies. In the present study, rosiglitazone increased PPARγ expression in a time-dependent manner. More importantly, the effect of fluorouracil-induced apoptosis induced by rosiglitazone was blocked by GW9662, suggesting that fluorouracil-induced apoptosis induced by rosiglitazone depends on PPARγ.

Fluorouracil has been known to cause cell injury by inhibiting thymidylate synthesis or by incorporating itself into DNA or RNA[23]. High level expression of thymidylate increases the activity of deoxyuridine triphosphatase[23], methylation of the MLH1 gene, and over expression of Bcl-2, Bcl-XL[24,25]. It was reported that Mcl-1 proteins lead to resistance to 5-Fu[26], suggesting that multiple factors contribute to 5-Fu resistance. It was reported that that ovarian tumors over-expressing Bcl-2 may not respond well to E1A gene therapy, but treatment with a combination of E1A and Bcl-2-ASO may overcome it[27]. Zhu *et al*[28] found that colon cancer cells resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can be re-sensitized by a combination therapy of TRAIL and 5-Fu. In our study, rosiglitazone decreased Bcl-2 expression in HT-29 cells, suggesting that fluorouracil-induced apoptosis may reduce HT-29 cell resistance by down regulating Bcl-2 in a time- and dose- dependent manner.

Bcl-2 family can positively and negatively regulate apoptosis[29]. Bcl-2 and Bax are two members of the Bcl-2 family, and play a different role in programmed cell

death[30]. When Bax is over-expressed in cells, apoptosis in response to death signals is accelerated, leading to its designation as a death agonist[31]. When Bcl-2 is over-expressed it heterodimerized with Bax and death is repressed[31]. Therefore, the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis[30]. In our study, rosiglitazone increased Bax expression in HT-29 cells in a time- and dose- dependent manner, suggesting that rosiglitazone-induced apoptosis may also reduce HT-29 cell resistance by up-regulating Bax expression.

On the other hand, the effect of rosiglitazone on decreasing Bcl-2 level and increasing Bax level in HT-29 cells was blocked by GW9662, suggesting that the enhancing effect of rosiglitazone on apoptosis of HT-29 cells is associated with decreasing Bcl-2/Bax expression by activating PPARγ.

In conclusion, a combination of rosiglitazone and fluorouracil induces strong inhibition of HT-29 cell proliferation and growth. However, the *in vivo* effect needs further study.

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Expression of mutant type-*p*53 products in *H pylori*-associated chronic gastritis

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Abstract

AIM: To investigate the mutation of *p*53 immunohistochemically in non-tumorous gastric mucosa with *H pylori* infection before and after *H pylori* eradication therapy.

METHODS: 53 subjects (36 male, 17 female, mean age ± SEM, 57.1 ± 12.1) undergoing endoscopic examination were included in this study. 42 of 53 patients were *H pylori*-positive, and 11 were *H pylori*-negative. All *H pylori*-positive patients had successful eradication therapy. Biopsy specimens were taken from five points of the stomach, as recommended by the updated Sydney system. Immunohistochemical studies were performed by using primary antibodies against *p*53 (DO-7 and PAb240).

RESULTS: *p*53 (DO-7 and PAb240) immunoreactivity was shown in the neck region of the gastric pits, however, quite a few cells were found to be immunopositive for *p*53 (PAb240) in the *H pylori*-infected gastric mucosa. The proportion of patients immunopositive for *p*53 (PAb240) was significantly reduced 6 mo after eradication [28/42 (66.7%) to 6/42 (14.3%)] ($P < 0.05$), while the biopsies taken from *H pylori*-negative patients showed no immunoreactivity for *p*53 (PAb240). *p*53 (PAb240)-positive patients were divided into two groups by the number of positive cells detected: one with more than six positive cells per 10 gastric pits (group A, $n = 12$), and the other with less than five positive cells per 10 gastric pits (group B, $n = 30$). Atrophy scores in group A were significant higher than those in group B at the greater curvature of the antrum (group A: 2.00 ± 0.14 *vs* group B: 1.40 ± 0.15, $P = 0.012$), the lesser curvature of the corpus (group A: 2.00 ± 0.21 *vs* group B: 1.07 ± 0.23, $P = 0.017$), and the greater curvature of the corpus (group A: 1.20 ± 0.30 *vs* group B: 0.47 ± 0.21, $P = 0.031$). Group A showed significant higher intestinal metaplasia scores than group B only at the lesser curvature of the antrum (group A: 2.10 ± 0.41 *vs* group B: 1.12 ± 0.29, $P = 0.035$).

CONCLUSION: *H pylori*-associated chronic gastritis expressed the mutant-type *p*53, which was significantly associated with more severe atrophic and metaplastic changes. *H pylori* eradication led to a significant reduction in the expression of the mutant-type *p*53. It is considered that *H pylori*-infected chronic gastritis is associated with a genetic instability that leads to gastric carcinogenesis, and *H pylori* eradication may prevent gastric cancer.

Key words: *H pylori*; *H pylori* eradication; Atrophic gastritis; Mutant-type *p*53; Gastric cancer; Updated Sydney system

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INTRODUCTION

Helicobacter pylori is one of the main pathogens that cause many kinds of gastroduodenal diseases. These include acute gastritis[1-3], chronic atrophic gastritis[3,4], intestinal metaplasia[5], peptic ulcer[6,7], mucosal associated lymphoid tissue (MALT) lymphoma[8], and other disorders[9,10]. Although many epidemiological studies and animal models revealed close association between gastric cancer and *H pylori* infection[11-13], there have been few studies that report on genetic alterations suggestive of gastric carcinogenesis associated with chronic *H pylori* infection[14-17]. Previously, we reported the expression of *p*53, a product of oncosupressor-gene and cell cycle regulator, in the *H pylori*-infected human gastric mucosa[18] as well as in a Japanese monkey model[19]. *p*53 expression was observed in the neck region of the gastric pit, and was reported to be reduced after *H pylori* eradication[18]. *p*53 mutation is among the major episodes in the multi-step process of gastric carcinogenesis, while

it has also been reported in pre-malignant lesions of the stomach, such as chronic gastritis, intestinal metaplasia, and dysplasia[20-22]. However, it remains unclear whether *p*53 expression in the gastric pit is mutant-type or wild-type. In this study, we used the primary antibodies reacting with both wild and mutant type-*p*53, and those reacting only with the mutant-type *p*53 in our immunohistochemical studies to clarify the *p*53 alterations in the *H pylori*-infected gastric mucosa.

MATERIALS AND METHODS

Subjects

A total of 53 subjects (36 male, 17 female, mean age SEM, 57.1 ± 12.1) who underwent endoscopic examination were included in this study. 42 out of 53 patients were *H pylori*-positive and 11 were *H pylori*-negative. In 42 *H pylori*-positive patients, 14 patients had gastric ulcers, 5 had duodenal ulcers, 5 had gastro-duodenal ulcers, and 15 had chronic gastritis. All *H pylori*-positive patients had successful eradication therapy, and underwent biopsies before and six months after eradication. Biopsies for examination were taken at the same biopsy sites in *H pylori*-negative subjects as in the *H pylori* positive patients.

Detection of H pylori in gastric biopsy specimens

H pylori in the stomach was detected by the rapid urease test, culture, and histological examination. For the urease test, biopsy specimens were immediately inserted into the rapid urease test solution. For culture detection, biopsy material was cultured on 7% sheep's blood agar plates under micro-aerobic conditions and at high humidity and at 37℃ for four days. *H pylori* was histologically detected by May-Giemsa stain. *H pylori* eradication was considered successful when the results of all three tests were found negative.

Histological evaluation

Biopsy specimens were taken from five points of the stomach, as recommended by the updated Sydney system[23], i.e. the lesser curvature of the antrum (A1), and the greater curvature of the antrum (A2), the smaller curvature of the angle (IA), and the lesser curvature of the middle corpus (B1), and the greater curvature of the upper corpus (B2). All biopsy materials were fixed in buffered formalin for 24 h and embedded in paraffin. Serial sections were stained with haematoxylin-eosin and with May-Giemsa stain. The status of the gastric mucosa was evaluated according to the updated Sydney system. The degree of inflammation, neutrophil activity, atrophy, and intestinal metaplasia were classed by four grades, with 0 being for 'normal', 1 for 'mild', 2 for 'moderate', and 3 for 'marked', respectively.

Immunohistochemical detection of p53

Serial paraffin sections were washed in 1/15 mol/L phosphate buffered saline (PBS, pH 7.4) three times for five minutes, and pre-incubated in normal rabbit serum (1:10 in PBS) for 20 min. Next, these sections were incubated with primary antibodies for 16 h at 4℃, followed by the avidin-biotin complex method. The sections were immersed in 0.05 mol/L Tris-HCl buffer containing 0.02% 3, 3'-diaminobenzidine tetrahydrochloride and 0.005% H_2O_2, and the nuclei were counterstained with hematoxylin. Control sections incubated with normal mouse IgG instead of the primary antibody showed no non-specific staining. The primary antibodies used in this study were mouse monoclonal anti-*p*53 protein (clone DO-7 and PAb240, Dako, Carpinteria, CA, USA), and were diluted to 1:50. Control sections incubated with normal mouse IgG instead of the primary antibody showed no non-specific staining.

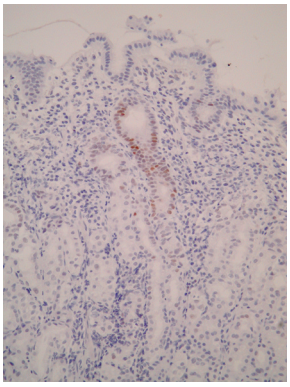

Figure 1 Immunohistochemistry for p53 (DO-7) in *H pylori*-infected gastric mucosa. Positive staining for p53 (DO-7) was observed in the gastric pit, especially in the neck region before eradication (× 200).

Evaluation of p53 (DO-7 and PAb240)

Cells immunopositive for *p*53 (DO-7) were counted in longitudinal sectioned foveolar pits and were found visible along their whole length. Labeling indices for *p*53 (DO-7) are indicated as the proportion of positive cells among the cell total of the gastric mucosa. At least 500 cells in the gastric epithelial cells were calculated in each specimen. Cells immunopositive for *p*53 (PAb240) were counted in per 10 longitudinal gastric pits.

Statistical analysis

Statistical analysis (Wilcoxon signed-rank test and Mann-Whitney's U test) was performed to analyze the updated Sydney system score and labeling indices.

RESULTS

Immunohistochemical detection of p53 (DO-7)

Immunoexpression of *p*53 (DO-7) was observed in the neck region of the *H pylori*-infected gastric pits from all biopsy sites (Figure 1). These *p*53-positive cells were significantly reduced 6 mo after *H pylori* eradication. In contrast, the gastric mucosa without *H pylori* infection showed very few positive cells in the gastric pits. The labeling index for *p*53 (DO-7) in the *H pylori*-infected group before eradication was significantly higher than that in the non-*H pylori*-infected group ($P < 0.001$) (Figure 2). After eradication, the labeling index for *p*53 was significantly reduced at all biopsy sites (A2; from 14.98% to 6.80%; $P < 0.001$, A1; from 12.63% to 4.96%; $P < 0.001$, IA; from 14.24% to 4.26%; $P < 0.001$, B1; from 17.49% to 6.41%; $P < 0.001$, B2; from 14.45% to 4.48%; $P < 0.001$) (Figure 2).

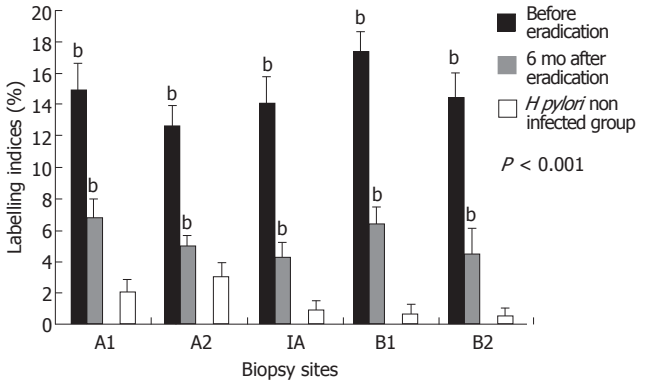

Figure 2 Labeling indices for *p*53 (DO-7) in 42 patients with *H pylori* infection, 6 mo after eradication, and 11 patients without *H pylori* infection. Results were shown as mean ± SEM. *p*53 (DO-7) indices were significantly reduced 6 mo after eradication at all biopsy sites. *p*53 indices for the *H pylori*-infected mucosa were significantly higher than those for the gastric mucosa without *H pylori* infection at all biopsy sites. $^{b}P < 0.001$, *vs H pylori* non infected group.

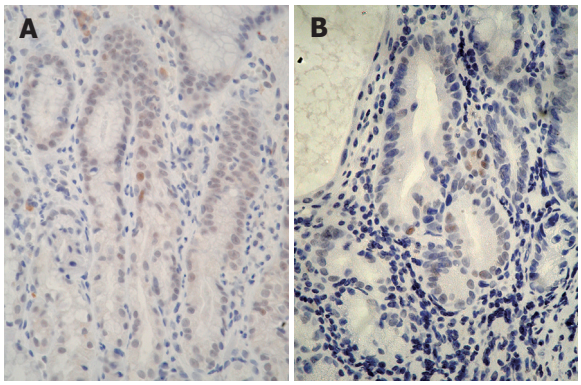

Figure 3 Immunohistochemistry for *p*53 (PAb240) in the *H pylori*-infected gastric mucosa. A small number of cells clearly showing the expression of *p*53 (PAb240) were detected in the neck region before eradication. **A**: a 53-yr-old male with gastric ulcer; **B**: a 51-yr-old male with chronic gastritis; × 400).

Immunohistochemical detection of p53 (PAb240)

Immunoreactivity of *p*53 (PAb240) was also shown in the neck region of the gastric pits; however, quite a few cells were found to be immunopositive for *p*53 (PAb240) in the *H pylori*-infected gastric mucosa (Figure 3A and 3B). Indices for *p*53 (PAb240) in all examined patients are indicated in Table 1. Immunopositive cells for *p*53 (PAb240) were found in 28 out of 42 (66.7%) *H pylori*-positive patients, while *H pylori*-negative patients showed no immunoreactivity for *p*53 (PAb240) in all five biopsy specimens. Six months after eradication, the ratio of cells immunopositive for *p*53 (PAb240) was significantly reduced [28/42 (66.7%) to 6/42 (14.3%)] ($P < 0.05$) (Table 2). In patients immunoreactive for *p*53 (PAb240), the total number of positive cells from all five biopsy specimens ranged from 0 to 19.1 per 10 gastric pits. When divided by the number of positive cells, 30 *H pylori*-positive patients had less than five cells, 8 patients had six to 10 cells, 1 patient had 11 to 15 cells, and 3 patients had more than 16 positive cells, respectively, with the majority of patients found to have less than five positive cells. Thus the patients were divided into two groups by the number of positive cells: one with more than six positive cells per 10 gastric pits (group A, $n = 12$); and the other with less than five positive cells per 10 gastric pits (group B, $n = 30$).

Before eradication, labeling indices of *p*53 (DO-7) were significantly higher in group A than in group B at all biopsy sites except the lesser curvature of the antrum (Figure 4). *H pylori* density, inflammation, and activity scores in the updated Sydney system showed no significant difference between the groups (Table 3). However, atrophy scores in group A were significantly higher than in group B at biopsy site A2 (group A: 2.00 ± 0.14 *vs* group B: 1.40 ± 0.15, $P = 0.012$), B1 (group A: 2.00 ± 0.21 *vs* group B: 1.07 ± 0.23, $P = 0.017$), and B2 (group A: 1.20 ± 0.30 *vs* group B: 0.47 ± 0.21, $P = 0.031$) (Table 3). There was no significant difference in atrophy scores between the two groups at biopsy site A1 and IA. group A showed significantly higher intestinal metaplasia scores than group B only at A1 (group A: 2.10 ± 0.41 *vs* group B: 1.12 ± 0.29, $P = 0.035$), with no significant difference in intestinal metaplasia scores seen at all the other biopsy sites.

DISCUSSION

Previously, we reported the significant over-expression of *p*53 in the *H pylori*-infected gastric pits, as well as its significant reduction after *H pylori* eradication[18]. Other researchers also reported that *p*53 was immunohistochemically detected in the non-neoplastic *H pylori*-infected gastric mucosa[24-28], and that *H pylori* eradication therapy also diminished the expression of *p*53[18,25,27].

*p*53 is thought to be one major player in the regulation of cell cycle[29] in which DNA damage activates *p*53 proliferation, and *p*53 binds to MDM2 and other molecules. This binding protein arrests cell proliferation and maintains the integrity of the cell by DNA repairing or cell apoptosis. In contrast, mutant *p*53 loses these abilities. In a previous report[18], we considered that immunodetection of *p*53 (DO-7) signified whether accelerated wild type-*p*53 or occurrence of mutant type-*p*53. Teh *et al*[28] reported that *p*53 detection in the non-neoplastic epithelium was considered to reflect an accumulation of wild-type *p*53 due to the sensitivity of the currently available antigens. Our study also suggested an accumulation of wild-type *p*53, especially in the *H pylori*-infected mucosa probably because of the DNA damage that occurred to *H pylori* infection. In addition, the present study showed that quite a few cells were found positive for mutant-type *p*53 (PAb240), instead of *p*53 (DO-7). There has been no study reported to date demonstrating the positive immunohistochemical staining for mutant-type of *p*53 in the non-neoplastic gastric mucosa. Our results indicated that while the majority of cells that reacted with *p*53 (DO-7) account for an accumulation of wild-type *p*53, mutant type *p*53 might also be expressed in some cells of the gastric pit. Murakami *et al*[14] and Morgan *et al*[31] reported the presence of point mutations in exon 5 to 8 of *p*53 in *H pylori*-positive gastritis, and several other studies have shown that gastric precancerous lesions, such as atrophic gastritis and intestinal metaplasia, are associated with *p*53 abnormalities[20-22,26, 32]. Murakami *et al*[14] reported that the point mutations are present in 52.4% of the gastritis, with Morgan *et al*[31] reporting the presence of *p*53 mutations in 35% of the gastritis and 45% of the intestinal

Table 1 Immunohistochemical detection agaisnt *p*53 (PAb 240) in gastric mucosa

	Case	Sex	Age (yr)	Clinical diagnosis	Number of positive cells per ten crypts against *p*53 (PAb240) before eradication					Number of positive cells per ten crypts against *p*53 (PAb240) 6 mo after eradication				
					A2	A1	IA	B1	B2	A2	A1	IA	B1	B2
H pylori positive	1	F	42	Gstric ulcer	0	0	0	0	0	0	0	0	0	0
	2	F	26	Duodenal ulcer	0	0	0	0	0	0	0	0	0	0
	3	M	64	Gastric ulcer	0	0	11.4	0	0	0.7	0	0	0	0
	4	M	64	Gastric ulcer	0	0	0	2.5	0	0	0	0	0	0
	5	M	56	Gastric ulcer	0	0.8	0	0	0	0	0	0	0	0
	6	M	50	Gastric ulcer	0	0	0	0	0	0	0	0	0	0
	7	M	45	Gastroduodenal ulcer	0	0	0	0	2.3	0	1.1	0	0	0
	8	M	52	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	9	M	60	Gastric ulcer	0	0	0	0.8	0	0.5	0	0	2.2	0
	10	F	71	Chronic gastritis	1.7	0	0.8	0.7	0	0	0	0	0	0
	11	M	60	Gastric ulcer	1.3	0	0	0	0	0	0	0	0	0
	12	M	55	Gastric ulcer	0	0	0	0	0	0	0	0	0	0
	13	M	42	Gastroduodenal ulcer	2.4	2.2	0	0	0	0	0	0	0	0
	14	M	68	Gastric ulcer	0	0	0	0	0	0	0	0	0	0
	15	M	67	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	16	M	57	Chronic gastritis	0	0	0	2	0	0	0	0	0	0
	17	F	77	Gastric ulcer	0	0	0	1.7	0	0	0	0	0	0
	18	F	51	Chronic gastritis	0	0	0	0.7	0	0	0	0	0	0
	19	M	64	Gastric ulcer	0	0	0	0	2.3	0	0	0	0	0
	20	F	57	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	21	M	51	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	22	F	75	Chronic gastritis	5.7	0	3.3	0	0.8	0	0	0	0	0
	23	F	65	Chronic gastritis	0.5	0	0	0	0	0	0	0	0	0
	24	F	51	Chronic gastritis	0.6	0	2.7	0	4.2	0	0	0	0	0
	25	M	53	Gastric ulcer	7.5	2.7	5	2.1	0	6	0	0	0	0
	26	M	68	Chronic gastritis	0.9	2.9	0	0	0	0	0	0	0	0
	27	M	50	Chronic gastritis	0	1.3	0.6	0	0	0	0	0	0	0
	28	F	78	Chronic gastritis	0	0	0	2.2	1.8	0	0	0	0	0
	29	M	58	Gastric ulcer	0	0	2.5	0	0	0	0	0	0	0
	30	M	74	Chronic gastritis	0	0	0	1.7	4.5	0	0	0	0	0
	33	M	48	Chronic gastritis	1.1	0	3.8	0	3.8	0	0	0	1.7	0
	34	F	68	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	35	F	46	Gastric ulcer	3.3	0	7.5	6	0	0	0	0	0	0
	36	M	64	Duodenal ulcer	0	0	0	6.7	0	2	1.7	0	0	5
	37	M	42	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	38	F	71	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	39	M	58	Chronic gastritis	0	1	0.8	0	0	0	0	0	0	0
	40	F	46	Chronic gastritis	0	0	0	0	0	0	0	0	0	0
	41	M	76	Chronic gastritis	0.9	3	2.2	1.7	1.7	0	0	0	0	0
	42	M	51	Chronic gastritis	2.9	0	3.1	5.4	7.7	0	0	0	0	0
H pylori negative	43	M	25		0	0	0	0	0					
	44	M	49		0	0	0	0	0					
	45	M	51		0	0	0	0	0					
	46	M	68		0	0	0	0	0					
	47	M	40		0	0	0	0	0					
	48	F	64		0	0	0	0	0					
	49	F	52		0	0	0	0	0					
	50	M	59		0	0	0	0	0					
	51	M	73		0	0	0	0	0					
	52	F	59		0	0	0	0	0					
	53	M	38		0	0	0	0	0					

A1: Lesser curvature of the antrum; A2: Greater curvature of the antrum; IA: Lesser curvature of the angle; B1: Lesser curvature of the lower body; B2: Greater curvature of the upper body.

Table 2 Positive ratio of Immunohistochemical detection against p53 (PAb 240)

	Before eradication	6 mo after eradication
H pylori positive	66.7% (28 out of 42)	14.3% (6 out of 42)[b]
H pylori negative	0% (0 out of 11)	

[b]$P < 0.01$ *vs* before eradication in *H pylori* positive group.

metaplasia. Unger *et al*[32] demonstrated that the expression of *p*53 and apoptotic indices in gastritis in the absence of intestinal metaplasia are significantly higher in *H pylori*-positive patients than in *H pylori*-negative patients. These studies are in agreement with our results indicating that in some *p*53 mutations are found mutant type-*p*53 products in the *H pylori*-infected gastric pits.

In a comparison between group A with more than six

Table 3 The differences of updated Sydney system score between the group showed more than six positive cells for p53 (PAb240) per 10 gastric pits (group A) and the group showed less than five cells for p53 (PAb240) per 10 gastric pits (Group B)

Contents of updated Sydney system	Group	Biopsy points				
		A2	A1	IA	B1	B2
H pylori	Group A	1.25 ± 0.25	0.50 ± 0.21	1.00 ± 0.21	0.88 ± 0.24	1.38 ± 0.21
	Group B	0.71 ± 0.18	0.88 ± 0.22	0.88 ± 0.21	1.13 ± 0.19	1.41 ± 0.17
Inflammation	Group A	2.20 ± 0.12	2.20 ± 0.12	2.10 ± 0.16	2.30 ± 0.13	1.90 ± 0.16
	Group B	2.05 ± 0.18	1.99 ± 0.17	2.00 ± 0.17	2.00 ± 0.12	1.82 ± 0.12
Activity	Group A	1.40 ± 0.15	1.00 ± 0.23	1.20 ± 0.23	1.20 ± 0.23	1.30 ± 0.23
	Group B	1.06 ± 0.20	0.94 ± 0.18	1.00 ± 0.18	1.05 ± 0.16	0.94 ± 0.17
Atrophy	Group A	2.00 ± 0.14	1.75 ± 0.13	2.13 ± 0.28	2.00 ± 0.21	1.20 ± 0.30
	Group B	1.40 ± 0.15[a]	1.57 ± 0.15	1.67 ± 0.18	1.07 ± 0.23[a]	0.47 ± 0.21[a]
Intestinal etaplasia	Group A	0.90 ± 0.31	2.10 ± 0.41	1.80 ± 0.40	1.30 ± 0.36	0.20 ± 0.12
	Group B	0.82 ± 0.27	1.12 ± 0.29[c]	1.47 ± 0.32	1.11 ± 0.29	0.12 ± 0.12

A1: the lesser curvature of the antrum; A2: the greater curvature of the antrum; IA: smaller curvature of the angle; B1: and the lesser curvature of the middle corpus; B2: the greater curvature of the upper corpus. [a]$P < 0.05$ *vs* atrophy scores in group A, [c]$P < 0.05$ *vs* intestinal metaplasia scores in group A.

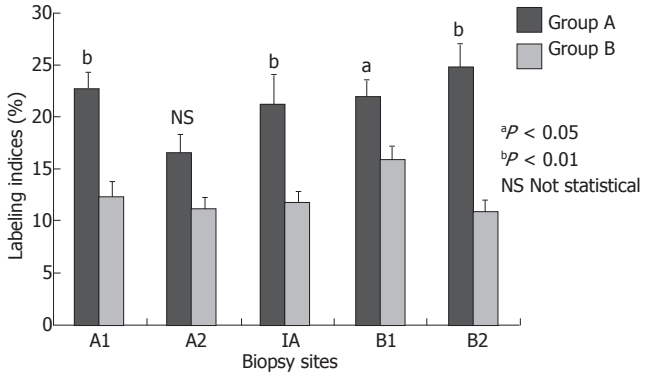

Figure 4 Labeling indices for p53 (DO-7) in group A with more than 6 positive cells for p53 (PAb240) per 10 gastric pits (n = 12) and group B with less than 5 positive cells for p53 (PAb240) per 10 gastric pits (n = 30) before eradication. Results are shown as mean ± SEM. Labeling indices for p53 (DO-7) were significantly higher for group A than for group B at all biopsy sites except for the lesser curvature of the antrum. [a]$P < 0.05$, [b]$P < 0.01$, NS, not statistical.

positive cells per 10 gastric pits and group B with less than five cells per 10 gastric pits, group A showed significantly higher labeling indices for *p*53 (DO-7) in all biopsy sites than group B. This result suggested an increase in wild-type *p*53 as it was caused by the greater DNA damage due to the gene instability in place that might increase mutant-type *p*53. Moreover, group A showed significantly higher atrophy and intestinal metaplasia scores in the stomach, suggesting that the gastric mucosa with more severe atrophic changes or extensive intestinal metaplasia has a greater number of mutant-type *p*53. Thus, it is likely that the development of atrophic changes and intestinal metaplasia gradually increased the occurrence of *p*53 mutations. Gastric cancer of the intestinal type follows a multi-step process of carcinogenesis, such as atrophic gastritis, intestinal metaplasia, and dysplasia[33]. Therefore, the present results indicate that *H pylori* infection in the gastric mucosa may be implicated in the pathway of gastric carcinogenesis. Nardone *et al*[26] reported that DNA aneuploidy was seen in 11 patients with *H pylori* infection and atrophy, with 8 of these found to show c-Myc expression, and 6 of these to have *p*53 expression, and they concluded that chronic *H pylori* infection may be responsible for genomic instability in a subset of *H pylori*-positive chronic atrophic gastritis. Unger *et al*[32] also reported that *H pylori*-positive gastritis accompanied with intestinal metaplasia showed a lack of increased apoptosis with a higher *p*53 expression, which suggests an increased genetic instability, thus concluding that *p*53 mutation is an early step in the multi-step process of gastric carcinogenesis. Our results confirm the relationship between atrophic and/or metaplastic gastric mucosa with *H pylori* infection and genetic instability, which lead to gastric carcinogenesis.

In the present study, *H pylori* eradication therapy led to a significant reduction in the expression of both *p*53 (DO-7) and *p*53 (PAb240). The number of mutant-type *p*53-positive patients was significantly decreased 6 mo after eradication. In our previous study, we exhibited that eradication reduces both *p*53 and MDM2 expression[18]. Hibi *et al*[25] and Satoh *et al*[27] also described a reduction in the expression of *p*53 after eradication. Nardone *et al*[26] reported that *H pylori* eradication reduced gastritis activity, atrophy, and complete metaplasia, accompanied by the disappearance of markers of genomic instability, thereby concluding that eradication can reverse inflammation, associated atrophy, metaplasia, and genomic instability. The present study also indicated that *H pylori* eradication diminished *p*53 abnormality, and this result accounted for the improvement of genetic instability.

Many researchers reported morphologic alterations of gastric mucosa, especially those of atrophic and metaplastic status after *H pylori* eradication[34], while their results are conflicting, with some of them reporting that eradication did not improve atrophy and/or intestinal metaplasia, and some concluding that eradication could improve the gastric atrophy and/or intestinal metaplasia. However, after the year 2000, the majority of papers reported improvements in the gastric mucosa with *H pylori* eradication.

The present study confirmed the hypothesis that *H pylori* eradication reverses the atrophic changes in the gastric mucosa and the genetic instability, thus preventing the development of gastric cancer.

In conclusion, in chronic gastritis associated with *H pylori* infection, the expression of mutant-type *p*53 was

seen significantly greater in more severe atrophic and metaplastic changes. *H pylori* eradication led to a significant reduction in the expression of mutant-type *p*53. It is suggested that chronic gastritis associated with *H pylori* infection has genetic instability, which leads to gastric carcinogenesis, and that *H pylori* eradication may prevent gastric cancer.

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Th immune response induced by *H pylori* vaccine with chitosan as adjuvant and its relation to immune protection

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Abstract

AIM: To study the immunological protective effect of *H pylori* vaccine with chitosan as an adjuvant and its mechanism.

METHODS: Female BALB/c mice were randomly divided into seven groups and orally immunized respectively with PBS, chitosan solution, chitosan particles, *H pylori* antigen, *H pylori* antigen plus cholera toxin (CT), *H pylori* antigen plus chitosan solution, *H pylori* antigen plus chitosan particles once a week for four weeks. Four weeks after the last immunization, the mice were challenged twice by alive *H pylori* (1×10^9 CFU/mL) and sacrificed. Part of the gastric mucosa was embedded in paraffin, cut into sections and assayed with Giemsa staining. Part of the gastric mucosa was used to quantitatively culture *H pylori*. ELISA was used to detect cytokine level in gastric mucosa and anti- *H pylori* IgG1, IgG2a levels in serum.

RESULTS: In the groups with chitosan as an adjuvant, immunological protection was achieved in 60% mice, which was significantly higher than in groups with *H pylori* antigen alone and without *H pylori* antigen ($P < 0.05$ or 0.001). Before challenge, the level of IFN and IL-12 in gastric mucosa was significantly higher in the groups with chitosan as an adjuvant than in the control group and the group without adjuvant ($P < 0.05$ or 0.005). After challenge, the level of IFN and IL-12 was significantly higher in the groups with adjuvant than in the groups without adjuvant and antigen ($P < 0.05$ or 0.001). Before challenge, the level of IL-2 in gastric mucosa was not different among different groups. After challenge the level of IL-2 was significantly higher in the groups with adjuvant than in the control group ($P < 0.05$ or 0.001). Before challenge, the level of IL-10 in gastric mucosa was significantly higher in the groups with chitosan as an adjuvant than in other groups without adjuvant ($P < 0.05$ or 0.01). After challenge, the level of IL-10 was not different among different groups. Before challenge, the level of IL-4 in gastric mucosa was significantly higher in the groups with chitosan as an adjuvant than in other groups without adjuvant ($P < 0.05$). After challenge, the level of IL-4 was significantly higher in the groups with chitosan particles as an adjuvant than in the group with CT as an adjuvant ($P < 0.05$), and in the group with chitosan solution as an adjuvant, the level of IL-4 was significantly higher than that in control group, non-adjuvant group and the groups with CT ($P < 0.05$ or 0.001). The ratio of anti- *H pylori* IgG2a/IgG1 in serum was significantly lower in the groups with chitosan as an adjuvant than in the groups with CT as an adjuvant or without adjuvant ($P < 0.01$).

CONCLUSION: *H pylori* vaccine with chitosan as an adjuvant can protect against *H pylori* infection and induce both Th1 and Th2 type immune response.

Key words: *H pylori*; Chitosan; Vaccine; Adjuvant; Th immune response

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INTRODUCTION

Colonization of *H pylori* in the stomach is associated with the risk of developing different gastroduodenal diseases including chronic gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma and gastric cancer. The current treatment against *H pylori* infection is a combination therapy with two different antibiotics plus a proton-pump inhibitor with or without colloidal bismuth, which can eradicate the bacteria in most cases. However, this therapy has some major drawbacks, including high cost and development of antibiotic resistance. So vaccination

would be a suitable alternative or complement to antibiotic treatment to eradicate the bacteria. A large number of animal experiments have shown that immunization with *H pylori* antigen in combination with certain adjuvants can prevent and even eliminate *H pylori* infection[1-3]. At present, the effective adjuvants are cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT), but they cannot be used in humans due to their toxicity and side effects. There is no effective and non-toxic adjuvant for humans. Chitosan is a deacetylated product of chitin, which is non-toxic, non-irritable, non-antigenic, bioadhesive, biocompatible and biodegradable[4]. Some studies showed that chitosan as an immune adjuvant could effectively enhance the immune response of local mucosa[5-7] and antigen presentation[8]. But there is no report about chitosan as an adjuvant for *H pylori* vaccine. In the present study, mice were vaccinated with a bacterial whole-cell sonicate of *H pylori* plus chitosan and then challenged by *H pylori*, in order to delineate its effect and possible mechanisms against *H pylori* infection.

MATERIALS AND METHODS

Reagents and bacterial strains

Chitosan and 88.5% deacetylated chitosan powder were purchased from Qisheng Biological Products Limited Company, Shanghai. Campylobacter agar base and Brucella broth were purchased from Shanghai Reagent Providing and Research Centre for Diarrhea Disease Control, China. ELISA kits for IL-2, IFN, IL-12, IL-4 and IL-10 were purchased from Bender MedSystem (USA). Sheep-anti-mouse IgG1 and IgG2a peroxidase conjugate was purchased from Zymed-Laboratories INC (USA). *H pylori* Sydney strain 1 (SS1) was kindly provided by *H pylori* Strain Pool, China.

Animals

Female BALB/c mice, 6-8 wk of age and 22.5 g of mean weight, were purchased from Animal Centre of China Academy of Sciences, Shanghai (licensing number: SCXK (HU) 2002-0010). The mice were housed in a specific pathogen-free environment with free access to food and water.

Culture of H pylori

The Sydney strain of *H pylori* was used throughout the experiments. *H pylori* was grown in Campylobacter agar base containing 7.5% sheep blood under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37℃ for 2-3 d, and harvested from the agar plates by eluting with broth culture. Bacterial density was detected at 660 nm. One OD was 10^8/mL.

Preparation of H pylori antigen

After cultured for 2-3 d, the Sydney strain of *H pylori* was eluted with phosphate buffer saline (PBS), and centrifuged at 1000 × *g* for 10 min. The pellet was washed three times with PBS, and cells were disrupted by sonication. After centrifugation at 8000 × *g* for 30 min at 4℃, supernatant was collected and stored at -85℃ until use. Protein concentration was determined.

Preparation of chitosan particles

Ddeacetylated (88.5%) chitosan powders were suspended in saline to the final concentration of 10 mg/mL and sonicated at 80 HZ output power two times for 5 min each with a sonicator, at 1 min intervals. Following a light centrifugation (50 r/min, 10 min), small particles in the supernatants were removed, filtered through a 400/2800 stainless steel mesh, and further centrifuged to collect the particles at 1400 r/min for 10 min as previously described[9].

Preparation of chitosan solution

Deacetylated (88.5%) chitosan stock solution was prepared at 3% (w/w) in 0.8% (v/v) acetic acid 0.9% (w/v) saline and heated at 37℃ to dissolve[10].

Vaccination and challenge of mice

BALB/c mice were randomly divided into 7 groups: (1) control (PBS alone), 15 mice; (2) chitosan solution alone, 12 mice; (3) chitosan particles alone, 13 mice; (4) *H pylori* antigen alone, 15 mice; (5) *H pylori* antigen plus chitosan solution, 15 mice; (6) *H pylori* antigen plus chitosan particles, 15 mice; (7) *H pylori* antigen plus CT, 12 mice.

BALB/c mice were orally immunized with *H pylori* antigen (1.2 mg/mouse), chitosan particles (500 μg/mouse), CT (5 μg/mouse), and 0.5% chitosan solution, once a week for four weeks. Chitosan particles were whipped into a stable emulsion by sonication with a sonifier at 20 HZ before immunization[9]. Four weeks after the last immunization, the mice were challenged twice by alive *H pylori* (1×10^9/mL, 0.5 mL/mouse).

Before challeng, five mice were randomly collected respectively from the control group, *H pylori* antigen group, *H pylori* antigen plus chitosan solution group and *H pylori* antigen plus chitosan particles group and killed. Samples were collected for further use. The other mice were killed 4 wk after the last challenge. Blood was collected by removing eyeballs immediately before the mice were killed. The stomach was isolated for histology, examination of *H pylori*, and determination of cytokine level. The stomach was washed in sterile 0.8% NaCl and cut longitudinally into two pieces. One was used for quantitative culture of *H pylori*, while the other was used for histology and determination of cytokine expression.

Assessment of bacterial load in stomach

The bacterial load in the stomach was determined by quantitatively culture of *H pylori* and improved Giemsa staining, when both of them negative was difined as *H pylori* negative and when anyone of them positive was difined as *H pylori* positive. For assessment of *H pylori* colonization, weighed stomachs were homogenized in 0.3 mL of Brucella broth, 1/4 and 1/8 serial dilutions were spread over the surfaces of serum plates containing 10 mg of vancomycin, 2500 IU of polymyxin and 5 mg of trimethoprim per liter. The plates were incubated for 3-7 d. Colonies were counted to determine the CFU per gram of stomach tissue. In Giemsa staining, the colonization was

Table 1 Rates of immune protection of *H pylori* vaccine with different kinds of adjuvant

Groups	*n*	*H pylori* positive *n* (%)	Rates of immune protection (%)
(1) Control	10	10 (100)	0
(2) Chi-solution	12	12 (100)	0
(3) Chi-particles	13	13 (100)	0
(4) *Hp* antigen	10	10 (100)	0
(5) *Hp* antigen + CT	12	5 (41.67)	58.33[d]
(6) *Hp* antigen + chi-solution	10	4 (40)	60[a,b]
(7) *Hp* antigen + chi-particles	10	4 (40)	60[a,b]
$P < 0.001$			

[a]$P < 0.05$ *vs* (1) and (4) groups; [b]$P < 0.01$ *vs* (2) and (3) groups; [d]$P < 0.01$ *vs* (1)-(4) groups; chi = chitoson.

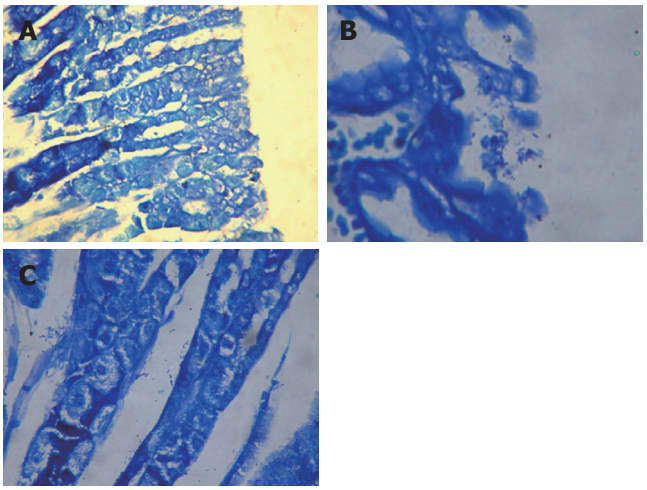

Figure 1 *H pylori* stained with Giemsa in gastric tissue of mice. No *H pylori* found in *H pylori* antigen + chi-particles group (**A**) , and lots of *H pylori* found on surface of gastric mucosa (**B**) and in gastric foveola (**C**) of control group (Giemsa dyeing × 400).

assessed by semiquantitative analysis of *H pylori* in gastric mucosa (nil = 0; 1-2 call/crypt = 1; 3-10 call/crypt = 2; 11-20 call/crypt = 3; > 21 call/crypt = 4)[11].

Determination of cytokines in gastric mucosa by ELISA

After weighed, the gastric mucosa was homogenized in 1.3 mL PBS and the homogenates were centrifuged at 3000 × *g* at 4℃ for 20 min. Supernatant was harvested and diluted at 1:2. For quantification of IL-2, IFN, IL-12, IL-4 and IL-10 in the supernatants, commercial enzyme-linked immunosorbent assay (ELISA) systems were used. The limit of detection was 3 pg/mL for IL-4, 22 pg/mL for IL-10, and 6 pg/mL for IL-12, 8 pg/mL for IL-2, and 8 pg/mL for IFN. The results were represented as pg/mg wet weight of gastric mucosa.

Determination of H pylori-specific antibodies in serum

H pylori-specific antibodies (IgG1 and IgG2) in serum were detected by indirect ELISA. Each well of microtiter plates was coated with 100 μL of *H pylori* antigen solution at the concentration of 20 μg/mL in 0.01 mol/L sodium carbonate-bicarbonate buffer (pH 9.6) overnight at 4℃. After suction, 200 μL of 0.1% BSA solution in PBS-Tween-20 was added to each well and further incubated for 1 h at 37℃. After washed three times with PBS-Tween-20, 100 μL of diluted (1/100) serum samples was added to wells and incubated for 1 h at 37℃. After washed three times with PBS-Tween-20, 100 mL of sheep-anti-mouse IgG1 and IgG2a peroxidase conjugate was added to wells and incubated further for 1 h at 37℃. After washed three times with PBS-Tween-20, 100 μL of o-phenylendiamine solution containing 0.01% H_2O_2 was added to wells and incubated for 30 min at room temperature. The reaction was stopped by the addition of 50 μL of 2 mol/L sulfuric acid, and color development was measured by a plate reader at 492 nm. The results were represented as A value of samples/A value of control.

Table 2 *H pylori* colonization score in gastric mucosa

Groups	*n*	*H pylori* colonization score			
		0	1	2	3
(1) Control	10	0	2	4	4
(2) Chi-solution[d]	12	1	8	2	1
(3) Chi-particles[d]	13	0	10	1	2
(4) *Hp* antigen[c]	10	1	5	2	2
(5) *Hp* antigen + CT[a,d]	12	7	2	1	2
(6) *Hp* antigen + chi-solution[b]	10	6	4	0	0
(7) *Hp* antigen + chi-particles[b]	10	7	3	0	0
$H = 57.181$, $P < 0.001$					

[b]$P < 0.01$ *vs* (1)-(4) groups; [a]$P < 0.05$ *vs* (4) group; [c]$P < 0.05$ *vs* (1) group; [d]$P < 0.01$ *vs* (1) group; chi = chitoson.

Statistical analysis

Differences in the protection rate against *H pylori* infection were analyzed by Fisher's exact test. Differences in *H pylori*-specific antibody and cytokine level in gastric mucosa among experimental groups were detected for statistical significance by analysis of variance or Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Rates of immune protection against H pylori infection

Significant difference was found in the rates of immune protection of vaccines with different adjuvants against *H pylori* infection ($P < 0.001$), and the protection rates were significantly higher in the groups with adjuvant than in the groups without adjuvant and antigen ($P < 0.05$ or 0.001, Table 1).

H pylori colonization score in gastric mucosa

Significant difference was observed in density of *H pylori* colonization among different groups ($P < 0.001$), and the density of *H pylori* colonization was significantly lower in the groups with adjuvant than in the groups without adjuvant or antigen ($P < 0.05$ or 0.001, Table 2, Figure 1).

CFU/g of H pylori in gastric mucosa

Significant difference was found in *H pylori* colonization among different groups, as indicated by the number of CFU/g of *H pylori* in gastric mucosa ($P = 0.001$). The number of CFU/g of *H pylori* was significantly lower in the groups with chitosan as an adjuvant than in other

Table 3 Density of *H pylori* colonization in gastric mucosa

Groups	*n*	Median of *H pylori* colony number (CFU/g)
(1) Control	10	4.74×10^5
(2) Chi-solution	12	0.73×10^5
(3) Chi-particles	13	1.83×10^5
(4) *Hp* antigen	10	0.48×10^5
(5) *Hp* antigen + CT	12	0
(6) *Hp* antigen + chi-solution	10	0
(7) *Hp* antigen + chi-particles	10	0
$H = 27.43$, $P = 0.001$		

chi = chitoson.

groups without adjuvant ($P < 0.05$ or 0.01). There was no significant difference in *H pylori* colonization among the groups with CT as an adjuvant or without adjuvant ($P > 0.05$, Table 3).

Cytokine level in gastric mucosa

Before challenge, there was no significant difference in IL-2 level among different groups. But there was significant difference in levels of IFN, IL-12, IL-10 and IL-4 among different groups ($P < 0.05$ or 0.005), which were significantly higher in the groups with chitosan as an adjuvant than in other groups without adjuvant ($P < 0.005$, Table 4). After challenge, there was significant difference in levels of IFN, IL-12, IL-2 and IL-4 among different groups. The level of IL-2 was significantly higher in the groups with chitosan solution as an adjuvant than in the groups without antigen ($P < 0.05$ or 0.001). Moreover, the level of IL-2 was significantly higher in the groups with chitosan particles and *H pylori* antigen alone than in the control group and the group with chitosan solution alone ($P < 0.05$). The level of IFN and IL-12 was significantly higher in the groups with adjuvant than in the groups without antigen or adjuvant ($P < 0.05$ or 0.001). The level of IL-4 was significantly higher in the groups with chitosan particles than in the groups with CT ($P < 0.05$). Moreover, the level of IL-4 in the groups with chitosan solution as adjuvant was significantly higher than in other groups with chitosan solution alone, *H pylori* antigen alone and CT as adjuvant ($P < 0.05$). There was no significant difference in the level of IL-10 among different groups ($P > 0.05$, Table 5).

There was significant difference in the levels of IL-2, IFN, IL-12, IL-10 and IL-4 before and after challenge by alive *H pylori*. The levels of IL-2, IFN and IL-12 were significantly higher in the groups with *H pylori* antigen after challenge than those before challenge ($P < 0.05$). The levels of IL-10 were significantly lower in the groups with adjuvant after than before challenge ($P < 0.05$). The level of IL-4 was significantly lower in the groups with chitosan particles as adjuvant after challenge than before challenge ($P < 0.05$). The level of cytokines before and after challenge was not significantly different in the other group ($P > 0.05$, Figure 2).

Level of anti-Hp IgG2a, IgG1 and ratio of IgG2a/IgG1 in serum

After challenge, there was significant difference in the levels of anti-Hp IgG2a, IgG1 and ratio of IgG2a/IgG1 in serum among different groups ($P < 0.001$). The level of anti-Hp IgG2a was significantly higher in the groups with chitosan particles than in other groups with *H pylori* antigen alone or chitosan alone and the control group ($P < 0.05$ or 0.001). The level of anti-Hp IgG2a was significantly higher in the groups with CT or chitosan solution than in the control group ($P < 0.05$). The level of anti-Hp IgG1 was significantly higher in the groups with adjuvant than in other groups with chitosan alone and in the control group ($P < 0.05$). Moreover, the level of anti-Hp IgG1 was significantly higher in the groups with *H pylori* antigen than in the control group ($P < 0.05$). The ratio of IgG2a/IgG1 was significantly lower in the groups with chitosan than in the groups with CT, *H pylori* antigen alone and in the control group ($P < 0.01$, Table 6).

DISCUSSION

All effective vaccines need a suitable antigen-presenting system that depends on adjuvant or vehicle[12]. *H pylori* antigen alone cannot induce protective immune response. Antigen-presenting system can introduce exogenous antigen into cells, and can enhance the immune response to antigen and even change the type of immune response. Many studies showed that chitosan can effectively promote local immune response and enhance antigen presentation[5-8]. In this study, we evaluated a vaccine delivery system with chitosan, the rate of immune protection of vaccine with chitosan as adjuvant against *H pylori* infection was 60%, which was significantly higher than with *H pylori* antigen alone or chitosan alone, indicating that chitosan can be used as a mucosa adjuvant of *H pylori* instead of CT.

$CD4^+$ helper T cells (Th) in mice can be divided into Th1 and Th2 subtypes. Th1 cells can synthesize and secrete IL-2, IL-12, IFN-γ, take part in cell-mediated immune response and promote the production of IgG2a by B cells. Th2 cells can also secrete cytokines such as L-4, IL-5, and IL-10, help B cells produce antibody, take part in humoral immune response and promote the production of antibodies such as IgG1, IgE and IgA. Negative feedback exists in the two types. Th can regulate immune response. Recently, different Th response types induced by *H pylori* vaccine and their effects in immune response are the main point in the mechanism of *H pylori* vaccine, indicating that the balance of Th1 and Th2 response is involved in the protection mechanism of *H pylori* vaccine. In natural *H pylori* infection, the presence of Th1 is the primary immune response. Th1-mediated cell immunity cannot protect against *H pylori* infection and is related to the severity of *H pylori* infection[13-15]. At the same time, $CD4^+$ Th2 secreting IL-4 and IL-10 is depressed, thus IgA secreted by B cells is reduced, leading to persistent *H pylori* infection. After immunization, the type of immune response has changed from Th1 to Th2. Hatzifoti *et al*[16] reported that immunization of mice with DNA vaccine encoding urease B genes could up-regulate the expression of Th2 cytokine IL-10. Mohammadi *et al*[17] found that stimulating immune response to Th2 could reduce the number of *H pylori* and the intensity of inflammation of gastric mucosa, indicating that if the type of immune response

Table 4 Levels of IL-2, IFN-γ, IL-12, IL-10 and IL-4 in gastric mucosa before challenge (mean ± SD)

Groups	*n*	IL-2	IFN-γ	IL-12	IL-10	IL-4
(1) Control	5	19.9 ± 12.6	28.6 ± 10.2	118.9 ± 46.2	67.2 ± 32.5	4.19 ± 2.95
(2) *Hp* antigen	5	20.6 ± 3.5	33.6 ± 13.7	165.3 ± 47.4	104.3 ± 19.1	6.49 ± 2.61
(3) *Hp* antigen chi-solution	5	28.6 ± 9.3	58.5 ± 12.2[b]	283.5 ± 93.7[c,d]	255.3 ± 131.8[c,d]	14.70 ± 8.73[a]
(4) *Hp* antigen+ chi-particles	5	27.6 ± 13.9	59.8 ± 15.2[b]	283.4 ± 99.6[c,d]	237.1 ± 98.3[c,d]	14.48 ± 6.84[a]
		F = 0.739	F = 7.948	F = 6.083	F = 6.228	F = 4.189
		P = 0.544	P = 0.002	P = 0.006	P = 0.005	P = 0.023

[a]P < 0.05 *vs* (1) and (2) groups; [b]P < 0.01 *vs* (1) and (2) groups; [c]P < 0.05 *vs* (2) group; [d]P < 0.01 *vs* (1) group; chi = chitoson.

Table 5 Levels of IL-2, IFN-γ, IL-12, IL-10 and IL-4 in gastric mucosa after challenge (mean ± SD)

Groups	*n*	IL-2	IFN-γ	IL-12	IL-10	IL-4
(1) Control	10	34.3 ± 11.8	48.7 ± 27.8	233.7 ± 125.7	86.4 ± 38.2	3.87 ± 1.99
(2) Chi-solution	11	31.8 ± 27.4	56.8 ± 26.1	308.5 ± 178.7	100.7 ± 52.6	4.47 ± 1.89
(3) Chi-particles	10	51.1 ± 42.6	58.8 ± 28.1	311.3 ± 129.6	88.4 ± 51.7	7.21 ± 4.02[g]
(4) *Hp* antigen	9	97.9 ± 64.2[a]	59.4 ± 15.0	319.4 ± 136.3	89.3 ± 29.01	4.89 ± 3.15
(5) *Hp* antigen + CT	10	80.9 ± 60.2[c]	93.6 ± 23.5[e,f]	487.0 ± 289.3[e,f]	81.8 ± 49.5	3.67 ± 1.76
(6) *Hp* antigen + chi-solution	10	124.3 ± 75.2[b]	107.5 ± 42.0[d]	525.6 ± 112.2[d]	108.5 ± 39.0	8.78 ± 4.96[h,i]
(7) *Hp* antigen + chi-particles	10	88.6 ± 57.0[a]	105.9 ± 48.1[d]	554.0 ± 164.4[d]	93.1 ± 39.2	6.59 ± 1.38[k]
		F = 3.370	F = 6.346	F = 5.448	F = 0.745	F = 3.214
		P = 0.002	P < 0.001	P < 0.001	P > 0.05	P = 0.003

[a]P < 0.05 *vs* (1) and (2) groups; [b]P < 0.001 *vs* (1)-(3) groups; [c]P < 0.05 *vs* (1) group; [d]P < 0.01 *vs* (1)-(4) groups; [e]P < 0.05 *vs* (2)-(4) groups; [f]P < 0.01 *vs* (1) group; [g]P < 0.05 *vs* (1), (2) and (5) groups, [h]P < 0.01 *vs* (1), (2) and (5) groups; [i]P < 0.05 *vs* (4) group; [k]P < 0.05 *vs* (5) group; chi = chitoson.

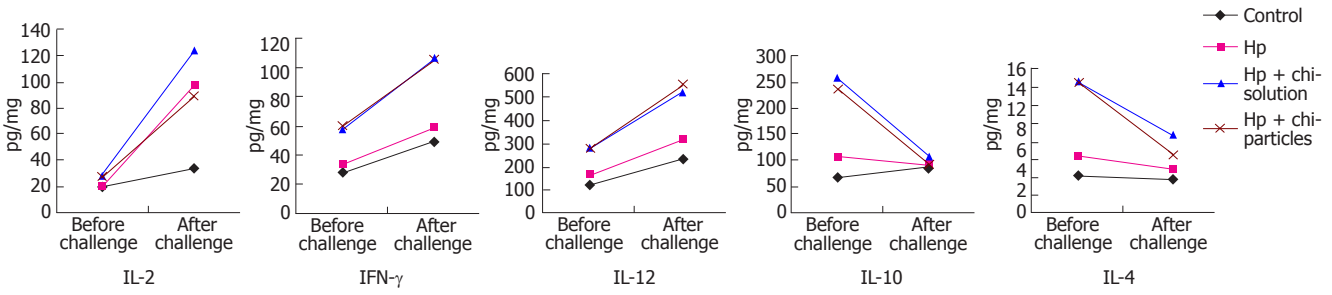

Figure 2 Levels of IL-2, IFN-γ, IL-12, IL-10 and IL-4 in gastric mucosa before and after challenge in different groups.

Table 6 Levels of anti-HpIgG2a, IgG1 and ratio of IgG2a/IgG1 in serum after challenge (mean ± SD)

Groups	*N*	IgG2a	IgG1	IgG2a/IgG1
(1) Control	10	4.44 ± 2.09	2.77 ± 1.18	2.13 ± 1.24
(2) Chi-solution	11	6.28 ± 3.62	3.96 ± 2.32	1.64 ± 0.85
(3) Chi-particles	10	5.56 ± 2.27	4.57 ± 2.09	1.43 ± 0.37[g,h]
(4) *Hp* antigen	9	9.21 ± 6.80[a]	6.43 ± 4.88[a]	2.356 ± 1
(5) *Hp* antigen + CT	10	11.22 ± 5.52[a]	7.71 ± 5.11[d,e]	2.19 ± 0.86
(6) *Hp* antigen + chi-solution	10	11.48 ± 5.55[a]	9.04 ± 5.35[b]	1.06 ± 0.4[f]
(7) *Hp* antigen + chi-particles	10	16.13 ± 10.20[b]	8.02 ± 5.29[c,d]	1 ± 0.37[f]
		F = 4.572	F = 10.308	F = 3.780
		P < 0.001	P < 0.001	P < 0.001

[a]P < 0.05 *vs* (1) group; [b]P < 0.01 *vs* (1)-(3) groups; [c]P < 0.05 *vs* (2) and (3) groups, [d]P < 0.01 *vs* (1) group; [e]P < 0.05 *vs* (2) group; [f]P < 0.01 *vs* (1), (4) and (5) groups; [g]P < 0.05 *vs* (5) group; [h]P < 0.01 *vs* (4) group.

induced by immunization has changed from Th1 to Th2, *H pylori* colonization in gastric mucosa can be inhibited by producing Th2 cytokines such as IL-4. Saldinger *et al*[18] immunized *H. felis* -infected mice with oral rUreB and CT, and found that infected mice were cured 3 wk after the 4th immunization. they also found that immunization could lead to the proliferation of $CD4^+$ T cells in the spleen of mice accompanying gradual decrease in IFN-γ and increase in IL-4, indicating that immunization of mice with rUreB and CT could induce gradual Th2 immune

response , thus eliminating *H. felis*. However, some studies showed that Th1 and Th2 response together is better than Th2 response only in preventing *H pylori* infection[19]. Gottweln *et al* reported that the two kinds of *H pylori* vaccine with complete Freund's or aluminum as adjuvant, which induce Th1 and Th2 immune response respectively, could induce protective immune response *in vivo* in mice, indicating that Th1 and Th2 immune response have the effect of immune protection. Eisenberg *et al*[21] immunized neonatal and adult mice with *H pylori* antigen and complete or incomplete Freund's adjuvant and found that the number of T cells producing Th1 cytokines like IFN-γ, IL-2 and Th2 cytokines like IL-4 IL-5 increased, have the effect of immune protection. Sommer *et al*[22] used CpG oligonucleotide as adjuvant to induce Th1 immune response, and found that it could not protect against *H pylori* infection but lead to more serious gastritis. Thus in the protective immune response to *H pylori*, Th1 and Th2 are needed. It must have a balance between Th1 and Th2 to achieve immune protection and to prevent tissue from damaging by serious inflammation[23].

In our study, before challenge by alive *H pylori*, the levels of IFN, IL-12, IL-4 and IL-10 were significantly higher in the groups with chitosan than in other groups without adjuvant, indicating that in the early stage of immune, they induce immune response to both Th1 and Th2. But 4 wk after challenge the levels of IL-2, IFN and IL-12 were significantly higher in the groups with adjuvant than in groups without adjuvant or in control group, indicating that after challenge they could promote the production of Th1 cytokines. The levels of IL-4 and IL-10 were significantly lower after challenge than before challenge. Chen *et al*[24] found that in the early stage of *H pylori* challenge (5 wk), the level of Th2 cytokines was significantly lower, even undetectable. Yu *et al*[25] found that after oral immunization with *H pylori* antigen plus LT, Th1 and Th2 immune response are induced in the early and advanced stage respectively, indicating that oral immunization can induce Th1 as well as Th2 immune response, which is in accordance to our study. In our study, after *H pylori* challenge the level of IL-4 was significantly higher in the group with chitosan particles as adjuvant than in the group with CT as adjuvant, and the level of IL-4 was significantly higher in the group with chitosan solution as adjuvant than in the groups with CT as adjuvant, chitosan solution alone and *H pylori* antigen along, indicating that *H pylori* vaccine with chitosan is better than that with CT in inducing Th2 cytokines especially IL-4. In addition, after *H pylori* challenge the levels of anti-Hp IgG2a and IgG1 were significantly higher in the groups with adjuvant than in the control group. IgG2a and IgG1 were induced by Th1 and Th2 immune response respectively, indicating *H pylori* vaccine can up-regulate Th1 and Th2 immune response. But the ratio of IgG2a/IgG1 in serum was significantly lower in the groups with chitosan as adjuvant than in other groups with CT as adjuvant or *H pylori* antigen alone, indicating that chitosan as an adjuvant can reverse the inhibition of Th2 induced by *H pylori* infection and return to the balance between Th1 and Th2, thus contributing to the immune protection against *H pylori* infection.

Chitosan can regulate immune response. Studies showed that chitosan as a mucosa adjuvant of the vaccine against meningococci and Bordetella bronchiseptica could successfully induce protective immune response[26,27]. Seferian *et al*[28] inoculated BALB/c mice with chitosan plus β- human chorionic gonadotropin, and found that the mixed immune response to IgG1, IgG2a and IgG2b antibodies could be observed in the groups with chitosan emulsion as adjuvant. Bivas-Benita *et al*[29] immunized mice with oral Toxoplasma gondii GRA1 protein and DNA vaccine-loaded chitosan particles, and successfully induced specialized anti- GRA1 IgG1 and IgG2a, indicating that it can enhance immune respons to Th1 and Th2. McNeela *et al*[30] found that immunization with chitosan plus diphtheria toxin could induce both systemic and local specific humoral immune response. At the same time, it could enhance immune response to Th1 and Th2.

In conclusion, *H pylori* vaccine with chitosan as adjuvant could induce Th1 and Th2 immune response, and partially reverse the inhibition of Th2 induced by *H pylori* infection and recover the balance between Th1 and Th2. As an adjuvant of *H pylori* vaccine, chitosan is better than CT in immune protection against *H pylori* infection.

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BASIC RESEARCH

Epithelial cells with hepatobiliary phenotype: Is it another stem cell candidate for healthy adult human liver?

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Abstract

AIM: To investigate the presence and role of liver epithelial cells in the healthy human adult liver.

METHODS: Fifteen days after human hepatocyte primary culture, epithelial like cells emerged and started proliferating. Cell colonies were isolated and sub-cultured for more than 160 d under specific culture conditions. Cells were analyzed for each passage using immunofluorescence, flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Flow cytometry analysis demonstrated that liver epithelial cells expressed common markers for hepatic and stem cells such as CD90, CD44 and CD29 but were negative for CD34 and CD117. Using immunofluorescence we demonstrated that liver epithelial cells expressed not only immature (α-fetoprotein) but also differentiated hepatocyte (albumin and CK-18) and biliary markers (CK-7 and 19), whereas they were negative for OV-6. RT-PCR analysis confirmed immunofluorescence data and revealed that liver epithelial cells did not express mature hepatocyte markers such as CYP2B6, CYP3A4 and tyrosine amino-transferase. Purified liver epithelial cells were transplanted into SCID mice. One month after transplantation, albumin positive cell foci were detected in the recipient mouse parenchyma.

CONCLUSION: According to their immature and bipotential phenotype, liver epithelial cells might represent a pool of precursors in the healthy human adult liver other than oval cells.

Key words: Epithelial-like cells; Liver stem cell; Hepatocyte; Differentiation; Cell therapy

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INTRODUCTION

Mammalian adult liver shows a high capacity to regenerate under different physiopathological circumstances thanks to mature and progenitor cells with stem-like properties[1-4]. Following cellular loss, fully differentiated hepatocytes and biliary epithelial cells can proliferate and completely repair the liver[5-8]. When proliferation of these cells is impaired during chronic or extensive damage of the liver, non-parenchymal epithelial progenitor cells, expressing both biliary and hepatocytic phenotypes, are activated and participate in liver regeneration[9-11].

Studies using hepato-toxins or chemical carcinogens treated animal models, revealed the existence of oval cells in the canals of Hering[12-14]. These cells express both hepatic [albumin (Alb), alpha fetoprotein (AFP)] and biliary [cytokeratin (CK)-7, CK-19, OV-6, γ-glutamyltranspeptidase (GGT)] markers, and some antigens traditionally associated with hematopoietic cells, including c-kit, Thy-1 and CD34[14,15] as well. During liver regeneration, stimulated oval cells express high levels of AFP, proliferate in the periportal area and migrate into liver parenchyma. Liver epithelial cells (LECs) differ from oval cells, and are as well candidate hepatic progenitor cells. Such liver epithelial cells were obtained from the livers of both normal[16] and carcinogen treated rats[17], normal pigs[18], and human subacute hepatic failure patient[10], but so far not from healthy human adult livers.

In the current study, we demonstrated the presence of LECs in healthy human liver and isolated these cells after primary culture. We successfully maintained their proliferation more than 160 d and analyzed their characteristics by flow cytometry, immunofluorescence, and RT-PCR. Furthermore, LECs were transplanted into SCID mice to evaluate their engraftment capacity.

MATERIALS AND METHODS

Cell isolation and culture

Liver parenchymal cell fraction was isolated from livers of

Table 1 Medical history of donors

Age (yr)	Sex	Cause of death
4	Male	Cranial trauma
9	Male	Cranial trauma
16	Female	Trauma
18	Male	Trauma
19	Male	Cranial trauma
36	Male	Cardiac arrest
43	Male	Euthanasia
43	Female	Cranial trauma + Cerebral hemorrhage
43	Female	Cerebral hemorrhage
44	Female	Cerebral hemorrhage
47	Female	Pulmonary embolism
51	Male	Suicide
61	Male	Cerebral hemorrhage

13 healthy cadaveric donors (Table 1) using the two-step perfusion technique[19,20]. Ten to twenty million isolated cells were suspended in hepatocyte growth medium (HGM) [Williams' E medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Perbio, Hyclone), 1% penicillin-streptomycin (Invitrogen), 100 nmol/L insulin (Lilly), 1 μmol/L dexamethasone (Organon), and 25 ng/mL epidermal growth factor (Peprotech)] and plated on dishes coated with acid soluble type I collagen at a density of 10^5 cells/cm^2 in a fully humidified atmosphere containing 5% CO_2. Cells were allowed to attach and the first medium change was performed after 24 h and then every 3 d.

Fifteen days later, hepatocytes died and small colonies of LECs emerged from the culture dishes. The culture medium was then switched to DMEM containing 10% FBS and 1% penicillin/streptomycin in order to stimulate the growth of these cells. When colonies were evident, the other cell types were mechanically eliminated and LECs were lifted using trypsin (Invitrogen). Isolated LECs were replated on 6 well collagen I - coated plates and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For each passage, cells were analyzed using immunofluorescence, flow cytometry and RT-PCR.

Immunofluorescence

Isolated cells were cultured on collagen I-coated glass cover-slips and fixed with paraformaldehyde 4% (Merck). Then cells were permeabilised for 15 min using 1% Triton X-100 (Sigma) in TBS buffer (50 mmol/L Tris-HCl pH 7.4 and 150 mmol/L NaCl). Non-specific immunostaining was prevented by 1 h incubation in TBS buffer containing 3% non-fat dry milk at 37℃. The primary antibodies [monoclonal mouse anti-human Alb (Sigma), polyclonal rabbit anti-human AFP (Dakocytomation), monoclonal mouse anti-human CK-7 (Chemicon), monoclonal mouse anti-human CK-19 (Dakocytomation) and monoclonal mouse anti-human oval cell (OV)-6 (donation from professor Tania Roskams-Katholic Universteit van Leuven)] were incubated with cells for 1 h at room temperature. The secondary antibodies used [Cy3 conjugated anti-mouse IgG (Jackson immunoresearch laboratories) and FITC-conjugated anti-rabbit IgG (Sigma)] were incubated for 1 h at room temperature. The nuclei were revealed by 4, 6-Diamidino-2-phénylindole (DAPI, Sigma) staining.

Periodic acid Schiff reaction

Cells were fixed with paraformaldehyde 4% (Merck) during 20 min at room temperature, incubated with 1% periodic acid solution for 10 min, rinsed in distilled water and placed in Schiff reagent for 15 min. After washing for 10 min with water, nuclei were revealed by hematoxylin staining.

Flow cytometry

Cells were harvested, suspended at a concentration of 500-1000/μL in PBS and incubated for 30 min at 4℃ with the following antibodies: CD45-APC, CD34-APC (Becton Dickinson), CD90-PC5, CD73-PE, CD29-PE, HLA-DR-FITC (BD Pharmingen); CD44-FITC, CD117-PC5 (Immunotech); HLA-ABC-PE (Dako Systems), CD13-PE (Beckham Coulter) and CD105 Endoglin-FITC (Immunokontact). The corresponding isotypes were used for evaluation of non-specific binding of monoclonal antibodies. Cells were then washed and suspended in Isoton® (Beckham Coulter) for reading with a Beckham Coulter Flow Cytometer.

Reverse transcription-polymerase chain reaction

Total RNA was prepared using Tripure isolation Reagent (Roche) and cDNA was generated using the Thermoscript™ RT kit (Invitrogen). PCR amplification was performed for 28 cycles using specific primers (Table 2) and Elongase™ Enzyme Mix (Invitrogen). PCR products were subject to 1% agarose gel electrophoresis and visualized after ethidium bromide staining.

Mouse transplantation and immunohistochemistry

One million LECs were suspended in 100 μL PBS and injected into the spleen of 6-8 wk SCID mice, half of them being subject to 2/3 hepatectomy. Thirty days after transplantation, livers were harvested, formalin-fixed and embedded in paraffin. Five μm liver sections were deparaffinized and hydrated to distilled water. Endogenous peroxidase activity was blocked by incubation for 15 min in a 3% hydrogen peroxide methanol solution. Slides were incubated overnight with polyclonal rabbit anti-human Alb antibody (Calbiochem) diluted at 1/2500 in PBS containing 0.1% bovine serum albumin. Staining detection was visualized by Envision Dako Anti-Rabbit (Dakocytomation) using diaminobenzidine (Sigma) as chromogenic substrate. The nuclei were revealed by hematoxylin staining.

RESULTS

Characterization of LECs

Isolated parenchymal cell fraction contained majority of hepatocytes with viability estimated at 90% using trypan blue exclusion technique. After 15 d culture in collagen type I - coated plates, hepatocytes died and small colonies of LECs emerged and proliferated (Figure 1A). The LECs appeared in polygonal shape with central nucleus, several

Table 2 Primers used for RT- PCR analysis of LECs

Gene name	Primers	PCR product size
AFP	5′-TGAAATGACTCCAGTAAACCC-3′ 5′-GATGAAGCAAGAGTTTCTCATT-3′	199 pb
Oct-4	5′-CGA CCA TCT GCC GCT TTG AG-3′ 5′-CCC CCT GTC CCC CAT TCC TA-3′	573 pb
Human hepatocyte nuclear factor 4 (HNF4)	5′-CCA AGT ACA TCC CAG CTT TC-3′ 5′-TTG GCA TCT GGG TCA AAG-3′	295 pb
ALB	5′-CCTTGGTGTTGATTGCCTTTGCTC-3′ 5′-CATCACATCAACCTCTGGTCTCACC-3′	308 pb
CK-8	5′-AAG GGC TGA CCG ACGAGA TC-3′ 5′-GCT TCC TGT AGG TGG CGA TC-3′	537 pb
Glucose 6 phosphatase (G6P)	5′-TCA GCT CAG GTG GTC CTC TT-3′ 5′-CCT CCT TAG GCA GCC TTC TT-3′	291 pb
α-antitrypsin (AAT)	5′-TCGCTACAGCCTTTGCAATG-3′ 5′-GGAACTCCTCCGTACCCTCAA-3′	142 pb
Tyrosine aminotransferase (TAT)	5′-TGA GCA GTC TGT CCA CTG CCT-3′ 5′-ATG TGA ATG AGG AGG ATC TGA G-3′	359 pb
Cytochrome P450 1B1 (CYP1B1)	5′-GAGAACGTACCGGCCACTATCACT-3′ 5′-GTTAGGCCACTTCACTGGGTCATGAT-3′	357 pb
CYP2B6	5′-CCT CTT CCA GTC CAT TAC CG-3′ 5′-TGA CTG CCT CTG TGT ATG GC-3′	551 pb
CYP3A4	5′-TGC TGT CTC CAA CCT TCA CC-3′ 5′-TAG CTT GGA ATC ATC ACC ACC-3′	802 pb
CK-19	5′-TTTGAGACGGAACAGGCTCT-3′ 5′-CAGCTCAATCTCAAGACCCTG-3′	426 pb
GGT	5′-GAC GAC TTC AGC TCT CCC AG-3′ 5′-CTT GTC CCT GGA TTG CTT GT-3′	489 pb
Multidrug resistance-associated protein 2 (MRP2)	5′-ACA CCA ACC AGA AAT GTG TC-3′ 5′-CCA AGG CCT TCC AAA TCT C-3′	660 pb
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	5′-CGGAGTCAACGGATTTGGTCGTAT-3′ 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′	307 pb

nucleoli and a high cytoplasm/nucleus ratio (Figure 1B). LECs colonies grew up, increased in size and had contact inhibition after filling free spaces in the culture dishes. LECs were obtained both from freshly isolated and cryopreserved cell suspensions. Purified LECs resisted to trypsin application and maintained their proliferation potential of more than 5 passages in DMEM medium supplemented with 10% FBS (Figure 1C and D) that leads to purification of the cell population, indicating the self-renewing ability of LECs *in vitro*.

As shown in Figure 2, the flow cytometry analysis of LECs immuno-phenotype revealed that the cell population strongly expressed common markers of haematopoietic and mesenchymal stem cells. Indeed, LECs population was positive for CD90 (Thy-1) and CD44, moderately expressed CD73 and was negative for CD105 and CD13. LECs expressed CD29, an immature liver cell marker, but were negative for the hematopoietic markers CD34, and CD117 whereas expression of CD45 was weakly noted. Most of LECs expressed HLA-ABC but not HLA-DR.

As demonstrated by immunofluorescence, LECs were homogenously positive for AFP, Alb and CK-18 (Figure 3A-C). Biliary markers such as CK-19 and CK-7 were also positive in all the cell population (Figure 3D and E).

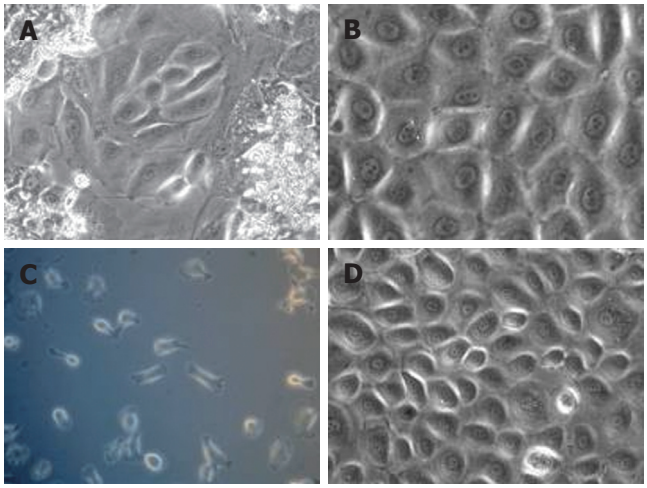

Figure 1 Morphology of LECs after liver cells primary culture (× 200). **A**: After 15 d primary culture, small colonies of LECs emerged; **B**: organized in monolayers; **C**: When colonies were evident, the other cells types were mechanically eliminated and LECs were lifted and replated; **D**: LEC population reaching 70% confluence analyzed for the other passages.

Up to now, oval cells are not unambiguously defined as precursors of hepatocyte and biliary cells in adult liver diseases. But morphology of LECs was distinguishable from oval cells and did not express oval cell marker (OV-6) (Figure 3F). Metabolic function analysis of LECs also demonstrated their immature status. Thus LECs did not secrete Alb and urea in the culture medium (data not shown) and periodic acid-Schiff staining showed the absence of glycogen storage (Figure 3G).

The data were reproducible in 3 different liver cell supensions and the bi-potential phenotype of LECs was confirmed using RT-PCR analysis. These cells strongly expressed Alb, CK-8, CK-19 and CYP1B1 (Figure 4). Furthermore, they expressed immature markers such as AFP and POU transcription factor octamer-binding protein 4 (Oct-4) but not HNF-4 (transcription factor expressed early in endoderm formation). Expression of mature hepatocyte markers such as G6P and AAT was weakly noted whereas hepatocyte markers of final maturation phase (CYP2B6, CYP3A4 and TAT) were not found. Expression of genes associated with biliary phenotype was also noted as for GGT and MRP2.

These parameters were maintained up to 5 passages. After that, LECs adopted a very large cytoplasm (Figure 5A and B). Whereas expression of Alb, CK-18 and CK-19 (Figure 5D-F) was maintained during all of cultured time, AFP (Figure 5C) and CK-7 (Figure 5G) staining was significantly decreased.

Intrasplenic transplantation of LECs

To investigate the *in vivo* outcome of LECs, transplantation of these cells was performed in the spleen of SCID mice, half of them were hepatectomized. One month after transplantation, mice were sacrificed and plasma analyzed for the detection of human Alb. No presence of this marker was noted after transplantation. In parallel, liver tissues were analyzed using immunohistochemistry. Foci or isolated cells positively stained for human Alb were

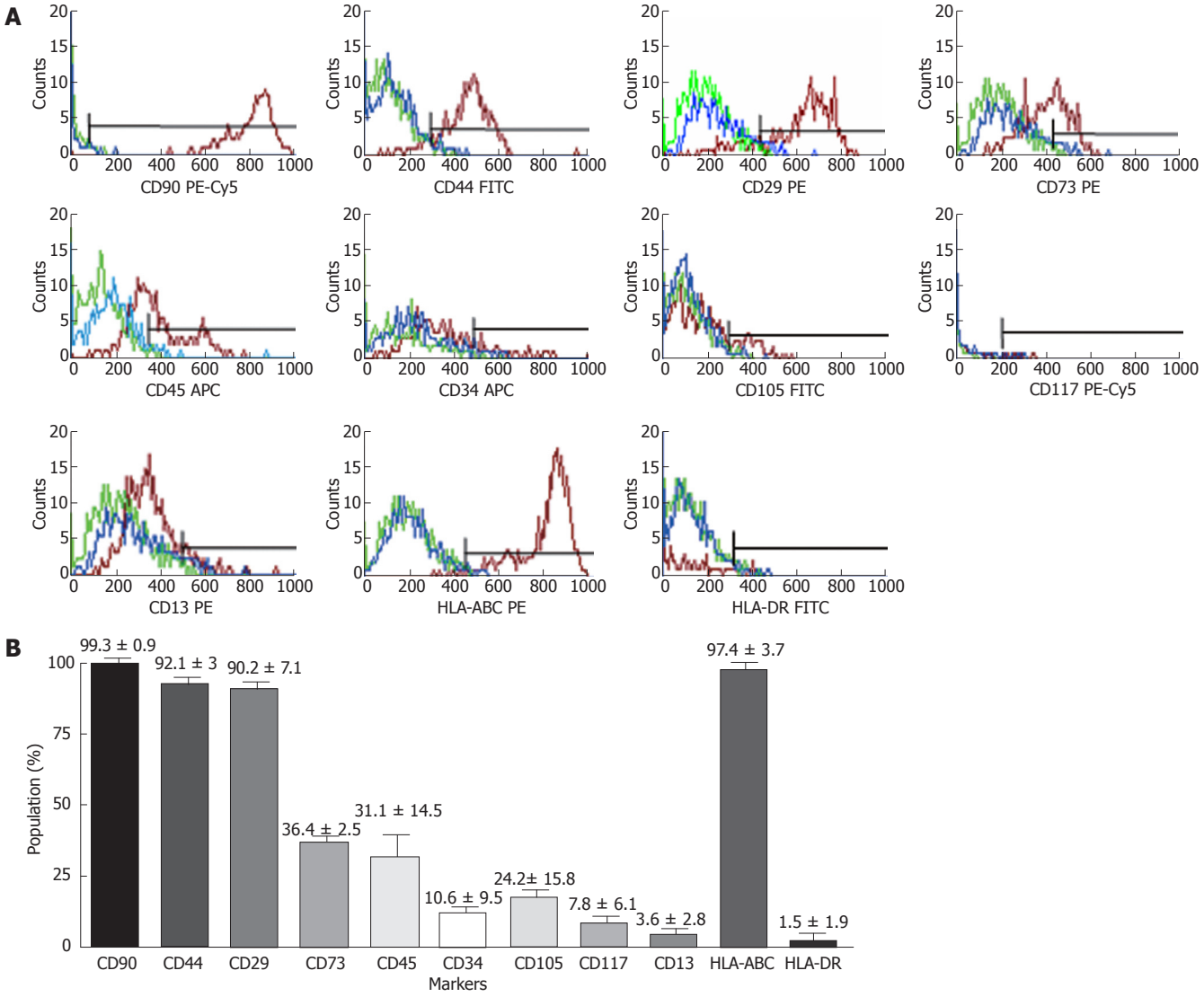

Figure 2 Flow cytometry analysis of purified LECs. **A**: Flow cytometry histograms showing three cell populations isolated from different livers. Antigens expression was detected with specific antibodies (red). Isotype-matched antibody controls (blue) and auto-fluorescence (green) showing the non-specific fluorescent labeling; **B**: Mean expression percentage of analyzed cell markers obtained from three different LEC populations after the third passage.

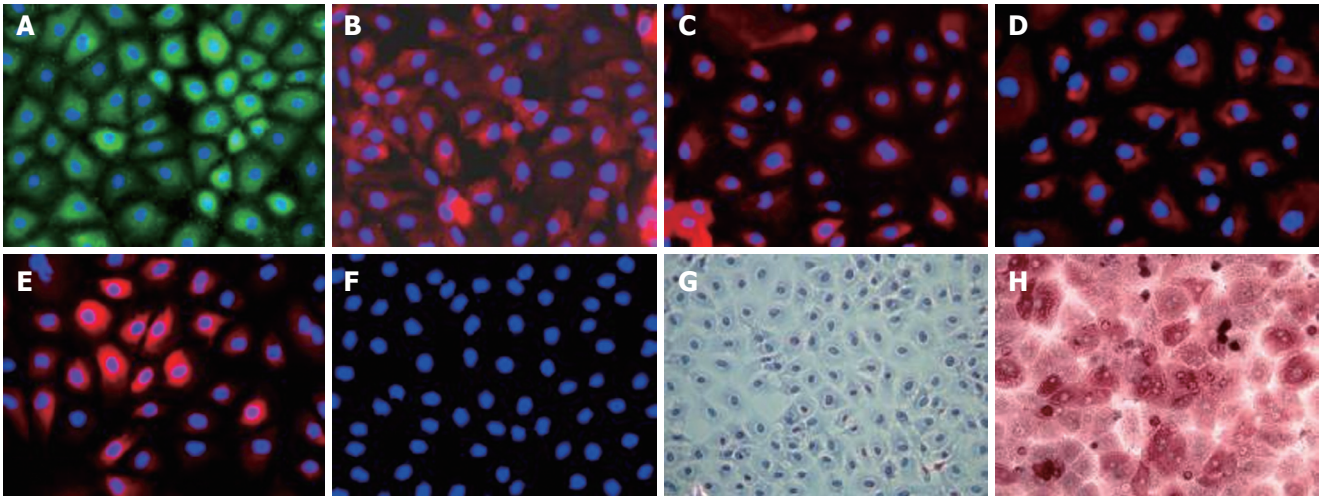

Figure 3 Immunofluorescence characterization of LECs in primary culture (× 200). Immunofluorescence was visualized using anti-rabbit FITC (green) and anti-mouse Cy3 (rouge). The nuclei, stained by DAPI, appeared in blue. **A**: The expression of AFP, fetal liver marker, reflected their immature status; LECs expressed both hepatocytic (**B**: ALB; **C**: CK-18) and biliary markers (**D**: CK-19; **E**: CK-7); **F**: LECs did not express oval cell marker OV-6; **G**, **H**: The representative photographs at phase contrast microscopy after PAS staining. LECs in the primary culture were not able to store glycogen (**G**) as compared to human hepatocytes (**H**). The nuclei were stained with hematoxylin.

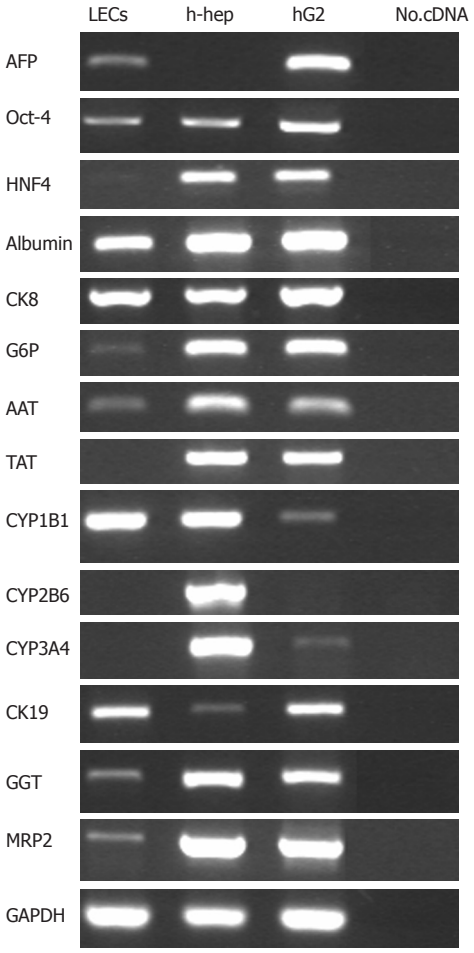

Figure 4 RT-PCR analysis of purified LECs. RT-PCR was assessed on mRNA extracted from purified LECs at the 3rd passage. Data shown are agarose gel electrophoresis of amplification products. Isolated human liver cells (h-hep) and HepG2 cells were used as positive controls. LECs expressed human fetal liver markers (α-fetoprotein (AFP) and transcription factor Oct-4) and adopted hepatocyte (positive for ALB, CK-8, G6P, AAT, and CYP1B1) and biliary (positive for CK-19, GGT and MRP2) phenotypes. These cells did not express HNF4, TAT, CYP2B6 and CYP3A4.

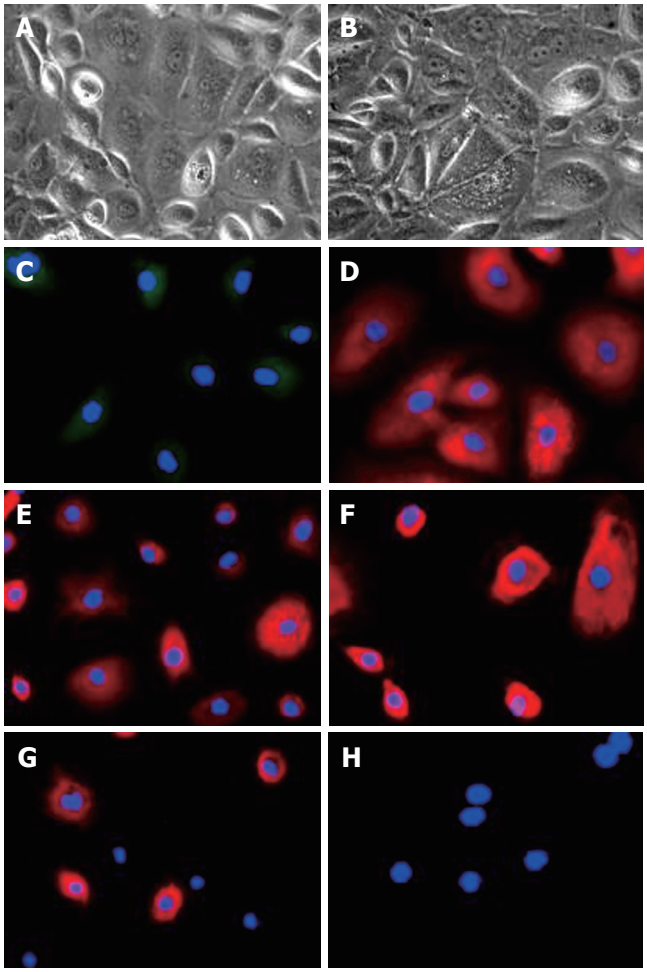

Figure 5 Characterization of LECs at the 5th passage (× 200). The phase contrast microscopy photographs of LECs at the 5th passage showing an increased size (**A** and **B**). Using immunofluorescence, in parallel to morphological changes, LECs lost the expression of AFP (**C**) and CK-7 (**G**) but maintained the expression of ALB (**D**), CK-18 (**E**) and CK-19 (**F**). LECs remained mostly negative for oval cell marker OV-6 (**H**).

detected in the recipient liver tissue of 3 transplanted mice (Figure 6A and B) (2 of them were hepatectomized). The detected cells were mostly localized near vascular structures.

DISCUSSION

In the present study, we report the *in vitro* isolation of LECs from normal adult human liver. The culture of the purified cells was maintained *in vitro* for more than 160 d (seven passages) leading to their characterization at both the immuno-cytochemical and genetic levels. LECs injected into the spleen of SCID mice showed their ability to migrate and engraft into the recipient liver tissue.

Cell suspension obtained after collagenase liver disaggregation might contain all the cell types in the liver, the largest part being hepatocytes whereas the presence of minority of non-parenchymal cells is not excluded. When majority of hepatocytes undergoes cell death, LECs spontaneously emerge and proliferate. These cells were closely associated in a monolayer and formed several

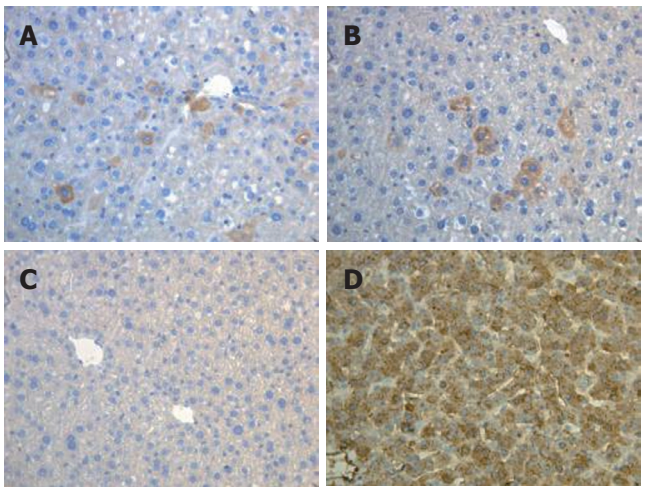

Figure 6 Immunohistochemical analysis of LECs in the liver of transplanted SCID mice. Foci or isolated cells stained for human Alb (brown) were detected around centrolobular vein (**A**) and portal area (**B**) of transplanted SCID mice (2 hepatectomized and 1 normal SCID mice; **C**: Human Alb cells were not detected in control mice; **D**: All of cells in human liver sections were stained for human Alb.

isolated colonies in the culture dishes. We hypothesized that these cells are resident in the normal adult liver and probably co-isolated with hepatocytes. Their detection in the intact liver may remain difficult because of absence of specific markers. Their proliferation may be stimulated due to chemical signals released in the culture medium after the death of mature hepatocytes (as proposed *in vivo* in liver injury animal models). Furthermore, LECs have been shown to survive *in vitro* after they were lifted in new culture dishes leading to the purification of cell population. LECs were evaluated both in the primary culture (heterogeneous environment) and after trypsin application, at the immunofluorescence level and showed that these cells homogenously expressed hepatocytic (Alb, CK-18) and biliary (CK-7 and CK-19) markers. The hepatobiliary phenotype was confirmed using RT-PCR analysis up to the 5th passage. LECs were also positive for AFP and expressed Oct-4 but not the final maturation phase markers CYP2B6, CYP3A4 and TAT, indicating their immature state. The expression of these markers was maintained while cells continue proliferating. The bi-potential phenotype of LECs has already been described for the oval cells that were presumed to be precursors of hepatocyte and biliary cells in adult liver[2,14,15,21]. LECs were morphologically different from oval cells (high cytoplasm/nuclear ratio) and did not express markers such as CD117, CD105 and OV-6[14,15]. The bi-potential phenotype of LECs may propose these cells as originating from another resident progenitor/stem cells pool within the normal adult liver. LECs were positive for CD90, a marker of hematopoietic and fetal liver stem cells[22-24]. They also expressed integrin β1 (CD29), a marker of hepatoblasts, which persists after maturation process of both hepatocyte and biliary epithelial cells[22,25].

The morphological and immunofluorescence analyses demonstrate that human LECs resemble those previously described in rats[2]. In young adult Fisher 344 rats, LECs were isolated from the non-parenchymal fraction after an incomplete collagenase-digestion of the liver. In this study, hepatocytes were eliminated by differential centrifugation[26]. In adult pig liver, epithelial cells have been described to emerge as clusters after the culture of parenchymal cell fraction. Nonetheless, a non-parenchymal cell fraction, rather than a parenchymal cell fraction, was recommended for obtaining such epithelial cells[18,27]. The LECs reported in these studies even positive for Alb, AFP and the oval cell marker OV-6 did not express biliary cell markers such as CK-19 and GGT. In human, Selden and colleagues have isolated epithelial cell population from the liver of a 6 wk subacute liver failure patient[10]. The authors hypothesized that this condition will enrich the recipient liver of progenitor liver cells. In our study, we demonstrate that LECs, although with low proportion, can be obtained from normal human liver tissue even when we plated a very limited initial cell suspension. Furthermore, we can observe that LECs isolated in our study express CK-8 in contrary to cells isolated from the subacute liver failure patient. The differences observed between these studies may be correlated to the use of liver isolation and cell culture techniques.

In our study, culture of purified LECs was maintained after incubation in DMEM medium supplemented with 10% FBS. The immunofluorescence analysis revealed that after the 5th passage, some LECs had a very large cytoplasm and lose CK-7 although expression of Alb and CK-18 was maintained. A decrease in AFP expression was also noted. These data could suggest spontaneous *in vitro* differentiation of these cells. Such phenomenon has been documented for porcine LECs[18,27]. Nevertheless, other studies demonstrated the ability of LECs to differentiate into hepatocytes and biliary cells after incubation with growth factors defined media[28,29]. In the rat model, normal LECs have also been shown to engraft and to differentiate into hepatocytes or biliary cells[30,31].

To evaluate this parameter, purified LECs were intrasplenically transplanted in 4 SCID mice, half of them being hepatectomized (70%). Evaluation of engraftment efficiency was analyzed 1 mo after transplantation. Although no human Alb was detected in serum, immunohistochemistry analysis demonstrated the presence of human cells within the recipient parenchyma mostly near vascular structures. These data suggest the ability of LECs to migrate and to engraft in the liver. In our study, hepatectomy did not stimulate the engraftment and proliferation potential of human LECs in SCID mouse liver suggesting a low ability of LECs to respond to murine differentiation signals. It is also possible that an inhibition of mouse liver cell regeneration induced by hepato-toxins is necessary for stimulating LECs proliferation.

In conclusion, we demonstrated the presence of LECs population within adult normal human liver. These cells expanded *in vitro*, presented bi-potential phenotype and were able to migrate and to engraft in the recipient parenchyma after intrasplenic transplantation. As being obtained after isolation of normal human liver, the pool of these progenitor/stem-like cells may reside in the liver, however, additional data regarding the progenitor's biology of this organ are needed.

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BASIC RESEARCH

Roles of the MEK1/2 and AKT pathways in CXCL12/CXCR4 induced cholangiocarcinoma cell invasion

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Abstract

AIM: To evaluate the expression of C-X-C motif chemokine receptor 4 (CXCR4) and its signaling cascades, which were previously identified as a key factor for cancer cell progression and metastasis, in cholangiocarcinoma cell lines.

METHODS: The expression of CXCR4 and its signaling cascades were determined in the cholangiocarcinoma cell lines (RMCCA1 and KKU100) by Western blotting. The invasion assays and the detection of actin polymerization were tested in these cholangiocarcinoma cells treated with CXC chemokine ligand -12 (CXCL12).

RESULTS: Expression of CXCR4 was detected in both cholangiocarcinoma cell lines and activation of CXCR4 with CXCL12 triggered the signaling *via* the extracellular signal-regulated kinase-1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K) and induction of cholangiocarcinoma cell invasion, and displayed high levels of actin polymerization. Addition of CXCR4 inhibitor (AMD3100) abrogated CXCL12-induced phosphorylation of MEK1/2 and Akt in these cells. Moreover, treatment with MEK1/2 inhibitor (U0126) or PI3K inhibitor (LY294002) also attenuated the effect of CXCL12-induced cholangiocarcinoma cell invasion.

CONCLUSION: These results indicated that the activation of CXCR4 and its signaling pathways (MEK1/2 and Akt) are essential for CXCL12-induced cholangiocarcinoma cell invasion. This rises Implications on a potential role for the inhibition of CXCR4 or its signal cascades in the treatment of cholangiocarcinoma.

Key words: Cholangiocarcinoma; CXCR4; CXCL12; MEK1/2; PI3K

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INTRODUCTION

Cholangiocarcinoma is a malignant tumor composed of cells resembling those of the biliary tract epithelium[1,2]. The incidence of and mortality rate for cholangiocarcinoma varied considerably in different geographic regions, with the incidence highest in Southeast Asia especially in Thailand[3]. Three-year survival rates of 35% to 50% are achieved in only a few numbers of patients when negative histological margins are attained at the time of surgery[2,4-6]. The causes of lethality of this disease are not only its rapid growth but also the tendency to invade adjacent organs and metastasize[4-6].

At present, a number of molecules implicated in the metastasis processes of cholangiocarcinoma cells have been identified[7-9]. However, there have been no studies exploring the precise mechanisms determining the directional of invasion of cholangiocarcinoma cells into specific organs. In this regard, chemokines are a superfamily of small proteins that bind to G protein-coupled receptors on target cells[10,11]. CXC chemokine ligand-12 (CXCL12)-or stromal cell-derived factor-1 (SDF1) is a member of CXC chemokine family, which was initially cloned from murine bone marrow and characterized as a pre-B-cell growth stimulating factor. CXCL12 exerts effects through its cognate receptor C-X-C motif chemokine receptor 4 (CXCR4), which is the only physiological receptor for CXCL12 and is known to play roles in leukocyte homing as well as metastasis of many kinds of cancer cells[12-15]. A previous study demonstrated that CXCL12 released from fibroblasts induced the increase in migration of cholangiocarcinoma cells[16]. However, the signal transduction pathways following CXCR4 activation and stimulation of cholangiocarcinoma cell invasion have not been delineated.

In this study, we have demonstrated the expression of

CXCR4 in human cholangiocarcinoma cell lines. Using *in vitro* model systems, we demonstrated the activation of CXCR4 by CXCL12 induced phosphorylation of the MEK1/2 and Akt and also enhanced cholangiocarcinoma cells invasion. In addition, administration of AMD3100, a bicyclam noncompetitive antagonist of CXCR4 or inhibition of its signal transduction intermediate molecules (MEK1/2 and PI3K) suppressed the invasiveness of cholangiocarcinoma cells.

MATERIALS AND METHODS

Materials

HAM's F12 medium and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). The recombinant human CXCL12, polyclonal antibodies to human CXCL12 and CXCR4 were purchased from Abcam (Cambridge, MA, USA). Polyclonal antibodies to MEK-1/2 (phosphorylated at Ser217/221 and total), Akt (phosphorylated at Ser473 and total) were purchased from Cell Signaling (Cell Signaling Technology, Beverly, MA, USA). 24-well Biocoat Matrigel invasion chambers (8 μm) were purchased from Becton Dickinson (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell cultures

Two human cholangiocarcinoma cell lines; KKU100 derived from Hilar-cholangiocarcinoma patient[17] (kindly provided by Dr. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University) and RMCCA1 derived from Peripheral-cholangiocarcinoma patient[18] were grown in HAM's F12 medium supplemented with 100 mL/1L fetal bovine serum at 37℃ in a 50 mL/L CO_2 humidified atmosphere. For signal transduction experiments with CXCL12, cells were starved overnight in serum-free medium.

Western blotting analyses

For Western blot analysis, 500 000 cells were seeded in a six-well culture plate, followed by treatment with 100 ng/mL of CXCL12. Cells were collected and then Western blot analyses were performed as previously described[18]. Chemiluminescent detection of antibody–antigen complexes revealed the target proteins on X-ray film.

Cell proliferation assay

Cells were seeded in 96-well culture plates at a density of 10 000 cells per well followed by the addition of CXCL12 in various concentrations. Then cells were incubated for indicated time before applying the WST-1 cell proliferation assay reagent (Roche Diagnostics, Laval, Quebec) according to the recommendation of the manufacturer. The percentage of proliferation was calculated based on the untreated cells.

Cell migration assay

The migration of cholangiocarcinoma cells was assayed using chamber with 8-μm pore filters (Transwell, 24-well cell culture, Coster, Boston, MA). 50 000 cholangiocarcinoma cells were added to the upper chamber. Then 0.5 mL serum-free media with 100 ng/mL of CXCL12 was added to the lower chamber. The chambers were incubated for 12 h at 37℃. After incubation, the filters were fixed and stained with hematoxylin and counted in five random high-power fields under a light microscope[19].

Cell invasion assay

The invasion of cholangiocarcinoma cells was assayed in 24-well Biocoat Matrigel invasion chamber (8 μm; Becton Dickinson, Franklin Lakes, NJ). Similar to the migration assays, 50 000 cells were seeded in the upper chamber while the bottom chamber contained with 100 ng/mL of CXCL12.

Detection of MMP9 activity by gelatin zymography assay

Cholangiocarcinoma cells were starved by culturing in serum-free medium containing with or without CXCL12 for 24 h before collection of the conditioned medium. The conditioned medium was stored at -80℃ and analyzed for MMP9 activity by gelatin zymography.

Detection of actin polymerization

Detection of actin polymerization was performed as described previously[18]. Cholangiocarcinoma cells were treated with AMD3100, U0126, LY294002 or control and incubated for 6 h. Then the cells were incubated in serum-free medium containing with 100 ng/mL of CXCL12 for 4 h. The cells were exposed to Alexa Fluor 488 Phalloidin (Molecular Probes, Eugene, OR) for 30 min and washed with PBS. The cells were examined under a Confocal laser scanning microscope (CLSM), (Olympus SV1000).

Statistical analysis

The experiments were all performed in triplicate and identical results were obtained. Values were expressed as the mean and SD. The student's t-test was used for analysis of the cell proliferation and invasion assay. The P value of less than 0.05 was considered significant.

RESULTS

Expression of CXCR4 and CXCL12 in cholangiocarcinoma cell lines

In order to utilize an *in vitro* system to study the influence of CXCR4 activation, the expression of CXCR4 and CXCL12 in two cholangiocarcinoma cell lines (RMCCA1 and KKU100) needed to be investigated. Western blot analysis demonstrated definite expression of CXCR4 but not CXCL12 in both cholangiocarcinoma cell lines (Figure 1).

The effect of CXCR4 on cholangiocarcinoma cell proliferation

Since the activation of CXCR4 with CXCL12 was known to play an important role in cell proliferation in many kinds of cancer cells, we investigated the role of CXCL12 in cholangiocarcinoma cell proliferation. Cell proliferation assay was performed in RMCCA1 and KKU100 cells treated with CXCL12 at concentrations of 0, 50, 100 and 200 ng/mL. After 48 h of incubation, the results showed that CXCL12 had no effect on cholangiocarcinoma cell

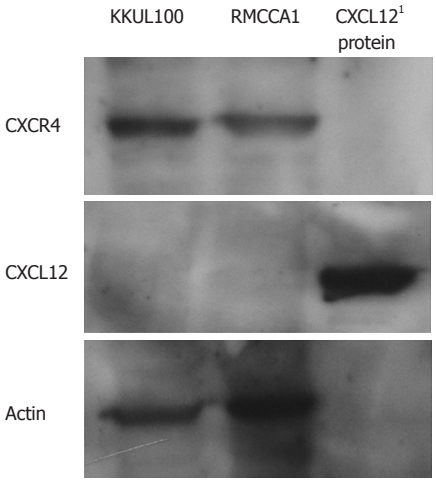

Figure 1 CXCR4 and CXCL12 expression in human colorectal carcinoma cell lines (RMCCA1 and KKU100). Western Blot analysis revealed the presence of CXCR4 specific bands in both cell lines. However, no expression of CXCL12 was identified in both cell lines. [1]The CXCL12 recombinant protein was used as a positive control for detection of CXCL12.

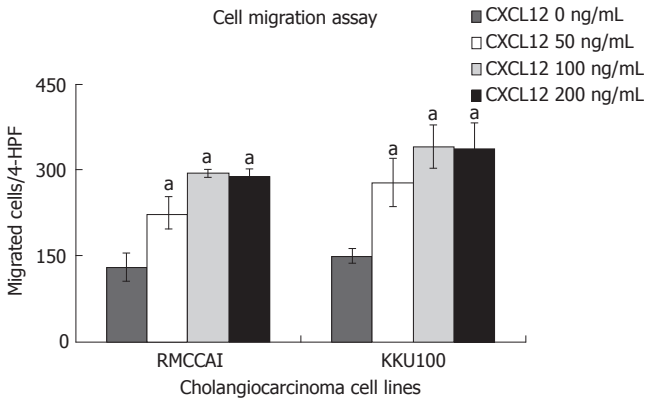

Figure 3 Effect of CXCL12 on the migration of cholangiocarcinoma cells. RMCCA1 and KKU100 were seeded in the 8-μm pore filters (Transwell, 24-well cell culture, Coster, Boston, MA). The bottom chamber contained 0 or 100 ng/mL of CXCL12. After 24 h, the cells on the lower surface were counted under a microscope at five random 100 x power fields. The experiment was repeated for 3 times and the data represent the average results from 3 individual experiments. CXCL12 induced the migration of cholangiocarcinoma cells ($^aP < 0.05$ *vs* 0 ng/mL of CXCL12).

proliferation (Figure 2).

The effect of CXCL12 on cholangiocarcinoma cell migration and invasion

We found that CXCL12 induced the migration of RMCCA1 and KKU100. Their maximum effect was identified at 100 ng/mL of CXCL12 (Figure 3). Therefore, the following cell invasion experiments were performed by using CXCL12 at a concentration of 100 ng/mL. CXCL12 significantly enhanced cholangiocarcinoma cell invasion when compared with untreated cells (Figure 4). To confirm the mechanism by which CXCR4 induced invasion of cholangiocarcinoma cells, RMCCA1 and KKU100 cells were pre-treated with AMD3100, a specific inhigitor for CXCR4, and then treated with CXCL12 for evaluation of cell invasion activity. The invasion induced by CXCL12

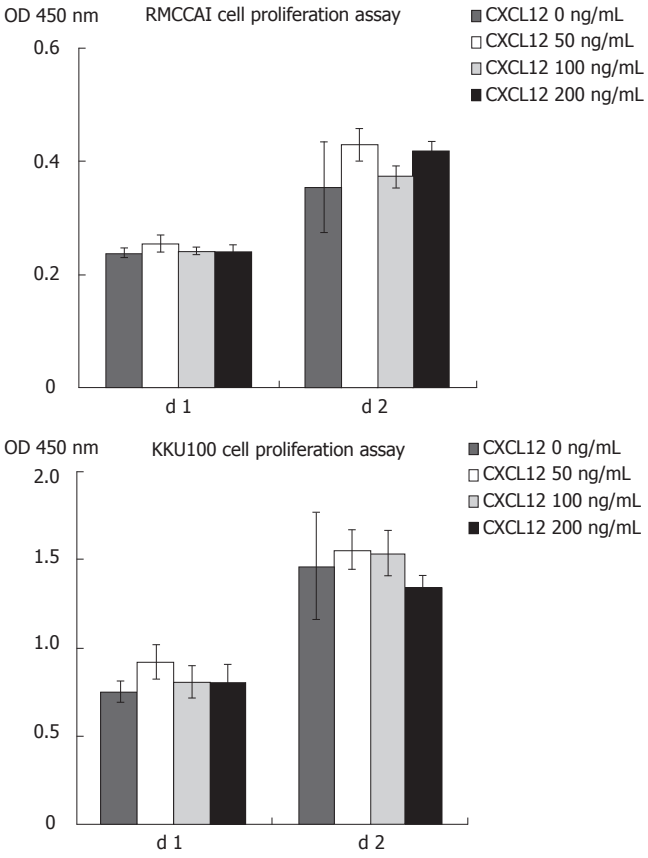

Figure 2 Effect of CXCL12 on the proliferation of cholangiocarcinoma cells. RMCCA1 and KKU100 were treated with CXCL12 at various concentrations (0, 50, 100 and 200 ng/mL). Cell proliferation assay was performed after 2 d by using WST-1. The absorbance at 450 nm, against a reference wavelength of 650 nm, was determined.

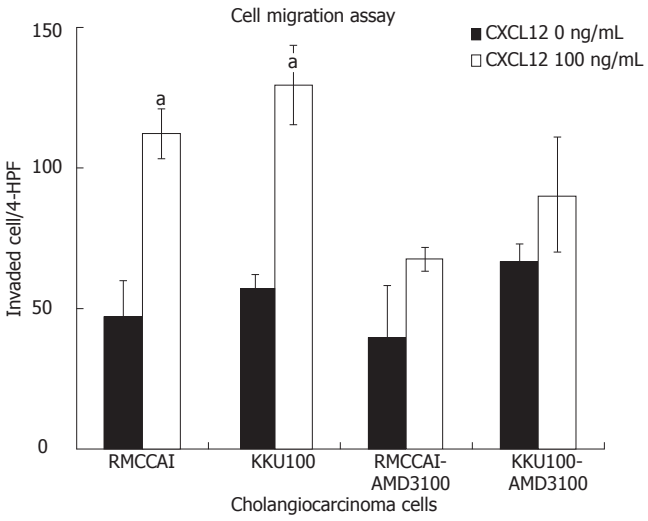

Figure 4 Effect of CXCL12 on the invasion of cholangiocarcinoma cells. RMCCA1 and KKU100 were pre-treated with or without AMD3100 for 12 h then were seeded in the 24-well Biocoat Matrigel invasion chamber. The bottom chamber contained 0 or 100 ng/mL of CXCL12. After 24 h, the cells on the lower surface were assayed as described previously. CXCL12 induced the invasion of cholangiocarcinoma cells. The effect of CXCL12 was decreased when the cells were pre-treated with AMD3100 ($^aP < 0.05$).

was inhibited by AMD3100 in both cholangiocarcinoma cell lines (Figure 4).

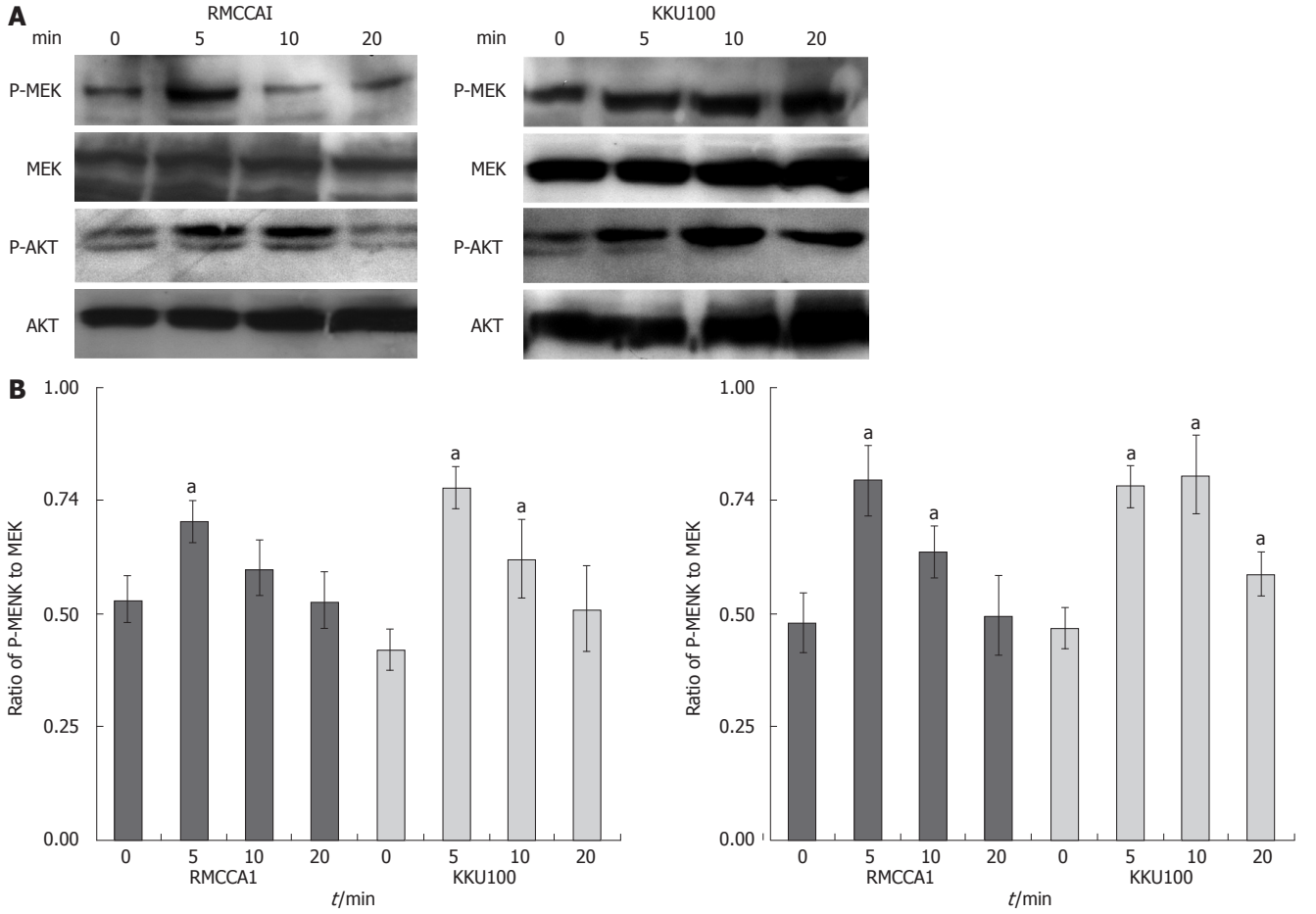

Figure 5 **A**: Western blot analysis of MEK1/2 and Akt phosphorylation in CXCL12-treated cholangiocarcinoma cells. RMCCA1 and KKU 100 were treated with 100 ng/mL of CXCL12 for indicated time. MEK1/2 and Akt phosphorylation were determined by Western blot as described; **B**: The average band intensity based on 3 biologically separate experiments, showing levels of MEK1/2 and Akt phosphorylation relative to the levels of MEK1/2 and Akt expression from the same experiment ($^aP < 0.05$).

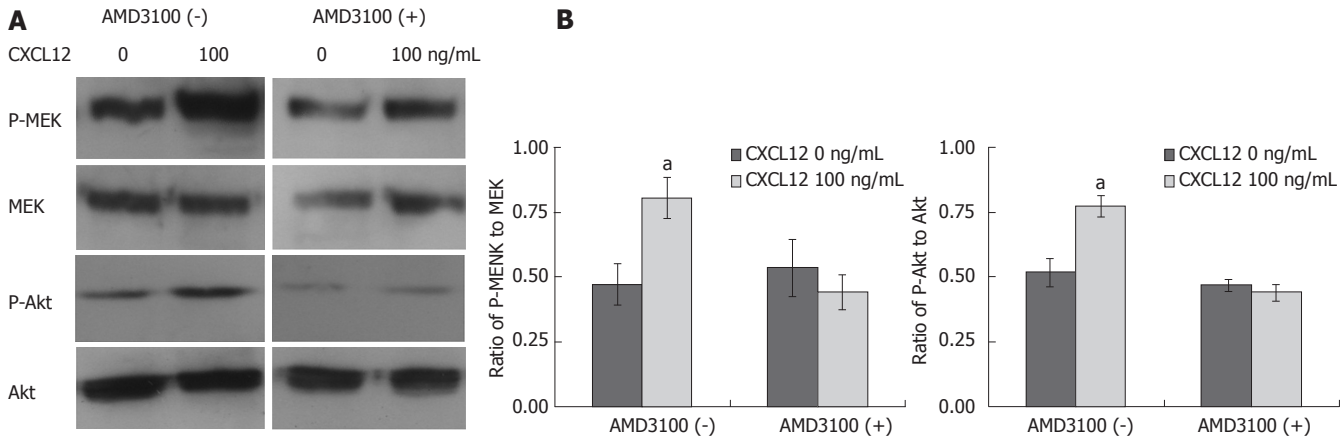

Figure 6 **A**: Effect of AMD3100 on CXCL12-induced phosphorylation of MEK1/2 and Akt in cholangiocarcinoma cells. RMCCA1 cells were pre-treated with or without AMD3100 before added CXCL12. Cells were collected at 5 min and MEK1/2 and Akt phosphorylation were determined by Western blot as described; **B**: The average band intensity based on 3 biologically separate experiments, showing levels of MEK1/2 and Akt phosphorylation relative to the levels of MEK1/2 and Akt expression from the same experiment ($^aP < 0.05$ *vs* 0 ng/mL of CXCL12).

The effect of CXCL12 on the phosphorylation of MEK1/2 and Akt in cholangiocarcinoma cells

We attempted to evaluate the signaling pathways relevant to the CXCL12 induction of cholangiocarcinoma cell invasion. The phosphorylation of molecules, which were previously demonstrated as CXCR4, mediated signaling molecules, was assayed by Western blot analysis. CXCL12-treated cells demonstrated a higher extent of the phosphorylated MEK1/2 and Akt than untreated cells (Figure 5). To determine whether the activation of CXCR4 induced phosphorylation of these signal transduction molecules, cells were pre-treated with AMD3100. The phosphorylation of MEK1/2, and Akt in AMD3100 pre-treated cells was significantly lower than in untreated cells (Figure 6).

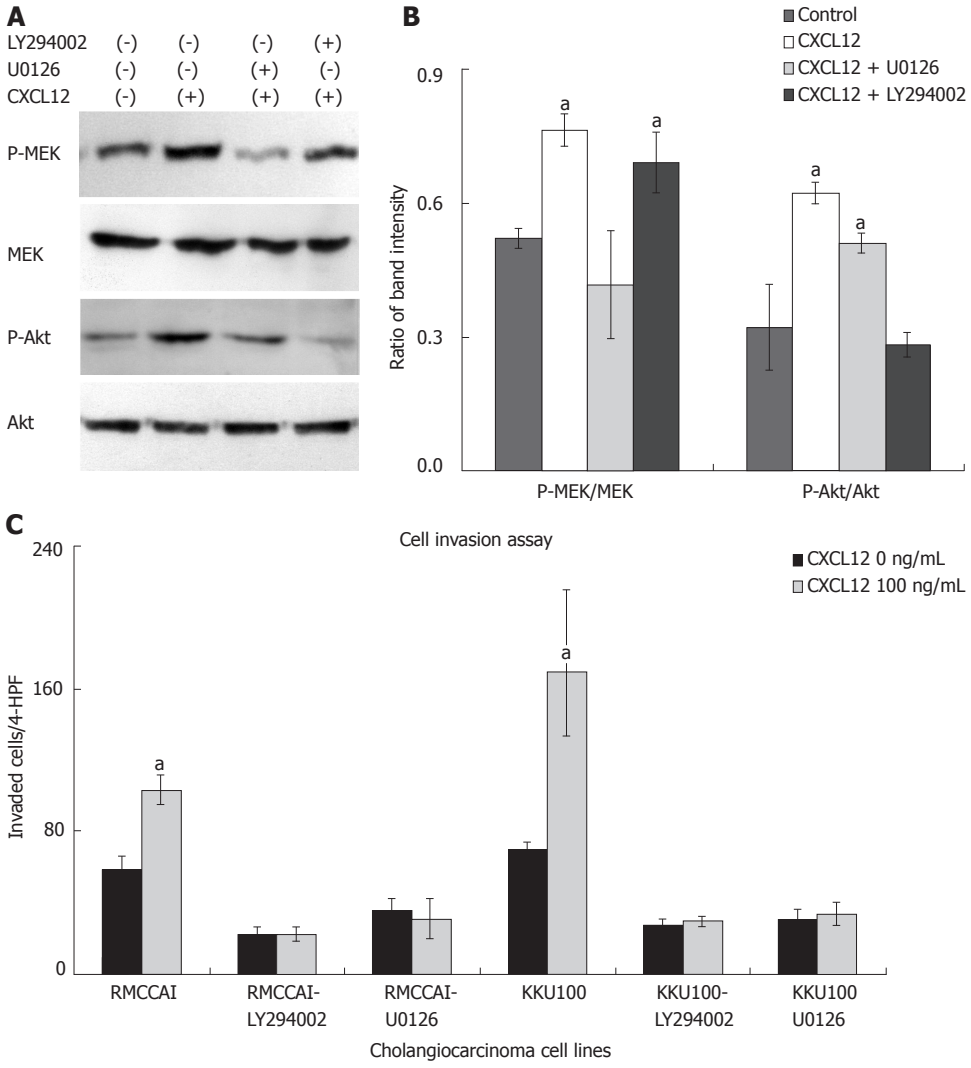

Figure 7 **A**: Effect of U0126 and LY294002 on CXCL12-induced phosphorylation of MEK1/2 and Akt in cholangiocarcinoma cells. RMCCA1 cells were pre-treated with U0126 or LY294002 before added CXCL12. Cells were collected at 5 min and MEK1/2 and Akt phosphorylation were determined by Western blot as described; **B**: The average band intensity based on 3 biologically separate experiments, showing levels of MEK1/2 and Akt phosphorylation relative to the levels of MEK1/2 and Akt expression from the same experiment ($^aP < 0.05$ compared with control); **C**: Effect of MEK1/2 and Akt phosphorylation induced by CXCL12 on the invasion of cholangiocarcinoma cells. RMCCA1 and KKU100 were pre-treated with or without LY294002 and U0126 for 12 h then were seeded in the 24-well Biocoat Matrigel invasion chamber. The bottom chamber contained 0 or 100 ng/mL of CXCL12. After 24 h, the cells on the lower surface were assayed as described previously. LY294002 and U0126 inhibited the effect of CXCL12 induced cholangiocarcinoma cells invasion ($^aP < 0.05$).

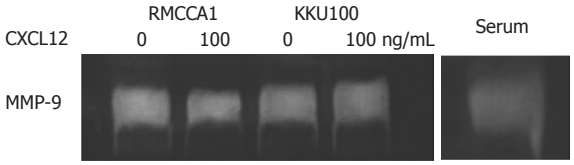

Figure 8 Gelatin zymography of the conditioned medium from cholangiocarcinoma cell lines revealed the proteolytic bands at molecular weight indicating them to be MMP-9. Levels of the proteolytic activity are not different between each sample.

Inhibition of the MEK1/2 or PI3K pathway attenuates CXCL12-induced cholangiocarcinoma cell invasion

The ability of MEK1/2 inhibitor (U0126) and PI3k inhibitor (LY294002) to decrease the effect of CXCL12-induced phosphorylation of MEK1/2 and Akt was assessed. The MEK1/2 inhibitor (U0126) suppressed CXCL12-induced MEK1/2 phosphorylation and the PI-3K inhibitor (LY294002) suppressed CXCL12-induced Akt phosphorylation (Figure 7A and B). To evaluate the contribution of the MEK1/2 or PI3K pathways to CXCL12-induced cholangiocarcinoma cell invasion, RMCCA1 and KKU100 cells were pre-treated with U0126 or LY294002 and then treated with CXCL12. The invasion induced by CXCL12 was inhibited by U0126 or LY294002 in both cholangiocarcinoma cell lines (Figure 7C). These results strongly suggest that the activation of MEK1/2 and PI3K signaling pathways is essential for CXCL12-induced cholangiocarcinoma cell invasion.

Activation of CXCR4 by CXCL12 had no influence on MMP-9 activation

Previous studies have demonstrated that CXCL12 induced MMP-9 activation. Therefore, we investigated the effect of CXCL12 on MMP-9 activation by gelatin zymography from condition medium of cholangiocarcinoma cells. The results showed that prominent constitutive MMP-9 activation was observed in both cholangiocarcinoma cell lines. However, activation of CXCR4 by CXCL12 had no influence on MMP-9 activation (Figure 8).

The effect of CXCL12 on the actin cytoskeleton of cholangiocarcinoma cells

The ability of cancer cell invasion requires coordinated activation of extracellular matrix degradation and cell motility mechanism. The cell motility was assessed by checking the actin polymerization. Cholangiocarcinoma cells were stained with phalloidin for detection of actin polymerization. Serum-starved cells showed low levels of actin polymerization. After the treatment with 100 ng/mL of CXCL12, cholangiocarcinoma cells displayed

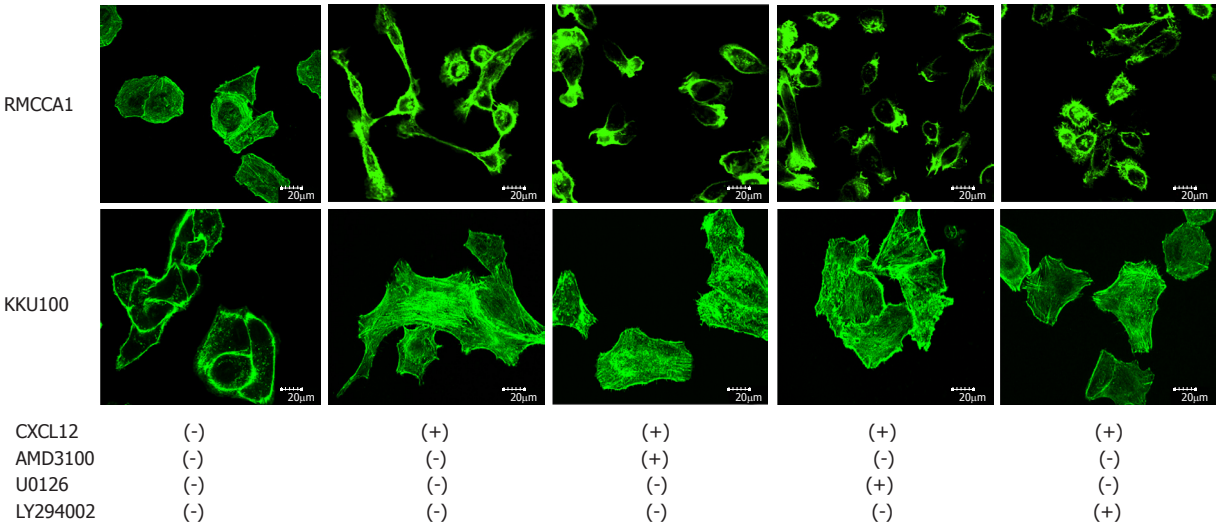

Figure 9 Effect of CXCL12 on the polymerization of actin cytoskeleton. Cholangiocarcinoma cells were pre-treated with vehicle, AMD3100, U0126 or LY294002 and incubated for 6 h in medium containing 0 or 100 ng/mL CXCL12. The cells were stained with Alexa Fluor 488 Phalloidin to visualize actin cytoskeleton under a Confocal laser scanning microscope (Olympus SV1000).

high levels of actin polymerization in the periphery of the cells and a distinct pseudopodia formation. Treatment of cholangiocarcinoma cells with AMD3100, U0126 or LY294002 before addition of CXCL12 caused eradication of actin polymerization (Figure 9).

DISCUSSION

Cholangiocarcinoma is a disease with dismal prognosis characterized by early vascular invasion and metastasis. Therapeutic options for cholangiocarcinoma have been limited due to poor response to chemotherapy and radiation therapy. Surgery is perhaps the only effective treatment for cholangiocarcinoma[2,4]. Previous studies suggested that the most important prognostic factor is a tumor-free surgical margin while other features that were associated with a poor prognosis include factors connected to the extent of disease that is caused by cancer cell invasion, such as bilobar distribution, lymph node involvement, vascular invasion and distant metastases[4,5]. Therefore, an understanding of the mechanism of cholangiocarcinoma cell invasion will be a decisive step towards the development of targeted tumor-specific therapies.

Chemokines and their receptors are involved in the process of cell migration during inflammation. Recently, studies implicated CXCR4 in chemotaxis, invasiveness and metastasis of tumors, particularly in metastasis of breast cancer, in an organ-specific manner[13,20]. In this present work, we report the results of our studies of CXCR4 and CXCL12 expression in two kinds of human cholangiocarcinoma cell lines; KKU100 derived from the hilar-cholangiocarcinoma patient and RMCCA1 derived from the peripheral-cholangiocarcinoma patient. Both cell lines are expressed CXCR4 but not CXCL12. These findings imply a paracrine effect of CXCR4/CXCL12 rather than an autocrine such in both cholangiocarcinoma cell lines.

In the present study, the effect of CXCL12 on CXCR4 in two cholangiocarcinoma cell lines was tested *in vitro* by using cell proliferation, cell migration and cell invasion assays. The findings provided interesting data on the possible molecules of significance involved in promoting cholangiocarcinoma cell invasion. Despite suggestions in previous reports that CXCL12 was a potent stimulator for small cell lung cancer cell proliferation[21], this present study included others arrived to exactly the opposite conclusions[22,23]. In cholangiocarcinoma cell lines, we identified that CXCL12 had no direct effect on cell proliferation. We suggested that these differences might be due to the different culture system or to different target cells. We identified that both cholangiocarcinoma cell lines expressed CXCR4 and stimulation of CXCR4 with CXCL12 promotes cancer cell migration and invasion. Moreover, we also found that KKU100 had a higher invasiveness property than RMCCA1. This result was related with the high expression of CXCR4 in KKU100. Our studies suggested that these events may involve the activation of the ERK1/2 and PI3K. Previous studies have demonstrated that activation of ERK1/2 by G-protein-coupled receptors occurred *via* the Raf/MEK1/2/ERK1/2 cascade while activated PI3K converted phosphatidylinositol 4, 5 phosphate (PIP2) into phosphatidylinositol 3, 4, 5 phosphate (PIP3), which results in the membrane localization of Akt[24,25]. The latter assertion is based on the finding that inhibition of CXCR4 by AMD3100 suppressed the phosphorylation of MEK1/2 and Akt and also inhibited the invasiveness properties of cholangiocarcinoma cells. Moreover, the addition of MEK1/2 inhibitor (U0126) or PI3K inhibitor (LY294002) also attenuated the effect of CXCL12-induced cholangiocarcinoma cell invasion. To the best of our knowledge, this present study is the first report demonstrating the signal transduction pathways of CXCR4 in cholangiocarcinoma. The targets of AMD3100, U0126 and LY294002 (CXCR4, MEK1/2 and PI3K, respectively) are shown in Figure 10. These results are consistent with the previous studies that demonstrating the activation of ERK and Akt signaling after stimulation of cancer cells

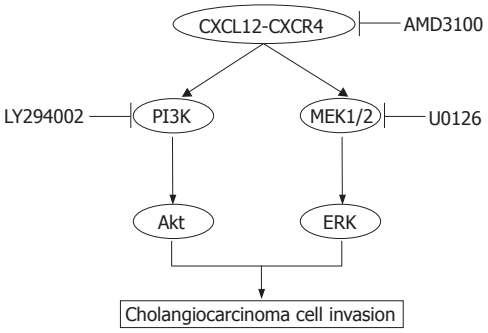

Figure 10 The pathway diagram identified the targets of AMD3100, U0126 and LY294002 (CXCR4, MEK1/2 and PI3K respectively). Inhibition of these pathways abrogates the invasion of cholangiocarcinoma cells.

with CXCL12[22,26,27].

In cancer cells, high level of actin polymerization is a key for the formation of pseudopodia, which in turn are implicated in the enhancement of cancer cell migration and invasion[28]. Treatment of cholangiocarcinoma cells with CXCL12 resulted in our study in the increase in actin polymerization. In addition, inhibiting CXCR4 with AMD3100 in our study resulted in a dramatic decrease in actin polymerization. Our findings suggest that CXCL12 and CXCR4 play an important role in the invasion as well as the metastasis in cholangiocarcinoma. Previous studies have demonstrated the influence of CXCL12 on MMP-9 secretion[26,27]. In cholangiocarcinoma cell lines, we showed that CXCL12 had no effect on MMP-9 secretion. The mechanisms responsible for MMP-9 activation in cholangiocarcinoma cells remain unclear. We suggested that the mechanism of cholangiocarcinoma cell invasion is not dependent on a single oncogenic pathway but possible complex networks of ligands and their receptors are implicated in cancer invasion such as c-Met/HGF[18], focal adhesion kinase[29] and TNFα/TNF receptor as well[30].

In conclusion, in this present experimental study, we show that the stimulation of CXCL12/CXCR4 plays an important role in cholangiocarcinoma cell invasion by the induction of MEK1/2 and Akt signal transductions and stimulation of actin polymerization. Inhibition of CXCL12/CXCR4 and its pathway may represent one of the potential approaches in cholangiocarcinoma therapy.

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RAPID COMMUNICATION

Usefulness of fecal lactoferrin and hemoglobin in diagnosis of colorectal diseases

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Abstract

AIM: To evaluate prospectively usefulness of fecal lactoferrin (Lf) and fecal hemoglobin (Hb) in the diagnosis of colorectal diseases.

METHODS: Fecal Lf and Hb were measured using ELISA in 872 patients before they underwent colorectal endoscopy.

RESULTS: Lf was positive in 18 (50%) of 36 patients with colorectal cancer, 25 (15.9%) of 157 with colorectal polyps, 29 (46.8%) of 62 with ulcerative colitis, and 25 (62.5%) of 40 (62.5%) with Crohn's disease. The Hb-positive rates were 50%, 12.1%, 41.9% and 32.5%, respectively. Of the 318 patients free of abnormalities by colorectal endoscopy, Lf was positive in 29 (9.1%) and Hb was positive in 15 (4.7%). Among patients with Crohn's disease, the Lf-positive rate was significantly higher than the Hb-positive rate. If either high Lf or Hb levels were considered positive, the positive rates rose to 61.1%, 51.6%, and 67.5% in the colorectal cancer group, ulcerative colitis group, and Crohn's disease group, respectively. If both high Lf and Hb levels were rated positive, the positive predictive values (PPV) were 21% for colorectal cancer, 33% for ulcerative colitis, and 17% for Crohn's disease, and PPV of high Hb level alone was 18%, 25% and 13%, respectively.

CONCLUSION: Fecal Lf and Hb were found useful in the detection of colorectal diseases, and the combination of the two measurements appears to increase the sensitivity and efficacy of diagnosis.

Key words: Feces; Lactoferrin; Hemoglobin; Diagnosis; Colorectal disease

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INTRODUCTION

In the United States, colorectal cancer is the second leading cause of death[1]. In Japan, the prevalence of colorectal cancer and the percentage of this cancer among all cancer deaths have recently been rising as well. Because the prognosis of patients with colorectal cancer depends on the stage of cancer at the time of detection, screening for early diagnosis of colorectal cancer is considered essential[2,3]. In the United States, fecal occult blood testing by the guaiac method has been used as a screening method for colorectal cancer. A randomized study found that this method reduced the mortality rate of colorectal cancer[4,5]. In Japan, immunological fecal occult blood testing with a target of hemoglobin (Hb) has often been used in screening for colorectal cancer[6]. Fecal occult blood testing is also useful in the diagnosis and evaluation of various colorectal diseases other than colorectal cancer[7-9]. However, Hb in feces is unstable, which can be a cause of false-negative cases. Furthermore, Hb is not useful in the detection of lesions not accompanied by bleeding. For these reasons, a fecal marker with a high sensitivity and specificity has to be developed. Lactoferrin (Lf), which is released from neutrophil-specific granules, is stable in feces and is an excellent marker of activity of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease[10]. It has also been reported that fecal Lf level is higher in the patients with not only inflammatory bowel diseases but also colorectal tumors than in healthy individuals[11]. In a pilot study comparing fecal Lf level with fecal occult blood testing (immunological qualitative method) in 351 patients, Lf was found as useful as fecal occult blood testing in the diagnosis of colorectal diseases[12].

This prospective study involved a larger number of subjects than in previous studies to compare the usefulness of fecal Lf with that of fecal Hb as a quantitative parameter for the diagnosis of colorectal diseases and to evaluate the combination of fecal Lf and Hb using fecal samples collected from patients on the day before colorectal endoscopy.

MATERIALS AND METHODS

Subjects and fecal sampling method

The subjects of this study were 872 patients scheduled to undergo colorectal endoscopy at the Second Department of Internal Medicine, Osaka Medical College University Hospital, who provided written informed consent to participate in the study. Their ages ranged from 12 to 90 years (14 > 20 years; 34, 20-29 years; 51, 30-39 years; 116, 40-49 years; 244, 50-59 years; 274, 60-69 years; 130, 70-79 years; and 9 over 80 years). Examination for colorectal cancers and polyps was also performed in 657 subjects over 50 years of age. Patients with severe diseases of liver, gallbladder or pancreas were excluded from this study. Feces were collected from each patient on the day before colorectal endoscopy. The container used for fecal sampling had a stick type, designed to allow collection of about 10 mg feces into 1 mL buffer solution by thrusting the stick into feces at several points. On the day of the test, each subject was pretreated with intestinal lavage in the morning and underwent colorectal endoscopy in the afternoon. During endoscopy, the entire large intestine was examined, with biopsy performed as needed.

Measurement of Lf and Hb

Lf and Hb levels in feces were measured by sandwich ELISA, using 96-well microplates. For measurement of Lf, rabbit anti-human Lf antibody (Dakopatts, Glostrup, Denmark) and peroxidase-labeled anti-human Lf antibody were used, with tetramethylbendizine as a color developer[13]. For Hb, anti-human Hb antibody and alkaline phosphatase-labeled anti-human Hb antibody were used by the Kind-King method for color development[7]. Concentrations were calculated, referring to the standard curves prepared from Lf originating from human colostrum (Cappel Co. Durham, NC) and Hb derived from the blood of healthy adults. All samples were subjected to measurement in blind fashion.

Statistical analysis

McNemar's test was used for statistical analysis. $P < 0.05$ was considered significant. For a given disease, sensitivity was defined as the positive rate, while specificity was defined as (number of true-negative cases)/(number of true-negative cases + number of false-positive cases). To compare the usefulness of fecal Hb and Lf, a operating characteristic analysis (ROC) was conducted. The ROC analysis involved preparing a sensitivity-false-positive rate curve and comparing areas under the curve (AUC), with an AUC of 0.5 uninformative and an AUC of 1 perfect[13].

Table 1 Comparison of fecal Lf and Hb between normal and abnormal groups

Group		Fecal Lf			Specificity	% (No.)
		+	-	Sum		
Normal group						
Fecal Hb	+	7	8	15	Lf	90.9 (289/318)
	-	22	281	303	Hb	95.3 (303/318)
	Total	29	289	318	$P = 0.0176$	
Abnormal group						
Fecal Hb	+	59	27	86	Lf	29.6 (164/554)
	-	105	363	468	Hb	15.5 (86/554)
	Total	164	390	554	$P < 0.0001$	

Table 2 Sensitivity of fecal Lf and Hb in various diseases

Diseases	*n*	Sensitivity	
		Hb > 100 % (No.)	Lf > 65 % (No.)
Colorectal cancer	36	50.0 (18/36)	50 (18/36)
Colorectal polyp	157	12.1 (19/157)	15.9 (25/157)
Ulcerative colitis	62	40.3 (25/62)	46.8 (29/62)
Crohn's desease	40	32.5 (13/40)	62.5[b] (25/40)
Colorectal diverticulum	73	1.4 (1/73)	19.2[b] (14/73)
Internal hemorrhoids	142	4.2 (6/142)	27.5[b] (39/142)
Nonspecific colitis	20	15.0 (3/20)	20 (4/20)
Other diseases1	24	4.2 (1/24)	41.7[b] (10/24)

[b]$P < 0.01$ *vs* Hb. 1Including 4 cases of previous intestinal tuberculosis, 4 of submucosal tumor, 2 of ischemic colitis, 2 of rectal carcinoid, 2 of Behcet disease, 2 of rectal mucosal prolapse syndrome, 2 of Cronkhite-Canada syndrome, 2 of Cowden disease, 1 of mucosa-associated lymphoma, and 1 of periappendiceal abscess.

RESULTS

Diagnosis

Of the 872 subjects, 554 were found to have abnormalities on colorectal endoscopy, while 318 were rated as free of abnormalities (Tables 1 and 2). Polyps were defined as adenomas over 5 mm in size. Individuals with colorectal polyps were classified as having colorectal polyps even accompanied with internal hemorrhoids and/or colorectal diverticula. Individuals with both internal hemorrhoids and colorectal diverticula were classified as cases of internal hemorrhoids.

Establishment of cut-off levels for fecal Lf and Hb

For the 25 healthy individuals, the mean + 2SD (the upper limit of the normal range) was 5.96 μg/g for fecal Lf and 9.18 μg/g for fecal Hb[10]. In this study, feces were diluted 1:100 with the buffer solution in the container, the mean + 2SD for healthy individuals was equivalent to 59.6 ng/mL for Lf and 91.8 ng/mL for Hb. We therefore set the cut-off levels for this study at 65 ng/mL (Lf) and 100 ng/mL (Hb).

The AUC of the ROC curve was greater for Lf (0.600) than for Hb (0.556), although this difference was not statistically significant.

Analysis of groups with and without abnormalities

In the abnormality-free group ($n = 318$), fecal Lf was

Table 3 Diagnosis value using combination of fecal Lf and Hb

Diseases	*n*	Sensitivity	
		Hb > 100 or Lf > 65 % (No.)	Hb > 100 and Lf > 65 % (No.)
Colorectal cancer	36	61.1 (22/36)	38.9 (14/36)
Colorectal polyp	157	25.5 (40/157)	2.5 (4/157)
Ulcerative colitis	62	51.6 (32/62)	35.5 (22/62)
Crohn's desease	40	67.5 (27/40)	27.5 (11/40)
Colorectal diverticulum	73	19.2 (14/73)	1.4 (1/73)
Internal hemorrhoids	142	28.9 (41/142)	2.8 (4/142)
Nonspecific colitis	20	20.0 (4/20)	15.0 (3/20)
Other diseases	24	45.8 (11/24)	0.0 (0/24)

Table 4 Positive predictive value (PPV) of fecal Lf and Hb in colorectal diseases

Diseases	*n*	PPV	
		Hb > 100 % (No.)	Hb > 100 and Lf > 65 % (No.)
Colorectal cancer	36	17.8 (18/101)	21.2 (14/66)
Colorectal polyp	157	18.8 (19/101)	6.1 (4/66)
Ulcerative colitis	62	24.8 (25/101)	33.3 (22/66)
Crohn's desease	40	12.9 (13/101)	16.7 (11/66)
Colorectal diverticulum	73	1.0 (1/101)	1.5 (1/66)
Internal hemorrhoids	142	5.9 (6/101)	6.1 (4/66)
Nonspecific colitis	20	3.0 (3/101)	4.5 (3/66)
Other diseases	24	1.0 (1/101)	0.0 (0/66)

Table 5 Comparison of fecal Lf and Hb in subjects aged over 50 yr

		Fecal Lf			Specificity	% (No.)
		+	-	Sum		
Normal group						
Fecal Hb	+	7	8	15	Lf	89.7 (217/242)
	-	18	209	227	Hb	93.8 (227/242)
	Total	25	217	242	$P = 0.0776$	
Abnormal group						
Fecal Hb	+	32	20	52	Lf	26.0 (108/415)
	-	76	287	363	Hb	12.5 (52/415)
	Total	108	307	415	$P < 0.0001$	

Table 6 Sensitivity of fecal Lf and Hb in colorectal cancer

Colorectal cancer	*n*	Sensitivity	
		Hb % (No.)	Lf % (No.)
Total	36	50.00 (18/36)	50.00 (18/36)
Early stage	17	11.80 (2/17)	29.40 (5/17)
Right side	5	20 (1/5)	40.00 (2/5)
Left side	12	8.30 (1/12)	25.00 (3/12)
Advanced stage	19	84.20 (16/19)	68.40 (13/19)
Right side	6	66.70 (4/6)	83.30 (5/6)
Left side	13	92.30 (12/13)	61.50 (8/13)

Note: *n*. number of cases.

positive in 29 cases (specificity: 90.9%) and fecal Hb was positive in 15 cases (specificity: 95.3%). Specificity was thus significantly higher for Hb than for Lf. In the group with abnormalities ($n = 554$), fecal Lf was positive in 164 cases (sensitivity: 29.6%) and fecal Hb was positive in 86 cases (sensitivity: 15.5%). Sensitivity was thus significantly higher for Lf than for Hb.

Analysis in each disease group

Sensitivities were compared between fecal Lf and Hb in each disease group (Table 2). The sensitivity of fecal Lf was significantly higher than that of fecal Hb in the Crohn's disease, internal hemorrhoid, and colorectal diverticulum. There was no significant difference in sensitivity between Lf and Hb in the colorectal cancer, colorectal polyp, and ulcerative colitis.

Diagnosis using a combination of fecal Lf and Hb

When either high Hb or high Lf (Hb > 100 or Lf > 65) was rated positive, sensitivity in disease detection rose to 61.1% for colorectal cancer, 25.5% for colorectal polyps, 51.6% for ulcerative colitis, 67.5% for Crohn's disease, 19.2% for colorectal diverticulum, 28.9% for internal hemorrhoids, 20.0% for nonspecific colitis, and 45.8% for other diseases (Table 3). When both high Hb and high Lf (Hb > 100 and Lf > 65) were rated positive, the number of individuals rated positive among the 318 abnormality-free individuals decreased to 7 (Table 1). The positive rate by each disease group also decreased (Table 3), though the magnitude of the decrease was relatively mild in the colorectal cancer, Crohn's disease, and ulcerative colitis. Positive predictive value (PPV) (number of positive cases in a given disease group divided by the number of all positive cases) rose to 21.2% (14/66) for colorectal cancer, 33.3% (22/66) for ulcerative colitis, and 16.7% (11/66) for Crohn's disease (Table 4).

Analysis of subjects over 50 years of age

Because examinations for colorectal cancer are usually performed in individuals aged 50 years or older, we performed an analysis confined to the 657 individuals aged over 50 years[14]. Among the 242 subjects free of abnormalities, Lf was positive in 25 (specificity, 89.7%) and Hb was positive in 15 (specificity, 93.8%), as shown in Table 5. Among the 415 patients with abnormalities, Lf was positive in 108 (sensitivity, 26.0%) and Hb was positive in 52 (sensitivity, 12.5%) (Table 5). We then examined the sensitivity in detection of colorectal cancer and polyps. In the 33 patients with colorectal cancer aged over 50 years, Hb was positive in 16 (sensitivity, 48.5%) and Lf was positive in 17 patients (sensitivity, 51.5%). When either high Lf or high Hb was rated positive, sensitivity rose to 60.6% for colorectal cancer and 25.2% for colorectal polyps. When both high Lf and high Hb were rated positive, PPV rose to 33.3% in the colorectal cancer group.

Analysis of patients with colorectal cancer

Of 36 patients aged 36-81 years with colorectal caner, 14 had both Lf and Hb levels below the cut-off levels. Twelve of the 14 patients had colorectal cancer at an early stage confined to the submucosal layer. When analyzed by location (left or right side of the colon) and stage of cancer (early and advanced), the Hb-positive rate (12/13) was higher than Lf-positive rate (8/13) in patients with advanced left side colon cancer (Table 6). Among patients with right side colon cancer, however, the Lf-positive rate was slightly higher than the Hb-positive rate in both

early and advanced patients(2/5 *vs* 1/5 and 5/6 *vs* 4/6, respectively). Macroscopically, bloody stool was noted in 10 of the 36 patients with colorectal cancer. Therefore, excluding these 10 cases, routine fecal test was enough for screening of colorectal cancer. Among the 26 cases, the Lf-positive rate (46.2%) was higher than the Hb-positive rate (38.5%).

Analysis of patients with ulcerative colitis or Crohn's disease

Ulcerative colitis was considered in active stage if active lesions were revealed by colorectal endoscopy. Among the 38 cases of active ulcerative colitis, Lf and Hb were positive in 26 and 22 cases, respectively. Among the 24 cases of inactive ulcerative colitis, Lf and Hb were positive in 3 and 3 cases, respectively.

Crohn's disease was considered active if the Crohn's disease activity index (CDAI) was over 150 or if active lesions were detected by colorectal endoscopy[15]. Among the 26 cases of active Crohn's disease, Lf and Hb were positive in 18 and 12 cases while in the 12 cases of inactive Crohn's disease, Lf and Hb were positive in 8 cases and 1 case, respectively. In patients with Crohn's disease, minor active lesions of the small intestine often persist, but the CDAI is likely to be lower than 150 during treatment, often leading to the judgment that the patient has inactive disease clinically.

DISCUSSION

Lf is an ironbound protein with a molecular weight of about 80000. It is found not only in neutrophil-specific granules but also in milk, tears, saliva, etc.[16,17]. The presence of Lf in the cytoplasm of colorectal cancer and adenoma cells has also been reported[18,19]. The high Lf level in the feces of patients with intestinal inflammation is believed to originate from the neutrophils which have infiltrated the intestinal mucosa[20-22]. The reason for elevation of fecal Lf level in patients with colorectal cancer has not yet been fully determined. It has been reported that some colorectal cancers are accompanied by local inflammatory reaction, and that leukocyte scintigraphy is sometimes positive in patients with colorectal cancer[23-25]. Neutrophil elastase and calprotectin, which are neutrophilic granular proteins, are absent in tumor cells, though their levels in feces of patients with colorectal cancer are high[26, 27]. Neutrophils thus appear to be a more important source of fecal Lf than exfoliated tumor cells in patients with colorectal cancer. In this study, the usefulness of fecal Lf in the diagnosis of various colorectal diseases was prospectively evaluated.

Among the subjects free of abnormalities, the LF-positive rate was 9.1% (29/318) and the Hb-positive rate was 4.7% (15/318), both of which were higher than those in the general screening. In patients with symptoms such as diarrhea and abdominal pain, fecal Lf may be rated positive due to transient or minor inflammation not detectable with colorectal endoscopy, even when they are considered free of abnormalities by colorectal endoscopy. In patients who have been found positive for fecal occult blood before admission to the hospital, the percentage of the patients who are fecal Hb-positive without any abnormality is high.

Many of our patients were positive in fecal occult blood prior to visiting the hospital or visited the hospital complaining of macroscopically bloody stool. This suggests that patients with colorectal lesions of hemorrhagic subtype accounted for a high percentage of the subjects of this study. For example, colorectal cancers which likely cause bleeding or positive findings for fecal occult blood were found in a high percentage of patients with colorectal cancer in this study. Thus, based on the fact that Hb is expected to be more useful than Lf, the sensitivity of Lf was significantly higher than that of Hb in patients with abnormalities. Because this study involved quantitative measurement of both Lf and Hb, we compared the usefulness of Lf and Hb in the diagnosis of colorectal diseases by means of ROC analysis. A greater AUC was found in Lf than in Hb, although this difference was not significant. These findings suggest that Lf is comparable to or more useful than Hb in detecting colorectal diseases.

By type of disease, the sensitivity of testing for Lf was comparable to that for Hb for colorectal cancer, colorectal polyps, ulcerative colitis, and nonspecific colitis. For Crohn's disease, internal hemorrhoids, colorectal diverticulum, and other colorectal diseases, the sensitivity of Lf testing was significantly higher than that of Hb testing.

In patients with Crohn's disease, the Lf-positive rate was high even the disease was rated as inactive. It seems more rational to interpret this result as that inflammation of the intestine persists in patients with Crohn's disease with high fecal Lf levels, rather than as a false-positive result for the following reasons: (1) in Crohn's disease, the intestine often remains inflamed even after the CDAI decreases to below 150 after treatment; and (2) complete evaluation of residual inflammation of the intestine is difficult even with colorectal endoscopy. The "other diseases" mentioned above include important diseases such as old intestinal tuberculosis, submucosal tumor, rectal carcinoid, Behcet disease, Cronkhite-Canada syndrome, Cowden disease, and mucosa-associated lymphoid tissue lymphoma (MALT). Significantly higher sensitivity of testing for Lf than that for Hb in the "other diseases" group is clinically important.

The results of this study suggest that it is possible to increase the accuracy of screening based on the conventional fecal occult blood test with a target set at Hb by combining Hb with Lf. For example, when immunological fecal occult blood testing is used for screening of colorectal cancer, feces are often sampled repeatedly to increase the sensitivity of detection. However, if a combination of Hb and Lf is used and results are considered positive in cases with high Lf or Hb, it is possible to perform a highly sensitive screening that requires only one fecal sampling. In addition, if results were considered positive in cases with both high Lf and Hb, PPV rose in the colorectal cancer, ulcerative colitis, and Crohn's disease. If PPV is high, it will be easier to convince patients to take further examinations such as colorectal endoscopy, etc. because of the likelihood of the presence of colorectal disease.

Hb is likely to be degraded by bacteria or enzymes contained in feces, resulting in loss of antigenicity. In buffer fluid, both fecal Lf and fecal Hb remained stable for 3 days, retaining 75% or greater activity compared to that recorded immediately after sampling[12]. In this study, feces were collected into a buffer fluid. For this reason, we believe that stability after sampling was not different between Lf and Hb in this study. In the intestine, however, Lf is believed to be more stable than Hb. We therefore, analyzed Lf- and Hb-positive rates by location of colorectal cancer, and found that Hb was highly sensitive in detecting advanced cancer of the left side of the colon, i.e., the sensitivity of Hb was lower in detection of colorectal cancer than in advanced cancer of the left side of the colon. This finding appears to be related to the following factors: (1) relatively low stability of Hb within the intestine, as noted above; and (2) the feces in the right portion of the colon are still too soft to cause bleeding. Although the sensitivity of Lf in detecting cancer of the left side of the colon was lower than that of Hb, it was higher in detecting cancers affecting other portions of the colon compared to Hb. The low sensitivity of fecal occult blood testing in the detection of early colorectal cancer has been noted in previous reports. In this study, only 2 of the 17 cases of early colorectal cancer were positive by this method. However, of these 17 patients with early colorectal cancer, 14 had been found in other clinics to be positive for fecal occult blood and visited our hospital for this reason. This suggests that in cases of early colorectal cancer, bleeding is often intermittent rather than continuous. On the other hand, Lf was positive in 5 of the 17 cases of early colorectal cancer, and its sensitivity in detecting early colorectal cancer was higher than that of Hb. Thus, the findings of the analyses by location and stage of colorectal cancer also suggest that a combination of Lf and Hb can compensate the shortcomings of these two parameters used individually for detection of colorectal cancer.

Examinations for colorectal cancer are usually performed in individuals over 50 years of age. When analysis was confined to this age group, specificity of Lf was slightly lower (89.7%) than that of Hb (93.8%), while sensitivity of Lf was slightly higher (51.5%) than that of Hb (48.5%). When results were considered positive if both Lf and Hb were high, PPV rose to 33.3%. The combination of Lf and Hb is thus useful in judging whether an individual aged over 50 years has a high risk for colorectal cancer.

Numerous studies have been published concerning noninvasive screening methods, which may replace the fecal occult blood test used for screening of colorectal caner[28,29]. A multi-target assay, designed to detect abnormal DNA in feces, exhibited a high sensitivity in detecting colorectal cancer, although no comparison of this assay had been made with fecal Hb measurement. Furthermore, this assay is too expensive for general use in clinics or health checkups[30]. Measurement of calprotectin, one of the neutrophil-specific granules, is highly sensitive in detecting colorectal cancer, though its specificity is low. Furthermore, no comparison of calprotectin with fecal Hb in detection of colorectal cancer has been performed[27,31].

In conclusion, our results suggest that the usefulness of fecal Lf measurement appears comparable to that of fecal Hb measurement in detection of colorectal diseases. Furthermore, the combination of Lf and Hb measurement appears to increase the sensitivity and efficacy of diagnosis.

COMMENTS

Background

The high Lactoferrin (Lf) level in the feces of patients with intestinal inflammation is believed to originate from the neutrophils which have infiltrated the intestinal mucosa. The presence of Lf in the cytoplasm of colorectal cancer and adenoma cells has also been reported. The reason for elevation of fecal Lf level in patients with colorectal cancer has not yet been fully determined. It has been found that some colorectal cancers are accompanied by local inflammatory reaction, and that leukocyte scintigraphy is sometimes positive in patients with colorectal cancer. Neutrophil elastase and calprotectin, which are neutrophilic granular proteins, are absent in tumor cells, although their levels are high in feces from patients with colorectal cancer. Neutrophils thus appear to be a more important source of fecal Lf than exfoliated tumor cells in patients with colorectal cancer. Therefore, the usefulness of fecal Lf in the diagnosis of various colorectal diseases was prospectively evaluated in this study.

Research frontiers

In the United States, fecal occult blood testing (FOBT) by the guaiac method has been used as a screening for colorectal cancer. A randomized study found that this method lowered the mortality rate of colorectal cancer. In Japan, immunological FOBT with a target of hemoglobin (Hb) has often been used in screening for colorectal cancer. FOBT is also useful in the diagnosis and evaluation of various colorectal diseases other than colorectal cancer. However, since Hb in feces is unstable, it may result in false-negative cases. Furthermore, Hb is not useful in the detection of lesions not accompanied by bleeding. For these reasons, development of a fecal marker with high sensitivity and specificity is needed. Lf, which is released from neutrophil-specific granules, is stable in feces and is an excellent marker of activity of IBD. Fecal Lf level has been reported to be higher in the patients with not only IBD but also colorectal tumors than in healthy individuals. In a pilot study comparing fecal Lf level with fecal occult blood testing in 351 individuals, Lf was found to be as useful as fecal occult blood testing in the diagnosis of colorectal diseases.

Innovations and breakthroughs

The measurement of fecal Lf and Hb was found to be useful in the detection of colorectal diseases, and the combination of the two measurements appears to increase the sensitivity and efficacy of diagnosis.

Applications

The combined measurement of fecal Lf and Hb should be useful for screening of colorectal diseases including colorectal cancer.

Terminology

Lf is an ironbound protein with a molecular weight of about 80 000. It is found not only in neutrophil-specific granules but also in milk, tears, saliva, etc. The high Lf level in the feces of patients with intestinal inflammation is believed to originate from the neutrophils which have infiltrated the intestinal mucosa.

Peer review

Hirata *et al* demonstrated the usefulness of fecal Lf and Hb levels for screening of colorectal cancer and IBD. This study might be clinically relevant. The reliability of their data is dependent on the presentation of determination method. More precise methods of their ELISA for lactoferrin and hemoglobin should be described.

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RAPID COMMUNICATION

A new iron free treatment with oral fish cartilage polysaccharide for iron deficiency chronic anemia in inflammatory bowel diseases: A pilot study

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Abstract

AIM: To investigate the effect of a new oral preparation, highly concentrated in fish cartilage, in a group of inflammatory bowel diseases (IBD) patients with chronic iron deficient anemia.

METHODS: In an open label pilot study, we supplemented a group of 25 patients (11 with Crohn's disease and 14 with ulcerative colitis) in stable clinical conditions and chronic anemia with a food supplement which does not contain iron but contains a standardized fraction of fish cartilage glycosaminoglycans and a mixture of antioxidants (Captafer Medestea, Turin, Italy). Patients received 500 mg, twice a day during meals, for at least 4 mo. Patients were suggested to maintain their alimentary habit. At time 0 and after 2 and 4 mo, emocrome, sideremia and ferritin were examined. Paired data were analyzed with Student's *t* test.

RESULTS: Three patients relapsed during the study (2 in the 3rd mo, 1 in the 4th mo), two patients were lost to follow up and two patients dropped out (1 for orticaria, 1 for gastric burning). Of the remaining 18 patients, levels of serum iron started to rapidly increase within the 2nd mo of treatment, $P < 0.05$), whereas serum ferritin and hemoglobin needed a longer period to significantly improve their serum levels (mo 4) $P < 0.05$. The product was safe, easy to administer and well tolerated by patients.

CONCLUSION: These data suggest a potential new treatment for IBD patients with iron deficiency chronic anemia and warrant further larger controlled studies.

INTRODUCTION

Inflammatory bowel diseases (IBD) represent chronic recurrent diseases of the gut with a typical negative balance of iron that leads to anemia.

Iron deficiency anemia affects approximately 30% of patients with IBD. The symptoms correlated with iron deficiency are very common in IBD and microcytic and hypocromic anemia has a significant impact on the quality of life of IBD affected patients[1-3].

Anemia from iron deficiency is due to inadequate intake or the loss of iron, whereas in chronic inflammatory conditions, anemia is also the result of decreased erythropoiesis, secondary to increase of proinflammatory cytokines, reactive oxygen metabolites and nitric oxide.

Patients with IBD are treated commonly for their disease, which can ameliorate the iron deficiency and the anemia. Oral iron supplements may have a negative effect on the inflammatory activity of the disease. Studies in animal models suggest that iron may increase the activity of the disease through activation of the NFkb pathway of inflammation[4]; moreover, iron supplementation may cause alterations in the immune function affecting the inflammatory state by increasing the level of inflammation itself, modulating the function of macrophages and Th1 cells[5]. Finally in animal models of ulcerative colitis (UC) and Crohn disease (CD) it has been shown that the iron not absorbed can reach the ileum and the colon increasing, at the site of ulceration, the oxidative stress and enhancing pro-inflammatory cytokines[6,7]. An alternative approach to treat iron deficient anemia in IBD patients is to improve bioavailability of the iron normally introduced from the diet.

A pilot study of a food supplement (Captafer trade mark) containing selected fish cartilage and a mixture of antioxidants (Table 1) has been recently tested in healthy women with iron deficiency at reproductive age, free from estroprogestinic therapy. A significant elevation in serum iron and ferritin levels after 30 and 60 d of treatment was noted, in contrast with the inefficacy of the placebo[8].

Our work focused on the understanding of the role of this food supplement in iron deficiency and chronic anemia that characterized patients with IBD. With this aim in an open pilot study, we selected a group of patients with IBD in clinical remission with chronic anemia and low levels of serum iron and ferritin. The bioavailability of iron was ascertained by analyzing three variables: serum iron, hemoglobin and serum ferritin.

MATERIALS AND METHODS

The study was an open-label, pilot clinical trial approved by the local ethical committee of the University of Bologna, S.Orsola Hospital. Twenty-five patients with IBD (11 Crohn's disease, 14 ulcerative colitis) were selected from our registered labeled archive. Patient characteristics at the time of enrollment are listed in Table 2. Patients in clinical remission, according to CDAI for CD and Simple Clinical Colitis Activity Index for UC, taking mesalamine 2.4 g/d for preventing relapse and having mycrocytic and hypocromic anemia were considered eligible for this study. Disease localization in patients with CD was: 6 in the ileocolon, 3 in the ileum, and 2 in the colon. The treatment consisted of a supplementation of 500 mg of Captafer™ twice a day during meals for at least 4 mo. The composition of Captafer tablet is shown in Table 1.

Patients were suggested to maintain their alimentary habit throughout all the study period. An hemocrome, sideremia and ferritin evaluation plus a normal screening laboratory exam was performed at time 0 (before the beginning of the treatment), and after 2 and 4 mo, respectively. Patients who had a relapse of the disease during the period of the treatment, who had adverse reactions, or who did not make all the blood evaluations were excluded from the final statistical analysis.

Statistical analysis

The serum iron level (μg/mL), ferritin level (ng/mL) and hemoglobin value (g/dL) were analyzed and compared with Student's *t* test for paired data. Three groups were considered: the baseline group (time 0), the mo 2 group and the mo 4 group. The three parameters (serum iron, serum ferritin and hemoglobin) were compared by pairing the baseline group with the mo 2 and mo 4 groups, respectively. Data were expressed for each group as geometric mean ± SD. A P value < 0.05 was considered to be statistically significant.

RESULTS

Eighteen of the 25 patients who began the treatment completed the study.

Reasons for not completing the study were: relapse of the disease (3/25), loss to follow up (2/25) and adverse reactions (2/25). Two patients relapsed during the third month and one in the fourth month of treatment. Of the patients who dropped out, one developed orticaria and one complained a gastric burning. The occurrence of these events was not linked to the treatment or was independent events. Ten of the patients who completed the study were affected by ulcerative colitis and eight had Crohn's disease.

Table 1 Captafer composition

	Twe tablets composition	% RDA	Per 100 g
Energy	1.3 Kcal 5.5 KJ		83 Kcal 347 kJ
Proteins (N × 6.25)	0.19 g		11.9 g
Carboydrates	0.01 g		0.8 g
Fat	0.05 g		3.5 g
Vitamin C	90 mg	150	5.62 g
Vitamin E	30 mg	300	1.87 g
Folic acid	150 mcg	75	0.01 g
Zinc	15 mg	100	0.93 g
Cupper	1.2 mg		0.07 g
Selected fish cartilage	800 mg		50 g

RDA: recommended daily allowance.

Table 2 Patient characteristics

Characteristics	Crohn's disease	Ulcerative colitis
Age (yr)	20-60 ± 12	30-70 ± 15
Sex (M/F)	9/2	8/6
Simple clinical colitis activity index		3-6 ± 1
Score on Crohn's disease activity index	59-120 ± 20	

Table 3 Serum iron, ferritin and hemoglobin levels before and after the treatment with captafer

	Baseline (time 0)	mo 2	mo 4
18 patients (10 UC/8CD)	mean ± SD	mean ± SD	mean ± SD
Serum iron (μg/dL)	26.7 ± 13.6	46 ± 27.2[1]	44.5 ± 21.2[1]
		P < 0.05	P < 0.05
Serum ferritin (ng/mL)	8.5 ± 6.8	14.5 ± 20.5	14.1 ± 10.3[1]
			P < 0.05
Hemoglobin (g/dL)	10.96 ± 0.90	11.07 ± 1.02	11.48 ± 0.94[1]
			P < 0.05

[1]Student's *t* test for paired data.

The results are shown in Table 3. With regard to serum iron levels, after two months of treatment, a significant increase was seen(P < 0.05). The increase was around 1.722 fold of baseline values (serum iron: baseline value 26.7 μg/dL; mo 2, 46 μg/dL). With regard to serum ferritin and hemoglobin, a trend towards increased value at mo 2 was noted (Figure 1).

Statistical analysis of the results obtained after 4 mo of treatment were consistent with the purpose of this study. There was a significant increase in serum iron, ferritin and hemoglobin (P < 0.05). The serum iron increased 1.66 fold, serum ferritin increased 1.65 fold and the hemoglobin increased 1.035 fold after 4 mo of treatment. Data analysis did not show any difference between UC and CD patients or between CD subgroups, according to disease localization. The product was safe, easy to administer and

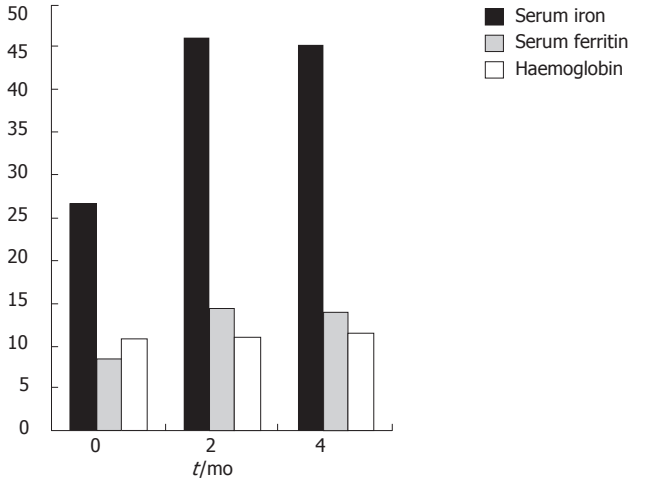

Figure 1 Serum iron, ferritin and hemoglobin levels before and after the treatment with captafer.

patients tolerated the treatment for all the period.

DISCUSSION

Dietary iron occurs as haeme and non-haeme iron (mostly from plants). The non-haeme iron is transformed to the ferric state in the presence of oxygen. After reduction to the ferrous state, iron is transferred to enterocytes by the apical transporter divalent metal transporter 1. If iron stores are replete, iron is trapped in ferritin and lost in the intestinal lumen when the cell desquamates.

In inflammatory bowel diseases, iron impairment may be due to several factors: poor absorption due to the activity of the disease localized in the small intestine or to the surgical resection, chronic loss of blood due to chronic intestinal bleeding and an inflammatory environment determined by the presence of proinflammatory and inflammatory cytokines[9-14]. In IBD the iron is lost because of the presence of chronic bleeding from the gut, which amount exceeds the iron that may be absorbed from the diet. Although iron absorption may be affected in Crohn's disease due to the localization of the disease, several treatments have been introduced to restore iron depletion.

As it is reported by large studies the efficacy of oral iron is limited by poor absorption, intolerance and induction of oxidative stress at the site of bowel inflammation[4-6].

Another therapeutical option is the treatment with iron i.v.[15], particularly iron sucrose together with erythropoietin; this combination is effective but its clinical use is limited by high cost and difficulty of administration[16].

In this study we proposed an alternative and potential new treatment for iron deficient anemia in inflammatory bowel disease using a new food supplement with high content of fish cartilage that was well tolerated and without major adverse reactions. The only previous clinical experience was in healthy women with iron deficiency with a substantial enhancement of blood values[8].

In a recent paper it has been shown that the carbohydratic component of the fish muscle is involved in determining a better bioavailability of iron by increasing absorption of iron. Huh *et al*[17] used an *in vitro* model to confirm and analyze the fish meat factors that enhance the non-haeme iron bioavailability, showing a 5 fold increase in iron uptake. They evidenced that low-molecular-weight carbohydrates of muscle tissue obtained from fish are responsible for increasing non-haeme iron uptake in caco-2 cells. These carbohydrates may be represented by oligosaccharides originating from glycosaminoglycans in the extracellular matrix of muscle tissue. This finding well correlates with our results. Our preparation is composed of glycosaminoglycans in a greater percentage, which may be involved in the enhancement of non-haeme iron absorption.

In our study, a significant increase in serum iron was seen after 2 mo of therapy, and a significant increase in serum iron, serum ferritin and hemoglobin was noted at the end of the treatment. These preliminary data seem to offer a new and alternative approach to treat iron deficiency in patients with IBD, because of the efficacies tested, absence of major adverse effects, and easy administration. A placebo controlled trial is needed to confirm the potential beneficial effects of this preparation.

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RAPID COMMUNICATION

Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease

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Abstract

AIM: To clarify whether nonalcoholic fatty liver disease (NAFLD) increases the risk of cardiovascular disease.

METHODS: We carried out a prospective observational study with a total of 1637 apparently healthy Japanese men and women who were recruited from a health check-up program. NAFLD was diagnosed by abdominal ultrasonography. The metabolic syndrome (MS) was defined according to the modified National Cholesterol Education Program (NCEP) ATP Ⅲ criteria. Five years after the baseline evaluations, the incidence of cardiovascular disease was assessed by a self-administered questionnaire.

RESULTS: Among 1221 participants available for outcome analyses, the incidence of cardiovascular disease was higher in 231 subjects with NAFLD at baseline (5 coronary heart disease, 6 ischemic stroke, and 1 cerebral hemorrhage) than 990 subjects without NAFLD (3 coronary heart disease, 6 ischemic stroke, and 1 cerebral hemorrhage). Multivariate analyses indicated that NAFLD was a predictor of cardiovascular disease independent of conventional risk factors (odds ratio 4.12, 95% CI, 1.58 to 10.75, P = 0.004). MS was also independently associated with cardiovascular events. But simultaneous inclusion of NAFLD and MS in a multivariate model revealed that NAFLD but not MS retained a statistically significant correlation with cardiovascular disease.

CONCLUSION: Although both of them were predictors of cardiovascular disease, NAFLD but not MS retained a statistically significant correlation with cardiovascular disease in a multivariate model. NAFLD is a strong predictor of cardiovascular disease and may play a central role in the cardiovascular risk of MS.

Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Coronary heart disease; Cardiovascular disease; Risk factors

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common clinical condition with histological features that resemble those of alcohol-induced liver injury, but occur in patients who do not abuse alcohol[1]. It is emerging as the most common chronic liver disease in Western countries and also in other parts of the world[2-4]. NAFLD encompasses a histological spectrum ranging from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis[1]. The problem of NAFLD is not confined to its potential to cause serious liver related morbidity and mortality. It frequently occurs with features of the metabolic syndrome including obesity, type 2 diabetes mellitus[5,6], dyslipidemia[7] and hypertension[8]. In fact, the metabolic syndrome is a strong predictor of NAFLD[9]. Although the metabolic syndrome is a well-known precursor of cardiovascular disease[9-13], the potential cardiovascular risk of NAFLD itself has not been well investigated. Therefore, we designed a prospective observational study in apparently

healthy Japanese people to assess whether subjects with NAFLD have an increased risk of cardiovascular disease.

MATERIALS AND METHODS

Study subjects

In order to investigate whether non-alcoholic fatty liver disease is a predictor of cardiovascular disease, a prospective observational cohort study was carried out in a setting of a medical health checkup program at Murakami Memorial Hospital, Gifu, Japan. The purpose and details of the health checkup program were described previously[9]. Briefly, the center at which the checkups were performed was founded in 1994 and evaluated about 5000 examinees annually at the beginning of this study. Of these examinees, 60% repeatedly participated in the program annually or biennially and 40% were new registrants. Most of the participants were employees of various companies and local governmental organizations and their spouses. These companies and organizations recruit the participants each year from their employees according to a contract with our center. The cost of the medical examination was largely paid by the employers. The ethics committee of Murakami Memorial Hospital approved the study. Between January and December 1998, the center evaluated a total of 5409 participants. Among them, there were 3835 participants who had repeated the checkups on an annual or biennial basis. These repeaters who were thought to be suitable for a follow up at our center were invited to join the study.

Data collection and measurements

The health checkup programs included the following: urinalysis, blood cell counts, blood chemistry, measurements of hepatitis B antigen and hepatitis C antibody, electrocardiography, chest radiography, barium examination of the upper gastrointestinal tract, and abdominal ultrasonography. Medical history and lifestyle factors, including physical activity, smoking and alcohol consumption were surveyed by a self-administered questionnaire. Regarding medical history, participants were asked whether they had any past or current illness, and if so they were asked to indicate the doctor's diagnosis and the time of diagnosis, medication, and any surgical operations undergone. Smoking status was expressed by using the Brinkman index, which is calculated as the number of cigarettes smoked per day multiplied by the number of years that the participant smoked. We asked participants about the amount and type of alcoholic beverages consumed per week, and then estimated the mean ethanol intake per day. When the participants had difficulty completing the questionnaire, trained nurses provided assistance. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of the participant's height in meters.

The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was done by trained technicians with Aloka SSD-650CL (Aloka Co., Ltd., Tokyo, Japan). All ultrasonographic images were stored as photocopies. One gastroenterologist reviewed the photocopies and made the diagnosis of fatty liver without reference to any of the participant's other individual data. Of 4 known criteria (hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring)[9,14], the participants were required to have hepatorenal contrast and liver brightness to be given a diagnosis of NAFLD.

We used the criteria of National Cholesterol Education Program Adult Treatment Panel Ⅲ (NCEP ATP Ⅲ)[15] to characterize the metabolic syndrome. Because waist measurements were not available for the entire study sample, we substituted a BMI of 25 kg/m^2 or greater for all participants as an index of obesity. A BMI of 25 kg/m^2 or greater has been proposed as a cutoff for the diagnosis of obesity in Asian people[16]. Individuals with 3 or more of the 5 abnormalities were considered to have the metabolic syndrome.

We excluded participants who reported previous myocardial infarction, angina pectoris, ischemic stroke, cerebral hemorrhage or cancer. Additional exclusion criteria were known liver disease and an alcohol intake of more than 20 g/d. Regarding liver disease, participants with a positive test for hepatitis B antigen or hepatitis C antibody and those who reported a history of known liver disease, including viral, genetic, autoimmune, and drug-induced liver disease, were also excluded[9,17].

Follow up study

Between January 2003 and December 2004, the incidence of cardiovascular events among the study participants was assessed by a self-administered questionnaire when they visited the center for their health check-ups. To those who did not come back to our center during the period of the follow-up study we sent the questionnaire by mail. Cardiovascular diseases were defined to include coronary heart disease, ischemic stroke and cerebral hemorrhage. Coronary heart disease includes unstable angina, acute myocardial infarction and silent myocardial infarction. Stable angina pectoris were not counted. The participants who reported a doctor's diagnosis compatible with the above definition of cardiovascular disease or those who reported signs and symptoms indicative of cardiovascular disease were interviewed at the time of their visits or by phone. Thereby, we identified hospitals where the diagnosis of cardiovascular diseases was made. Through contacts with the hospitals the diagnosis of cardiovascular disease was confirmed. We counted only first-ever events in this study.

Statistical analysis

The SPSS statistical package, version 11.0.1 J (SPSS, Inc., Chicago, Illinois) was used for all statistical analyses, and a P value less than 0.05 was considered statistically significant. Two groups of participants were compared by the unpaired t-test and the chi-square test. Logistic regression was used to analyze correlations between the incidence of cardiovascular disease and NAFLD or the metabolic syndrome while controlling for conventional cardiovascular risk factors. Unadjusted and adjusted odds ratios and 95% CI were calculated. Data are expressed as means and SD for continuous variables.

Table 1 Baseline characteristics of the study participants (*n* = 1647)

Variables	Subjects without NAFLD (*n* = 1335)	Subjects with NAFLD (*n* = 312)	*P*
Asparate aminotransferase, U/L	17.4 ± 5.2	23.9 ± 11.2	< 0.001
Alanine aminotransferase, U/L	17.7 ± 9.6	38.8 ± 27.8	< 0.001
Gamma-glutamyltransferase, U/L	17.8 ± 18.2	34.0 ± 31.5	< 0.001
Men, *n* (%)	730 (54.7)	250 (80.1)	< 0.001
Age (yr)	47.8 ± 8.6	49.1 ± 8.7	0.017
The Brinkman index[1]	223.8 ± 359.3	358.6 ± 461.9	< 0.001
LDL-C, mmol/L	3.4 ± 0.8	3.7 ± 0.9	< 0.001
The metabolic syndrome, *n* (%)	87 (6.5)	119 (38.1)	< 0.001
BMI, kg/m^2	21.9 ± 2.6	25.1 ± 2.5	< 0.001
Fasting plasma glucose, mmol/L	5.1 ± 0.6	5.7 ± 1.3	< 0.001
Systolic blood pressure, mmHg	114.6 ± 16.2	123.7 ± 15.9	< 0.001
Diastolic blood pressure, mmHg	71.6 ± 10.2	77.8 ± 9.7	< 0.001
HDL-C, mmol/L	1.3 ± 0.3	1.0 ± 0.2	< 0.001
Triglycerides, mmol/L	1.2 ± 0.7	1.9 ± 1.0	< 0.001
C reactive protein, mg/dL	0.1 ± 0.4	0.2 ± 0.3	0.19

NAFLD: nonalcoholic fatty liver disease; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol. We applied unpaired *t*-test and the chi-square test to unpaired values between two groups. [1]The Brinkman index is the number of cigarettes per day multiplied by years of smoking. A higher value indicates increased smoking-associated health hazard.

RESULTS

Between January and December 1998, a total of 2429 participants (1680 men and 748 women) gave informed consent to be included in the study. Fifty subjects (44 men and 6 women) reported a previous history of myocardial infarction, angina pectoris, stroke, or cancer, and were excluded from the study. In addition, we excluded 108 subjects (80 men and 28 women) who had known liver disease, 531 subjects (510 men and 21 women) who consumed more than 20 g of ethanol per day, and 93 subjects (66 men and 27 women) who were taking medication concurrently. As a result, there were 1647 study subjects (980 men and 667 women). Mean age and BMI were 47.8 years (SD, 8.5) (range, 22 to 83 years) and 22.0 kg/m^2 (SD, 2.4) (range, 15.1 to 32.6 kg/m^2), respectively.

At baseline there were 312 subjects (18.9%) with NAFLD (Table 1). Mean age, BMI, and the Brinkman index were higher and systolic and diastolic blood pressures were also higher in the subjects with NAFLD. The NAFLD group included more men. The subjects with NAFLD had higher liver enzymes, fasting plasma glucose, LDL-cholesterol and triglycerides, and lower HDL-cholesterol than those without NAFLD. The metabolic syndrome was diagnosed in 206 of the 1647 study participants (12.5%), and the prevalence of the metabolic syndrome was higher in the subjects with NAFLD (38.1%) than those without (6.5%).

Incidence of cardiovascular disease

One thousand two hundred twenty one of 1647 participants (74.1%) completed the follow-up investigations. One thousand thirty three of them filled out the questionnaire at the time of their visits to our center and 188 subjects responded to the questionnaire mailed to them. Four hundred twenty six subjects did not send back the mailed questionnaire and were lost to the follow up analyses. Of 1221 participants available for follow up analyses, 231 subjects had NAFLD and 162 subjects had the metabolic syndrome at baseline. When the baseline characteristics were compared between the participants who completed and those who were lost to the follow-up study, there were more men among those who completed the follow-up than those who were lost to it (% of men, 61.3% *vs* 54.5%). LDL-cholesterol was slightly lower in the subjects who completed the follow-up than in those who did not complete it (3.4 ± 0.8 mg/dL *vs* 3.6 ± 0.9 mmol/L). The prevalence of NAFLD (18.9% *vs* 19.0%) or the metabolic syndrome (13.3% *vs* 10.3%) and the other parameters listed in Table 1 except for LDL-cholesterol were not different between the two groups.

Table 2 Univariate analyses of factors associated with cardiovascular disease among 1221 subjects

	Odds ratio (95% CI)	*P*
NAFLD	5.37 (2.29-12.58)	< 0.001
Gamma -glutamyltransferase, U/L	1.02 (1.01-1.03)	< 0.001
Age, 10 yr	2.30 (1.46-3.61)	< 0.001
Men	1.70 (0.66-4.38)	0.27
The Brinkman index, 100 index[1]	1.08 (1.00-1.17)	0.049
LDL-C, mmol/L	1.48 (0.89-2.46)	0.13
The metabolic syndrome	3.14 (1.26-7.83)	0.014
The components of metabolic syndrome		
BMI, kg/m^2	1.14 (1.00-1.31)	0.044
Fasting plasma glucose, mmol/L	1.42 (1.01-2.01)	0.046
Systolic blood pressure, 10 mmHg	1.72 (1.4-2.12)	< 0.001
Diastolic blood pressure, 10 mmHg	2.31 (1.57-3.39)	< 0.001
HDL-C, mmol/L	0.16 (0.03-0.84)	0.031
Triglycerides, mmol/L	1.61 (1.11-2.32)	0.012

NAFLD: nonalcoholic fatty liver disease; LDL-C: low dense lipoprotein cholesterol; HDL-C: high dense lipoprotein cholesterol. [1]The Brinkman index is the number of cigarettes per day multiplied by years of smoking. A higher value indicates increased smoking-associated health hazard.

During 7115 person-years of follow-up, we identified 22 events (1.8%) of nonfatal cardiovascular disease among 1221 subjects who completed the follow-up study: 8 events of coronary heart disease (7 events of acute coronary syndrome and 1 event of silent myocardial infarction), 12 events of ischemic stroke and 2 events of cerebral hemorrhage. A death due to carcinoma, the details of which were unknown, was reported from the family of a participant. There was no death due to cardiovascular disease (CVD) during the study period. In the 231 subjects with NAFLD, 12 events of cardiovascular disease occurred (5.2%), which comprised 5 events of coronary heart disease, 6 events of ischemic stroke and 1 event of cerebral hemorrhage. On the other hand, 10 events of CVD (1.0%) occurred in the 990 subjects without NAFLD, which comprised 3 events of coronary heart disease, 6 events of ischemic stroke and 1 event of cerebral hemorrhage. The incidence of cardiovascular disease was higher in the subjects with NAFLD than in those without ($P < 0.001$).

Table 3 Multivariate analyses of factors associated with cardiovascular disease among 1221 subjects (748 men and 473 women)

Total (n = 1221)	Model 1		Model 2		Model 3	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
NAFLD	3.57 (1.47-8.67)	0.005	(-)	(-)	4.12 (1.58-10.75)	0.004
The metabolic syndrome	(-)	(-)	2.61 (1.03-6.60)	0.042	1.38 (0.49-3.86)	0.54
Age, 10 yr	1.61 (0.97-2.66)	0.064	2.08 (1.30-3.33)	0.002	2.12 (1.32-3.41)	0.002
The Brinkman index, 100 index[1]	1.06 (0.97-1.16)	0.18	1.05 (0.96-1.14)	0.28	1.03 (0.95-1.12)	0.48
Systolic blood pressure, 10 mmHg	1.60 (1.26-2.04)	< 0.001	(-)	(-)	(-)	(-)
LDL-C, mmol/L	1.19 (0.70-2.03)	0.52	1.35 (0.8-2.3)	0.26	1.24 (0.73-2.08)	0.43
Men (n = 748)	Model 1		Model 2		Model 3	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
NAFLD	2.78 (0.98-7.89)	0.054	(-)	(-)	3.56 (1.16-10.95)	0.027
The metabolic syndrome	(-)	(-)	2.06 (0.70-6.11)	0.19	1.13 (0.34-3.78)	0.84
Age, 10 yr	1.59 (0.90-2.80)	0.11	1.90 (1.11-3.23)	0.019	1.96 (1.15-3.34)	0.014
The Brinkman index, 100 index[1]	1.07 (0.97-1.18)	0.19	1.05 (0.95-1.15)	0.35	1.04 (0.95-1.13)	0.44
Systolic blood pressure, 10 mmHg	1.51 (1.10-2.07)	0.011	(-)	(-)	(-)	(-)
LDL-C, mmol/L	1.42 (0.74-2.71)	0.29	1.53 (0.81-2.90)	0.19	1.42 (0.76-2.65)	0.27
Women (n = 473)	Model 1		Model 2		Model 3	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
NAFLD	14.33 (1.98-103.57)	0.008	(-)	(-)	7.32 (1.22-43.84)	0.029
The metabolic syndrome	(-)	(-)	4.59 (0.78-27.10)	0.093	2.86 (0.42-19.39)	0.28
Age, 10 yr	1.87 (0.60-5.78)	0.28	3.28 (1.09-9.86)	0.035	3.04 (0.99-9.31)	0.051
The Brinkman index, 100 index[1]	0.46 (0.03-6.76)	0.57	0.55 (0.04-7.63)	0.65	0.41 (0.03-6.44)	0.53
Systolic blood pressure, 10 mmHg	1.88 (1.23-2.86)	0.003	(-)	(-)	(-)	(-)
LDL-C, mmol/L	0.62 (0.21-1.85)	0.39	0.87 (0.30-2.52)	0.80	0.77 (0.27-2.16)	0.62

NAFLD: nonalcoholic fatty liver disease; LDL-C: low dense lipoprotein cholesterol. [1]The Brinkman index is the number of cigarettes per day multiplied by years of smoking. A higher value indicates increased smoking-associated health hazard.

Among 162 subjects with the metabolic syndrome, 7 events of CVD (4.3%) comprising 1 event of coronary heart disease and 6 events of ischemic stroke occurred, while 15 events of CVD (1.4%) comprising 7 events of nonfatal coronary heart disease, 6 events of ischemic stroke, and 2 events of cerebral hemorrhage occurred among 1059 subjects without the metabolic syndrome. The incidence was higher in the subjects with the metabolic syndrome than in those without (P = 0.019). Correlations of CVD events with NAFLD and the metabolic syndrome in addition to conventional CVD risk factors were first analyzed by univariate analyses (Table 2). NAFLD, gamma-glutamyltransferase, age, smoking (Brinkman index) and the metabolic syndrome showed statistically significant correlations with CVD. Each component of the metabolic syndrome also had a statistically significant correlation with CVD. Male gender and LDL-cholesterol were not correlated with CVD.

We constructed 3 models for multivariate analyses of the correlation of CVD events with NAFLD and other variables. Model 1 included NAFLD, age, smoking (Brinkman index), systolic blood pressure and LDL-cholesterol as independent variables. In model 2 the metabolic syndrome in place of NAFLD was selected as an independent variable. NAFLD as well as the metabolic syndrome was simultaneously included in model 3. Although gender was not correlated with CVD events in the univariate analyses, multivariate analyses were performed with subjects as a whole and men and women separately in order to examine a possible gender difference with respect to the relation between NAFLD and cardiovascular disease.

Analyses with subjects as a whole revealed that NAFLD and systolic blood pressure showed statistically significant correlations with CVD events in model 1. The respective odds ratios were 3.57 (95% CI, 1.47 to 8.67, P = 0.005) and 1.60 (95% CI, 1.26 to 2.04, P < 0.001) (Table 3). In model 2, the metabolic syndrome and age retained statistically significant correlations with CVD events. In a separate analysis, we replaced the metabolic syndrome with 5 components of the metabolic syndrome to evaluate the role of each component as a risk factor of CVD. In this analysis, only systolic blood pressure was correlated with CVD events. In model 3, NAFLD and age retained statistically significant correlation with CVD events and the metabolic syndrome lost an independent association with CVD events.

When men and women were separately analyzed, results were generally the same as described above. Although the correlation between NAFLD and CVD events appeared to be stronger in women than in men, a clear gender difference was not concluded. Some variables lost statistical significance, probably because a decrease in the number of CVD events weakened statistical power.

DISCUSSION

In this study, we found that NAFLD was a predictor of cardiovascular events among apparently healthy Japanese men and women. The association between NAFLD and future CVD events was independent of conventional cardiovascular risk factors.

Several previous studies demonstrated associations between NAFLD and intima-media thickness and/or

plaques of carotid artery that were used as measures of early atherosclerosis[18-20]. These were all cross-sectional studies in nature and it was difficult to determine a cause-effect relationship. Recently, Targher *et al*[21] reported in a prospective nested case-control study that NAFLD is a strong predictor of future cardiovascular events among type 2 diabetic patients. The subjects of their study were all patients with type 2 diabetes, which constitute a very high-risk population for cardiovascular disease. Therefore, it was uncertain whether the study could be extrapolated to the general population. We extended their study and showed that NAFLD can serve as a strong predictor of cardiovascular disease in apparently healthy people.

The metabolic syndrome defined by the modified National Cholesterol Education Program (NCEP ATP Ⅲ) criteria was also a predictor of CVD events in this study. Since an accumulating body of evidence suggests that there is a close association between the metabolic syndrome, which is a well-known atherogenic condition, and NAFLD, the mechanisms linking NAFLD with CVD events are at least partly mediated by the atherogenic abnormalities of the metabolic syndrome, that is, obesity, hyperglycemia, hypertriglyceridemia, low HDL-cholesterol and hypertension[15]. In fact, a correlation between the severity of liver histology of NAFLD and early carotid atherosclerosis was reported[19], while the association between liver histology and severity of the metabolic syndrome[22] has been noted as well. In a multivariate analysis based on the model that included NAFLD and the metabolic syndrome simultaneously as covariates, we found that NAFLD but not the metabolic syndrome retained an independent correlation with CVD events. This may suggest that NAFLD plays a central role in the pathway connecting the metabolic syndrome and cardiovascular diseases. The biological mechanism by which NAFLD promotes atherosclerosis is not known. Possible mechanistic pathways include increased oxidative stress, subclinical inflammation, an abnormal adipocytokine profile, endothelial dysfunction and lipid abnormalities[23].

Limitations of our study should be noted. First, although ultrasonography has a relatively high sensitivity (82%-94%) and specificity (66%-95%) in detecting fatty liver, it may give an incorrect diagnosis in 10%-30% of cases[24-28]. Moreover, it cannot distinguish steatohepatitis from simple steatosis. Second, our analysis is based on the incidence of self-reported cardiovascular disease. It is possible that selection biases could have masked a true association between NAFLD and cardiovascular disease. Finally, although the incidence rate of coronary heart disease in our study population was comparable to that of a much larger scale study reported recently from Japan[29], it was far lower than those reported in Western countries[13], and so the generalizability of our study to non-Japanese populations is uncertain.

In conclusion, our study confirms that NAFLD is a strong predictor of cardiovascular disease. The clinical implications of our study are twofold. Since ultrasonography of the liver is a non-invasive and easily applicable clinical test, it may be a useful tool for risk evaluation of cardiovascular disease. In patients with NAFLD, in addition to lifestyle modifications to reduce fat deposition, it may be important to explore the patients for risk factors of cardiovascular disease, and if found, treat them aggressively.

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RAPID COMMUNICATION

Risk factors for Barrett's oesophagus and oesophageal adenocarcinoma: Results from the FINBAR study

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Abstract

AIM: To investigate risk factors associated with Barrett's oesophagus and oesophageal adenocarcinoma.

METHODS: This all-Ireland population-based case-control study recruited 224 Barrett's oesophagus patients, 227 oesophageal adenocarcinoma patients and 260 controls. All participants underwent a structured interview with information obtained about potential lifestyle and environmental risk factors.

RESULTS: Gastro-oesophageal reflux was associated with Barrett's [OR 12.0 (95% CI 7.64-18.7)] and oesophageal adenocarcinoma [OR 3.48 (95% CI 2.25-5.41)]. Oesophageal adenocarcinoma patients were more likely than controls to be ex- or current smokers [OR 1.72 (95% CI 1.06-2.81) and OR 4.84 (95% CI 2.72-8.61) respectively] and to have a high body mass index [OR 2.69 (95% CI 1.62-4.46)]. No significant associations were observed between these risk factors and Barrett's oesophagus. Fruit but not vegetables were negatively associated with oesophageal adenocarcinoma [OR 0.50 (95% CI 0.30-0.86)].

CONCLUSION: A high body mass index, a diet low in fruit and cigarette smoking may be involved in the progression from Barrett's oesophagus to oesophageal adenocarcinoma.

Key words: Barrett's oesophagus; Oesophageal adenocarcinoma; Smoking; Gastro-oesophageal reflux; Body mass index; Diet; Case-control

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INTRODUCTION

Barrett's oesophagus, a condition of the distal oesophagus in which the normal stratified squamous epithelium is replaced by specialised intestinal metaplasia, is a recognized precursor of oesophageal adenocarcinoma[1], a cancer that has been increasing in incidence in many Western societies over recent decades[2-6]. It is unknown if all oesophageal adenocarcinomas arise from Barrett's oesophagus but there is some evidence to suggest that this is the case[7]. In surveillance programs of Barrett's oesophagus only a minority of patients develop oesophageal adenocarcinoma[8-12] raising the question of what factors are implicated in the development of oesophageal adenocarcinoma from Barrett's oesophagus.

Several case-control studies have investigated lifestyle factors associated with oesophageal adenocarcinoma[13-19] but few studies of factors associated with Barrett's oesophagus have been reported[20-22]. Gastro-oesophageal reflux is strongly associated with oesophageal adenocarcinoma[14-17,23,24] and is thought to be the main predisposing factor for Barrett's oesophagus[20]. A small proportion of gastro-oesophageal reflux sufferers develop Barrett's oesophagus[25-28] and approximately 0.5% of Barrett's oesophagus patients progress to oesophageal adenocarcinoma each year[8,29-31] indicating that factors apart from gastro-oesophageal reflux are involved in the development of Barrett's oesophagus and in its progression to oesophageal adenocarcinoma. Several risk factors for oesophageal adenocarcinoma have been established, including a high body mass index (BMI)[15,16,24,32-34], smoking[13,15,19,34,35] and possibly a diet low in fruit and vegetables[16,24,36-38]. Engel *et al*[39] estimated that these three factors, in combination with gastro-oesophageal reflux, have a population attributable risk for

oesophageal adenocarcinoma of 78.7%. However, it is not clear at which stage along the oesophageal inflammation-metaplasia-adenocarcinoma sequence these factors exert their effect. Studies comparing risk factors for Barrett's oesophagus and oesophageal adenocarcinoma provide the opportunity to examine whether these risk factors are important in the development of Barrett's oesophagus or in its progression to oesophageal adenocarcinoma. This is crucial to the targeting of preventive efforts aimed at reducing the morbidity and mortality associated with these conditions. We undertook a population-based case-control study of Barrett's oesophagus and oesophageal adenocarcinoma within Ireland.

MATERIALS AND METHODS

Study details have been described in detail elsewhere[40]. Briefly, the Factors Influencing the Barrett's Adenocarcinoma Relationship (FINBAR) study recruited three groups of subjects: (1) patients with oesophageal adenocarcinoma, (2) patients with long-segment Barrett's oesophagus, and (3) normal population controls between March 2002 and December 2004.

Oesophageal adenocarcinoma cases had histological confirmation of adenocarcinoma within the oesophagus. Northern Ireland cases were identified from electronic pathology records from all pathology laboratories within the province. Republic of Ireland cases were identified from the main hospitals involved in the diagnosis and treatment of oesophageal cancer. All available relevant clinical and histological records including endoscopy, surgical and radiological reports were reviewed by LAA, SJM, JM and a pathologist, to confirm that the tumour was located in the oesophagus and to assign tumours into two groups: (1) oesophageal tumours (including tumours encroaching on the esophagogastric junction) and (2) junctional tumours (tumours involving the oesophagus, esophagogastric junction and gastric cardia).

Barrett's oesophagus patients were eligible for inclusion if $\geqslant$ 3 centimetres of typical Barrett's mucosa were seen at endoscopy and the presence of specialised intestinal metaplasia was confirmed by histological examination of biopsy specimens. Incident and prevalent cases were included and subjects were frequency matched to the age and 5-year sex distribution of oesophageal adenocarcinoma patients. Patients with dysplasia on histological examination were excluded. In Northern Ireland, cases of Barrett's oesophagus were initially identified from pathology reports gathered from throughout Northern Ireland. Endoscopy note review was necessary in most patients to confirm the length of the segment of Barrett's oesophagus, as length was infrequently recorded on the pathology report. In the Republic of Ireland, clinicians in the Dublin and Cork areas sent details of Barrett's oesophagus patients who met the inclusion criteria to the research personnel.

Eligible control subjects were adults without a history of oesophageal or other gastro-intestinal cancer or a known diagnosis of Barrett's oesophagus and were frequency matched, by sex and 5-year age band, to the distribution of oesophageal adenocarcinoma patients. Northern Ireland controls were selected at random from the General Practice Master Index (a province-wide database of all persons registered with a general practitioner) and Republic of Ireland controls were selected at random from four General Practices (two urban and two rural) in the Dublin and Cork areas chosen by the researchers to reflect the urban/rural distribution of oesophageal adenocarcinoma patients in the Republic of Ireland.

Participants underwent a structured interview with trained interviewers after giving informed written consent. Information obtained included data on symptoms of gastro-oesophageal reflux (questions based on a translation of those used by Lagergren *et al*[14] in their Swedish case-control study), weight 5 years before the interview, height and weight at age 21, maximum and minimum weight during adulthood, smoking history, education, occupation and alcohol consumption. Anthropometric measures (height, weight, waist and hip circumference) were taken at the time of interview.

Frequent gastro-oesophageal reflux was defined as symptoms of heartburn and/or acid reflux occurring more than 50 times per year (at least once per week), more than 5 years prior to the interview. Frequent gastro-oesophageal reflux, which prevented subjects from going to sleep or awoke them from sleep, was classified as nocturnal gastro-oesophageal reflux symptoms. The reflux symptom score used by Lagergren *et al*[14] was applied to the FINBAR dataset but scores 1-4 were combined in the analyses because of the small number of subjects in the first 2 categories.

Current BMI and BMI 5 years before the interview date were calculated by dividing weight in kilograms (current measured and 5-year self-reported, respectively) by current height in metres squared. BMI at age 21 was calculated by dividing self-reported weight in kilograms at age 21 by self-reported height in meters at age 21 squared.

Current smoking status was defined as having smoked at least one cigarette per day for 6 mo or longer, 5 years before the interview date. Previous smokers were those who had quit smoking more than 5 years prior to the interview date. People who had never smoked, and those who had smoked less than 100 cigarettes in their lifetime, or less than one cigarette per day for 6 mo or longer were defined as never smokers. Pack years of smoking were calculated by multiplying the number of cigarettes smoked per day by the number of years of smoking, and dividing by 20. Cigar and pipe smokers were those who had ever smoked at least one cigar or one pipe-full of tobacco per week irrespective of whether they smoked cigarettes.

Fruit, vegetable and energy intake were measured using the European Prospective Investigation of Cancer (EPIC) food frequency questionnaire used in the Norfolk area of England[41] as part of a large European cancer cohort study. This validated questionnaire was modified for the Irish population by including foods commonly eaten in Ireland as identified in the recent North/South Food Consumption Survey[42]. Fruit and vegetable consumption 5 years prior to the interview date were quantified in terms of the sum of the frequencies that each item of fruit/vegetable was eaten per week.

Table 1 Characteristics of controls, Barrett's and oesophageal adenocarcinoma patients

Variables	Controls	Barrett's oesophagus	*P* (BO *vs* controls)	Oesophageal adenocarcinoma	*P* (OAC *vs* controls)
Sex , *n* (%)			0.548		0.992
Male	220 (84.6)	185 (82.6)		192 (84.6)	
Female	40 (15.4)	39 (17.4)		35 (15.4)	
Age			0.567		0.276
Mean years	63	62.4		64.2	
Education			0.013		< 0.001
Years (full-time)	12	11.3		10.7	
Job type			0.016		0.013
Manual	119 (48.0)	130 (59.1)		128 (59.5)	
Non-manual	129 (52.0)	90 (40.9)		87 (40.5)	
Alcohol, *n* (%)			0.135		0.004
Never	69 (26.5)	57 (25.6)		65 (28.9)	
Ever	191 (73.5)	166 (74.4)		160 (71.1)	
Mean (grams/d)	26.1	22.3		19.2	
GOR frequent, *n* (%)			< 0.01		< 0.001
Never	211 (81.2)	60 (26.8)		117 (51.5)	
Ever	49 (18.8)	164 (73.2)		110 (48.5)	
Body mass index			0.895		< 0.001
Mean	27	27		28.7	
Fruit			< 0.001		< 0.001
Frequency per week	14.2	11.9		11.2	
Vegetables			0.04		0.348
Frequency per week	20	18.7		20.6	
Total energy intake (kcal)			0.036		0.023
Mean	2573.9	2727.2		2744.2	
Smoking status, *n* (%)			0.4		< 0.001
Never	102 (40.2)	87 (39.2)		45 (20.4)	
Ex-smoker	107 (42.1)	85 (38.3)		99 (44.8)	
Current	45 (17.7)	50 (22.5)		77 (34.8)	

BO: Barrett's oesophagus; OAC: oesophageal adenocarcinoma; GOR: gastro-oesophageal reflux.

Statistical analysis

Throughout the article exposures of interest are presented in tertiles. Tertiles of BMI, for example, were calculated from the normal control group with all subjects categorised according to these tertiles. Statistical analyses were performed using STATA 8.0[43]. Chi-square tests were used to examine differences between groups for categorical variables and t-tests were used for continuous variables. Unconditional maximum-likelihood multinomial (polytomous) logistic regression analyses were undertaken to examine the associations between the exposure variables of interest and case/control status adjusting for potential confounders; odds ratios (OR) with 95% confidence intervals (CI) were calculated. Analyses are shown adjusted for potential confounders, including sex, age at interview, job type (manual, non-manual work), education (years full-time) and alcohol consumption (grams per week). Further adjustment was made for gastro-oesophageal reflux symptoms (never, ever), smoking (current, ex-, never), BMI (5 years before diagnosis/interview) and total energy intake (in kilocalories) where appropriate.

Ethical approval for the FINBAR study was obtained from the Research Ethics Committee of the Queen's University Belfast, the Clinical Research Ethics Committee of the Cork Teaching Hospitals and the Research Ethics Committee Board of St. James's Hospital, Dublin.

RESULTS

Two hundred and twenty-four Barrett's oesophagus patients, 227 oesophageal adenocarcinoma patients and 260 controls were recruited. One hundred and thirty-one (57.7%) of the oesophageal adenocarcinoma patients were classified as having oesophageal tumours, ninety-two (40.5%) were classified as junctional tumours and insufficient evidence was available to classify the position of 4 (1.8%) tumours. Characteristics of patients and controls are shown in Table 1.

The participation rate of eligible, alive oesophageal adenocarcinoma patients was 74.2% and the overall response rate was 63.9%. The participation rates among Barrett's oesophagus patients and controls were 82.4% and 41.8%, respectively.

Gastro-oesophageal reflux

Symptoms of gastro-oesophageal reflux more than 5 years prior to the interview date were strongly associated with Barrett's oesophagus and to a lesser extent with oesophageal adenocarcinoma (Table 2). In total 72.8% of Barrett's patients, 48.5% of oesophageal adenocarcinoma patients and 18.9% of controls reported at least weekly symptoms of gastro-oesophageal reflux. Barrett's oesophagus and oesophageal adenocarcinoma patients were more likely than controls to report nocturnal gastro-oesophageal reflux symptoms, although in the oesophageal adenocarcinoma group the association did not hold for those with junctional tumours (chi-square test, $P = 0.001$).

Using the symptom scoring system developed by Lagergren *et al*[14] Barrett's patients were 18 times, and oesophageal adenocarcinoma patients more than 3 times,

Table 2 Comparison of gastro-oesophageal reflux symptoms between controls and Barrett's oesophagus and oesophageal adenocarcinoma patients and between oesophageal and junctional tumour subgroups

	Controls No.	BE No.	Adjusted1 OR (95% CI)	EAC No.	Adjusted[1] OR (95% CI)	Oesophageal tumours No.	Adjusted[1] OR (95% CI)	Junctional tumours No.	Adjusted[1] OR (95% CI)
Frequent GOR[2]									
No	211	60	1.00	117	1.00	64	1.00	51	1.00
Yes	49	164	12.0 (7.64 to 18.7)	110	3.48 (2.25 to 5.41)	67	3.73 (2.27 to 6.13)	41	3.12 (1.79 to 5.43)
Nocturnal symptoms[3]									
No	237	110	1.00	167	1.00	86	1.00	79	1.00
Yes	23	113	10.9 (6.49 to 18.5)	60	3.16 (1.81 to 5.51)	45	4.53 (2.49 to 8.22)	13	1.45 (0.67 to 3.14)
Reflux symptom score[4]									
No GOR	211	54	1.00	116	1.00	63	1.00	51	1.00
1-4 points	25	63	10.4 (5.90 to 18.5)	53	3.63 (2.06 to 6.40)	22	2.59 (1.30 to 5.15)	30	4.99 (2.58 to 9.62)
4.5-6.5 points	24	107	18.4 (10.5 to 32.3)	58	3.55 (2.02 to 6.27)	46	5.11 (2.78 to 9.38)	11	1.52 (0.66 to 3.47)
Symptom frequency in tertiles (*t*/yr)									
No GOR	211	61	1.00	117	1.00	64	1.00	51	1.00
< 208	17	44	9.34 (4.88 to 17.9)	33	2.94 (1.50 to 5.77)	18	2.96 (1.38 to 6.35)	14	2.81 (1.23 to 6.41)
208-365	23	50	7.86 (4.36 to 14.2)	35	2.75 (1.49 to 5.07)	18	2.53 (1.23 to 5.19)	16	2.94 (1.38 to 6.29)
> 365	9	69	29.2 (13.3 to 64.5)	42	6.54 (2.93 to 14.6)	31	8.20 (3.51 to 19.1)	11	4.50 (1.69 to 12.0)
Duration of symptoms in tertiles (yr)									
No GOR	211	54	1.00	116	1.00	63	1.00	51	1.00
< 10	18	64	11.9 (6.37 to 22.1)	39	3.67 (1.94 to 6.97)	16	2.67 (1.25 to 5.72)	22	5.00 (2.38 to 10.5)
10-22.6	15	38	8.09 (4.09 to 16.0)	21	2.28 (1.08 to 4.83)	18	3.57 (1.62 to 7.86)	3	0.78 (0.21 to 2.88)
> 22.6	16	68	16.0 (8.38 to 30.5)	51	4.41 (2.30 to 8.48)	34	5.11 (2.51 to 10.4)	16	3.41 (1.52 to 7.65)

BO: Barrett's oesophagus; OAC: oesophageal adenocarcinoma; GOR: gastro-oesophageal reflux; OR: odds ratio; CI: confidence interval. [1]Adjusted for sex, age at interview date, body mass index (5 yr prior to the interview date), smoking status (never, ex-, current), alcohol intake (grams), years of full-time education and job type (manual, non-manual). [2]Symptoms of heartburn and/or reflux more than 50 times per year. [3]Symptoms of GOR that prevented sleep or woke the person from sleep. [4]Reflux symptom score[14]. Heartburn or regurgitation only = 1 point, GOR symptoms 2 to 6 times per week = 1 point, heartburn and regurgitation = 1.5 points, nightly symptoms = 2 points, GOR symptoms 7 to 15 times per week = 2 points, symptoms more than 15 times per week = 3 points.

as likely as controls to have a score in the highest gastro-oesophageal reflux category. Patients defined as having oesophageal tumours experienced more severe gastro-oesophageal reflux than patients defined as having junctional tumours. Barrett's oesophagus and oesophageal adenocarcinoma patients were more likely to have suffered from gastro-oesophageal reflux on a more frequent basis and for a longer duration of time than controls (Table 2).

Body mass index

The associations between BMI (in tertiles) and risk of Barrett's oesophagus and oesophageal adenocarcinoma at the time of interview are displayed in Table 3. No associations were observed between Barrett's oesophagus and BMI at any stage (current, at 5 years prior to the interview date or at age 21). Current BMI was significantly lower in oesophageal adenocarcinoma patients than in controls, most likely due to cancer-associated weight loss. However, high BMI 5 years prior to the interview date was associated with a more than 2-fold increased risk of oesophageal adenocarcinoma. The association was similar (highest tertile vs lowest) for tumours classified as oesophageal [OR 2.54 (95% CI 1.44 to 4.48)] or junctional [OR 2.95 (95% CI 1.52 to 5.72]. Oesophageal adenocarcinoma patients were also more likely than controls to be in the highest tertile of BMI at age 21 and to have a higher maximum and minimum weight than controls. Adjusting any of the BMI analyses for a history of gastro-oesophageal reflux symptoms did not significantly alter the observed associations (Table 3).

Similar results were observed when BMI was categorised according to the World Health Organisation classification system[44]. Barrett's oesophagus patients were no more likely than controls to be currently overweight (25-30 kg/m^2) [OR 0.86 (95% CI 0.55 to 1.36)] or obese (> 30 kg/m^2) [OR 1.06 (95% CI 0.62 to 1.81)] or to have been overweight or obese 5 years before the interview date [OR 0.95 (95% CI 0.62 to 1.45)] and [OR 0.80 (95% CI 0.47 to 1.38) respectively]. Oesophageal adenocarcinoma patients were currently less overweight/obese than controls [OR 0.36 (95% CI 0.23 to 0.57) and OR 0.39 (95% CI 0.22 to 0.69), respectively], but were more likely to have been overweight or obese 5 years before the interview date [OR 1.55 (95% CI 0.96 to 2.50) and OR 2.55 (95% CI 1.47 to 4.41) respectively].

Waist-hip ratio was measured at the time of interview, and no relationship was observed between waist-hip ratio and oesophageal adenocarcinoma [(highest tertile vs lowest) OR 0.80 (95% CI 0.50 to 1.28)] or Barrett's oesophagus [OR 1.09 (95% CI 0.68 to 1.73)].

Fruit and vegetable intake

Barrett's oesophagus patients appeared to be less likely than controls to consume fruit and/or vegetables (Table 4). However, following adjustment for gastro-oesophageal reflux symptoms neither fruit nor vegetable intake alone was significantly associated with Barrett's oesophagus. Compared to controls oesophageal adenocarcinoma patients had a lower intake of fruit, but not vegetables (Table 4). There was no significant difference in the consumption of fruit and vegetables between the oesophageal and junctional subgroups (chi-squared test, $P = 0.691$).

Table 3 Body mass index and weight comparisons between controls and Barrett's oesophagus and oesophageal adenocarcinoma patients

	Controls	Barrett's oesophagus			Oesophageal adenocarcinoma		
	No.	No.	Adjusted[1] OR (95% CI)	Further adjusted for GOR OR (95% CI)	No.	Adjusted[1] OR (95% CI)	Further adjusted for GOR OR (95% CI)
Current BMI in tertiles (kg/m^2)							
< 25.8	86	77	1.00	1.00	115	1.00	1.00
25.8-29.0	87	64	0.75 (0.47 to 1.19)	0.61 (0.36 to 1.03)	54	0.38 (0.24 to 0.62)	0.35 (0.21 to 0.58)
> 29.0	85	82	0.97 (0.61 to 1.52)	0.75 (0.44 to 1.25)	50	0.38 (0.23 to 0.62)	0.33 (0.20 to 0.56)
BMI 5 yr ago in tertiles (kg/m^2)							
< 25.0	86	75	1.00	1.00	51	1.00	1.00
25.0-28.1	87	78	1.02 (0.65 to 1.61)	0.84 (0.50 to 1.42)	55	1.23 (0.72 to 2.08)	1.74 (0.66 to 1.97)
> 28.1	86	71	0.89 (0.56 to 1.41)	0.85 (0.51 to 1.44)	120	2.70 (1.65 to 4.41)	2.69 (1.62 to 4.46)
BMI age 21 in tertiles (kg/m^2)							
< 22.1	81	70	1.00	1.00	55	1.00	1.00
22.1-24.1	88	80	0.96 (0.61 to 1.52)	1.17 (0.70 to 1.99)	64	0.97 (0.59 to 1.60)	1.10 (0.65 to 1.85)
> 24.1	84	69	0.83 (0.51 to 1.33)	1.04 (0.61 to 1.79)	96	1.59 (0.97 to 2.59)	1.81 (1.08 to 3.02)
Maximum weight in tertiles (kg)[2]							
< 73	87	72	1.00	1.00	53	1.00	1.00
73-86	91	89	1.21 (0.76 to 1.95)	1.04 (0.61 to 1.79)	67	1.54 (0.90 to 2.61)	1.42 (0.83 to 2.45)
> 86	80	62	0.98 (0.58 to 1.66)	0.91 (0.50 to 1.66)	104	3.44 (1.97 to 5.98)	3.32 (1.88 to 5.86)
Minimum weight in tertiles (kg)[2]							
< 64	108	102	1.00	1.00	79	1.00	1.00
64-70	81	61	0.83 (0.52 to 1.34)	1.00 (0.58 to 1.72)	52	1.10 (0.65 to 1.86)	1.20 (0.70 to 2.08)
> 70	70	60	0.97 (0.57 to 1.64)	1.25 (0.69 to 2.27)	95	2.83 (1.63 to 4.89)	3.22 (1.82 to 5.71)

GOR: gastro-oesophageal reflux; OR: odds ratio; CI: confidence interval. [1]Adjusted for sex, age at interview date, smoking status (never, ex-, current), alcohol intake (grams), years of full-time education and job type (manual, non-manual). [2]Also adjusted for height (centimetres). [b]$P \leq 0.001$.

Table 4 Comparison of fruit and vegetable consumption (5 yr before interview date) between controls and Barrett's oesophagus and oesophageal adenocarcinoma patients

	Controls	Barrett's Oesophagus			Oesophageal adenocarcinoma		
	No.	No.	Adjusted[1] OR (95% CI)	Adjusted[1] + GOR OR (95% CI)	No.	Adjusted[1] OR (95% CI)	Adjusted[1] + GOR OR (95% CI)
Fruit and vegetables (portions/wk)							
<20	85	105	1.00	1.00	98	1.00	1.00
20-34	86	58	0.58 (0.36 to 0.92)	0.50 (0.30 to 0.84)	61	0.64 (0.39 to 1.04)	0.60 (0.36 to 1.00)
> 34	86	60	0.61 (0.38 to 0.98)	0.67 (0.40 to 1.15)	65	0.67 (0.41 to 1.12)	0.71 (0.43 to 1.19)
Fruit (portions/wk)							
< 5	83	94	1.00	1.00	109	1.00	1.00
5-20	87	76	0.78 (0.49 to 1.23)	0.91 (0.55 to 1.51)	63	0.52 (0.32 to 0.85)	0.56 (0.34 to 0.92)
> 20	87	53	0.57 (0.35 to 0.94)	0.64 (0.37 to 1.12)	53	0.47 (0.28 to 0.80)	0.50 (0.30 to 0.86)
Vegetables (portions/wk)							
< 12	83	90	1.00	1.00	66	1.00	1.00
12-17	85	68	0.76 (0.48 to 1.21)	0.75 (0.45 to 1.25)	71	1.13 (0.68 to 1.86)	1.12 (0.67 to 1.87)
> 17	89	65	0.72 (0.44 to 1.15)	0.82 (0.48 to 1.39)	87	1.38 (0.84 to 2.28)	1.49 (0.89 to 2.48)

GOR: gastro-oesophageal reflux; OR: odds ratio; CI: confidence interval. [1]Adjusted for sex, age at interview date, smoking status (never, ex-, current), alcohol intake (grams), energy intake (kilocalories), body mass index (5 yr prior to interview), years of full-time education and job type (manual, non-manual).

Smoking

Cigarette Smoking was not significantly associated with Barrett's oesophagus; however there was a strong relationship between smoking and oesophageal adenocarcinoma (Table 5). The findings remained significant regardless of the method of smoking categorisation. Adjusting for symptoms of gastro-oesophageal reflux did not significantly alter the observed associations. Ex-smoking and current smoking status 5 years prior to interview were similar in both the oesophageal [OR 1.86 (95% CI 1.05 to 3.30) and OR 4.62 (95% CI 2.40 to 8.91) respectively] and junctional subgroups [OR 1.80 (95% CI 0.95 to 3.42) and OR 4.68 (95% CI 2.40 to 8.91) respectively, chi-square test, $P = 0.951$]. Neither pipe smoking nor cigar smoking was significantly associated with oesophageal adenocarcinoma or Barrett's oesophagus (Table 5).

DISCUSSION

This is the first reported population-based case-control study to compare risk factors for both Barrett's oesophagus and oesophageal adenocarcinoma. The study confirms established risk factors for oesophageal adenocarcinoma and demonstrates important differences between Barrett's oesophagus and oesophageal adenocarcinoma in their

Table 5 Smoking in controls, Barrett's oesophagus and oesophageal adenocarcinoma patients

	Controls	Barrett's oesophagus			Oesophageal adenocarcinoma		
	No.	No.	Adjusted[1] OR (95% CI)	Further adjusted for GOR OR (95% CI)	No.	Adjusted[1] OR (95% CI)	Further adjusted for GOR OR (95% CI)
Smoking status (5 yr prior to interview)							
Never[2]	102	87	1.00	1.00	46	1.00	1.00
Ex-smoker	107	85	0.97 (0.63 to 1.49)	0.86 (0.53 to 1.40)	99	1.85 (1.15 to 2.97)	1.72 (1.06 to 2.81)
Current smoker	45	50	1.27 (0.74 to 2.17)	1.41 (0.77 to 2.58)	77	4.64 (2.64 to 8.17)	4.84 (2.72 to 8.61)
No. of cigarettes smoked per day							
Never[2]	102	87	1.00	1.00	46	1.00	1.00
< 15	54	41	0.94 (0.57-1.57)	0.98 (0.55 to 1.75)	44	1.85 (1.07-3.20)	1.87 (1.06 to 3.28)
15-20	51	41	0.98 (0.58-1.65)	0.94 (0.52 to 1.69)	67	3.14 (1.83-5.37)	3.05 (1.76 to 5.30)
> 20	49	53	1.32 (0.78-2.22)	1.16 (0.65 to 2.09)	66	2.92 (1.68-5.10)	2.72 (1.54 to 4.80)
Duration of smoking (yr)							
Never[2]	102	87	1.00	1.00	46	1.00	1.00
< 20	47	28	0.72 (0.41 to 1.28)	0.72 (0.38 to 1.38)	38	1.92 (1.07 to 3.44)	1.91 (1.05 to 3.48)
20-35	55	57	1.21 (0.74 to 1.98)	1.09 (0.62 to 1.90)	54	2.08 (1.21 to 3.58)	1.94 (1.11 to 3.38)
> 35	53	51	1.18 (0.70 to 1.98)	1.18 (0.66 to 2.11)	85	3.74 (2.18 to 6.40)	3.71 (2.14 to 6.42)
Years since quitting smoking							
Never[2]	102	87	1.00	1.00	46	1.00	1.00
< 26	27	29	1.27 (0.74 to 2.19)	1.42 (0.78 to 2.60)	36	4.72 (2.68 to 8.31)	4.89 (2.74 to 8.71)
26-41	40	32	1.26 (0.68 to 2.33)	1.11 (0.56 to 2.20)	29	2.68 (1.42 to 5.07)	2.51 (1.30 to 4.83)
> 41	40	24	1.02 (0.58 to 1.81)	0.89 (0.47 to 1.70)	34	1.52 (0.82 to 2.85)	1.40 (0.74 to 2.66)
Pack years of smoking							
Never[2]	102	87	1.00	1.00	46	1.00	1.00
< 15	48	36	0.92 (0.54 to 1.57)	0.94 (0.51 to 1.72)	30	1.39 (0.76 to 2.55)	1.39 (0.75 to 2.58)
15-40	55	47	1.01 (0.61 to 1.67)	0.92 (0.52 to 1.63)	68	2.95 (1.74 to 5.00)	2.84 (1.66 to 4.86)
> 40	51	52	1.28 (0.76 to 2.17)	1.23 (0.68 to 2.21)	79	3.48 (2.01 to 6.02)	3.34 (1.91 to 5.83)
Cigar							
Never	234	204	1.00	1.00	194	1.00	1.00
Ever	22	18	0.84 (0.41 to 1.71)[3]	0.73 (0.33 to 1.61)[3]	29	1.30 (0.67 to 2.53)[3]	1.22 (0.61 to 2.43)[3]
Pipe							
Never	233	194	1.00	1.00	182	1.00	1.00
Ever	23	28	1.68 (0.88 to 3.20)[3]	1.31 (0.64 to 2.68)[3]	41	1.64 (0.88 to 3.06)[3]	1.45 (0.77 to 2.75)[3]

GOR: gastro-oesophageal reflux; OR: odds ratio; CI: confidence interval. [1]Adjusted for sex, age at interview date, body mass index 5 yr prior to the interview date, alcohol intake (grams), years of full-time education and job type (manual, non-manual). [2]Never smoked, smoked less than 100 cigarettes in lifetime or smoked less than 1 cigarette per day for 6 mo in their lifetime [3]Adjusted for use of other tobacco products e.g. cigarettes, pipe or cigar respectively.

association with these factors. Gastro-oesophageal reflux symptoms were strongly associated with Barrett's oesophagus and to a lesser extent with oesophageal adenocarcinoma. A high BMI, 5 years prior to the interview date, and smoking were significantly associated with an increased risk of oesophageal adenocarcinoma but not Barrett's oesophagus. Barrett's oesophagus patients appeared to eat less fruit and vegetables than controls. A diet high in fruit but not in vegetables was associated with a reduced risk of oesophageal adenocarcinoma. These data may suggest that gastro-oesophageal reflux symptoms and possibly a diet low in fruit and vegetables are initially responsible for the development of Barrett' s oesophagus, and that obesity and smoking are involved in the progression of Barrett's oesophagus to oesophageal adenocarcinoma.

The strengths of the FINBAR study are its population-based design, the rapid case ascertainment and stringent inclusion criteria for Barrett's oesophagus (specialised intestinal metaplasia, length $\geqslant$ 3 centimetres) which minimise the inclusion of subjects with a biopsy from an unrecognized hiatus hernia.

In this study, cancers were divided into two subgroups: oesophageal tumours (which could encroach on, but not involve, the oesophagogastric junction) and tumours involving the oesophagus, oesophagogastric junction and gastric cardia (termed junctional tumours). There was a potential weakness for some misclassification of oesophageal adenocarcinoma patients in this study as it was impossible to determine whether junctional tumours are truly gastric or oesophageal in origin.

A potential weakness of the study was the low response rate amongst controls, which may have introduced selection bias. However, controls were similar to the general population with regards to symptoms of gastro-oesophageal reflux and BMI. In a study from Bristol, 15.6% of people aged 20-59 had weekly symptoms of heartburn[45] compared to 18.4% of controls within this age range in the FINBAR study. If gastro-oesophageal reflux symptoms were over-represented in controls then the actual associations between gastro-oesophageal reflux, Barrett's oesophagus and oesophageal adenocarcinoma may be stronger than observed in this study. The mean weight and the proportion of the obese controls were similar to those seen in the all-Ireland Food Consumption Survey (1997 to 1999)[46]. Mean BMI in males aged 51-64 years in the survey was 27.6 kg/m^2 (s.d. 3.6 kg/m^2) and in FINBAR 28.0 kg/m^2 (s.d. 4.5 kg/m^2). Similarly, 20% of men were obese (BMI > 30.0 kg/m^2) in the Food Consumption Survey compared to 22% of FINBAR male

controls. However, in the 2001 Northern Ireland health and social wellbeing survey[47] 23.6% of males at the age of 55 years or over were current smokers, 53.1% ex-smokers and 23.3% non-smokers compared to 18.9%, 48% and 31.1% respectively in FINBAR controls. Non-smokers may be overrepresented among FINBAR controls which could lead to an overestimation of the positive association between oesophageal adenocarcinoma and smoking.

The main predisposing risk factor for Barrett's oesophagus is gastro-oesophageal reflux. Our finding of a strong association between gastro-oesophageal reflux symptoms and Barrett's oesophagus is in agreement with a previous case-control study by Conio *et al*[20]. Since the main presenting symptom for Barrett's oesophagus is gastro-oesophageal reflux it is possible that diagnosed Barrett's oesophagus patients are not representative of all Barrett's patients with regards to gastro-oesophageal reflux symptoms. The association is likely causal in nature; however there may be an overestimation of the true association between gastro-oesophageal reflux and Barrett's oesophagus. Although the exact mechanisms by which gastro-oesophageal reflux causes Barrett's oesophagus are still not fully understood, reflux of acid and/or bile into the distal oesophagus is believed to damage the native squamous epithelium and result in re-epithelisation with columnar mucosa. The strength of the relationship between gastro-oesophageal reflux symptoms and oesophageal adenocarcinoma, is in keeping with the findings of several studies on Barrett's oesophagus[17,23,48] except that of Lagergren *et al*[14] who reported an OR of 7.7. Our data suggest that although gastro-oesophageal reflux is common in patients with tumours classified as either oesophageal or junctional, those with junctional tumours seem to have less severe symptoms. In particular, nocturnal symptoms of gastro-oesophageal reflux are more strongly associated with oesophageal but not with junctional tumours.

Obesity has been linked with the development of gastro-oesophageal reflux[45,49,50], increased intra-abdominal pressures[54,55] and relaxation of the lower oesophageal sphincter[56] which may worsen gastro-oesophageal reflux symptoms. Obesity has been increasing in incidence[51-53], paralleling the increasing incidence of oesophageal adenocarcinoma[2-6].

Some studies have suggested that a high BMI is associated with an increased risk of Barrett's oesophagus[22,57,58], although Caygill *et al*[58] suggested that obesity is only a risk factor for Barrett's oesophagus in young people. No associations were observed between current BMI, BMI 5 years prior to the interview date, or BMI at age 21, and Barrett's oesophagus in the FINBAR study. It is possible that the BMI of controls was higher than that of the population which could explain the fact that no association was observed. However, a high BMI 5 years prior to the interview date was associated with a 2.5 fold increased risk of oesophageal adenocarcinoma, which is similar to reports in other case-control studies[24,32-34,59]. If BMI is not associated with Barrett's oesophagus then one possible mechanism for the association between BMI and oesophageal adenocarcinoma may be through the increased production of free insulin-like growth factor-1 in obese subjects, which stimulates cell proliferation and inhibits apoptosis[60,61]. Sohda *et al*[62] suggested that increased free insulin-like growth factor-1 may be associated with the development of oesophageal cancer. In Barrett's patients increased expression of insulin-like growth factor-1 receptor is associated with neoplastic progression[63].

Fruit, although not vegetable intake was significantly associated with a reduced risk of oesophageal adenocarcinoma in this study. A diet high in fruit and vegetables has been shown to be able to protect against a number of cancers[64], including cancers of the digestive tract[65]. Several case-control studies have specifically reported positive associations between high fruit and/or vegetable intake and a reduced risk of oesophageal adeno carcinoma[16,24,36-38] and a cohort study recently reported a non-significant inverse association between oesophageal adenocarcinoma and vegetables and citrus fruit[66]. In the FINBAR study vegetable consumption was not associated with a reduced risk of oesophageal adenocarcinoma in fact the OR was raised [OR 1.49 (95% CI 0.89 to 2.48)]. One possible explanation for the apparent protective effect of fruit against oesophageal adenocarcinoma may be that patients with gastro-oesophageal reflux avoid certain fruits which can aggravate their symptoms. However, the protective association between fruit (and overall fruit/vegetable) consumption and oesophageal adenocarcinoma remains after adjustment for gastro-oesophageal reflux symptoms. Fruit and vegetables are high in anti-oxidants, especially in vitamin C, dietary intake of which is reduced in oesophageal adenocarcinoma patients[38,67-70]. Tissue levels of vitamin C are also lower in areas of specialised intestinal metaplasia than in squamous mucosa suggesting that oxidative stress may be implicated in the neoplastic progression of Barrett's oesophagus[71]. Reflux of gastric contents into the oesophagus can enhance the production of free radicals which may cause damage to lipids, proteins and DNA through oxidative stress and may be implicated in the development of Barrett's oesophagus and/or oesophageal adenocarcinoma.

Smoking has been associated with an increase in gastro-oesophageal reflux symptoms in some studies[50,72,73], but not in others[20,58,74,75]. Unlike Smith *et al*[57] we observed no significant association between smoking and Barrett's oesophagus in the FINBAR study. There was a strong relationship between smoking and oesophageal adenocarcinoma with a slightly higher OR than observed in previous studies[13,15,16,18,19,35,76]. The under-representation of current smokers among FINBAR controls may tend to overestimate the association between smoking and oesophageal adenocarcinoma/Barrett's oesophagus but should not affect the difference in ORs seen between the two conditions. Our data suggest that smoking may influence the progression of Barrett's oesophagus to oesophageal adenocarcinoma and not the initiation of Barrett's oesophagus. One possible explanation may be that the higher rate of cell division and proliferation of columnar epithelial cells[77] and the malignant potential that such cells possess[78], may be promoted by carcinogenic (or DNA damaging) compounds from cigarette smoke. Olliver *et al*[79] showed that Barrett's mucosa has higher levels of DNA damage

than squamous epithelium and smoking is associated with increased DNA damage in Barrett's mucosa[80].

In conclusion, our data indicate that gastro-oesophageal reflux is a risk factor for oesophageal adenocarcinoma and demonstrate the high proportion of diagnosed Barrett's patients with gastro-oesophageal reflux symptoms. In the FINBAR study oesophageal adenocarcinoma differs from Barrett's oesophagus by being associated with high BMI and smoking. These factors could be implicated in the development of oesophageal adenocarcinoma from Barrett's oesophagus although further observational and interventional studies are required to confirm or refute our findings. It is hoped that these findings will help direct future research into the mechanisms underlying oesophageal adenocarcinoma and the development of prevention strategies.

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RAPID COMMUNICATION

Influence of *H pylori* on plasma ghrelin in patients without atrophic gastritis

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Abstract

AIM: To determine the association between *H pylori* infection and serum ghrelin levels in patients without atrophic gastritis.

METHODS: Fifty consecutive patients (24 males and 26 females) with either *H pylori*-positive gastritis (*n* = 34) or *H pylori*-negative gastritis (*n* = 16) with normal gastric acid secretion determined by 24-h pHmetry and without atrophic gastritis in histopathology were enrolled in this study. Thirty-four *H pylori*-infected patients were treated with triple therapy consisting of a daily regimen of 30 mg lansoprazole bid, 1 g amoxicillin bid and 500 mg clarithromycin bid for 14 d, followed by an additional 4 wk of 30 mg lansoprazol treatment. *H pylori* infection was eradicated in 23 of 34 (67.6%) patients. *H pylori*-positive patients were given eradication therapy. Gastric acidity was determined *via* intragastric pH catethers. Serum ghrelin was measured by radioimmunoassay (RIA).

RESULTS: There was no signifficant difference in plasma ghrelin levels between *H pylori*-positive and *H pylori*-negative groups (81.10 ± 162.66 ng/L *vs* 76.51 ± 122.94 ng/L). In addition, there was no significant difference in plasma ghrelin levels and gastric acidity levels measured before and 3 mo after the eradication therapy.

CONCLUSION: *H pylori* infection does not influence ghrelin secretion in patients with chronic gastritis without atrophic gastritis.

Key words: *H pylori*; Ghrelin; Gastric acidity; Gtrophic gastritis

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INTRODUCTION

H pylori is a Gram-negative bacteria that colonizes on gastric mucosa and is the important etiological agent for gastric ulcer and carcinomas[1]. However, it has been reported that long-term persistent *H pylori* infection leads to atrophic gastritis, which increases the risk of gastric adenocarcinomas[2]. The rate of peptic ulcer in *H pylori*-infected population is approximately 3% in USA and 25% in Japan[3]. The rate of *H pylori* infection is 50% in worldwide[4]. The *H pylori* prevalence varies according to the socioeconomic status. The prevalence of *H. pylori* is approximately 67.6%-81.3% in Turkey[5]. For developing clinical disease, host genetics, host immune response, and bacterial virulence factors appear to play critical roles[6].

Ghrelin is a recently discovered growth hormone-releasing peptide which is mostly produced by gastric X-like cells[7] and it has been shown to increase food intake and body mass gain, alters gastric motility and acid secretion[8]. Ghrelin is also released from the small intestine, kidney, hypothalamus, pancreas, placenta and pituitary gland[9]. Plasma ghrelin concentrations increase before meals and decrease postprandially. Ghrelin is the first circulating hormone shown to promote feeding and adiposity following systemic administration in animals[10] and it has been demonstrated that ghrelin potently stimulates appetite and food intake in man[8]. Animal studies have shown that administration of ghrelin into intracerebroventricular compartment or *via* peripheral vein decreases gastric acid secretion in rats[11,12].

Gastric acid secretion is affected by nervous system, several hormones like ghrelin and somatostatin as well as *H pylori* infection[13]. There are a few data in the literature about the relationship between *H pylori* and ghrelin. Masaoka *et al*[14] reported on a case of 31-year-old man with *H pylori*-positive gastritis. In this case, the plasma levels of total and active ghrelin showed no marked changes after successful eradication therapy on the long-term follow-up[14]. Several other studies demonstrated that in *H pylori*-positive individuals, the plasma ghrelin levels

were significantly lower compared with the patients without *H pylori* infection[15,16]. According to these reports, *H pylori* infection decreased plasma ghrelin concentrations and ghrelin levels were increased after eradication[17]. On the contrary, Gokcel *et al*[18] reported *H pylori* infection had no effect on plasma ghrelin concentration. In the aforementioned studies, patients with decreased plasma ghrelin levels and *H pylori* infection were suffering from atrophic gastritis, leading to gastric hypoacidity. The effect of *H pylori* infection on gastric acidity independent of atrophic gastritis is not thoroughly investigated. For this reason, we aimed to determine the effect of *H pylori* infection on plasma ghrelin levels in patients with normal gastric acidity and without atrophic gastritis.

MATERIALS AND METHODS

Patients

Fifty consecutive patients (24 males and 26 females) with normal 24-h pHmetry analysis were enrolled in the present study. All patients were performed upper gastrointestinal endoscopy and biopsy. In accordance with the Helsinki declaration,written informed consent was taken from all patients. The study was approved by Local Ethics Committee. The inclusion criteria were normal 24-h gastric pHmetry analysis and absence of gastric atrophy. Exclusion criteria were: age < 18 or > 70 year, abnormal gastric acidity, presence of atrophic gastritis in the histopathological evaluation, history of prior *H pylori* eradication therapy, acid suppressive therapy in the last 6 wk, prior gastrectomy, gastric cancer, diabetes mellitus, chronic renal disease, liver disease, drug addiction, alcohol abuse, pregnancy, and body mass index (BMI) > 30 kg/m^2. Fifty patients (24 males, 26 females) with either *H pylori*-positive gastritis (n = 34) or *H pylori*-negative gastritis (n = 16) were enrolled.

H pylori was detected by histological evaluation of gastric biopsy samples (two biopsies from antrum and corpus) and ^{13}C-urea breath test. Any positive result was accepted as presence of *H pylori* infection.

Thirty-four *H pylori*-infected patients were treated with triple therapy consisting of a daily regimen of 30 mg Lansoprazole bid, 1 g amoxicillin bid and 500 mg clarithromycin bid for 14 d, followed by an additional 4 wk of 30 mg lansoprazol treatment. *H pylori* infection was eradicated in 23 out of 34 (67.6%) patients. Body mass and height were measured while subjects wore light clothing without shoes. A complete medical history and physical examination were carried out to exclude endocrine disorder, gastrointestinal disease and operations or any other condition known to affect endocrine and gastric function.

H pylori eradication was re-evaluated six weeks after completion of the therapy by endoscopic biopsies (2 samples from antrum and corpus) and ^{13}C-urea breath test. Any positive test was regarded as therapy failure. Endoscopy was performed after overnight fasting and two specimens were taken from intact mucosa in the gastric antrum and corpus. The specimens were stained with hematoxylin and eosin and Giemsa to demonstrate *H pylori*. Gastritis was described according to the Sydney Classification[19].

Table 1 Demographic and clinical characteristics of gastritis patients

Clinical features	Male (n = 24)	Female (n = 26)
Age (yr)	40.9 ± 10.7	41.2 ± 14.5
BMI (kg/m^2)	28.7 ± 0.9	23.9 ± 3.4
H pylori, *n* (%)		
Positive	19 (77.8)	16 (61.5)
Negative	5 (22.2)	10 (28.5)
Topoghrapy of gastritis, *n* (%)		
Antrum	20 (83.3)	15 (86.7)
Corpus	4 (16.7)	11 (13.3)

Gastric acidity assays

Patients with and without *H pylori* infection were evaluated with 24-h ambulatory pH monitoring. Patients with *H pylori* infection had a control pHmetry 3 mo after the cure of *H pylori* infection, in order to rule out any late effects of *H pylori* eradication on gastric acidity. After overnight fast, in our motility laboratory pH electrode with two channels was inserted through the nose into the esophagus. The pH electrode was positioned 10 cm below the lower esophageal sphincter. The pH signal was recorded by Digitrapper MK Ⅲ (Medtronics Functional Diagnostic). Gastric acidity levels were assessed by monitoring gastric pH during 24 h and the time with pH below 4 was calculated to determine the individual's gastric acidity levels.

Ghrelin concentrations

On the day of endoscopy, blood samples were taken between 8:00 am and 10:00 am after an overnight fast, transferred into tubes containing EDTA and centrifuged, then plasma was separated and stored at -80℃ until assay. Blood samples were taken before and 3 mo after the cure of *H pylori* infection. Plasma ghrelin concentrations were measured by radioimmunoassay (RIA) at Gazi University Hormone Research Laboratory.

Statistical analysis

Data were expressed as means ± SE. Fisher-exact test, Cruscall Wallis, Mann-Whitney U test, Wilcoxon tests and correlation analysis were used to determine significant differences between the values in various groups of patients. $P < 0.05$ was considered statistically significant. Data were analyzed using SPSS for Windows (version 11.0; SPSS).

RESULTS

The study population included 26 females and 24 males with normal gastric acidity levels evaluated with 24-h ambulatory pH monitoring (Table 1). None of these patients had atrophic gastritis in the histological examination. Thirty-five of the patients had antrum predominant and 16 of the patients had corpus predominant gastritis. There were no significant differences in the baseline characteristics of the patients. Mean fasting serum ghrelin level was higher in the patients with higher

Table 2 Plasma ghrelin levels in gastritis patients

Group	*n*	Plasma ghrelin levels (pg/mL)
Gender	Male: 24	62.3 ± 48.6
	Female: 26	84.3 ± 164.7
BMI (kg/m^2)	-25 (24)	60.4 ± 111.2[a]
	+25 (26)	99.0 ± 176.3[a]
Age (yr)	-35 (13)	89.6 ± 133.8
	36-49 (21)	59.2 ± 46.7
	50+ (16)	83.3 ± 194.8

[a]$P < 0.05$.

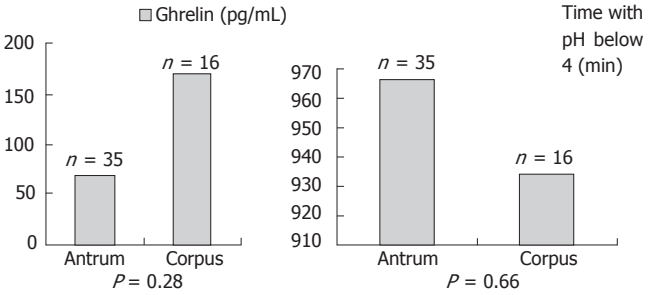

Figure 1 Acid secretion and plasma ghrelin levels according to the topography of gastritis.

BMI (> 25 kg/m^2) than the patients with BMI < 25 kg/m^2 (99.0 ± 176.3 ng/L *vs* 60.4 ± 111.2 ng/L, $P = 0.04$; Table 2). Age and gender had no effect on ghrelin levels. There were no significant differences in mean plasma ghrelin levels between *H pylori*-positive and *H pylori*-negative groups (81.10 ± 162.66 ng/L *vs* 76.51 ± 122.94 ng/L). Plasma ghrelin levels before and after eradication of *H pylori* infection were also similar in *H pylori*-positive patients (78.34 ± 101.45 ng/L *vs* 88.67 ± 111.21 ng/L). When compared between antrum predominant and corpus predominant group, plasma ghrelin levels did not differ significantly (Figure 1).

H pylori infection was cured in 23 patients who received eradication therapy. There was no significant difference in plasma ghrelin levels and gastric acidity measured before and after treatment.

DISCUSSION

Ghrelin is synthesized from gastric mucosa[1] and also released from the small and large intestine, hypothalamus, and pancreas[16]. Plasma ghrelin levels change in a narrow spectrum because of the secretion from other tissues. It has been shown in controlled studies that ghrelin increases food intake and eating/appetite and leads to increased body mass[20]. In the present study, we found that mean fasting serum ghrelin level was higher in the patients with BMI > 25 kg/m^2 than the patients with BMI < 25 kg/m^2.

To date, there are few studies discussing the association between ghrelin level, which is produced from the gastric oxyntic cells, and *H pylori* infection. In addition, available data in the literature have contradiction regarding the association between ghrelin level and *H pylori* infection. Nwokolo *et al*[21] reported that after *H pylori* eradication therapy, plasma ghrelin levels significantly increased, hypothesizing that this elevation in plasma ghrelin levels led to obesity by raising appetite. Ghrelin production was assumed to be reduced as a result of *H pylori* infection. *H pylori* infection led to chronic gastritis and atrophy, thereby reducing number of oxynthic cells, the main cells producing ghrelin. In a case of 31-year-old man with *H pylori*-positive gastritis, the plasma levels of total and active ghrelin showed no marked changes after successful eradication therapy on the long-term follow-up[14]. Suzuki *et al*[22] investigated gastric and plasma ghrelin dynamics in *H pylori*-infected Mongolian gerbils. Although expressions of preproghrelin mRNA in the gastric mucosa and total ghrelin were reduced after 17 and 23 wk of *H pylori* infection, a compensatory increase in ghrelin secretion was noted at wk 17. Isomoto *et al*[15] reported variable plasma ghrelin levels in 257 patients with various upper gastrointestinal diseases. In this study, the patients with chronic gastritis had lowest level of ghrelin than the patients with acute gastritis, duodenal or gastric ulcer, gastric cancer, benign gastric polyp, reflux esophagitis and normal gastric mucosa. Pepsinogen Ⅰ/Ⅱ ratio was found to be significantly correlated with plasma ghrelin levels, especially in *H pylori*-positive patients. These findings indicate the significant effect of gastric atrophy on impaired ghrelin secretion in *H pylori*-infected patients. The improvement of gastric acidity and ghrelin levels may be parallel to the improvement of atrophic gastritis in previous reports. Gokcel *et al*[18] failed to demonstrate any association between *H pylori* and plasma ghrelin levels. However, in this study, the authors did not describe the degree of gastric atrophy in the study group. For this reason, we are not able to comment about gastric atrophy-induced impaired secretion of ghrelin in this study. *H pylori* species leading to atrophic gastritis, such as cag A, is less prevalent in Turkey[23]. For this reason, it is probable that geographical differences might influence the variable ghrelin levels after *H pylori* eradication.

In our study, we enrolled patients without atrophic gastritis and with normal gastric acidity. In this special subgroup of patients, *H pylori* infection and its eradication had no impact on the serum levels of ghrelin. A study, which aimed to determine plasma ghrelin level variations according to topographical distribution of gastritis, showed that in the antrum dominant gastritis, ghrelin levels were lower than the corpus predominant gastritis[15]. In contrast, we did not observe any significant difference between the antrum dominant and corpus dominant gastritis for plasma ghrelin levels.

Although other studies preferred at least 6 mo of duration to accurately assess the relevant gastric acid recovery, previous studies have shown that gastric acid secretion changes rapidly, even after one month, in patients with duodenal ulcer[24,25]. For this reason, we checked the gastric acidity levels 3 mo after *H pylori* eradication.

In conclusion, *H pylori* infection has no effect on plasma ghrelin concentration in Turkish population without atrophic gastritis.

COMMENTS

Background

Many studies demonstrated that in *H pylori*-positive individuals, the plasma ghrelin levels were significantly lower than that in patients without *H pylori* infection. *H pylori* infection decreased plasma ghrelin concentrations and ghrelin levels were increased after *H pylori* eradication. However, none of the studies evaluated the ghrelin-*H pylori* relationship in the patients without atrophic gastritis.

Research frontiers

Previous studies on ghrelin and *H pylori* infection were performed on heterogenous group of patients. Ghrelin levels in various gastric diseases, including duodenal ulcer, gastric ulcer, reflux disease or bile acid reflux should be investigated as a seperate entity.

Innovations and breakthroughs

In this study, it has been shown that patients without atrophic gastritis and with normal gastric acidity had similar serum ghrelin levels irrespective of their *H pylori* status.

Applications

Atrophic gastritis and gastric hypoacidity are main factors affecting serum ghrelin level changes associated with *H pylori* infection. For this reason, early detection and eradication of *H pylori* is crucial for the prevention of irreversible changes of gastric mucosa.

Terminology

To concisely and accurately describe, define or explain the specific, unique terms that are not familiar to majority of the readers, but are essential for the readers to understand the article.

Peer review

The article provides relevant data about the role of *H pylori* infection in ghrelin metabolism, a growth hormone-releasing peptide mostly produced by gastric X-like cells and shown to increase food intake and weight gain among other minor gastrointestinal effects.

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RAPID COMMUNICATION

Prevalence of coeliac disease in patients with autoimmune thyroiditis in a Turkish population

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Abstract

AIM: To investigate the prevalence of coeliac disease in a series of Turkish patients with autoimmune thyroiditis.

METHODS: Sera from 136 consecutive patients with newly diagnosed autoimmune thyroiditis and 119 healthy blood donors were tested for IgA tissue transglutaminase antibody with enzyme-linked immunosorbent assay. Endoscopic mucosal biopsy from the second part of duodenum was performed in patients with positive antibody test.

RESULTS: Eight patients (5.9%) and one control subject (0.8%) were positive for IgA tissue transglutaminase antibody (OR: 7.38, 95% CI: 0.91-59.85, P = 0.04). Six patients and one control agreed to take biopsies. Histopathological examination revealed changes classified as Marsh Ⅲa in one, Marsh Ⅱ in one, Marsh Ⅰ in two, and Marsh 0 in two patients with autoimmune throiditis, and Marsh Ⅰ in one blood donor.

CONCLUSION: Turkish patients with autoimmune thyroiditis have an increased risk of coeliac disease and serological screening may be useful for early detection of coeliac disease in these patients. Our findings need to be confirmed in a larger series of patients.

Key words: Coeliac disease; Autoimmune thyroiditis; Tissue transglutaminase

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INTRODUCTION

Coeliac disease (CD) is characterized by malabsorbsion of nutrients, chronic inflammation and damage of the small intestinal mucosa caused by the ingestion of gliadin fraction of wheat gluten and similar alcohol-soluble proteins of barley and rye in genetically susceptible subjects[1]. While the elimination of gluten from the patient's diet results in clinical and complete histological recovery, reintroduction of gluten leads to relapse[1,2].

The clinical presentation of CD is extremely heterogeneous. Typical symptoms include chronic diarrhea and abdominal distension[2,3]. However, only few patients with CD show clinical malabsorption, while most patients have subtle symptoms, if any[1]. Therefore, the disease is clearly underdiagnosed[3]. Diagnostic tests of anti-endomysial antibodies (EMA) and the anti-tissue transglutaminase (tTG) for CD with a sensitivity and specificity of over 95% were recently introduced[3-5]. The prevalence of CD has changed over the past 30-40 years with the availability of new sensitive serologic tests which have increased the possibility of the diagnosis of subclinical cases[1]. Screening studies show a high prevalence of CD (between 1/80-1/300) among both healthy children and adults in European countries[6,7].

It is known that CD is associated with some autoimmune disorders, especially type- I diabetes mellitus, autoimmune thyroid diseases, collagen disorders, Addison's disease, pernicious anaemia, alopecia and autoimmune hepatitis[8-10]. Among adult patients with autoimmune thyroiditis, the prevalence of CD has been reported to be 3.3-4.8 times more than in the general population[11-14]. In contrast, a study reported a CD prevalence of 0% in patients with autoimmune thyroiditis[15].

To our knowledge, there is no study in Turkey about prevalence of CD in patients with autoimmune thyroiditis. The aim of this study was to define the prevalence of CD in a series of Turkish patients with autoimmune thyroiditis.

MATERIALS AND METHODS

Between January and September 2006, 136 consecutive patients aged between 17 and 65 years (mean age 43.1 ± 10.5), including 118 female (86.8%) and 18 males (13.2%) with newly diagnosed autoimmune thyroiditis (AT) and 119 healthy blood donors aged between 18-64 years (mean age 41.5 ± 10.1), including 109 female (91.6%) and 10 males (8.4%) as control group, were included into the study. The diagnosis of AT was made based on clinical and biochemical findings, including positive titres of anti-thyroid peroxidase antibody and/or anti-thyroglobulin antibodies, and a positive ultrasound scan defined by either diffuse marked hypoechogenicity or a focal non-homogeneous pattern with hypoechogeneous areas. None of the patients had other autoimmune diseases such as Addison's disease, vitiligo, and diabetes mellitus.

Antibody test

Sera from patients with AT and controls were stored at -70℃. IgA anti-tTG assays were carried out by enzyme-linked immunosorbent assay (ELISA), (Aida Gmbh, Germany). Cut-off values were 15 IU/mL set by the manufacturer. In all subjects who were positive for IgA anti-tTG serum iron, ferritin, folate and vitamin B12 levels were assayed, and endoscopic mucosal biopsy was performed from the second part of duodenum in patients who agreed to the procedure. Samples of duodenal mucosa were graded according to the modified Marsh classification[16,17].

The study protocol was approved by the ethics committee of the Kirikkale University Hospital.

Statistical analysis

Fisher's exact test was used to compare CD prevalence and gender differences between the two groups. The comparison of mean ages between the two groups was done with Student's *t* test. The statistical analyses were performed using a statistical program for PC (SPSS 11.0 for Windows, SPSS Inc., IL, USA). *P* values of less than 0.05 were considered statistically significant.

RESULTS

The age and gender were similar in both groups ($P = 0.21$ and $P = 0.32$, respectively). Clinical, serologic and histological features of the patients with AT are shown in Table 1.

IgA anti-tTG antibody was found to be positive in 8 (7 female) patients (5.9%) with AT (Table 1) and in one female blood donor (0.8%) (OR: 7.38, 95% CI: 0.91-59.85, $P = 0.04$).

Six patients (5 female and 1 male) and one control subject agreed to endoscopy and duodenal biopsy. Four patients (3 female and 1 male) and one control subject had histological findings of CD, and two patients with AT had normal duodenal histology. Histopathological examination revealed changes classified as Marsh IIIa in one, Marsh II in one, Marsh I in two patients with AT, and Marsh I in one blood donor.

Among the 8 patients who were positive in antibody tests, one patient (case 7), a 61 years old female with histological CD as Marsh IIIa, had iron-deficiency anemia and osteopenia, and one patient (case 8), a 40 years old female with histological CD as Marsh II, had iron-deficiency, but no anemia (Table 1). The other 6 patients with AT and one control subject who had positive antibody test did not have any symptoms, signs or laboratory findings of malabsorbtion. All subjects with histologically proven CD were prescribed gluten-free diet.

Table 1 Clinical, antibody and histological features of 8 autoimmune throiditis patients with positive IgA anti-tTG test

Patient	Gender	Age (yr)	Anti-tTG IgA (> 15 IU/mL)	Duodenal biopsy	BMI (kg/m^2)	Malabsorption
1	F	31	79.6	Not performed	26.0	Absent
2	F	56	55.1	0	29.9	Absent
3	F	18	57.8	0	22.3	Absent
4	F	34	26.2	I	23.7	Absent
5	F	44	17.9	Not performed	35.3	Absent
6	M	35	43.4	I	27.6	Absent
7	F	61	39.1	IIIa	24.0	Present
8	F	40	27.9	II	30.1	Present

BMI: body mass index. Modified Marsh classification: IIIa = mild atrophy, II = intraepithelial lymphocytosis + crypt hyperplasia, I = intraepithelial lymphocytosis (> 40/100 epithelial cells).

DISCUSSION

The association between coeliac disease and autoimmune thyroiditis has been previously reported. An increased prevalence of coeliac disease has been found in patients with AT and Graves' disease[11-14]. Moreover, it has been demonstrated that many coeliacs are prone to autoimmune thyroid dysfunction[18,19]. This association could be related to a common genetic background (HLA-DQ2 and HLA-DQ8).

Serologic tests developed in the past decade provide a non-invasive tool to screen both individuals at risk for the disease and general population. IgA anti-tTG assays by ELISA are highly sensitive (90%-98%) and specific (94-97) for diagnosis of coeliac disease. It is now widely available, less costly, and easier to perform than the immunofluorescence assay used to detect IgA EMA[2].

Screening studies show a high prevalence (between 1/80-1/300) of CD among both healthy children and adults in European countries[6,7]. The prevalence of coeliac disease in 2000 healthy blood donors has recently been found to be 1.3% (1/77) in Turkey[20]. This study shows that the prevalence of CD in the Turkish population is relatively high in comparison to Western world.

In our study, the prevalence of positive IgA anti-tTG test in patients with AT was significantly higher than the controls (5.9% *vs* 0.8%). Additionally, in our study, the prevalence rate of CD in patients with AT was higher than in the previous studies (mean value 3.7%)[11-14]. This might be associated with the higher prevalence rate of CD in Turkish population.

In the literature, there are some studies with different results. Ravaglia *et al*[21] reported that only the patients

aged 65 and older with AT had an increased risk of CD. In our study, the risk increased in patients under 66 years of age. In contrary to our findings, in a recent study from Italy, CD prevalence was reported as 0% in patients with autoimmune thyroiditis[15]. Racial and regional differences may explain these opposing findings.

It is of great importance to identify early CD in patients with AT, since a strict adherence to a gluten-free diet not only helps prevent the severe complications of untreated gluten-sensitive enteropathy such as ulcerative jejunoileitis, intestinal lymphoma and neoplasm[22], but also helps improve the associated autoimmune disease[12].

This is the first study conducted about association of CD with AT in a Turkish population. The relatively small sample size may be a limitation for our study. Studies with larger populations would bring more accurate results.

In conclusion, this study suggests that Turkish patients with AT have an increased risk of CD. Serological screening may be useful for early detection of CD in these patients. However, our findings need to be confirmed in a larger series of patients.

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RAPID COMMUNICATION

Screening and cloning for proteins transactivated by the PS1TP5 protein of hepatitis B virus: A suppression subtractive hybridization study

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Abstract

AIM: To clone and identify human genes transactivated by PS1TP5 by constructing a cDNA subtractive library with suppression subtractive hybridization (SSH) technique.

METHODS: SSH and bioinformatics techniques were used for screening and cloning of the target genes transactivated by PS1TP5 protein. The mRNA was isolated from HepG2 cells transfected with pcDNA3.1(-)-myc-his(A)-PS1TP5 and pcDNA3.1(-)-myc-his(A) empty vector, respectively, and SSH technique was employed to analyze the differentially expressed DNA sequence between the two groups. After digestion with restriction enzyme *Rsa* I, small size cDNAs were obtained. Then tester cDNA was divided into two groups and ligated to the specific adaptor 1 and adaptor 2, respectively. The tester cDNA was hybridized with driver cDNA twice and subjected to nested PCR for two times, and then subcloned into T/A plasmid vectors to set up the subtractive library. Amplification of the library was carried out with *E. coli* strain DH5α. The cDNA was sequenced and analyzed in GenBank with Vector NTI 9.1 and NCBI BLAST software after PCR amplification.

RESULTS: The subtractive library of genes transactivated by PS1TP5 was constructed successfully. The amplified library contained 90 positive clones. Colony PCR showed that 70 clones contained 200-1000-bp inserts. Sequence analysis was performed in 30 clones randomly, and the full-length sequences were obtained by bioinformatics technique. Altogether 24 coding sequences were obtained, which consisted of 23 known and 1 unknown. One novel gene with unknown functions was found and named as PS1TP5TP1 after being electronically spliced, and deposited in GenBank (accession number: DQ487761).

CONCLUSION: PS1TP5 is closely correlated with immunoregulation, carbohydrate metabolism, signal transduction, formation mechanism of hepatic fibrosis, and occurrence and development of tumor. Understanding PS1TP5 transactive proteins may help to bring some new clues for further studying the biological functions of pre-S1 protein.

Key words: Hepatitis B virus; Pre-S1 protein; Suppression subtractive hybridization

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INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic infections of the liver. Acute infections may result in serious disease with approximately 0.5% of cases developing into fatal, fulminant hepatitis. Chronic infections may also have remarkable consequences. In addition to causing acute and chronic hepatitis, HBV is considered a major etiological factor in the development of human hepatocellular carcinoma (HCC), one of the most frequent fatal malignancies worldwide and worldwide deaths of HCC exceed one million per year. Epidemiological studies have demonstrated an approximately 10-fold increase in the relative risk of HCC among HBV carriers compared to non-carriers[1-3].

The precise role of HBV in the etiology of HCC is not well understood. Only occasionally are genes controlling cell growth and differentiation disturbed by integration of HBV DNA sequences. An alternative mechanism of chronic infection and hepatocarcinogenesis may be the

key steps to mutual interaction between viral proteins and hepatocellular proteins. This action may mediate the virus to enter into the liver cells and affect the activities and function of these proteins. Moreover, proteins from hepatocytes infected with HBV inversely disturb virus replication and reduce immunity of the host, resulting in chronic liver diseases and HCC. Understanding of the interaction among these proteins may help to bring some new clues for discovering the pathogenesis of viral hepatitis and related HCC.

The first full-length nucleotide sequence of HBV was published in 1979[4]. The four open reading frames (ORF) defined in the HBV genome at that time were named as the regions of S, C, P and X. The region of S was divided into the sub-regions of pre-S1, pre-S2 and S according to different initial code ATG in frame. Dong *et al*[5] have shown that there is an ORF before the pre-S1 region in the genome of HBV and they amplified it from the serum of patients infected with HBV by long and accurate polymerase chain reaction (LAPCR). This region is of 135 bp and tentatively named the pre-pre-S and its promoter activity has been confirmed in a 277-bp upstream nucleotide sequence before the pre-S1 gene[6]. Thus, the complete S region of HBV includes pre-pre-S, pre-S1, pre-S2 and S subregions.

The function of the pre-S1 protein in the life cycle of HBV remains unknown. Its transactive function has been demonstrated by some recent studies[7,8]. Human gene 5 transactivated by pre-S1 protein of HBV (PS1TP5) is a novel target gene that has been screened and cloned with a suppression subtractive hybridization (SSH) technique in our laboratory (GenBank accession number: AY427953)[9]. The full length of the coding rank of PS1TP5 contains 438 nucleotides, and the protein product consists of 145 amino acid residues. To further investigate the biological significance of the PS1TP5 and pre-S1 protein, we screened and identified the proteins transactivated by PS1TP5 protein using the SSH technique.

MATERIALS AND METHODS

Materials

HepG2 cell line of hepatoblastoma and *E. coli* strains DH5α were conserved in our laboratory. Eukaryotic expression vector pcDNA3.1(-)-myc-his(A), Quickprep mico mRNA Purification kit, PCR-Select cDNA Subtraction kit, 50 × PCR Enzyme Mix kit and Advantage PCR Cloning kit were purchased from Clontech Co., America. FuGENE6 transfection kit was purchased from Roche Co., America. pGEM-T vector, pGEM-T-easy vector and High Pure PCR Product Purification kit were from Promega Co., America. *Eco*RI, *BgI*Ⅱ and DNA Marker were from Takara Company, Japan. DNA sequencing was performed by Invitrogen Company, China.

Plasmid construction

The reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify the gene of PS1TP5 from the mRNA of HepG2 cells. The sequences of the primers containing the *Eco*RI and *BgI*Ⅱ restriction enzyme sites were: 5'-CGG AAT TCA TGG GCT TGA AGA GCC AC-3' (sense) and 5'-CGA GAT CTA GTG AAG ATA TGC AGA GG-3' (anti-sense). Samples were amplified through 35 cycles, each amplification cycle consisting of denaturation at 94℃ for 45 s, primer annealing at 60℃ for 45 s, and extension at 72℃ for 1 min. Ten nanograms of the 438-bp PCR product was cloned into pGEM-T vector. The primary structure of insert was confirmed by direct sequencing. The fragment of encoding PS1TP5 was released from the pEGM-T-PS1TP5 by digestion with *Eco*RI and *BgI*Ⅱ, and ligated to the *Eco*RI/*BgI*Ⅱ sites of pcDNA3.1(-)-myc-his(A) empty vector. Recombinant eukaryotic expression vector pcDNA3.1(-)-myc-his(A)-PS1TP5 was obtained, then identified by PCR and digested with *Eco*RI/*BgI*Ⅱ.

Cell culture and DNA transfection

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 kU/L penicillin, 100 mg/L streptomycin, and 100 mL/L heat-inactivated fetal bovine serum (FBS), and incubated at 37℃ in a humidified atmosphere consisting of 50 mL/L CO_2 in air. About 1.5×10^6 cells were seeded in 35-mm plates 12 h prior to transfection, which reached 50% confluence at the time of transfection. Cells were transfected with FuGENE6 transfection reagent using 2.0 μg of pcDNA3.1(-)-myc-his(A) empty (driver) and pcDNA3.1(-)-myc-his(A)-PS1TP5 plasmid DNA (tester) according to the manufacturer's protocol.

mRNA and cDNA isolation

The mRNA from HepG2 cells transfected with pcDNA3.1(-)-myc-his(A)-PS1TP5 (tester) and pcDNA3.1(-)-myc-his(A) empty vector (driver) was isolated using a micro mRNA Purification kit, respectively, and cDNAs were reverse-transcribed from total RNA. Quantitative analysis of mRNA was carried out with an ultraviolet spectrophotometer. Identification was done by PCR with PS1TP5 sequence-specific primers.

Suppression subtractive hybridization

Genome comparison was performed by suppression subtraction hybridization (SSH) technique according to the manufacturer's instructions of PCR-select cDNA subtraction kit. Briefly, 2 μg of mRNA from the tester and the driver was subjected to cDNA synthesis. Tester and driver cDNAs were digested with *Rsa*I. The tester cDNA was split into two groups, and each was ligated with a different cDNA adapter. In the first hybridization reaction, an excess of driver was added to each sample of the tester. The samples were heat-denaturated and allowed to anneal. Because of the second-order kinetics of hybridization, the concentration of high- and low-abundance sequences is equalized among the single-stranded tester molecules. At the same time, single-stranded tester molecules were significantly enriched for differentially expressed sequences. During the second hybridization, the two primary hybridization samples were mixed together without denaturation. Only the remaining equalized and subtracted single-stranded tester cDNAs can re-associate

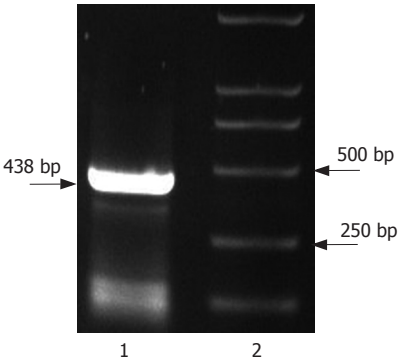

Figure 1 PS1TP5 RT-PCR product electrophoresis on 10 g/L agarose gel. Lane 1: PS1TP5 PCR products; lane 2: DNA marker 2000.

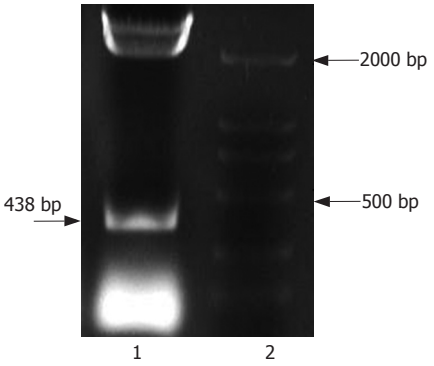

Figure 2 Digestion and identification of the recombinant plasmid pGEM-T-PS1TP5 by *Eco*RI/*BglⅡ*. Lane 1: Restriction analysis of pGEM-T-PS1TP5; lane 2: DNA marker 2000.

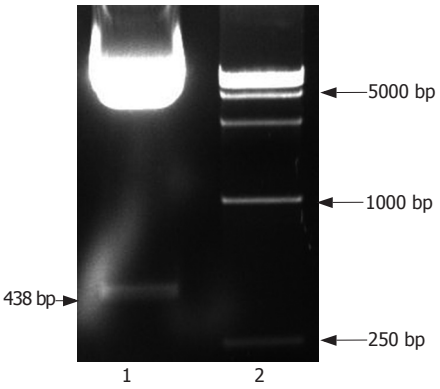

Figure 3 Restriction analysis of the recombinant plasmid pcDNA-3.1(-)-myc-his(A)-PS1TP5 by *Eco*RI/*BglⅡ*. Lane 1: Restriction analysis of pcDNA-3.1(-)-myc-his(A)-PS1TP5; lane 2: DNA marker 15 000.

forming double-stranded tester molecules with different ends. After filling in the ends with DNA polymerase, the entire population of molecules was subjected to nested PCR with two adapter-specific primer pairs. Then secondary PCR products were used as templates for PCR amplification of G3PDH (a housekeeping gene) at 18, 23, 28, 33 cycles to assure subtracted efficiency.

Cloning of subtracted cDNA libraries

Products of these amplified overhangs containing a subtracted cDNA library (6 μL) were ligated into a pGEM-T-easy vector. Subsequently, the plasmid was transformed into *E. coli* strain DH5α. Bacteria were cultured in 800 μL of LB medium and allowed to incubate for 45 min at 37℃ and 225 r/min. After incubation, bacteria were plated onto agar plates containing ampicillin (100 mg/L), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 20 μg/cm^2) and isopropyl-β-D-thiogalactoside (IPTG, 12.1 μg/cm^2) and incubated overnight at 37℃. White colonies were selected and identified by PCR. Primers were T7/SP6 primer of pGEM-T-easy vector. After sequencing the plasmids DNA of positive colonies, nucleic acid homology searches were performed using Vector NTI 9.1 and NCBI BLAST software.

RESULTS

Amplification of PS1TP5 and identification of the recombinant eukaryotic expression vector pcDNA3.1(-)-myc-his(A)-PS1TP5

The full-length sequences of PS1TP5 were generated by RT-PCR amplification of the mRNA of HepG2 cells, subcloned into pGEM-T vector, analyzed restrictively with *Eco*RI/*BglⅡ* and sequenced by comparing to vector NTI 9.1 and conducting a BLAST database homology search. Analysis of the PS1TP5 PCR reaction products (Figure 1) and recombinant plasmid pGEM-T-PS1TP5 (Figure 2) by agarose gel electrophoresis showed the clear bands with the expected size of 438 bp. Sequences of the PCR products were corrected using bioinformatic analysis. After being cut from pGEM-T-PS1TP5 by *Eco*RI/*BglⅡ*, the fragment was ligated in-frame into a pcDNA3.1(-)-myc-his(A) *Eco*RI/*BglⅡ* site. Restriction enzyme analysis of the pcDNA3.1(-)-myc-his(A)-PS1TP5 plasmid with *Eco*RI/*BglⅡ* yielded two bands: approximately 5500-bp empty pcDNA3.1(-)-myc-his(A); and a 438-bp PS1TP5 (Figure 3).

Table 1 Quantitative analysis of mRNA

Transfected plasmid	A_{230}	A_{260}	A_{280}	A_{260}/A_{280}	mRNA quantitation (mg/L)
pcDNA3.1(-)-myc-his(A)-PS1TP5	0.173A	0.377A	0.172A	2.192	15.08
pcDNA3.1(-)-myc-his(A)	0.191A	0.378A	0.173A	2.185	14.84

Quantitative analysis of mRNA

The mRNA isolated from HepG2 cells transfected with pcDNA3.1(-)-myc-his(A)-PS1TP5 (tester) and pcDNA3.1(-)-myc-his(A) empty vector (driver) was detected using an ultraviolet spectrophotometer (Table 1). Quantitative results of mRNA suggested that isolated mRNA could be used for suppression subtractive hybridization.

Result of PCR analysis of subtraction efficiency

The G3PDH (a housekeeping gene) primers were used to confirm the reduced relative abundance of G3PDH following the PCR selection procedure. The result displayed that the G3PDH abundance of the subtracted secondary PCR products significantly decreased compared to the unsubtracted one, indicating that the subtractive library had a high subtraction efficiency (Figure 4).

Analysis of the subtraction library

Using SSH technique, we obtained a total of 90 positive clones. These clones were prescreened by PCR

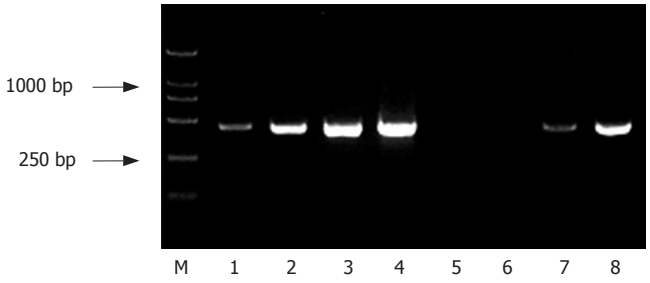

Figure 4 PCR analysis of substraction efficiency. M: DNA marker 2000; lanes 1-4: PCR of 18, 23, 28, 33 cycles in unsubstracted group, respectively; lanes 5-8: PCR of 18, 23, 28, 33 cycles in substracted group, respectively.

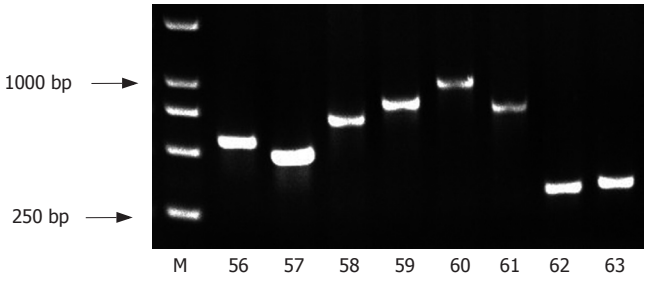

Figure 5 PCR amplification of part of clones (56-63) in substracted cDNA library. M: DNA marker 2000.

Table 2 Homolog searching of sequenced cDNA fragments from SSH library

High similarity proteins to known genes	*n*	Homology (%)
Homo sapiens ribosomal protein S16 (RPS16)	2	98
Homo sapiens ribosomal protein LP0 (RPLP0)	1	98
Homo sapiens ribosomal protein LP2 (RPLP2)	1	100
Homo sapiens ribosomal protein S7 (RPS7)	1	100
Homo sapiens ribosomal protein L31 (RPL31)	1	100
Homo sapiens transmembrane 4 superfamily member 4-CD81 (TM4SF4)	1	99
Homo sapiens inositol monophosphatase 2 (IMPA2)	2	99
Homo sapiens protein kinase BRPK	1	100
Homo sapiens clusterin (CLU)	1	100
Homo sapiens adenosine deaminase	2	100
Homo sapiens solute carrier family 7 member 5 (SLC7A5)	1	99
Homo sapiens cytochrome c oxidase subunit 8A	2	100
Homo sapiens enolase 1	1	99
Homo sapiens H3 histone family 3B	1	100
Homo sapiens replication factor C	2	98
Human cytoskeletal gamma-actin gene	1	100
Homo sapiens S100 calcium-binding protein A6 (calcyclin)	1	99
Homo sapiens eukaryotic translation elongation factor 1	1	100
Homo sapiens epoxide hydrolase 1	1	98
Homo sapiens dehydrogenase 1	1	99
Homo sapiens signal sequence receptor-β	2	100
Homo sapiens β5-microtubulin	1	99
Homo sapiens pyruvate dehydrogenase	1	98
New genes with unknown function	1	100

amplification to ensure that only clones with different inserts were subjected to sequencing (Figure 5). Among these clones, 70 clones contained 200-1000-bp inserts. A total of 30 clones from the cDNA library were randomly chosen and sequenced. Using the BLAST program at the National Center for Biotechnology Information, 23 sequences from 29 true positive colonies had a high similarity to known genes and one sequence failed to match with a homologous gene in GenBank (Table 2).

Splicing and reporting of the new gene

According to Kozak regulation of start codon and signal sequences of downstream conservative poly adenine, one sequence with unknown function was electronically analyzed, spliced, and concluded with Vector NTI 9.1 and

```
  1 ATG CCG AGG GTC GGG GAA GGA GGA GTC ACA GGC AAG ACA GGG ACT
    M   P   R   V   G   E   G   G   V   T   G   K   T   G   T
 46 CAG CGC AGT GCT CCC GCC ATC ATC GGG GAC AGG TGC CAG GGT CAG
    Q   R   S   A   P   A   I   I   G   D   R   C   Q   G   Q
 91 CTG CAG CTC TCC TTC CGG GAG CAG GGT GTG GTG GCC CTG GAT GGT
    L   Q   L   S   F   R   E   Q   G   V   V   A   L   D   G
136 CCC AGG GAG CAG AGG GAG GCA GGC AGC TGT CAC AGC CAC AGC GGC
    P   R   E   Q   R   E   A   G   S   C   H   S   H   S   G
181 TCT GAA TGG CTT GAG CTT TTA GTA TGT GTT CAA GTG CAG GAT ACT
    S   E   W   L   E   L   L   V   C   V   Q   V   Q   D   T
226 ACA AAC TGG TAA
    T   N   W   *
```

Figure 6 The nucleotide sequence of PS1TP5TP1 gene and relevant amino acid sequences (GenBank accession number: DQ487761).

NCBI BLAST software. A new gene was obtained and named PS1TP5TP1. The full length of the coding rank of PS1TP5TP1 contained 237 nucleotides, and the coding product consisted of 78 amino acid residues (GenBank accession number: DQ487761, Figure 6).

DISCUSSION

The open reading frame (ORF) of the HBV complete S gene consists of four coding regions: pre-pre-S, pre-S1, pre-S2 and S, each starting with an ATG codon in frame. Through in-frame translational initiation at each of the four ATG codons, complete S (pre-pre-S + pre-S1 + pre-S2 + S), large (LHBs; pre-S1 + pre-S2 + S), middle (MHBs; pre-S2 + S) and small (SHBs; S) envelope glycoproteins can be synthesized[10,11]. Interaction between viral and hepatocellular proteins plays an important role in the pathogenesis of the virus and may mediate virus to enter into hepatocytes. Their network interaction can change normal biological function of proteins, influence self-replication of the virus, and result in disease.

Suppression subtractive hybridization (SSH) is a new and highly effective gene analysis method designed by Diatchenko *et al*[12,13], and has been developed for the generation of subtracted cDNA libraries. It is based primarily on a technique called suppression PCR, and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver populations. As a result only one round of subtractive hybridization is needed and the

subtracted library is normalized in terms of abundance of different cDNAs. It dramatically increases the probability of obtaining low-abundance differentially expressed cDNA and simplifies analysis of the subtracted library. The SSH technique is applicable to many molecular genetic and positional cloning studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes.

In this study, we cotransfected HepG2 cells with pcDNA3.1(-)-myc-his(A)-PS1TP5 (tester) and pcDNA3.1(-)-myc-his(A) empty vector (driver). The mRNA was isolated from transfected HepG2 cells, and total RNA was reverse-transcribed into cDNAs. The subtractive library of genes transactivated by PS1TP5 was set up successfully, and 30 clones from the cDNA library were selected randomly and sequenced, and the full-length sequences were obtained with bioinformatics method. By sequence analysis using Vector NTI 9.1 and NCBI BLAST software, we obtained the sequences of the 23 genes with known functions and one gene with unknown functions. After electronic splicing, this new gene with unknown function was named PS1TP5TP1 and deposited in GenBank (accession number: DQ487761).

The 23 genes with known functions are closely correlated with immunoregulation, carbohydrate metabolism, signal transduction, formation of hepatic fibrosis, and initiation and development of tumor. Homo sapiens protein kinase BRPK, a novel protein kinase, is a kind of phosphorylation. BRPK has a serine/threonine-type protein kinase domain, and the recombinant proteins of BRPK are capable of autophosphorylation. Experiments performed by Nagajima *et al*[14] revealed that BRPK was expressed at a higher level in three carcinoma cell lines with higher metastatic potential, thereby indicating a possible link of BRPK to initiation and development of tumor. Clusterin, also known as apoprotein J, is a kind of glycoprotein heterodimers residing in most mammalian tissue and body fluid. Several studies reported that clusterin was exorbitantly expressed in many kinds of malignant tumors, including breast[15], kidney[16], bladder[17], pancreas[18], colon[19], lymph[20], liver[21], and so on. The formation and expansion of clusterin-nuclear matrix may be related to the activated cell growth. Therefore, it may play an important role in alarm reaction, apoptosis and tumorigenesis. Adenosine deaminase is a nucleic acid metabolic enzyme that is connected with immunological activity of somatocyte, and exists extensively in various kinds of tissues. Some reports suggested that adenosine deaminase deficiency was associated with acquired immunodeficiency syndrome (AIDS) and hepatitis B infection[22,23]. Homo sapiens S100 calcium-binding protein A6 (calcyclin), a cell cycle-regulated protein, is a member of the S100A family of calcium-binding proteins. The calcyclin gene has been localized to the long arm of human chromosome 1. While the precise function of calcyclin was unknown, some experimental observations suggested that the functional role of calcyclin was associated with cell proliferation, signal transduction, pulmonary fibrosis and several types of cancer phenotypes through the cell cycle[24-26]. Homo sapiens replication factor C (RFC) is located in eukaryotic cell. A functional mode of RFC through its interaction with proliferation cell nuclear antigen (PCNA) and cyclin by structural changes is called molecular switch extending DNA. This active characteristic is necessary for not only duplication and recovery of DNA as well as signal checkpoint of cell life, but also intracellular multifunction[21,22]. Mammalian transmembrane 4 superfamily (TM4SF) proteins (also known as tetraspans or tetraspanins) include at least 16 core members and a number of additional proteins with sequence similarities. Almost all mammalian cells contain one or more TM4SF proteins[29]. TM4SF protein CD81 may function in cell migration, proliferation and tumor cell metastasis. Most TM4SF proteins are located at plasma membrane, and several are located in cell lamellipodia and lopodia, consistent with their role in cell motility. TM4SF proteins including CD81 are also found in various intracellular granules and vesicles. A specific subset of TM4SF proteins may recruit PI 4-kinase to specific membrane locations, and thereby influence phosphoinositide-dependent signaling[30]. Ribosomal protein is a group of organella involving in cell structure and cell cycle, and possess the important ability to control cell growth, differentiation and adherence. The ribosomal protein is certainly related to cell energy or substance metabolism, and has critical physiological roles in nutrient transport[31].

HBV PS1TP5 protein also interacts with Homo sapiens inositol monophosphatase 2 (IMPA2), Homo sapiens solute carrier family 7 member 5 (SLC7A5), Homo sapiens cytochrome c oxidase subunit 8A, Homo sapiens enolase 1, Homo sapiens H3 histone family 3B, Human cytoskeletal gamma-actin gene, Homo sapiens eukaryotic translation elongation factor 1, Homo sapiens epoxide hydrolase 1, Homo sapiens dehydrogenase 1, Homo sapiens signal sequence receptor-β, Homo sapiens β5-microtubulin and Homo sapiens pyruvate dehydrogenase. How the interaction between PS1TP5 protein and the aforementioned interacting proteins affects the initiation and development of chronic hepatitis B, hepatic fibrosis and hepatocarcinoma needs to be further studied.

COMMENTS

Background

The hepatitis B virus (HBV) genome includes S, C, P and X regions. The S region is divided into four subregions of pre-pre-S, pre-S1, pre-S2 and S. PS1TP5 is a novel target gene transactivated by the pre-S1 protein (GenBank accession number: AY427953) and its precise function in the life cycle of HBV remains unknown. In order to investigate the biological function of the PS1TP5 protein, we performed a suppression subtractive hybridization technique to screen and identify proteins interacting with the PS1TP5 protein by constructing a cDNA subtractive library.

Research frontiers

Suppression subtractive hybridization (SSH) is a new and highly effective gene analysis method and is applicable to many molecular genetic and positional cloning studies for differentially expressed genes. Understanding PS1TP5 transactive proteins may help to bring some new clues for discovering pathogenesis of viral hepatitis and related hepatocellular carcinoma.

Innovations and breakthroughs

In this study, we found 23 genes with known functions that were closely correlated with immunoregulation, carbohydrate metabolism, signal transduction, formation of hepatic fibrosis, and initiation and development of tumor. A new gene with

unknown function was obtained and named PS1TP5TP1 (GenBank accession number: DQ487761).

Applications

Network interaction between viral and hepatocellular proteins plays an important role in the pathogenesis of viral hepatitis and related hepatocellular carcinoma. Studying of PS1TP5 precise role may help to further understand the network interaction and establish foundation for clinical gene therapy.

Terminology

Trans-activating: Activated mode of gene expression includes cis-activating and trans-activating. The former is intramolecular activating and the latter is intermolecular activating.

Peer review

Using the suppression subtractive hybridization technique the authors have identified the proteins transactivated by PS1TP5 protein. The study is well conducted and well written.

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RAPID COMMUNICATION

H pylori exist in the gallbladder mucosa of patients with chronic cholecystitis

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Abstract

AIM: To study whether *H pylori* locate in the gallbladder mucosa of patients with chronic cholecystitis.

METHODS: Using Warthy-Starry (W-S) silver stain and immunohistochemistry stain with anti-*H pylori* antibodies, we screened paraffin specimens in 524 cases of cholecystitis. *H pylori* urease gene A (HPUA) and *H pylori* urease gene B (HPUB) were analyzed by polymerase chain reaction (PCR) in the fresh tissue specimens from 81 cases of cholecystitis.

RESULTS: *H pylori*-like bacteria were found in 13.55% of the gallbladders of the cholecystitis patients using W-S stain. Meanwhile, bacteria positive for *H pylori* antibodies were also found in 7.1% of the gallbladders of patients with cholecystitis by immunohistochemistry. Of 81 gallbladders, 11 were positive for both HPUA and HPUB, 4 were positive for HPUA only and 7 were positive for HPUB only.

CONCLUSION: *H pylori* exist in the gallbladders of patients with chronic cholecystitis.

Key words: *H pylori*; Gallbladder mucosa; Chronic cholecystitis; Polymerase chain reaction; *H pylori* urease gene

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INTRODUCTION

H pylori closely correlate with chronic gastritis[1-5], peptic ulcer[6-8], gastric carcinoma and malignant lymphoma of gastric mucosa-related lymphoid tissues (MALToma)[9-11]. Recently, it has been found that *H pylori* have certain relationship to some diseases in the organs besides the stomach and duodenum[12]. However, it is still unclear whether *H pylori* have any correlation with adjacent structures of the stomach[13], the liver and the gallbladder[14,15]. In the present study, we compared the detection rate of *H pylori* in the stomachs of patients with chronic cholecystitis using Warthy-Starry (W-S) silver stain, immunohistochemistry and polymerase chain reaction (PCR) and showed that *H pylori* exist in the gallbladders.

MATERIALS AND METHODS

Subjects

A total of 142 patients who had chronic cholecystitis confirmed by pathologic examination since 1995 in our hospital and received gastroscopy and *H pylori* urease test were included. Randomly chosen patients without diseases of the gallbladder, who received gastroscopy due to symptoms of digestive tract were used as control group. The paraffin sections of 524 cholecystitis specimens were stained with W-S to observe *H pylori*-like bacteria. From fresh gallbladder specimens of 81 patients receiving cholecystectomy due to cholelithiasis and chronic cholecystitis in our hospital, DNA was extracted for PCR amplification.

Methods

Paraffin specimens of cholecystitis were sliced into 4 μm sections, stained with W-S and sealed with D.P.X for an optic microscopic observation for the existence of *H pylori*-like bacteria. Immunohistochemistry stain of *H pylori* was made using streptovitacin peroxidase (SP). Anti-*H pylori* antibody (Dako Corporation) was diluted to 1:10. At the same time, PBS was used as blank control, normal blood serum as negative control and the sections of gastric mucosa with positive *H pylori* as positive control. PCR amplification of *H pylori* urease genes A and B was carried out on 81 specimens. The gallbladder was placed in a prepared bottle for DNA extraction from gallbladder mucosa and frozen at -20°C. The procedures of PCR amplification were as follows: (1) Primer synthesis of urease gene A (HPUA) 1 and HPUA2 was based on nucleic

Table 1 Results of gastroscopy in both groups

Group	*n*	Bile reflux *n* (%)	Duodenal ulcer *n* (%)	Gastric ulcer *n* (%)	*H pylori* (+) *n* (%)
Control	123	36 (29.3)	7 (5.7)	6 (4.9)	61 (49.6)
Chronic cholecystitis	142	76 (53.5)[b]	8 (5.6)	13 (9.2)[b]	81 (57.0)

[b]$P < 0.01$ *vs* control group.

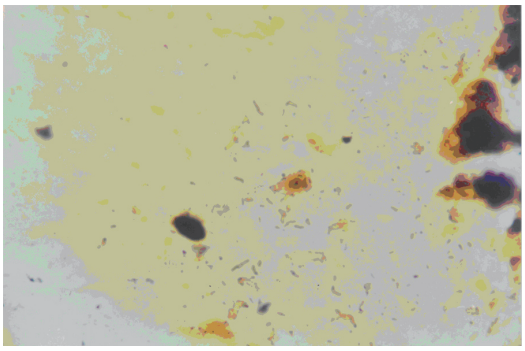

Figure 1 Curved positive stain bacteria in the surface mucus of gallbladder mucosa (WS × 200).

acids 304-714 of urease gene A, with an amplification portion of 411 bp. While the primers of urease gene B (HPUB) 1 and HPUB2 were designed according to nucleic acids 1971-2102 of urease gene B, with amplification part of 132 bp. These two primers maintained a high specificity and primer arrays that were HPUA1: 5'-GCCAATGGTA AATTAGTT-3'; HPUA2: 5'-CTCCTTAATTGTTTAC-3'; HPUB1: 5'-TGGGATTAGCGAGTATGT-3'; HPUB2: 5'-CCCATTTGACTCAATG-3', respectively, the primers of which were synthesized by Shanghai Bioengineering Corporation. (2) Reaction systems and parameters were as described previously[16]; (3) Gel electrophoresis analysis of the products of PCR amplification was performed under ultraviolet light and compared with the marker.

Statistical analysis

The data were processed with Chi-square test and $P < 0.05$ was considered a significant difference.

RESULTS

H pylori in the stomach of patients with chronic cholecystitis

There were 142 patients with chronic cholecystitis including 13 with gastric ulcer detected by gastroscopy, 8 with duodenal ulcer and 121 with chronic gastritis, of which 76 had bile reflux. A total of 123 randomly chosen patients with digestive symptoms who were examined by gastroscopy and without gallbladder diseases were used as control, of which 6 cases were with gastric ulcer, 7 with duodenal ulcer, 2 with gastric carcinoma and 108 with chronic gastritis. Bile reflux occurred in 36 cases in the control group. In both groups, the detection rates (61/123 *vs* 81/142) of *H pylori* in the stomach were similar, but the detection rate of the bile reflux in the cholecystitis group was significantly higher than that in the control group (36/123 *vs* 76/142 $P < 0.01$, Table 1). There was no significant difference with respect of detection rate of *H pylori* in both groups (16/36 *vs* 35/76, $P > 0.05$).

Figure 2 Positive stain bacteria of *H pylori* antibody in the surface mucus of gallbladder mucosa (SP × 200).

W-S silver stain of the filed cholecystitis paraffin

Under optic microscopes, in contrast with the yellow staining of the gastric mucosa, there could be seen bended, curved and spiral brown bacteria in the epithelial cells of the gastric mucosa that were used as positive control. Using W-S silver stain, *H pylori*-like bacteria could be seen in 71 (13.6%) out of 524 cholecystitis specimens under optic microscopes, showing curved, pole-like, bended, spiral and fusiform bacteria, and also some spherical shaped bacteria. In 34 specimens, besides positive *H pylori*-like bacteria, other bacteria could be seen (Figure 1).

Immunohistochemical observation of anti-H pylori antibody

After 71 specimens positive for *H pylori*-like bacteria were stained immunohistochemically with anti-*H pylori* antibody, bacteria positive for *H pylori* antibody could be seen in only 37 cases (52.1%) under optic microscopes. That is to say, bacteria positively stained with anti-*H pylori* antibody only accounted for 7.1% (37/524) of 524 cholecystitis specimens under optic microscopes. These positive bacteria were brown in color, bended with curved-poles, and spiral in shape. They were mainly located on the epithelial cell surface and within the mucosal glands, scattered or aggregated (Figure 2).

PCR amplification of HPUA and HPUB

Of 81 cholecystectomy specimens, positive amplification zone of HPUA or HPUB was seen in 22 (27.2%), including 11 (13.6%) with positive amplification zones of HPUA and HPUB, 4 with positive HPUA only and 7 with positive HPUB only. However, in 6 gastric mucosa specimens as positive control, the two primers had positive amplification zones of HPUA and HPUB (Figure 3).

DISCUSSION

By means of gastroscopy, we have found that the incidence rate of bile reflux in patients with chronic cholecystitis was significantly higher than that in the control group. It

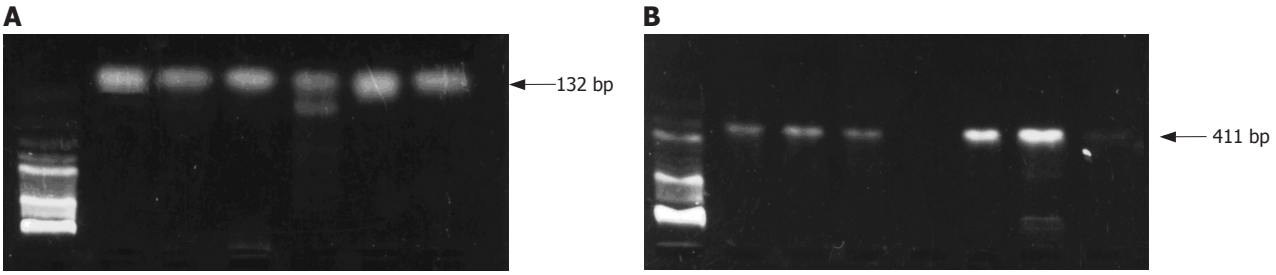

Figure 3 **A**: PCR amplification of primers of gallbladder mucosa DNA urease gene B (HPUB) and the positive amplification zone of 132 bp; **B**: PCR amplification of primers of gallbladder mucosa DNA urease gene A (HPUA) and the positive amplification zone of 411 bp.

indicates that patients with chronic cholecystitis are apt to bile regurgitation, which occurs in 80% of patients with chronic cholecystitis while in only 32% of normal persons[17,18]. *H pylori* are sensitive to bile salts, especially the unconjugated bile salts that exert poisonous effect on *H pylori*. The latter cannot live in an environment with bile salts[19]. We also found that in the stomachs of patients with chronic lithic cholecystitis and a bile reflux, *H pylori* were present in up to 46.1% (35/76), higher than 44.4% (16/36) in patients without bile reflux. It indicates that a high incidence rate of *H pylori* infection still existed in the stomach although there was a bile reflux. The results of our study are consistent with that of Caldwell *et al*[20], who studied the infection rate of gastritis and *H pylori* in patients with chronic calculus cholecystitis before and after cholecystectomy and found that the duodenogastric reflux increased after cholecystectomy. The gastritis was aggravated and the infection rate of *H pylori* in the stomach increased from preoperative 32% to postoperative 68%, with a significant difference ($P < 0.05$). Therefore, they concluded that *H pylori* could live in the basic condition and even aggravate gastritis. Our study indicates that the patients with chronic lithic cholecystitis were apt to bile regurgitation; however, there was still a high infection rate of *H pylori* in their stomachs, suggesting that a kind of *H pylori* that can resist bile salts exists. Bile regurgitation may play a role in selecting *H pylori* so that *H pylori* resistant to bile salts can survive and, in combination with the bile, aggravate the injury of gastric mucosa[21]. Thus, *H pylori* that resist the bile salts and survive under basic conditions can enter the gallbladder *via* an inverse infection route through the common passage.

In 524 chronic cholecystitis specimens, 71 showed positive bacteria by W-S silver stain, while only 37 had positive bacteria by immunohistochemistry stain using anti-*H pylori* antibodies. It suggests that W-S silver stain to screen *H pylori* in gallbladder specimens is suitable for an initial selection. In patients with chronic calculus cholecystitis, the infection rate of *H pylori* was 7.1% (37/524), a rather low level, which was confirmed by immunohistochemistry using anti-*H pylori* antibodies. *H pylori* were mainly present in the surface of epithelial cells of the gallbladder mucosa, sometimes in intercellular zone or within the mucous gland. The *H pylori*, spiral, U- and S-typed in morphology, are distributed in scattered or aggregated fashion. All these characteristics are similar to that of *H pylori* in the stomach.

After the fresh specimens of 81 patients with chronic cholecystitis treated with cholecystectomy were amplified by PCR with specific primers of HPUA and HPUB, the positive amplification zone existed in 27.2% (22/81), significantly higher than the positive rate (13.6%) detected by W-S silver stain and that (7.1%) by immunohistochemistry stain using *H pylori* antibodies. This is partly because the template DNA amplified by PCR was extracted from tissues (0.5 cm × 0.5 cm) of gallbladder mucosa and the PCR with a high sensitivity had a high detection rate of *H pylori*. In the meantime, the PCR was conducted to detect DNA of *H pylori*; therefore, under many conditions, the positive amplification zone would appear only if *H pylori* DNA existed in the gallbladder mucosa, whether the *H pylori* were dead or the remains from previous infections. In the present study, in specimens with positive PCR amplification, 11 were positive for HPUA and HPUB; while in the control group, 6 gastric mucosa specimens infected by *H pylori* were positive for HPUA and HPUB. It proved that *H pylori* exist in the gallbladder. However, it still remains unclear whether *H pylori* are present in the gallbladder of patients with positive HPUA or HPUB, whether the gallbladder is infected by *H pylori* and whether the genes of *H pylori* change in the gallbladder[22]. We conclude that *H pylori* are present in the gallbladder and may relate to the incidence of cholecystitis, which requires further research.

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RAPID COMMUNICATION

Clinical and molecular analysis of hereditary non-polyposis colorectal cancer in Chinese colorectal cancer patients

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Abstract

AIM: To analyze the frequency of hereditary non-polyposis colorectal cancer (HNPCC) in Chinese colorectal cancer (CRC) patients, and to discuss the value of microsatellite instability (MSI) and/or immunohistochemistry (IHC) for MSH2/MLH1 protein analysis as pre-screening tests in China.

METHODS: The Amsterdam criteria Ⅰ and Ⅱ (clinical diagnosis) and/or germline hMLH1/hMSH2 mutations (genetic diagnosis) were used to classify HNPCC families. Genetic tests, including microsatellite instability, immunohistochemistry for MSH2/MLH1 proteins and hMSH2/hMLH1 genes, were performed in each proband.

RESULTS: From July 2000 to June 2004, 1988 patients with colorectal cancer were analysed and 114 CRC patients (5.7%) from 48 families were categorized as having HNPCC, including 76 from 26 families diagnosed clinically and 38 from the other 22 families diagnosed genetically. The sensitivity and specificity of high MSI and IHC for predicting mutations were 100% and 54%, and 79% and 77%, respectively.

CONCLUSION: The frequency of HNPCC is approximately 10% among all Chinese CRC cases. The MSI and IHC detections for hMSH2/hMLH1 proteins are reliable pre-screening tests for hMLH1/hMSH2 germline mutations in families suspected of having HNPCC.

Key words: Hereditary non-polyposis colorectal cancer; Colorectal cancer; Mismatch repair gene; Immunohistochemistry; Microsatellite instability

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INTRODUCTION

In 1966, Henry Lynch and his colleagues described familial aggregation of colorectal cancer with stomach and endometrial tumors in two extended kindreds and termed it Cancer Family Syndrome[1], and then designated this constellation as "Lynch syndrome"[2]. In 1991, this condition was renamed "hereditary non-polyposis colorectal cancer (HNPCC)" by the International Collaborative Group on HNPCC (ICG-HNPCC), and the Amsterdam diagnosis criteria were published (Amsterdam criteria Ⅰ)[3]. In 1993, 1996 and 1997, the modified Amsterdam criteria[4,5], the Japanese criteria[6] and the Bethesda guidelines[7] were developed respectively for different purposes. In 1999, ICG-HNPCC proposed its own modification of the Amsterdam criteria Ⅰ, and termed it the Amsterdam criteria Ⅱ[8].

Since 1993, HNPCC has been confirmed to be associated with germline mutations in several DNA mismatch repair genes (MMR), including hMSH2, hMLH1, hMSH6, hPMS1, and hPMS2 (hMSH2 and hMLH1 mutations are the most common)[9-17]. The elucidation of the mismatch repair genes has added more diagnostic complexity[18-20]. The tumor tissue from HNPCC patients harbouring pathogenic mutations in the MMR genes is frequently characterized by microsatellite instability (MSI). Actually, carriers of pathogenic hMLH1/hMSH2 germline mutations show high MSI (H-MSI) in tumor tissues[21,22]. MSI and/or IHC analysis has been used as pre-screening tests to select individuals eligible for DNA mutation analysis in blood, and sequence analysis can avoid unnecessary, expensive and time-consuming DNA analyses[23].

Hereditary colorectal tumors registry, Tianjin, China was established in 1998. This study was conducted

to analyze the frequency of HNPCC among Chinese patients with colorectal cancer, and to discuss the value of microsatellite instability (MSI) and/or immunohistochemistry (IHC) for MSH2/MLH1 protein analysis as pre-screening tests in Chinese patients.

MATERIALS AND METHODS

Subjects

All colorectal cancer patients from the In-patient Department, Tianjin Binjiang Hospital and Tianjin Medical University Hospital seen between July 2000 and June 2004 were identified prospectively and included in the study. The inclusion criteria were: (1) patients with pathologically identified colorectal cancer, (2) self-reported Chinese born and living in mainland China, and (3) patients who agreed to participate in this study. We chose to categorise HNPCC patients using the following criteria: (1) patients from families meeting the Amsterdam criteria Ⅰ or Ⅱ and/or (2) patients who met less strict criteria for suspected HNPCC, but with pathogenic hMLH1/hMSH2 germline mutations. The suspected HNPCC was established in this study using the Japanese criteria and the top six guidelines of the Bethesda guidelines (the 7th guideline for young colorectal adenoma patients diagnosed before 40 years of age was not used in this study).

Each newly diagnosed HNPCC family was referred to Hereditary Colorectal Tumors Registry, Tianjin, China for the genealogic study, genetic counselling and DNA test in order to identify all the individuals of the family at risk and to ensure a lifelong follow-up.

Questionnaire

Surgeons completed a questionnaire covering malignancies and age at onset of cancer in the colorectal cancer patients and their first-degree relatives.

Genetic tests

hMSH2/hMLH1 germline mutation detection: Only HNPCC proband patients and suspected HNPCC proband patients underwent this test. Genomic DNA was isolated from peripheral blood using a kit from Dingguo Technologies. The 35 coding exons of hMLH1 and hMSH2 were amplified from purified DNA for SSCP analysis. The primers included part of the introns to detect possible splice mutations. In order to keep the length of the polymerase chain reaction (PCR) products for SSCP below 300 base pairs, hMLH1 exon 12, and hMSH2 exons 3, 12, and 14 were divided into two overlapping segments, giving a total of 39 PCR products. The PCR products were kept at -20℃ until SSCP analysis which was performed at a fixed gel temperature of 20℃. Sequencing was performed on PCR products with BigDye Terminator Cycle Sequencing Ready Reaction kits (Perkin Elmer) using standard conditions and the same sequencing primers as used for PCR. The PCR products were sequenced in both sense and antisense directions. SSCP and sequencing were performed using an ABI prism 377 sequencer, and analysed using the software programs GeneScan and Sequence Navigator (PE Applied Biosystems).

Microsatellite instability: Only HNPCC proband patients and suspected HNPCC proband patients underwent this test. MSI was determined by PCR of genomic DNA isolated from formalin-fixed, paraffin-embedded normal and colorectal cancer tissues from each proband. Tissue sections were deparaffinized in xylene, digested with proteinase K overnight at 55℃, and DNA was isolated using DNA kit (Dingguo Technologies, Beijing, China). PCR was carried out in 10 μL reactions containing 1 × manufacturer's PCR buffer, 1.5 mmol $MgCl_2$, 200 μmol/L deoxynucleotide triphosphates, 0.5 units of Taq polymerase (Dingguo Technologies), and 0.5 μmol/L of each primer. The forward primer was end-labeled using γ33P-ATP and polynucleotide kinase. The cycles were as follows: 8 min at 94℃, then 30 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, and a final extension at 72℃ for 10 min. The products were subjected to electrophoresis in 2% denaturing polyacrylamide gels, which were subsequently dried and exposed to X-ray film (Kodak, BioMax) at room temperature overnight. To determine the extent of MSI, nine microsatellite markers were examined (BAT25, BAT26, BAT40, D2S123, D18S58, D10S197, D5S36, D18S69, and MYCL). Microsatellite was found to be unstable if one or more novel bands were present in the PCR product of the tumor sample as compared with the PCR product of normal tissues from the same individual. A tumor was considered to be H-MSI if 4 or more of the markers were unstable, L-MSI if fewer than 4 of the markers were unstable, and MSS if none of the nine markers was unstable.

Immunohistochemistry for MLH2/MSH1 proteins: HNPCC proband patients and suspected HNPCC proband patients underwent this test. Mouse anti-human monoclonal antibodies were used for the detection of mismatch repair proteins MSH2 (Sigma, Saint Louis, Missouri, USA) and MLH1 (EMD Biosciences, San Diego, CA, USA). Freshly cut paraffin sections were dewaxed in xylene, rehydrated in graded alcohols, and washed in TRIS buffer. Heat-induced epitope retrieval (HIER; 600 W microwaves twice for 15 min in pre-warmed 10 mmol/L sodium citrate buffer, pH 6) was employed for MSH2 and MLH1 staining. Primary antibodies were added (dilution: MSH2 1:50; MLH1 1:75) and slides were incubated overnight at 4℃. Slides were then processed on an immunostainer. Antigen antibody binding was visualized by the avidin-biotin-complex method using 3-amino-9-ethylcarbazole as a chromogen. The first antibody was replaced by phosphate-buffered saline as a negative control to assess the specificity of the antibodies. Haematoxylin-counterstained sections were mounted in aqueous mounting medium and observed under light microscopy. Surrounding normal colonic mucosa, stromal cells, and lymphocytes served as internal controls. Tumors were considered not to express either protein when the nuclei showed no immunostaining, but internal controls were positive.

Statistical analysis

SPSS Release 11.5 software was used for all analyses. The clinical diagnosis of HNPCC was established using each

Table 1 Diagnosis criteria of hereditary non-polyposis colorectal cancer

Amsterdam criteria Ⅰ

There should be at least three relatives with histologically verified CRC; and all the following criteria should be met:

1) One should be a first degree relative of the other two;
2) At least two successive generations should be affected;
3) At least one CRC should be diagnosed before age 50;
4) FAP should be excluded in CRC cases;
5) Tumors should be verified by pathological examination.

Amsterdam criteria Ⅱ

There should be at least three relatives with an HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter or renal pelvis); and all the following criteria should be met:

1) One should be a first-degree relative of the other two;
2) At least two successive generations should be affected;
3) At least one cancer should be diagnosed before age 50;
4) FAP should be excluded in CRC cases (if present)
5) Tumors should be verified pathologically.

Japanese criteria

1 A case with 3 or more colorectal cancers within the first-degree relatives
2 A case with 2 or more colorectal cancers within the first-degree relatives meeting the following criteria:
a) Age of onset of colorectal cancer(s) being lower than 50;
b) With right colon involvement;
c) With synchronous or metachronous multiple colorectal cancers;
d) Associated with synchronous or metachronous extracolorectal malignancies.

Bethesda Guidelines

1 Individuals from families that fulfil the Amsterdam criteria;
2 Individuals with two HNPCC-related cancers, including synchronous or metachronous cancers;
3 Individuals with colorectal cancer, plus colorectal cancer and/or HNPCC-related cancer and/or colorectal adenoma in a first-degree relative; at least one of the cancers diagnosed before 45 yr of age and the adenoma diagnosed before age 40;
4 Individuals with colorectal or endometrial cancer diagnosed before age 45;
5 Individuals with right-sided colorectal cancer with an undifferentiated histopathological pattern (solid/cribiform) diagnosed before age 45;
6 Individuals with signet-ring cell type colorectal cancer diagnosed before age 45;
[1]7 Individuals with colorectal adenomas diagnosed before age 40.

[1]Condition 7 was not included in this study.

of the four defined criteria, and the detections of MSI and IHC were used as tests in two by two contingency tables to determine the sensitivity and the specificity. We evaluated the strength and weakness of each criterion by analyzing the clinical characteristics of the families with hMSH2 and hMLH1 mutations that were identified and missed by each of the respective criteria, and the same with detection of MSI and IHC. *P* values less than or equal to 0.05 were considered significant.

RESULTS

A total of 1988 patients with colorectal cancer were identified. Clinically and genetically diagnosed HNPCC patients and families are shown in Table 1 and 2.

One hundred and fourteen CRC patients (5.7%) from 48 families were categorized as having HNPCC, including: 76 clinically diagnosed HNPCC patients from 26 families (9 families with the Amsterdam criteria Ⅰ, 21 families with the Amsterdam criteria Ⅱ and 4 families with both) and 38 genetically diagnosed HNPCC patients from the other 22 families (all were clinically suspected HNPCC).

We found 391 clinically suspected HNPCC patients in 150 families in this study, including 116 colorectal cancer patients from 65 families, who fulfilled the Japan criteria, and 378 colorectal cancer patients from 145 families who fulfilled at least one of the top six Bethesda guidelines for the identification of HNPCC (Table 2).

Table 2 Number and size of families classified according to different clinical criteria for hereditary non-polyposis colorectal cancer

Clinical criteria	No. of families	Family size	Mean variance
Amsterdam I	9	15.2	37.2
Amsterdam II	21	14.3	20.5
Japanese criteria	65	6.7	13.2
Set A	26	8.7	9.3
Set B	61	6.9	10.6
Bethesda	145	8.3	9.6
Condition 1	9	15.2	37.2
Condition 2	58	13.6	20.7
Condition 3	89	11.7	8.9
Condition 4	105	10.9	7.6
Condition 5	9	7.2	8.5
Condition 6	2	9.9	12.6

In this study, 105 cases were diagnosed under 45 years of age, 29 cases (28%) were categorized as having HNPCC, including 12 clinically diagnosed HNPCC patients and 17 genetically diagnosed HNPCC patients.

hMSH2/hMLH1 germline mutation

In 26 clinically diagnosed HNPCC proband patients, 50% (13/26) showed germline mutation of hMSH2 or hMLH1 and 15% clinically suspected HNPCC proband

patients had positive results.

Microsatellite instability

Table 3 shows the results in each kind of families. The H-MSI phenotype was demonstrated in all 48 hMSH2/hMLH1 mutation carriers, and its sensitivity to mutations was 100%, and specificity was 54%.

Immunohistochemistry (IHC) for MLH2/MSH1 proteins

Among the 176 proband patients, 68 showed loss of staining for either MLH1 (28 cases) or MSH2 (40 cases). No case showed absence of both MLH1 and MSH2. For MLH2/MSH1 mutations, its sensitivity and specificity were 79% and 77%, respectively .

DISCUSSION

Diagnosis criteria

The diagnosis criteria of HNPCC are controversial due to the variety of clinical phenotypes associated with the syndrome in different areas or countries[4,5,7,30]. The International Collaborative Group on HNPCC established the Amsterdam criteria Ⅰ to provide a basis for uniformity in collaborative studies[3]. The criteria were restrictive, since extracolonic malignancies were not considered, and small families were unlikely to fulfil the criteria[31-33]. In this study, less than 19% patients were classified as the Amsterdam Ⅰ families. However, when Amsterdam Ⅰ criteria were met, the chance of HNPCC was high as it aimed at specificity rather than sensitivity. The Amsterdam criteria Ⅱ were also developed by the International Collaborative Group on HNPCC[4], and besides colorectal cancer, extracolonic malignant tumors were also included, such as endometrial, small bowel, ureter, renal pelvis cancers and other HNPCC-related malignant tumors, but the requirements were kept to three cases in at least two successive generations in the families. And small families were also unlikely to fulfil the second criteria. In this study, about 44% families were classified according to the Amsterdam criteria Ⅱ.

As the two Amsterdam criteria were considered to be too strict, the alternatives have been developed, such as the modified Amsterdam criteria required only two diagnosed relatives, and considered early-onset endometrial cancer or unusually early-onset neoplasm, and one of its two sets does not mention the number of generations[4,5]. The Japanese criteria did not mention the number of generations and also classified families with two cases, and additionally take into account the clinical features of HNPCC (such as early onset, multiple synchronous or metachronous colorectal cancers, and right colon involvement)[30] and 65 families were classified in this study. The Bethesda guidelines targeted the evaluation of colorectal tumors for microsatellite instability or mismatch repair gene testing, which are less restrictive and more sensitive, but less specific than the Amsterdam criteria[7], and 145 families were classified in this study.

The elucidation of the mismatch repair genes which are the genetic basis for many HNPCC families, has added more complexity[18,34,35].

Table 3 Microsatellite instability of HNPCC and suspected HNPCC probands *n* (%)

	Amsterdam Ⅰ (9 probands)	Amsterdam Ⅱ (21 probands)	Japanese (65 probands)	Bethesda (145 probands)
MSI-H	67 (6)	71 (15)	71 (46)	68 (99)
MSI-L	3 (3)	19 (4)	14 (9)	10 (14)
MSS	0 (0)	10 (2)	15 (10)	22 (32)

There was no significant difference among all these four kinds of patients.

HNPCC frequency in CRC

In China, this is the first population-based prospective study on a large series of consecutive CRC patients (n = 1988) whose family histories of malignant tumors were obtained from 114 HNPCC patients (5.7% of CRC patients) in 48 families and 105 patients diagnosed before 45 years of age, and 29 (28%) HNPCC patients.

Up to now, several study groups have estimated the frequency of HNPCC among consecutive CRC patients based on family histories without testing for hMLH1/hMSH2 germline mutations in Western countries. But estimation of the frequency of HNPCC based entirely on the Amsterdam criteria Ⅰ shows different results in different populations even in Western countries, ranging from 0.3% (Finnish) to 4.5% (Northern Italian)[25-27], even in the same Italian population, researches showed different frequency between 0.5% to 4.5%[27,28]. And 1.3% was found in this study. Differences in the frequency of HNPCC between populations may reflect the actual population differences but may also reflect differences in diagnoses among family members and differences in the level of proof that is accepted to verify diagnosis. Another approach to diagnose HNPCC was adopted by screening for germline mutations in hMLH1 or hMSH2. A Danish study group estimated the HNPCC frequency among 1200 consecutive colorectal cancer patients based on family histories and/or testing for hMLH1/hMSH2 germline mutations, and approximately 1.7% cases were diagnosed with HNPCC[29] which was much lower than our study (10%). And in our study, about 46% families with hMLH1 and hMSH2 germline mutations did not fulfill the two Amsterdam criteria, however only 2 of 20 families (10%) were found in the Danish study.

MSI and immunohistochemistry for MSH2/MLH1

MSI analysis was first described in 1993[36-38]. A H-MSI phenotype is reported in 85%-92% of colorectal carcinoma associated with HNPCC and in 10%-15% of sporadic CRC[21,39-42]. MSI analysis has a sensitivity of around 90% in detecting MMR deficiency in carriers of a pathogenic MMR mutation[43,44]. Several laboratories have reported H-MSI in all MSI- analysed patients with pathogenic hMLH1/hMSH2 mutations[21,22,45,46]. These results are in agreement with our findings as all patients with hMLH1/hMSH2 mutations in our study had H-MSI tumors using our panel of markers.

Immunohistochemical staining for the presence of the mismatch repair proteins offers a cheaper and simpler test,

and it is sensitive in predicting a truncating MMR defect in one of the genes[44,47]. IHC has additional advantages when compared with MSI analysis, indicating that the MMR gene is most eligible for DNA analysis. Since the mismatch repair proteins form heterodimeric complexes, distinct IHC patterns can be expected. Individuals can be selected for DNA mutation analysis, and the assessment of which gene to test first can be made. Previous studies have shown a high sensitivity in predicting mutations in MSH2 (92%) by applying IHC in colorectal tumors, but a lower sensitivity in MLH1 (48%)[44,45]. We got the likely results in this study.

Based on the results of our study, we propose that besides the two Amsterdam criteria, the Japan criteria and the Bethesda guidelines should also be used in clinical practice as the most inclusive clinical criteria for the diagnosis of HNPCC and genetic analysis should also be considered, and the detection of MSI and IHC for hMSH2 or hMLH1 protein is a reliable pre-screening test for hMLH1/hMSH2 mutations in families suspected of having HNPCC.

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CASE REPORT

Recurrence of autoimmune hepatitis after liver transplantation without elevation of alanine aminotransferase

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Abstract

It is controversial whether steroid therapy should be continued to prevent the recurrence of autoimmune hepatitis (AIH) in patients who have undergone liver transplantation (LTx) due to AIH. We report a case of recurrent autoimmune hepatitis after LTx despite a persistently normal range of alanine aminotransferase (ALT). A 50-year-old woman was admitted to our hospital because of jaundice and severe liver dysfunction, where she was diagnosed with liver failure due to AIH. Steroid therapy was not effective enough and the patient received living-donor LTx in 1999. Following the operation, the level of ALT was maintained within a normal range and anti-nuclear antibody (ANA) became negative, however, the serum level of IgG gradually elevated and ANA became positive, while platelets decreased. A liver biopsy performed 6 years after LTx showed histological findings of AIH and she was diagnosed with recurrent AIH. A recurrence of AIH may occur after LTx even if the level of ALT remains within a normal range. We consider that a protocol liver biopsy should be performed in patients who undergo LTx due to AIH to decide the indication for steroid therapy.

Key words: Autoimmune hepatitis; Liver transplantation; Recurrence; Steroid; Protocol liver biopsy

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INTRODUCTION

Autoimmune hepatitis (AIH) is characterized by the existence of autoantibodies, liver dysfunction and active hepatitis with infiltration of lymphocytes and plasma cells in the liver, and occurs mainly in females[1-3]. Steroid therapy is the first choice of treatment and most patients respond well, though some patients with AIH progress to chronic or acute liver failure[4]. Liver transplantation (LTx) is a therapeutic option for those patients, as the prognosis following LTx is relatively good (a 5-year survival rate of 80%-90%)[5]. However, some studies have reported that recurrent AIH ranges from 20% to 42% after LTx[6-10]. Such recurrence is usually accompanied with an elevation of alanine aminotransferase (ALT), though that does not occur in all cases[6]. Herein, we report a patient with AIH recurrence after living-donor LTx whose ALT level remained within a normal range.

CASE REPORT

A 50-year-old woman was presented with general fatigue and jaundice, and admitted to a local hospital on January 10, 1999. Because of a severely abnormal liver function test, the patient was then transferred to our hospital. Based on physical findings and laboratory data (total bilirubin 11.1 mg/dL, ALT 1145 IU/L, prothrombin time 35%, anti-nuclear antibody: anti-nuclear antibody (ANA) 1:160, IgG 3600 mg/dL), she was diagnosed with acute AIH. Steroid therapy including pulse therapy was initially employed, however, the patient did not respond well and her general state became worse. We assessed her condition as indicating LTx.

The living-donor LTx operation was performed on March 5, 1999 using the right lobe donated by the patient's elder sister, who had no history of autoimmune diseases, with a normal range of liver function tests, and was negative for the anti-nuclear antibody. We previously reported the clinical course from onset to just after LTx of this case[11]. The patient experienced episodes of transient mild acute rejection and a liver abscess at 3 mo and 1 year, respectively, following LTx, which were resolved by immunosuppression against the rejection and administration of antibiotics and discontinuation of prednisolone (PSL) for the liver abscess. Tacrolimus was

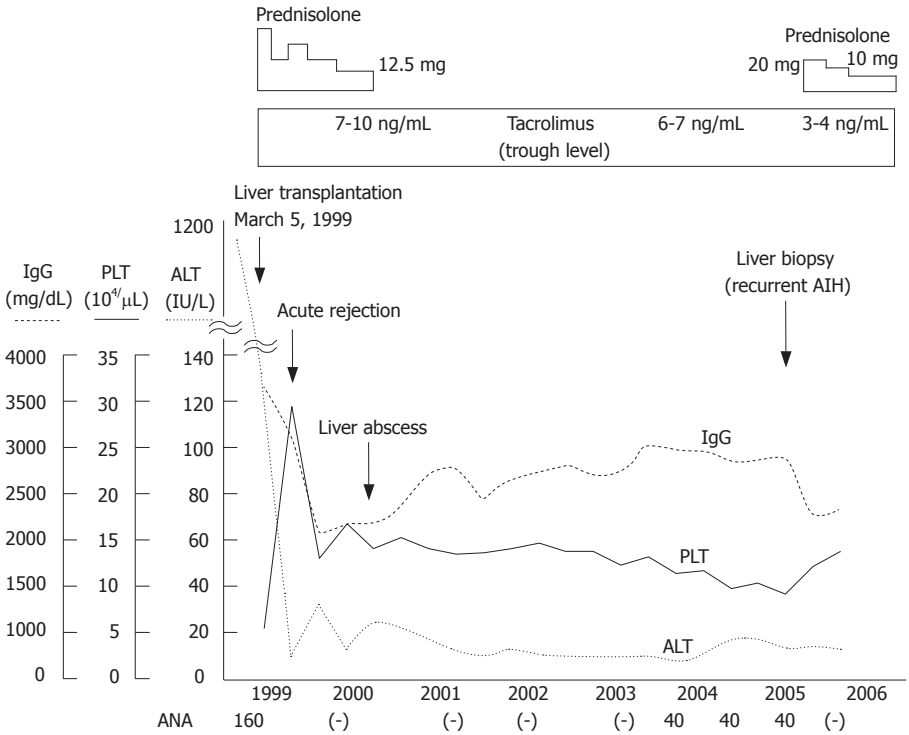

Figure 1 Clinical course and liver transplantation on March 5, 1999. Follow-up after liver transplantation from June 1999 to December 2005. ALT remained within a normal range after LTx. PLT was initially decreased and then increased, while IgG increased and then decreased after treatment. PLT: platelet; ALT: alanine aminotransferase; ANA : anti-nuclear antibody.

continued for immunosuppression, with the trough level kept at 6-10 ng/mL. Her clinical course after LTx is shown in Figure 1.

Steroid therapy was discontinued in 2000 and the serum levels of aspartate aminotransferase (AST), ALT, ALP, and γ-GTP remained within normal ranges. ANA remained negative following LTx, however, became positive in 2003. Although the patient had no subjective symptoms, she was admitted to our hospital again in March, 2005 for further examinations, because of elevated serum IgG and decreased platelet count. The laboratory data obtained upon admission were: AST 32 IU/L, ALT 14 IU/L, platelet 9.3 × 104/μL, PA-IgG 67.5 ng/10^7C, IgG 2720 mg/dL, ANA 1:40, and anti-smooth muscle antibody 1:40 with other autoantibodies negative (Table 1). Various viral markers, including hepatitis B surface antigen, anti-hepatitis B core antibody, and anti-hepatitis C virus antibody, were negative, while hepatitis B virus DNA, hepatitis C virus RNA, and hepatitis G virus RNA were undetectable by polymerase chain reaction assays. There was no history of alcohol abuse and no drug toxicity. Moderate splenomegaly was demonstrated by a computed tomography examination. Histopathological findings of the liver showed infiltrations of lymphocytes and plasma cells in the portal area, and a mild necroinflammatory change in the parenchyma (Figure 2). Bridging fibrosis was also observed. Acute or chronic rejection, such as biliary or vascular lesions, was not found. The patient was diagnosed histologically as chronic hepatitis at stage 3 and grade 1 (CH F3/G1) due to AIH. Based on the histological changes and clinical course, she was diagnosed with a recurrence of AIH.

We prescribed prednisolone (PSL) at 20 mg/d plus tacrolimus, which maintained a trough level of 3-4 ng/mL. PSL daily dosage tapered gradually to 10 mg. Upon admission, the IgG level was 2720 mg/dL, while after treatment it was reduced to 2100 mg/dL and ANA became negative. ESR, ALT and platelets were 60 mm/h, 14 IU/L, and 9.3 × 10^4/μL, respectively before treatment

Table 1 Laboratory data on admission in 2005

CBC		Renal function	
WBC	4400/μL	Na	140 mEq/L
RBC	3.72 × 10^6/μL	K	3.9 mEq/L
Hb	11.9 g/dL	Cl	106 mEq/L
Ht	36%	BUN	21 mg/dL
Platelet	9.3 × 10^4/μL	CRE	0.7 mg/dL
ESR	60 mm/h	UA	4.6 mg/dL
Coagulation test		HbA1c	5.00%
Prothrombin time	80.40%	Urinalysis	
Bleeding time	5.0 min	Protein	(-)
Blood chemistry		Blood	(-)
Total protein	8.1 g/dL	Sugar	(-)
Albumin	3.9 g/dL	Urobilinogen	(±)
Globulin	4.2 g/dL	Serological test	
Total bilirubin	1.1 mg/dL	HBs Ag	(-)
Direct bilirubin	0.1 mg/dL	Anti-HBc	(-)
AST	32 IU/L	Anti-HCV	(-)
ALT	14 IU/L	ANA	× 40
LDH	182 IU/L		(Diffuse pattern)
ALP	210 IU/L	AMA	(-)
γ-GTP	25 IU/L	ASMA	(-)
LAP	54 IU/L	Anti-LKM-1	(-)
CH.E	135 IU/L	LE test	(-)
ZTT	19 U	IgG	2720 mg/dL
TTT	6 U	IgA	259 mg/dL
Total Cholesterol	161 mg/dL	IgM	94 mg/dL
ICG R15	27%	PA IgG	67.5 ng/10^7C
K-ICG	0.094	Anti-platelet antibody	(-)
		HLA-DR	DR4 DR5
		Tacrolimus	5.2 ng/mL

ALT: alanine aminotransferase, AST: aspartate aminotransferase; ESR: erythrocyte sedimentation rare, ANA: anti-nuclear antibody, AMA: anti-mitochondrial antibody; HBc: hepatitis B core, HBs Ag: hepatitis B virus surface antigen; UA: uric acid, HLA: human leukocyte antigen.

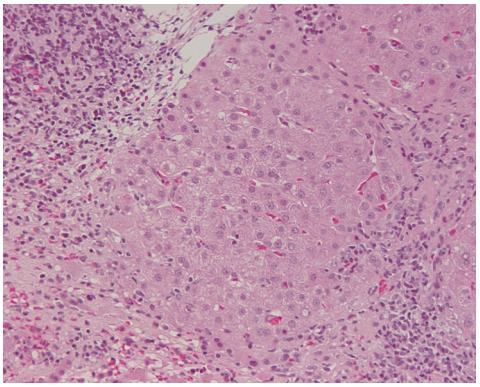

Figure 2 Microphotographic image of liver specimen. Infiltration of mononuclear cells including plasma cells and interface hepatitis can be seen (HE x 100).

and became 22 mm/h, 13 IU/L and 14.5 × 10^4/μL respectively thereafter.

DISCUSSION

Quality of life and graft survival rates are generally satisfactory in patients with end-stage liver failure following LTx, however, recurrence is possible in patients who develop various liver diseases, including AIH. The criteria used to diagnose a recurrence of AIH are based on increased serum transaminase levels, positivity of autoantibodies, marked hypergammaglobulinemia with elevation of serum IgG, portal lymphocytic and plasmacytic infiltration, interface hepatitis, and steroid dependency, while appropriate investigations are also necessary to exclude other possible causes of liver disease, such as rejection, viral infection, drug-induced liver disease, or biliary tract problems[1,6,12,13]. The present patient fulfilled those criteria, except for a normal ALT range, and was diagnosed with recurrent AIH based on the histological findings. A few reports have described some asymptomatic patients who underwent liver biopsies and showed abnormalities compatible with recurrent AIH without biochemical evidence of hepatitis[6,10]. It has also been reported that classic clinical, laboratory, immunoserological and histological manifestations can be absent in patients with recurrent AIH[13, 14].

It has been reported that the discontinuation of steroid administration may increase the risk of AIH recurrence[7]. However, long-term steroid therapy can cause multiple side-effects, and reintroduction of steroids was effective against recurrent AIH[7]. Successful outcomes in patients who underwent LTx due to AIH and withdrew from steroids have been reported by some centers[7,15]. Therefore, it remains controversial whether steroids should be continuously administrated following LTx or only reintroduced after recurrence.

In the present case, a gradual decrease in the number of platelets was observed. It is well known that platelet level is related to the degree of portal hypertension caused by advanced liver diseases. It was suspected that the decrease in platelet count in the present patient was caused by hypersplenism due to advanced fibrosis of the liver caused by the recurrence of AIH, because the liver biopsy findings showed advanced fibrosis (F3). It is important to monitor the platelets count even if the ALT level remains normal. In addition, increase in serum IgG and ESR were observed in our patient, which may be markers suggestive of AIH recurrence, though they are not specific.

Our findings confirmed that recurrent AIH should not be excluded even if the level of ALT remains normal after LTx in patients with AIH. We consider that a protocol liver biopsy should be performed in those cases.

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CASE REPORT

Intraductal papillary mucinous carcinoma with atypical manifestations: Report of two cases

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Abstract

Intraductal papillary mucinous neoplasms (IPMNs) are a well-characterized group of mucin-producing cystic neoplasms of the clear malignant potential type. We report here two cases of intraductal papillary mucinous carcinoma (IPMC) with atypical manifestations. In one case, we discussed a pseudomyxoma peritonei caused by a ruptured IPMC. In the other case we discussed the fistulization of IPMC into the stomach and duodenum. These two cases suggest that IPMN can either spontaneously rupture causing mucinous materials to spill into the free abdominal cavity or directly invade adjacent organs resulting in fistula development.

Key words: Intraductal papillary mucinous neoplasm; Intraductal papillary mucinous carcinoma; Pseudomyxoma peritonei; Fistula

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INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) is a rare and relatively slow growing pancreatic tumor characterized by papillary growth and mucin production within the pancreatic duct system[1,2]. Recently the number of publications regarding IPMN has risen sharply and contributed significantly to our current understanding of its clinical behavior. As a result, it is well established that IPMNs represent a spectrum of mucin-containing tumors that range from benign histology to invasive carcinoma[3,4]. While non-invasive IPMNs display slow growth and good prognosis after resection, the outcome can become poor if these tumors transform into invasive forms. A meeting of international experts on precursor lesions of pancreatic cancer held at The Johns Hopkins Hospital speculated on the possibility that some invasive IPMNs may begin as PanINs that then progress faster along a different pathway of neoplasia than conventional pancreatic ductal adenocarcinoma[5].

Here, we report two cases of intraductal papillary mucinous carcinoma (IPMC) with atypical manifestations which show aggressive features of this disease. In one scenario, a pseudomyxoma peritonei was caused by a ruptured IPMC. In the other case, we discussed the fistulization of IPMC into the stomach and duodenum.

CASE REPORT

Case 1

A 55-year-old man was admitted to our hospital due to incidentally detected ruptured pancreatic cystic mass and large amounts of fluid collection around the mass. His past medical history was significant for an appendectomy secondary to an acute appendicitis 35 years ago. At that time, there were no abnormal findings in the abdomen except for acute suppurative appendicitis. On admission, physical examination was unremarkable. Laboratory studies, including the level of serum tumor markers, were normal. The ultrasonography-assisted aspirated fluid was gelatinous with a high concentration of CEA at 1870 μg/L and CA19-9 at 8 U/L. Computed tomography showed massive ascites throughout the peritoneal space and a 5 cm-sized ruptured cystic mass in the pancreatic body and tail junction (Figure 1A and B). Endoscopic retrograde cholangiopancreatography (ERCP) showed a small outpouching lesion of the pancreatic duct in the body portion which was thought to be the same cystic lesion seen on the previous CT. During the operation, mucinous ascites was detected in the entire abdominal cavity. The patient was diagnosed with a pseudomyxoma peritonei (Figure 2A). In the body of the pancreas, there was an 8.5 cm × 6 cm-sized outpouching cystic mass, which ruptured and spread yellow-whitish gelatinous fluid around the mass (Figure 2B). Distal pancreatectomy and debulking of the mucinous material were performed. On microscopic examination, the resected specimen showed a growing papillary tumor characterized by adenocarcinoma

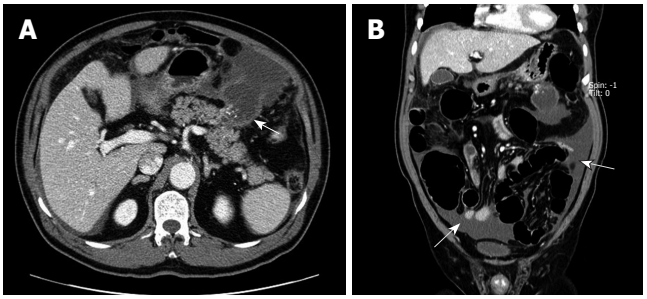

Figure 1 CT scan of case 1 showing a 5 cm-sized ruptured cystic mass in the pancreatic body and tail junction (arrow) (**A**) and massive ascites throughout the peritoneal space (arrow) (**B**).

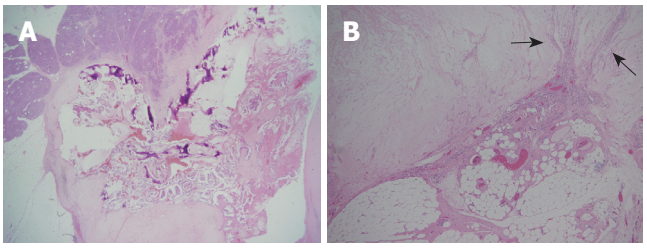

Figure 3 Microscopic examination of case 1 displaying papillary growth in the pancreatic duct (HE, × 12.5) (**A**) and infiltration of malignant cells into the pancreas parenchyma (HE, × 40, arrows) (**B**).

cells in the intrapancreatic duct with infiltration of malignant cells into the pancreatic parenchyma (Figure 3A and B). The patient's postoperative course was uneventful. The patient received postoperative systemic chemotherapy with gemcitabine (1000 mg/m^2) and cisplatin (60 mg/m^2) twice among planned six sessions. The patient was alive and well for 3 mo after operation without any evidence of disease progression.

Case 2

A 68-year-old man was admitted for epigastric discomfort. He was diagnosed with IPMN. Abdominal CT showed a huge mass in the pancreatic head with the possibility of stomach invasion. Clinicians recommended surgery as a potential treatment strategy but the patient refused and was later discharged. After 1.5 years, he again presented with epigastric discomfort and jaundice. Laboratory data showed 11.0 mg/dL total bilirubin, 68 IU/L aspartate aminotransferase, 105 IU/L alanine aminotrasferase, 609 IU/L alkaline phosphatase. The level of serum tumor marker CA 19-9 was 39 U/mL. Abdominal CT revealed a huge mass in the pancreatic head with diffuse pancreatic duct dilatation and bile duct dilatation with large fistulae between the mass and duodenal bulb and between the mass and posterior wall of the stomach antrum (Figure 4A and B). These findings were worse than his initial presentation 1.5 years ago. Gastroduodenoscopy showed a deep and huge ulcer in the antrum of the stomach with dirty mucinous material discharged from the patulous orifice of papilla and a suspicious communication between the pancreatic mass

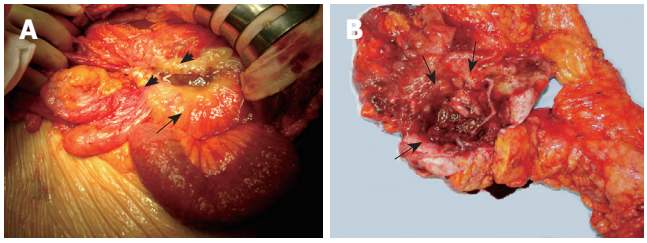

Figure 2 Operation of case 1 revealing a ruptured cystic mass at pancreatic tail (arrow) and spreaded mucinous materials around the mass (arrow heads) (**A**) and a ruptured mucinous cystic mass of pancreas (arrows) (**B**).

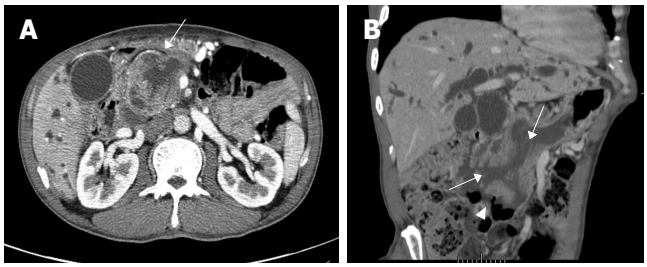

Figure 4 CT scan of case 2 demonstrating a 10 cm-sized cystic mass in pancreatic head (arrow) (**A**) and two communications between stomach and mass and between bulb and mass (arrows) and patulous ampulla of Vater (arrow head) (**B**).

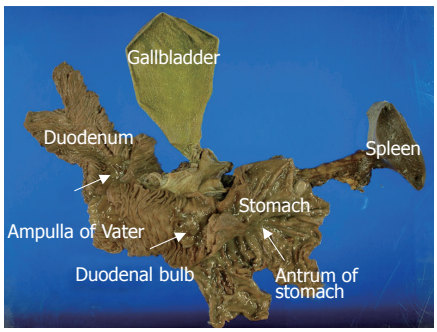

Figure 5 Specimen of case 2 showing two fistulous openings at stomach, duodenal bulb and patulous ampulla of Vater (arrows).

and duodenal bulb. At the time of operation, a 10 cm × 5.5 cm- sized huge cystic mass was found in the head of pancreas. Firm fibrous tissue was observed between the mass and the antrum of stomach. An en bloc resection of the pancreas, spleen, and distal stomach was performed. The distal stomach and duodenum were filled with sticky mucinous material. There were two fistulous openings in the specimen, one at posterior wall of the antral stomach and the other at the duodenal bulb (Figure 5). The diameter of the openings was approximately 2 to 3 finger breadths. The histological features were consistent with invasive intraductal papillary mucinous carcinoma and the fistula tract between the mass and duodenal bulb contained cancer cells. Granulation tissue was only found in the fistula tract between the mass and the antrum of stomach. The patient was discharged after an uneventful postoperative recovery. He received two cycles of concurrent chemoradiation therapy (a total of 20 Gy in 10 fractions with a concurrent intravenous infusion of 5-fluorouracil 500 mg/m^2 on d 1-3) and showed no signs of recurrence 3 mo after surgery.

Table 1 Summary of reported cases of pseudomyxoma peritonei combined with IPMN of pancreas

Reference	Age/Sex	Pathologic diagnosis	Pancreatectomy	Survival (mo)	
15	49/M	IPMN	Partial- > total	> 17	Recurrence 14 mo after total pancreatectomy
16	53/M	Unknown	None	> 24	
17	64/M	IPMC	Distal	> 6	
Present case	55/M	IPMC	Distal	> 3	

DISCUSSION

IPMN has been documented to infrequently result in acute pancreatitis and fistula formation[6-8]. However, there are no reports of a ruptured IPMN without any signs of acute pancreatitis and resulting in pseudomyxoma peritonei. Pseudomyxoma peritonei is a clinical condition characterized by the presence of large amounts of gelatinous tumor implants in the peritoneum. This disease was first reported by Rokitansky in 1842[9]. The most common underlying causes of this condition are mucinous neoplasms of the appendix and ovary[10,11]. Pseudomyxoma peritonei is rarely reported to be associated with IPMN. To date, there are only three reports of pseudomyxoma peritonei originating from IPMN of the pancreas (Table 1). The first case report was a 49-year old man who underwent total pancreatectomy for positive resection margins after pancreaticoduodenectomy due to chronic pancreatitis and was unexpectedly diagnosed histopathologically with IPMN[12]. Fourteen months later after total pancreatectomy, a bulky retroperitoneal mass was detected and a multilocular, thin-walled cystic mass was excised. The histological feature was consistent with pseudomyxoma peritonei. The second case was a 53-year old man who underwent a debulking operation due to a pseudomyxoma peritonei and was diagnosed with IPMN by imaging tools rather by histological confirmation[13]. In this case, the possibility of another source of pseudomyxoma peritonei could not be excluded. The third case was a 64-year old man who underwent distal pancreatectomy due to pseudomyxoma peritonei caused by acute pancreatitis in IPMC of the pancreas[14]. This case is similar to ours because the pseudomyxoma peritonei was associated with IPMC. However, in that case, the cause of pseudomyxoma peritonei was the fistula triggered by recurrent acute pancreatitis. Our case had no history of acute pancreatitis and spontaneous rupture was the cause of pseudomyxoma peritonei. The risk factors for spontaneous rupture of IPMN could be the high pressure in the main pancreatic duct filled with mucinous materials as in our case or the inflammatory stimulation by others factors such as acute pancreatitis.

Several treatment modalities including surgery, radiotherapy and chemotherapy have been utilized in the management of pseudomyxoma peritonei with varying degrees of success. The primary treatment of pseudomyxoma peritonei is surgery. Aggressive debulking of an intra-abdominal tumor is correlated with improved survival rates[15] and the effectiveness of subsequent adjuvant intraperitoneal or systemic chemotherapy[16]. Modern treatments include peritonectomy and intraperitoneal hypothermic chemoperfusion[10,11,17]. A recent report by Sugarbaker[18] suggested that an aggressive approach consisting of maximal surgical debulking and maximal regional chemotherapy has an impact on the outcome of the disease, but the optimal management remains controversial. In our case, debulking was done but it was not possible to remove the mucinous material completely. The patient also underwent adjuvant systemic chemotherapy with gemcitabine and cisplatin. Despite a typical pattern of progression of pseudomyxoma peritonei, the clinical course and individual survival depend significantly on the nature of the underlying neoplasm[15,19]. The natural history of IPMN is not well understood and therefore, controversies persist regarding treatment.

The first case suggested that IPMN could rupture spontaneously and cause pseudomyxoma peritonei. Additionally, dissemination of mucinous adenocarcinoma may also be caused iatrogenically not only by surgical intervention but also by endoscopic ultrasonography-guided fine needle aspiration biopsy[20,21]. Therefore, in patients with potentially resectable IPMN, the risk/benefit ratio of endoscopic ultrasonography-guided fine needle aspiration biopsy should be carefully considered.

Since fistula formation associated with an IPMN was first reported in 1980 as a pancreatobiliary fistula, several cases have been reported[6-8]. According to these reports, the organs most frequently affected by fistula formation are the duodenum, common bile duct, and stomach. Apparent cancer invasion is the main cause of fistula in most cases. There is only one case of IPMN associated with pancreatogastric fistula without invasive cancer[22]. The mechanism of such fistula formation without cancer spread could be explained by a combination of high pressure in the main pancreatic duct and inflammatory stimulation.

In the treatment of IPMN associated with fistula, the fistula should be removed regardless of cancer invasion to avoid dissemination. As usual, the extent of resection should depend on the extent of cancer invasion.

In conclusion, the two cases suggest that IPMN can either spontaneously rupture enabling mucinous materials to spread into the free abdominal cavity or directly invade adjacent organs resulting in pancreatic fistula development.

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CASE REPORT

Splenic abscess in a patient with fecal peritonitis

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Abstract

Splenic abscess is a rare entity normally associated with underlying diseases. We report a case of splenic abscess with large gas formation in a non-diabetic and non-immunosuppressed patient after surgery for colon perforation. The most frequent cause of splenic abscess is septic embolism arising from bacterial endocarditis. Splenic abscess has a high rate of mortality when it is diagnosed late. Computed tomography resolved any diagnostic doubt, and subsequent surgery confirmed the diagnosis.

Key words: Splenic abscess; Colon perforation

Delis SG, Maniatis PN, Triantopoulou C, Papailiou J, Dervenis C. Splenic abscess in a patient with fecal peritonitis. *World J Gastroenterol* 2007; 13(10): 1626-1627

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INTRODUCTION

The incidence of septic complications, such as abscess formation, in fecal peritonitis due to colon perforation has been reported in various organs[1,2]. The spleen is one of the least involved organs especially in non-diabetic and non-immunosuppressed patients[3,4]. In a recent review[5], out of 67 cases of splenic abscess, only one was due to colon perforation. We present a patient with persistent high fever two weeks after surgery due to colon perforation despite optimal treatment. An occult splenic abscess was found during diagnostic work up.

CASE REPORT

An 84-year-old Caucasian non-diabetic and non-immunosuppressed woman presented with abdominal tenderness and peritoneal irritation. Chest radiography revealed free air at the left subdiaphragmatic area confirming a hollow visceral perforation. An exploratory laparotomy revealed traumatic perforation of the sigmoid due to chicken bone with diffuse fecal peritonitis. The postoperative period was uneventful with complete recovery and the patient was discharged on the 8th postoperative day. Two weeks later the patient complained of high fever (40℃) and vague left upper quadrant pain. The chest X-ray documented left pleural effusion associated with a left subdiaphragmatic abscess by ultrasound. The white cell count was 13000 cells/mL with neutrophil predominance (85%). Computed tomography with contrast enhancement revealed a large splenic abscess associated with a vast amount of gas formation (Figure 1). The patient underwent exploratory laparotomy with splenectomy and recovered completely on the 7th postoperative day. Culture of the purulent material showed Enterococcus sensitive to vancomycin.

DISCUSSION

Splenic abscess is a very rare manifestation of fecal peritonitis especially in non-diabetic and non-immunocompromised patients[3,4]. Successful management requires a combination of early diagnosis and early surgery or imaging-guided intervention[6,7].

Splenic abscess can be identified by ultrasonography. However, ultrasonography can not discriminate between abscess and infarct in some cases, while large gas formation within the abscess may lead to technical difficulties. Gas formation when noted within splenic abscesses is associated mainly with gram negative bacillus infection. In a recent review[5], gas formation in splenic abscess was found in 11.9% of patients.

Computed tomography is the examination of choice. Splenic abscesses appear as focal areas of low attenuation. Usually there is no inflammatory rim. Gas collections are very rarely seen. Many abscesses remain uniformly hypoattenuating after contrast administration and do not show peripheral enhancement. In atypical cases, clinical presentation must be considered in order to make an accurate diagnosis.

In conclusion, after fecal peritonitis although an intra-abdominal abscess in the proximity of the primary lesion is rather common, an unexpected splenic abscess formation,

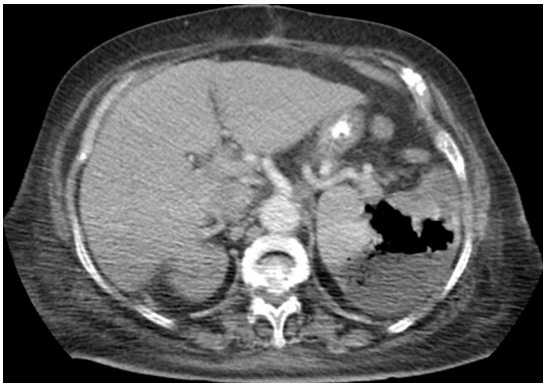

Figure 1 Computed tomography with contrast enhancement revealed a large splenic abscess associated with a vast amount of gas formation.

despite the patient immune status, could also be a rare but possible complication.

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CASE REPORT

Gynura root induces hepatic veno-occlusive disease: A case report and review of the literature

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Abstract

Gynura root has been used extensively in Chinese folk medicine and plays a role in promoting microcirculation and relieving pain. However, its hepatic toxicity should not be neglected. Recently, we admitted a 62-year old female who developed hepatic veno-occlusive disease (HVOD) after ingestion of Gynura root. Only a few articles on HVOD induced by Gynura root have been reported in the literature. It is suspected that pyrrolizidine alkaloids in Gynura root might be responsible for HVOD. In this paper, we report a case of HVOD and review the literature.

Key words: Hepatic veno-occlusive disease; Gynura root

Dai N, Yu YC, Ren TH, Wu JG, Jiang Y, Shen LG, Zhang J. Gynura root induces hepatic veno-occlusive disease: A case report and review of the literature. *World J Gastroenterol* 2007; 13(10): 1628-1631

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INTRODUCTION

Hepatic veno-occlusive disease (HVOD) is a clinical syndrome characterized by hyperbilirubinemia, painful hepatomegaly and weight gain due to fluid retention, after hematopoietic stem cell transplantation (HSCT), HVOD is a well-recognized life threatening complication, with an incidence rate of 10% to 60%[1]. In 1920, Willmot and Robertson[2] reported that HVOD is associated with the ingestion of Senecio tea, which contains pyrrolizidine alkaloids (PA). Other herb or plant medicine containing PA has been reported to cause hepatic injury and hepatic sinusoidal-obstruction syndrome[3]. A few cases of HVOD relating to Gynura root usage have been reported in Chinese literature[4-9]. Gynura root has been used extensively in Chinese folk medicine and plays a role in promoting microcirculation and relieving pain and curing injury.

Recently, we admitted a 62-year old female who presented with ascites, abdominal distention and was finally confirmed to have HVOD. This patient had a history of ingestion of Gynura root and experienced series of diagnostic approaches and various therapies. Since Gynura root or its analog may be used elsewhere in the world, we report the case and review the literature.

CASE REPORT

A 62-year old woman was admitted on January 23, 2006 to Hepato-gastroenterology Department of our hospital with abdominal complaints. She had abdominal distention after eating for about 10 mo. An abdominal ultrasound examination at a local hospital showed no remarkable findings. She had upper abdominal pain and weight gain in recent 3 mo. One week ago, abdominal pain became more severe, and repeated ultrasound examination revealed hepatomegaly and ascites. She had no fever and night sweats, no contact with sick persons or animals. Past medical history revealed asthma for over 20 years, which was treated occasionally with inhaler. She had no history of liver disease, alcohol abuse, pulmonary tuberculosis (TB). Family history was not significant.

Her initial vital signs were normal. Physical examination showed abdominal distention with upper abdominal tenderness, mildly dilated superficial abdominal veins, hepatomegaly (liver 7 cm below the xiphoid process), but no palpable spleen. Lower extremities showed no peripheral edema.

Laboratory tests showed normal hemoglobin (13.7 g/dL), normal WBC count (4700/μL), normal differentiatiation, but low platelets of 75000/μL. Serum total protein (62.1 g/L) and albumin (31.9 g/L) were slightly below normal (protein normal range 63-82 g/L, Albumin 35-53 g/L). Other liver function tests showed normal total bilirubin, direct bilirubin, alanine aminotransferase and gammaglutamyl transferase, but

slightly elevated aspartate aminotransferase 38 U/L (normal 5-35 U/L) and alkaline phosphatase 122 U/L (normal 30-110 U/L). Prothrombin time was normal and hepatitis B/C serology was negative. Serum tumor markers of alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and CA-199 were all normal but cancer antigen 125 (CA-125) was elevated (49.87 U/mL, normal range < 35 U/mL). Examination of ascetic fluid showed that the fluid was like exudate and transudate in appearance. The albumin level was elevated (20 g/L). The serum-ascites albumin gradient (SAAG) was 11.9 g/L. The ascitic cytology was negative for malignancy. Ziehl-Neelsen stain of the ascites was negative for TB. PPD test was negative.

An abdominal computerized tomography (CT) revealed hepatomegaly and residual ascites. No hepatic veins were visualized, suggesting that there was obstruction of hepatic vein outflow. No abnormality was seen in the pancreas, spleen, mesentery, retroperitoneal and pelvic organs. A digital subtraction angiography (DSA) showed normal left and right hepatic veins. There was no stricture or obstruction of the inferior vena cava, hepatic artery and portal vein. The patient was treated symptomatically with fluid restriction, diuretics,and albumin.

On her 23rd day of hospitalization (Feb 14), because of lack of clinical improvement and uncertainty of her diagnosis, exploratory laparoscopy was performed. At operation, about 3000 mL ascites was removed. Pelvic organs (uterus, ovaries and tubes), small and large intestines, stomach, omentum and diaphragm were normal. The liver appeared diffusely congested and the left lobe lateral segment was enlarged. Portal venous pressure of about 24 mmHg was measured in a branch of the mesenteric vein. Two biopsies of the liver were taken and histology showed that the central and sublobular veins and hepatic sinus were prominently congested and dilated. Hepatic cells appeared atrophic and degenerated. The walls of sublobular veins were thickened, and the hepatic venules revealed significant fibrosis. Based on the clinical manifestations and these histological findings, HVOD was confirmed.

One week later, because of increased abdominal distension and edema of lower legs and perineum, contrast enhanced timing robust angiography (ceMRA) was done showing narrowing of hepatic veins, uneven distribution of contrast material in hepatic parenchyma, suggesting venous congestion. The inferior vena cava, portal vein, abdominal aorta, and bilateral renal arteries appeared to be normal.

She had ingested Gynura root (*tu san qi*) for 3 mo before admission to our hospital for neck pain secondary to cervical osteophyte. She was treated with 3 slices of fresh Gynura root a day (about 2 g/d) soaked in rice wine in a bowl. Then she placed the bowl in a pot with water and steamed it. She ate the Gynura root and drank the wine. During the three months, she did not receive any Western medication and any other herbal remedy. We planted the Gynura root till it was full grown (Figure 1). The plant was identified as gynura segetum.

The patient was then treated with low molecular weight heparin and prostaglandin E1 and diuretics, *etc*, but her symptoms did not improve. Since methylprednisolone was tried for three weeks without much better effect, a transjugular intrahepatic portosystemic shunt (TIPS) was attempted on Mar 17. The puncture wire and needle could be passed through the hepatic veins into the portal vein branches. However, the stent could not be inserted because of the narrowing portal branches. TIPS failed. After a series of supportive therapy and consultations, the patient was transferred to the Surgery Department to undergo laparotomy and a mesocaval shunt was performed on Mar 29, 2006. At operation, the liver was found to be diffusely congested, the superior mesenteric vein was connected to the inferior vena cava with a 10 mm Gore-Tex graft. Initially, mesenteric venous pressure was 26 mm Hg. After the shunt was in place, the venous pressure decreased to 17 mmHg. After surgery, the patient received treatment with antibiotics, anticoagulant and tapering of her steroids. Anticoagulant was started from the 3rd post-operative day for 5 d, oral medication for two weeks, then subcutaneously for two more weeks. Two weeks post-operation, the patient developed pneumonia, treated and was discharged one month later. During follow-up examination in June of 2006, she had no abdominal distention nor pain. Repeated abdominal ultrasound study showed no ascites and the liver size was normal.

Figure 1 Gynura root and its plant gynura segetum (Lour.) Merr.

DISCUSSION

Since Gynura root is widely used in Chinese folk medicine, we searched the main Chinese medical database and found 6 Chinese publications with 21 cases of Gynura root-related HVOD[4-9], 11 of them were confirmed by liver biopsy or autopsy. The clinical characteristics of the Gynura root-related HVOD patients are summarized in Table 1. All the patients presented with hepatomegaly and ascites. Two patients died after ingestion of high doses of Gynura root (450 g and 1800 g within 10 d and 50 d respectively)[4]. Most of the patients had elevated total bilirubin, and only a few cases had a normal total bilirubin.

HVOD which was first described in 1920 by Willmot and Robertson[2] is associated with the ingestion of Senecio tea containing pyrrolizidine alkaloids (PA). In 1953, Hill *et al*[10] reported a large series of 150 Jamaican children who developed hepatic HVOD after drinking Senecio tea. More than 300 kinds of PA have been identified in over 6000 plants of the Compositae, Boraginaceae and Leguminosae

Table 1 Clinical characteristics of patients with Gynura root-related HVOD

Reference	Sex	Age (yr)	Reason for use of gynura root	duration	Total gynura (gram)	Total bilirubin (nl range[4])	Special tests of HVOD	Therapy for HVOD	Outcome
Hou JG, 1980[4]	M	57	Coronary heart disease	About 10 d	450	Normal	Autopsy	Symptomatic	Died 5-d after admission
	F	48	Diabetes	About 50 d	1800	4.5 mg%	Ultrasound, liver scan, autopsy	Symptomatic	Died
Li JP 2001[5]	M	53	No detail	No detail	No detail	53.1 μmol/L (0-20.4)	Ultrasound, MRI, Biopsy	*In situ* liver transplantation	Well
Li ZM, 2005[6]	M	43	Back ache	20 d	150	No report	Ultrasound MRI, DSA, Biopsy	Methylprednisolone 40 mg Ⅳ q 12 h	Well
Chen WX, 2005[7]	M	52	After a fall	4 mo	360[1]	27 μmol/L (0-20.4)	Ultrasound, CT, Biopsy	*In situ* liver transplantation	Well 45-d after operation
	F	39	Trauma	3 mo	180[2]	25 μmol/L (0-20.4)	Ultrasound CT, Biopsy	Symptomatic treatment	Discharged without improvement
Yan Hong 2005[8]	M	29	nephrolith	20 d	200	No report	Ultrasound, CT, DSA, Biopsy	Prednisone	Well
Our case	F	62	Neck pain	3 mo	180[3]	17 μmol/L (0-20.4)	Ultrasound, CT, DSA, Biopsy, ceMRA	Mesocaval shunt	Well 2-mo later
Zhang GH 2006[9]	M6, F8	41-73	Trauma or fracture	5 d (mean)	300-700	Elevated in 12 cases	All had ultrasound, CT; 4 cases biopsy; 2 cases DSA	Low molecular weight heparin, aspirin and diuretics	Well

[1]Calculated from author's data of 3 gm/d for four months; [2]Calculated from author's data of 2 gm/d for three months; [3]Estimated from patient's description of 3 slices a day for three months; [4]nl range: abbreviation of normal level range.

families[3]. Gynura root belongs to the Compositae family.

Yuan *et al*[11] isolated six alkaloids from Gynura segetum (Lour.) Merr. Two of the six were identified as senecionine and seneciphylline, which are known to have hepatic toxicity. These PAs, which have minimal toxicity in their original form, are metabolized in the liver through CYP (P450 cytochrome) and become toxic metabolites. The latter can act on local liver cells to cause damage by cross-linking DNA[12,13]. Furthermore, PA can decrease glutathione (GSH) in sinusoidal endothelial cells[14]. This enhanced oxidative stress also can affect collagen α1 transcription directly and/or through the activation of hepatic stellate cells[15], finally leading to HVOD. If the liver becomes damaged, the pyrrolizidine metabolites can overflow and infiltrate the lung fluids and cause damage there. Pulmonary edema and pleural effusions may occur, sometimes resulting in fatalities with very high levels of PA ingestion[13].

Confirmation of HVOD is based on the histology examination of liver tissue. The hepatic sinus, central and sublobular vein are significantly congested and dilated in Gynura root-related HVOD, while the venular walls are thickened with collagen deposition, with or without infiltration of lymphocytes, monocytes and neutrophils with fatty degeneration and necrosis presented in hepatic cells[4-9].

Currently, various strategies are used for the treatment of HVOD. For herb-related HVOD, plants containing PA should be avoided and discontinued. Some patients may recover after symptomatic treatment such as fluid restriction, diuretics and albumin. Administration of methylprednisolone seems to be another favorable alternative for some HVOD patients[6,16]. For patients with serious HVOD who do not respond to medical therapy, early use of transjugular intrahepatic portosystemic shunt (TIPS) can be considered[17]. However, it was reported that TIPS should not recommended for patients with HVOD[17,18]. In our patient, TIPS was not performed and she recovered well after undergoing meso-caval shunt. The value of porto-systemic shunt in HVOD remains to be further investigated. For those critically ill patients without response to porto-systemic shunt, liver transplantation might be considered with a survival rate of about 30%[19]. Li *et al*[6] and Chen *et al*[7] reported that two male cases of Gynura-root related HVOD recovered well over 40 d after liver transplant for hepatic failure.

Some researchers have suggested that PAs related safety problems are more widespread in certain areas of the world, such as Africa and South America[13]. Gynura root-related HVOD may be more widespread in China as well. Eighteen of these 22 patients (including our patient) were residents of Zhejiang Province, eastern coastal China, where more concerns are paid on Gynura root-related HVOD through organized case discussions between hospitals. Supervision and instruction of PA content in herbal medicine are crucial. The German Health Administration has set a standard for the use of herb petasites[13]. Further measures should be taken to determine and supervise herbs containing PAs to reduce their toxic effect, and make plentiful use of the Chinese traditional herbal medicine.

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CASE REPORT

A rare case of pregnancy complicated by mesenteric mass: What does chylous ascites tell us?

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Abstract

Mesenteric fibromatosis is a rare benign nonmetastatic neoplasm that appears as a sporadic lesion or occurs in patients with familial polyposis, while chylous ascites associated with aggressive mesenteric fibromatosis during pregnancy has never been reported thus far. Here we present the case of a 28-year old pregnant woman, in whom an aggressive mesenteric fibromatosis with chylous ascites was detected, involving the jejunum, superior mesenteric artery (SMA) and superior mesenteric vein (SMV) and pancreas. One year after a successful surgical excision, the patient had no signs of recurrence. The authors report the case for its rarity and emphasize on combining clinicopathological, radiological and immunohistochemistry analysis for management of the disease.

Key words: Mesenteric fibromatosis; Pregnancy; Chylous ascites

Sun L, Wu H, Zhuang YZ, Guan YS. A rare case of pregnancy complicated with mesenteric mass: What does chylous ascites tell us? *World J Gastroenterol* 2007; 13(10): 1632-1635

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INTRODUCTION

Chylous ascites is a rare clinical manifestation characterized by ascitic chylomicrons resulted from mechanical obstruction of or leakage from the lymphatic channel. chronic disorders, especially malignancies, accounting for most cases of chylous ascites. Mesenteric fibromatosis is a rare benign mesenteric tumor, characterized by infiltrative growth and high rates of recurrence[1]. There is no case report about mesenteric fibromatosis with chylous ascites, which is difficult to diagnose and differentiate from other tumors, thereby leading to diagnostic as well as therapeutic dilemmas[2]. Even when the diagnosis is well-established, mesenteric fibromatosis with chylous ascites still presents a management challenge for the surgeon to make an optimal therapeutic decision for the patient suffering from this disease[3,4].

CASE REPORT

A 28-year old woman was admitted 1 mo ago for a caesarean section operation and a healthy boy was born. However, 250 mL chylous ascites was found in her abdominal cavity during the operation. Sonographic examination detected a tumor in the left upper quadrant of the abdomen after her delivery. Multidetector spiral computed tomography (MDCT) implied that a mass was arising from the mesentery, semi-surrounding the superior mesenteric artery (SMA) and superior mesenteric vein (SMV) (Figure 1). Magnetic resonance imaging (MRI) revealed a tumor with low to intermediate mixed signal intensity in the lesion's periphery on T1 weighted images and bands of high signal intensity in the central region of the lesion on T2 weighted images (Figure 2). Digital subtraction angiography (DSA) demonstrated that the tumor's blood supply was poor and the endangium of SMA was smooth without lumen stenosis. A tumor was found about 6 cm beneath the suspensory ligament of duodenum during another laparotomy. The mesenteric vessels were almost semi-surrounded and the pancreas was adhered to the tumor tissue (Figure 3). The entire tumor with a loop of 86 cm jejunum was resected completely. The tumor measured 12.2 cm × 11.5 cm × 8.5 cm and was closely related to the bowel wall (Figure 4). The cut surface was tan-colored, whorled and firm, without necrosis, cystic change or haemorrhage. Microscopy of the resected slices showed loosely and haphazardly arranged spindle cells with bland, oval nuclei and minimal cytoplasm. There were also plump spindle cells with tapering ends containing oval, vesicular nuclei and a moderate amount of eosinophilic cytoplasm. The pancreas and bowel wall were infiltrated by the mass (Figure 5A). Subsequent immunohistochemistry

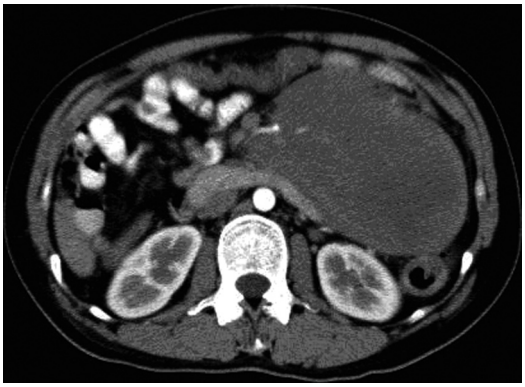

Figure 1 Contrast-enhanced axial scan of MDCT revealing a heterogeneous mesenteric huge soft tissue mass, which displaces the SMA.

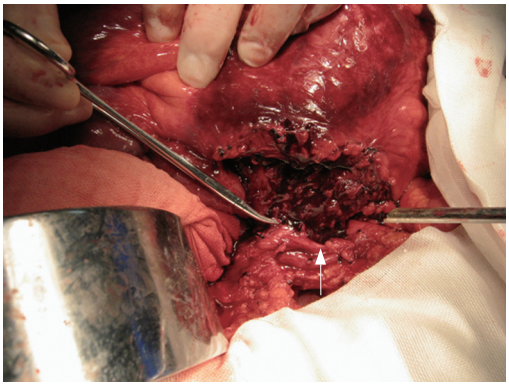

Figure 3 Semi-surrounding SMA and SMV (arrow) demonstrated during operation.

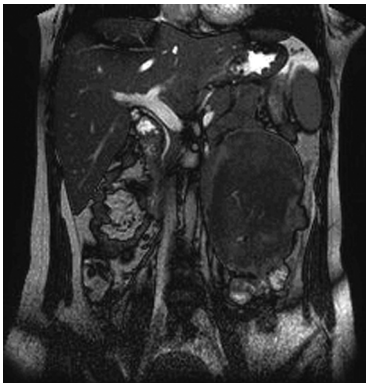

Figure 2 Coronal MRI image demonstrating a tumor with low to intermediate mixed signal intensity in the lesion's periphery and low signal intensity in the central region of the lesion on T2 weighted images.

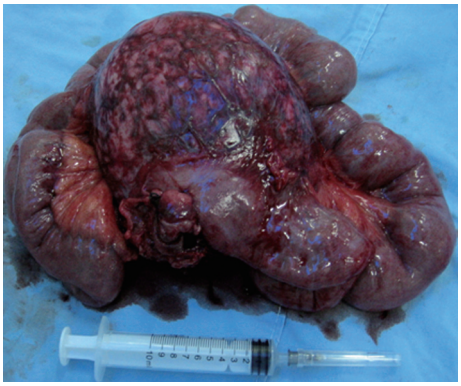

Figure 4 Resected small bowel with a well-circumscribed firm mass exhibiting an expanding growth pattern.

analysis had the following results: CD117 (-), CD34 (-), focal ACT (+), DES (-), S-100 (+++), β-catenin (-) (Figure 5B). A 12-mo follow-up period revealed a good condition of the patient.

DISCUSSION

Chylous ascites is brought about by lymph leaking into the abdominal cavity. Although chylous ascites sometimes may be caused by trauma, abdominal surgery, tuberculosis or other peritoneal infections, it is usually found as a symptom of tumor especially malignancies. There are few cases reporting chylous ascites during pregnancy, mainly caused by acute pancreatitis, small intestine volvulus and other conditions[5]. However, the symptoms and signs of an abdominal mass that grows during pregnancy are not easy to detect and identify. Aggressive mesenteric fibromatosis during pregnancy was rarely reported and aggressive mesenteric fibromatosis associated with chylous ascites has never been reported. Most cases of aggressive mesenteric fibromatosis reported in elderly individuals, are usually associated with familial polyposis coli, previous trauma, and hormonal imbalance[6]. Our case was a woman suffering from this disease during her pregnancy. The onset of the disease may be due to hormonal imbalance. The high level of serum lutin and estrin may induce the initiation of the disease in her gestation period[7]. Due to compression of the alveus ampullescens by the mass at the root of mesentery, lymphatic return was blocked as a result of chylous ascites formation.

Surgery provides good results in limiting the disease and non-surgical modalities are applied to cases with unresectable or residual lesions. In the preoperative evaluation of mesenteric fibromatosis, a careful investigation of its clinicopathological features is very important in selecting an appropriate approach to the surgical candidates[8]. Mesenteric fibromatosis is a rare, benign fibrous lesion originating from the bowel mesentery or the retroperitoneum. Characteristics its biological behavior present in the intermediate between benign fibrous tissue proliferation and fibrosarcoma. Fibromatosis has a typical inclination towards involving the visceral abdominal structures in its growth, and tends to recur but does not metastasize[9]. During the laparotomy, our case revealed a large mesenteric mass arising from the mesentery. The lesion was surrounded by a segment of intestinal canal and adhered to the pancreas. Semi-enclosed by the tumor tissue, the mesenteric vessels were displaced from their normal position. The tumors were excised together with a segment of jejunum.

Radiological studies play a significant role in establishing the diagnosis, working out the operation plan and evaluating the situation in follow-up of mesenteric fibromatosis. MDCT has an advantage of characterizing the mass in showing the homogeneity of attenuation, exhibiting slight enhancement after contrast medium administration. MDCT also provides a concise evaluation of the extension of the lesion. Images of reconstructed

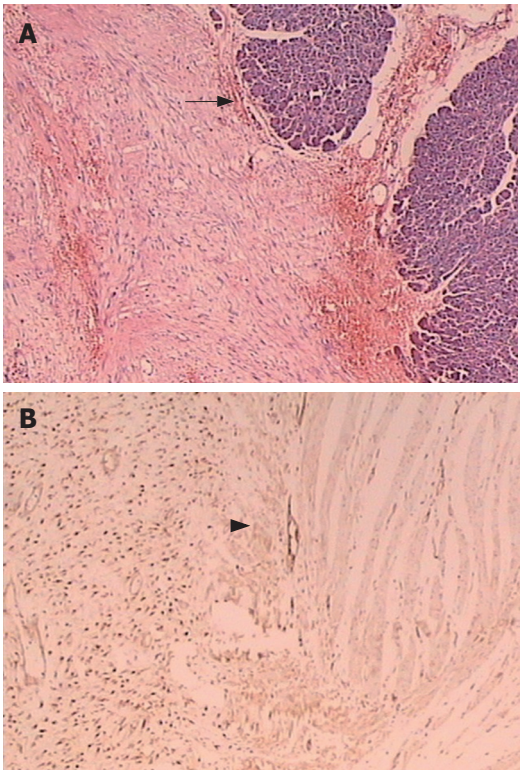

Figure 5 Tumoral lesion consisted of spindle cells growing in sweeping fascicles, with eosinophilic cytoplasm and occasional focal infiltration of plump nuclei in pancreas (arrow) (**A**) and bowel wall (arrow head) (**B**). Most of the tumor cells showed immunoreactivity for β-catenin (+++).

sagittal, coronal, or curved planes are very useful in most cases[10]. MRI may show characteristic features in prominent low to intermediate signal intensity and bands of low signal intensity representing highly collagenized tissue. However, fibromatoses with less collagen and more cellularity may have nonspecific high signal intensity on T2-weighted images[11]. DSA clearly depicts the bole and branch of SMA, which extend from the root of the mesentery towards the pelvis. Furthermore, it provides a clear description of the SMA and SMV lumen condition, confirming whether the main vasculature has been infiltrated[12]. Such unique imaging approaches may ultimately improve lesion detection, characterization, and surgical planning of the case.

Immunohistochemical analysis is very important for distinguishing mesenteric fibromatosis from gastrointestinal stromal tumor, which is clinically important in the Gleevec era[13]. Pathologists must differentiate this condition from a host of fibroblastic and myofibroblastic lesions or from smooth muscle neoplasms. Almost all-deep fibromatoses have somatic beta-catenin or adenomatous polyposis coli gene mutations leading to intranuclear accumulation of beta-catenin[14]. Since low-grade sarcomas in general lack beta-catenin and reactive proliferations are not expected to have it, nuclear beta-catenin expression would be detected in deep fibromatoses but absent in other entities in the differential diagnosis[15]. Beta-catenin immunohistochemistry separates deep fibromatosis from entities in the differential diagnosis, a finding that can be exploited for diagnosis[13,16]. Most fibromatoses have diffused nuclear staining and focal staining may be occasionally seen.

In conclusion, aggressive mesenteric fibromatosis with chylous ascites during pregnancy has never been reported, and it is not easy to make a diagnosis before and after operation. Radiological studies have a great value in designing management of the disease. In preoperative evaluation of mesenteric fibromatosis, its clinicopathological features are very important for working out an appropriate operation plan for surgical candidates. Immunohistochemical analysis of specimens offers the final diagnosis of the disease that may be confused with other kinds of tumor. Our report highlights the need of combining clinicopathological, radiological, and immunohistochemistry analysis in management of the disease.

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symposia@falkfoundation.de

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easl2007@easl.ch
www.easl.ch/liver-meeting/

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