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INTRODUCTION

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EDITORIAL

Influence of genes, sex, age and environment on the onset of autoimmune hepatitis

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Abstract

The pathogenesis of autoimmune hepatitis (AIH) is complex. However, it is believed that a susceptible individual, owing to his genetic background, sex and age, can develop the disease following exposure to an environmental trigger. Autoimmune hepatitis does not follow a Mendelian pattern of inheritance; hence no single causative genetic locus has been identified. However, several genes, inside and outside the HLA locus, have been linked to an increased susceptibility to AIH. Epidemiological evidence also suggests that the sex and age of the patient plays a role in AIH pathogenesis as the disease onset occurs mainly in the two first decades of life and a higher disease incidence is observed in females. No environmental trigger has been identified, but several have been proposed, mainly viruses and xenobiotics. This article aims at reviewing the current knowledge on susceptibility factors leading to AIH and putative triggers, emphasizing fundamental mechanisms responsible for the break of liver immunological tolerance.

Key words: Autoimmune hepatitis; Genetic; Environment; Sex; Virus

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INTRODUCTION

Autoimmune hepatitis (AIH) was described more than 50 years ago by Jan Gösta Waldenström and Henry George Kunkel[1] and patients were referred to as "Kunkel-Waldenström girls". In 1959, following the observation by Ian Mackay of lupus erythematosus (LE) cells in AIH patients, the disease was referred to as "lupoid hepatitis" as it was believed to be a form of lupus erythematosus[1]. As knowledge of symptoms, natural course of disease, pathogenesis and treatment progressed, the name "chronic active hepatitis" was chosen and finally "autoimmune hepatitis" was adopted at the first meeting of the International Autoimmune Hepatitis Group.

It is now believed that this autoimmune disease results from the progressive destruction of the hepatic parenchyma through a loss of immune tolerance towards hepatocytes. While the origin of the immune system dysregulation is still unknown, recent fundamental and clinical research have shed some light on predisposing factors and immune mechanisms involved. The current hypothesis for AIH pathogenesis is that this immune dysregulation is a consequence of an environmental triggering event in a genetically predisposed individual of a particular sex and age (Figure 1).

AUTOIMMUNE HEPATITIS: AN OVERVIEW

Autoimmune hepatitis is a disease with a chronic, but fluctuating course. Although primarily a female pediatric disease, autoimmune hepatitis is not limited to patients of a particular sex, age or ethnic group. Epidemiologic studies estimate the prevalence of AIH to be between 50 to 200 cases per million in Caucasian populations of Europe and North-America[2,3]. It is characterized by hypergammaglobulinemia, circulating autoantibodies, low levels of complement factor 4a (C4A) and a high prevalence of a HLA B8, DR3 and DR4 haplotype[4]. AIH clinical presentation can vary, from an acute to chronic hepatitis. Nonspecific symptoms include fatigue,

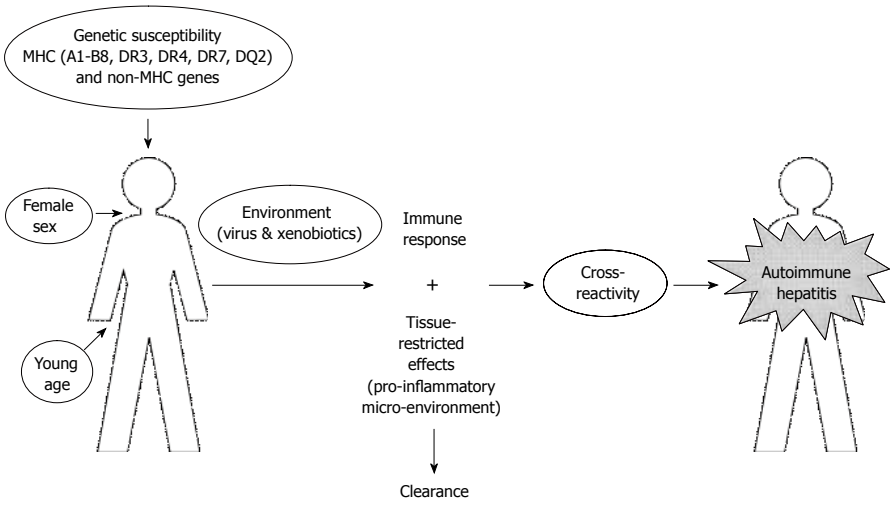

Figure 1 Pathogenesis of autoimmune hepatitis (molecular mimicry hypothesis). AIH can occur in an individual of a particular sex and age with a genetic background of susceptibility. To develop AIH, this individual must encounter an environmental trigger such as an infection or exposure to a xenobiotic. This will induce an immune response and secretion of pro-inflammatory molecules resulting in the elimination of the pathogen. However, this response may also lead to an immune cross-reactivity with liver proteins with an efficient activation of autoreactive cells leading to a break of immunological tolerance towards the liver.

Table 1 Clinical and biochemical characteristics of type 1 and type 2 autoimmune hepatitis

	Characteristics
Type 1 AIH	Anti-SMA, anti-ANA and/or anti-SLA
	Mean age of onset: 10 yr old
	Female:Male ratio 3:1
	Higher incidence
	Frequently associated with IBD and sclerosing cholangitis
Type 2 AIH	Anti-LKM1 and/or anti-LC1
	Mean age of onset: 6.5 yr old
	Female:Male ratio 9:1
	Associated with HLA DR3, DR7 and DQB1*0201
	More severe disease
	More likely to be resistant to treatment

anorexia and weight loss. Most AIH patients respond well to immunosuppressive treatments. The diagnosis of AIH is made according to a scoring system established by the International Autoimmune Hepatitis Group[4,5] which encompasses several clinical parameters and predisposing factors.

Liver histology in autoimmune hepatitis patients is usually characterized by portal and periportal inflammation (interface hepatitis) and lobular hepatitis. Approximately 50% of biopsies show some degree of bridging necrosis[4]. Infiltrates are mainly composed of a mononuclear cells with an abundance of plasma cells. Ten to twenty percents of liver biopsies also show multinucleated giant hepatocytes[4]. At diagnosis, portal fibrosis can be present ranging from an enlargement of the portal tract to cirrhosis.

Autoimmune hepatitis has been classified into two types according to circulating autoantibodies present in patients' sera. Type 1 AIH is characterized by the presence of anti-nuclear antibodies (ANA) and/or anti-smooth muscle antibodies (SMA) and/or anti-soluble liver antigen (SLA)[6,7]. Type 2 AIH patients are defined by the presence of circulating anti-liver-kidney microsome antibodies (LKM1) and/or anti-liver cytosol 1 antibodies (LC1)[8,9]. Anti-LKM1 antibodies recognize the cytochrome P4502D6 (CYP2D6)[10] and anti-LC1 antibodies react against the formiminotransferase cyclodeaminase (FTCD)[9]. Both CYP2D6 and FTCD are mainly expressed by hepatocytes. The incidence ratio of type 1 to type 2 AIH is 1.5 to 2:1 in Europe and reaches 7:1 in North and South America and Japan[11]. Several clinical differences exist between type 1 and 2 AIH (Table 1). The mean age of onset is 10 years in type 1 AIH and 6.5 years in type 2 AIH, and the female to male ratio is higher in type 2 AIH[6,12]. Type 2 AIH patients frequently present a more severe disease course and are more likely to be resistant to treatment[13]. In addition, associated extrahepatic autoimmune diseases are different in type 1 and 2 AIH. For example, type 1 AIH is more frequently associated with inflammatory bowel diseases and sclerosing cholangitis, diseases that are never observed in type 2 AIH[6,12].

GENETIC SUSCEPTIBILITY

Autoimmune hepatitis does not follow a Mendelian pattern of inheritance and no single genetic locus has been identified as responsible for the disease. It is generally believed that one or more genes, acting alone or in concert, reduce or increase susceptibility to AIH.

The strongest association between genes and autoimmune hepatitis has been found at the human leukocyte antigen (HLA) locus on chromosome 6. Susceptibility alleles have been identified in several populations (Table 2). In North America and Europe, HLA-A1-B8, HLA-DRB1*0301 and HLA-DRB1*0401 (DR3 & DR4) have been associated with a susceptibility to AIH[14,15]. Through a linkage disequilibrium study in families of AIH patients, HLA-DRB1*03 (DR3) and DRB1*1301 (DR13) as well as HLA-DQB1*0201 were found to be preferentially transmitted to patients compared to unaffected siblings in type 1 and type 2 AIH, respectively[16]. Another genetic study proposed that HLA-DR13 could be a risk factor in the absence of HLA-DR3 or HLA-DR4[17]. However, the size of the population studied did not allow reaching statistically significant conclusions. Hence this association needs confirmation.

Other HLA alleles have also been described as risk

Table 2 Specific susceptibility genes in type 1 and type 2 autoimmune hepatitis

Genes	Population	Type of AIH	Linkage disequilibrium	References
MHC genes				
HLA-A1-B8	North-America, Europe	1	Yes	[14, 15]
HLA-DRB1*0301	North-America, UK, Spain, Argentina	1 and 2	Yes	[14, 15, 25]
HLA-DRB1*0401	North-America, Europe		ND	[14, 15]
HLA-DRB1*0404	Mexico	1	ND	[18]
HLA-DRB1*0405	Argentina, Japan	1	-	[19, 20]
HLA-DRB1*1301	North-America, Europe, Brazil, Argentina	1	Yes	[16, 21]
HLA-DRB1-07	Germany, Brazil, UK	2	Yes	[23, 24]
HLA-DRB3*01	Brazil			[22, 23]
HLA-DQB1*0201	North-America, Europe	2	Yes	[16]
HLA-DQB1*0603	North-America, Europe	2	Yes	[16]
Non-MHC genes				
IgA	Europe	1		[28, 29]
C4A	Europe, North-America	1 and 2	Yes	[30, 31]
CTLA4	North-America, Europe	1	Yes	[35, 36]
Fas	Japan, North-America	1	ND	[37, 38]
Vitamin D receptor	Germany	1 and 2	ND	[39]
TNFa*2	North-America, UK	1	Yes	[43, 44]

ND: Non-determined.

factors for autoimmune hepatitis in other populations (Table 2). Among Mestizo Mexicans, HLA-DRB1*0404 is predominant in adult AIH patients[18]. In Japan and Argentina, HLA-DRB1*0405 has been associated with AIH[19,20] while in Brazil, HLA-DRB1*1301 and DRB3*01 are associated with the disease[19,21,22]. In type 2 autoimmune hepatitis, HLA-DRB1*07 has been associated in German, Brazilian and British populations while HLA-DRB1*03 was found as a risk factor in Spanish patients[23-25]. These differences in susceptibility alleles among various ethnic groups could be explained by the shared motif hypothesis which proposes that multiple alleles can encode for similar motifs within HLA class Ⅱ. In 94% of type 1 AIH patients, susceptibility alleles encode the LLEQKR or LLEQRR motifs at position 67-72 of class Ⅱ HLA[15,26]. In contrast, HLA-DB1*1501, which is associated with a reduced risk to develop type 1 AIH, encodes for the ILEQAR motif[15,26]. Substitution of a lysine or arginine to alanine at position 71, which changes both polarity and charge, possibly modifying peptide binding and orientation in the MHC, could influence autoantigen presentation to T cell receptors (TCR).

HLA alleles have also been found to influence the autoantigenic humoral response. In a recent study, HLA-DQB1*0201 was described as the main allele in association with susceptibility to type 2 AIH[27]. DQ2 is in linkage disequilibrium with DR3 or DR7, both associated with type 2 AIH. Interestingly, HLA-DRB1*03 was found associated with type 2 AIH patients which show both LKM1 and LC1 antibodies in their sera, while HLA-DRB1*07 was predominant amongst type 2 AIH patients, whose sole serological marker was anti-LKM1[27]. In addition, children carrying the HLA-DRB1*07 allele developed a more restricted repertoire of anti-LKM1 epitopes compared to those carrying the HLA-DRB1*03 allele[27].

Other genes located at the HLA locus are linked with AIH susceptibility, such as the IgA and complement factor 4A genes[12]. IgA deficiency is common in AIH patients. This deficiency is genetically linked to the MHC locus, especially with HLA susceptibility alleles such as HLA-DR1 and HLA-DR7[28,29]. Also, low levels of C4a are found in 69% of children with AIH[30]. Complement factor 4a (C4a) has also been linked with AIH pathogenesis since deletions in the C4A gene were found in patients who develop AIH at a younger age[31].

Genes outside the HLA locus have also been linked with AIH using single nucleotide polymorphism (SNP) screening techniques. These genes encode proteins which influence either the innate or adaptive immune system. As in Graves' disease[32], multiple sclerosis[33] and coeliac disease[34], cytotoxic T-lymphocytes antigen 4 (CTLA-4) gene polymorphisms have been found in adult and children with type 1 AIH[35,36]. A linkage disequilibrium was also found in affected children compared to non-affected siblings[36]. A FAS gene promoter polymorphism (position -670) was found to influence susceptibility to AIH[37] and its progression, leading to a more aggressive disease with an early development of cirrhosis[38]. Recently, polymorphisms in the vitamin D receptor was shown to contribute to development of autoimmune liver diseases[39]. This receptor was found to have immunomodulatory functions such as macrophage and monocyte activation, inhibition of Th1 functions and prevention of dendritic cells differentiation[40-42]. Therefore, polymorphisms in the vitamin D receptor could influence the immune response towards autoantigens. Polymorphisms in the tumor necrosis factor-α (TNF-α) gene (TNFA*2) confers a susceptibility to AIH and influences the natural course of the disease. A G to A substitution at position -308 is believed to influence gene transcription and result in higher induced or constitutive levels of circulating TNF-α[43,44]. AIH patients who possess this polymorphism are prone to early disease development, are less likely to enter into remission and more prone to develop liver cirrhosis[44].

Mutations in the autoimmune regulator gene (AIRE)

responsible for the development of Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) in patients can also lead to AIH in 10% to 20% of cases[45]. The AIRE gene encodes for a transcription factor involved in the thymic negative selection of lymphocytes. Thus, mutations that impair this function could cause multiple autoimmune manifestations. However, studies on known AIRE mutations in patients with autoimmune liver diseases showed that the AIRE gene does not play a major role in their pathogenesis[32,46].

Genetic background influence on the development of an AIH has also been observed in an animal model of type 2 AIH[47]. Xenoimmunisation with plasmid DNA coding for type 2 human autoantigens was performed in three mouse strains which differ in their MHC and/or non-MHC genes[48]. C57BL/6 mice developed a severe AIH while 129/Sv mice, who share the same MHC alleles as with C57BL/6, but on a different non-MHC genetic background, had a mild AIH. In contrast, BALB/c mice, which differ in both MHC and non-MHC genetic background, did not develop AIH. These results highlight the importance of both MHC and non-MHC genes in the initiation and progression of an autoimmune response towards the liver after an environmental triggering event, xenoimmunisation in this case, occurs[48].

INFLUENCE OF SEX AND AGE ON AUTOIMMUNITY

Most autoimmune diseases show a striking sex difference in their incidence, women being affected more frequently than men[49]. Differences in incidence between women and men range from 20:1 in Sjogren's syndrome to 3:2 in multiple sclerosis[49]. Less frequently, the female to male ratio approaches 1:1, as in ulcerative colitis and diabetes[49].

In AIH, the female to male ratio ranges from 3:1, in type 1, to 9:1, in type 2 AIH[11]. Sex differences in the immune response are also observed in other liver diseases. For example, men are more likely to become chronic carriers of hepatitis B than women[50]. In addition, several studies have investigated the effect of donor:recipient sex matching in the outcome of orthotopic liver transplantation (OLT) and have found that male to female grafts have the most, and female to male the least favourable, outcome, both in terms of patient and graft survival[51].

This gender discrepancy could be the result of existing differences in basic immune responses between females and males. In fact, higher levels of antibodies and stronger T cell activation are observed in women after vaccination[52]. Women have higher absolute numbers of $CD4^+$ T-cells and produce higher levels of Th1 cytokines than men[53]. Interestingly, *in vitro* oestrogen increases Th1 cytokine production by T lymphocytes, while a decrease is observed in presence of androgen[54].

Age also influences the incidence of several autoimmune diseases suggesting the role of hormones in the pathogenesis of these diseases. AIH is primarily a pediatric disease; 40% of type 1 and 80% of type 2 AIH cases are diagnosed before the age of 18[6,12]. A second peak of incidence of AIH has also been reported in women after menopause[55]. The hormonal status of patients could be related to these prepubertal and post-menopausal peaks of incidence. In fact, sexual hormones are known to directly modulate immune responses and, by doing so, alter the development of autoimmune diseases. 17β-estradiol has been shown to suppress IL-2 secretion by T cells and inhibit IL-2 receptor expression in activated peripheral blood T cells[56]. *In vivo,* 17β-estradiol (E2) protects C57BL/6 mice from experimental autoimmune encephalomyelitis (EAE)[57]. However, 17β-estradiol was also shown to enhance susceptibility to experimental myasthenia gravis[58], experimental autoimmune uveoretinitis[59] and lupus[60]. Therefore, the effects mediated by estradiol on autoimmunity are diverse and not fully understood. Male sex hormones also affect immune responses. Testosterone can directly affect $CD4^+$ T cells *via* androgen receptors and induce increased secretion of IL-10, an anti-inflammatory cytokine[61]. Testosterone was also found to protect female SJL mice from developing EAE[62].

Hormonal status of AIH patients during pregnancy can also impact the disease course, with both improvement and exacerbations reported[63,64]. Patients who experience a remission of their disease during pregnancy generally have a disease flare-up after delivery[64]. In some cases, AIH is diagnosed in the first few months of pregnancy or post-partum[65].

Currently, no pathological mechanism and/or direct hormonal effect can explain these observations. Although epidemiological studies show the impact of sex and age on AIH, more research will be needed to understand the interaction of sex, age and autoimmunity.

ENVIRONMENTAL FACTORS

Environmental factors are thought to be the triggering event for the development of an AIH in genetically predisposed individuals of a particular sex and age. These environmental factors could be drugs, chemicals or viruses. They are believed to initiate the autoimmune response through several means: (1) non-specific activation of resting T cells; (2) modification or release of sequestered proteins; (3) cross-reactivity between virus and self-protein (molecular mimicry); and (4) modulation of gene expression.

Non-specific activation of resting T cells

Non-specific activation of resting T cells has been reported after various virus infections, e.g. Epstein-Barr virus (EBV). It could be speculated that resting autoimmune T cells become activated and proliferate leading to an AIH development. EBV infection preceding the onset of AIH has been reported in some patients[66-69]. While this mechanism could be involved in AIH development, more evidence is needed to confirm its role in AIH pathogenesis.

Xenobiotics could also be a non-specific activator of lymphocytes, as observed in a murine model of

immune-mediated hepatic injury induced by injections of Concanavalin A (ConA)[70]. Concanavalin A is a leptin that stimulates the release of various cytokines by lymphocytes, mainly INF-γ and TNF-α[71]. It can also directly stimulate T cells by binding the of MHC and induction of their proliferation[72]. This massive non-specific T cell activation results in hepatitis through a bystander effect mediated by INF-γ and TNF-α[70,71]. Although this murine model does not rely on an autoimmune reaction against the liver *per se*, it has allowed a better understanding of how xenobiotics could lead to a T cell dependent autoimmune disease.

Modification or release of sequestered protein

An ever growing list of drugs and chemicals has been linked with AIH development in humans. Among these, minocycline, a drug used to treat acne, has been frequently associated with liver autoimmunity[73-75]. Interestingly, when minocycline treatment is stopped, the AIH-like syndrome disappears. Herbal agents such as black cohosh[76], a herbal medicine used to treat menopausal symptoms, and dai-saiko-to[77], a herbal medicine used in Japan, have also been proposed as causative agents for AIH. Recently, a case report of 3 adults and meta-analysis of previous case reports has associated atorvastatin and simvastatin with AIH[78]. This report is significant since statins are amongst the most widely prescribed drugs. However, these patients were also genetically predisposed for AIH, being HLA-DR3, DR4 or DR7[78]. No mechanisms have been proposed to explain the autoimmune effects of these drugs and chemicals. Explanations may lie in the hepatotoxic effect of these chemicals, which could release autoantigens, up-regulate proteins expression (P450s, immunoregulatory proteins) or act as a hapten by modifying the hepatic protein, making them immunogenic.

In experimental models, potential mechanisms for xenobiotics resulting in an immune-mediated liver disease have been described. A model of primary biliary cirrhosis (PBC) in guinea pigs was developed by the injection of 6-bromohexoanate to mimic the lipoate moiety of PDC-E2, the main epitope recognized by anti-mitochondrial antibodies from sera of patients with PBC[79]. In this model, the disease becomes evident 18 mo after being exposed to 6-bromohexoanate. This may suggest that exposition to a xenobiotic and induction of a clinically apparent disease could be a long-term process, and should be taken into consideration in future cause-effect studies on xenobiotic exposition and AIH development.

Another mechanism which could explain the development of an AIH is that a hepatotropic viral infection could result in a release of sequestered autoantigens from hepatocytes within a pro-inflammatory environment. This would lead to an autoimmune reactivity towards hepatic antigens. Also, since hepatocytes can express the MHC class Ⅱ molecule during the course of a clinical hepatitis[80], a function normally reserved to antigen presenting cells (APC), they acquire the ability to specifically activate $CD4^+$ T cells and induce an immune response[80]. This hypothetic mechanism implies that hepatotropic viruses may trigger an autoimmune reactivity in a non-specific manner by giving activated immune cells access to autoantigens in a pro-inflammatory environment.

However, the liver is involved in the development of immunological tolerance towards oral antigens[81] and thus, has a tolerogenic immune environment resulting in poor immune cell activation and response. Experimental evidence shows that $CD4^+$ T cells activated by hepatocytes are more likely to become $CD4^+$Th2 cells and impair the $CD8^+$ T cell response[82]. Some studies suggest that efficient immune responses are difficult to elaborate in the liver compared to lymph nodes[83]. Furthermore, work from Bowen *et al*[84] showed that specific T cells directed against a liver antigen are not properly activated if this antigen is uniquely expressed in the liver. A break of tolerance towards liver antigens could only be observed when the liver antigen was also expressed in the periphery[47,84,85]. In light of these results, both hepatotropic and non-hepatotropic viruses should be considered as potential triggering events leading to the development of an AIH, through release of autoantigens in a pro-inflammatory environment and/or by molecular mimicry between viruses and autoantigens.

Molecular mimicry

In many autoimmune disorders, molecular mimicry between a virus and a self-protein has been hypothesized to be the key event leading to the disease. Molecular mimicry occurs when a virus protein sequence, structure or motif is shared with a self-protein. The immune system will mount a response against the virus but, in the process, will cross-react with a homologous self-protein. This immune cross-reactivity could evolve, under certain circumstances, into an autoimmune disease. This hypothesis has been proposed in several autoimmune disorders such as multiple sclerosis where homologies between several infectious agents and the myelin basic protein were found[86].

In two animal models, molecular mimicry was proven to be a possible triggering mechanism for AIH. In the TTR-nucleoprotein (NP) transgenic mouse, which expresses the lymphocytic choriomeningitis virus (LCMV) NP under the control of a liver-specific promoter, DNA vaccination with a plasmid coding for the LCMV-NP led to a liver-specific immune response and a progressive destruction of the hepatic parenchyma[85]. In this case, a molecular identity between the self-protein and the injected antigen was the triggering factor for AIH development. In a model of type 2 AIH, DNA-vaccination of wild-type C57BL/6 mice with a plasmid coding for human type 2 autoantigens, CYP2D6 and FTCD, led to a break of immune tolerance towards the murine homologues of these proteins (the CYP2D9 and murine FTCD). While the initial immune response was directed against the foreign human proteins (CYP2D6 and FTCD), a molecular mimicry with murine homologous proteins led to the development of an autoimmune response against

Table 3 Putative candidate viruses as triggering event in autoimmune hepatitis

Putative virus as AIH trigger event	Evidences	References
Hepatitis A virus	Case reports	[94-96]
Hepatitis B virus	Case reports	[97, 98]
Hepatitis C virus	Specific AIH autoantibodies	[87-89]
	Cross-Reactivity at T and B-cell level	[90-92]
Epstein-Barr virus	Case reports	[66-69]
Human herpes virus 6	Sequence similarities	[99]
Herpes simplex virus	Sequence similarities	[102]
	Cross-reactivity at B-cell level	[102]

the antigens. These mice developed anti-LKM1 and anti-LC1 autoantibodies and an AIH which shows striking similarities with human type 2 AIH[47]. This murine model of type 2 AIH proved that exposure to a foreign protein can break immunological tolerance against a hepatic self-protein and this, without prior liver damage. The fact that a hepatitis is not necessary to break the immunological tolerance towards liver antigens argues in favor of non-hepatotropic virus(es) being able to trigger AIH in humans.

In patients, links between specific viruses and an autoimmune disease are difficult to establish in part due to the hit-and-run effect. The triggering viral infection could have been cleared months or even years before clinical signs of an autoimmune disorder become apparent. The identification of a virus as a causative agent for AIH must therefore rely on epidemiological studies to establish links between a specific infection and the autoimmune disease. A major obstacle in the elaboration of these studies is the necessity of very large cohorts of patients. Since AIH is a disease of very low prevalence, these studies are very difficult, if not impossible, to perform. Therefore, current candidate viruses as putative causative agent for AIH result from published case reports and homologies between liver autoantigens and virus proteins (Table 3).

The hepatitis C virus (HCV) is probably the most studied infection present in AIH patients. Five to 10% of HCV infected patients show autoimmune features that are generally associated with AIH such as anti-LKM1, anti-LC1 and/or anti-SLA autoantibodies[87-89]. Purified anti-LKM1 antibodies from HCV-infected patients cross-reacted with the NS3 and NS5a purified proteins suggesting that CYP2D6 and those viral proteins shared similar structures[90]. A molecular mimicry at the B-cell level between a structural motif of CYP2D6 and HCV proteins could explain the production of anti-LKM1 antibodies in HCV-infected patients[90,91]. Another link between P450 and HCV was found at the T cell level by Kammer *et al*[92] who reported T cell cross-reactivity between P450 and the HCV core protein. Although HCV infection in some patients elicits autoimmune hepatitis-like immune responses through molecular mimicry mechanisms, HCV infection is not the triggering event in AIH patients. In fact, a study showed that very few patients with AIH had specific antibodies against HCV suggesting that most of them had never encountered this virus[93].

Other hepatitis-causing viruses such as hepatitis A and B viruses (HAV, HBV) have been proposed as triggers for AIH[94-98]. The Epstein-Barr virus (EBV), has been associated with AIH in several case reports and in a clinical follow-up of 13 patients[66-69]. These associations occurred in type 1 AIH patients; therefore, no molecular mimicry could be established since no specific autoantigens in type 1 AIH were identified. However, specific markers for these viral infections are not found in the majority of AIH patients.

In type 2 AIH, a putative molecular mimicry was found between two B cell epitopes of FTCD, the target of anti-LC1 autoantibodies, and sequences of the 101K antigenic virion protein and U50 protein from human herpes virus type 6 (HHV-6)[99]. Several homologies were also found between known epitopes of CYP2D6, targeted by anti-LKM1 antibodies, and proteins from HHV-6[99]. HHV-6 hepatotropism and its association with chronic and autoimmune hepatitis in children[100,101] makes this virus a plausible candidate for AIH onset.

A molecular mimicry has also been proposed between the main antigenic site of anti-LKM1 on CYP2D6 and herpes simplex virus (HSV-1) based on similarities between proteins sequences[102]. Cross-reactivity has been found between CYP2D6 and a HSV-1 protein using purified anti-LKM1 autoantibodies from an AIH patient[102]. However, in this study, 4 out 20 patients had not encountered HSV-1 prior to the development of autoimmunity as determined by an antibody assay against HSV-1[102]. These data suggest that HSV-1 could be a trigger\for the development of AIH in some patients, although this remains to be confirmed.

Modulation of gene expression: the role of the innate immune system in autoimmunity

Environmental factors could trigger an autoimmune reaction by creating a pro-inflammatory immunological micro-environment in which autoantigens could be presented. Such a pro-inflammatory micro-environment could result from toll-like receptor (TLR) engagement. Members of the toll-like family of receptors are able to bind pathogen-associated molecular patterns (PAMP) present in most pathogens. By doing so, TLRs can induce a quick and efficient response against those pathogens through the up-regulation of key pro-inflammatory genes, such as type 1 interferons. When the liver undergoes an infection, TLR stimulation could result in the presentation of autoantigens in a pro-inflammatory environment which would result in an efficient activation of specific autoreactive cells.

The role of TLRs in autoimmune liver disease has been studied in PBC. It was shown that patients with PBC have higher levels of TLR3, TLR4 and TLR9 receptors in the liver[103,104]. *In vitro* stimulation of monocytes from PBC patients with several TLR-binding molecules resulted in higher levels of cytokine secretion[105]. PBMCs from PBC patients, when cultivated

with CpG, a TLR9 stimulator, secreted more IgM suggesting a role for TLR9 stimulation in the hyper-IgM observed in PBC patients[106]. Altogether, these studies suggest that TLRs could be involved in the pathogenesis of this autoimmune liver disease.

A break of immune tolerance towards the liver in a mouse model has been achieved by repeated CpG injections into a double-transgenic mouse expressing MHC class I molecule H-2K^b exclusively on hepatocytes and having T cells bearing specific TCR for MHC H-2K^b[107]. CpG injections were sufficient to break immune tolerance and induce a transient AIH, which faded when CpG injections were stopped. These data suggest that TLR9 stimulation is sufficient to activate pre-existing autoreactive T cells and initiate an autoimmune response, but is not sufficient to induce a self-perpetuating autoimmune response. Recently, Lang *et al*[108] were able to induce a liver inflammation by transferring liver-antigen specific CD8$^+$ T cells in combination with TLR9 and TLR3 stimulation. These results led to the speculation that the immunoprivileged status of the liver could be controlled by TLR signaling[108]. Altogether these data suggest that the innate immune system could be involved in the development of autoimmune processes in the liver possibly through expression up-regulation of pro-inflammatory genes.

CONCLUSION

The pathogenesis of AIH is a complex process. Development of this disease requires a series of events (viral infection and/or chemical exposure) in a suitable environment (genetic background of susceptibility, female sex and young age) (Figure 1). Further research, both clinical and fundamental, will be needed before the pathogenesis of AIH is fully understood. A better comprehension of the disease would allow the development of specific immunotherapies with fewer side effects.

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EDITORIAL

Update on autoimmune hepatitis

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Abstract

Autoimmune hepatitis (AIH) is a necroinflammatory liver disease of unknown etiology that occurs in children and adults of all ages. Characteristics are its autoimmune features, hyperglobulinemia (IgG), and the presence of circulating autoantibodies, as well as a response to immunosuppressant drugs. Current treatment consists of prednisone and azathioprine and in most patients this disease has become very treatable. Over the past 2 years, a couple of new insights into the genetic aspects, clinical course and treatment of AIH have been reported, which will be the focus of this review. In particular, we concentrate on genome-wide microsatellite analysis, a novel mouse model of AIH, the evaluation of a large AIH cohort for overlap syndromes, suggested novel criteria for the diagnosis of AIH, and the latest studies on treatment of AIH with budenoside and mycophenolate mofetil.

Key words: Autoimmune hepatitis; Autoimmune liver disease; Budesonide; Genetics; Mycophenolate mofetil; Overlap syndromes

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INTRODUCTION

Autoimmune hepatitis (AIH) is a necroinflammatory liver disease of unknown etiology that occurs in children and adults of all ages[1]. Most patients are female. Characteristics of the disease are a fluctuating spontaneous course of activity, hyperglobulinemia (IgG), and the presence of circulating autoantibodies, as well as a response to immunosuppressant drugs. However, AIH shows considerable heterogeneity[1]. No single clinical or biochemical test proves the presence of AIH. An exception may be the presence of soluble liver antigen/liver-pancreas (SLA/LP) autoantibodies. In 1992, the International Autoimmune Hepatitis Group recommended a scoring system for the diagnosis of AIH to allow reliable diagnosis of the disease, and this was further updated in 1999[2]. The sensitivity of the scoring system for AIH ranges from 97% to 100%, and its specificity for excluding chronic hepatitis C ranges from 66% to 92%[2]. Besides, variant, overlapping, or mixed forms of AIH, it shares common features with other putative autoimmune liver diseases such as primary biliary cirrhosis and primary sclerosing cholangitis[3]. Although some patients do present with acute liver failure and may need liver transplantation[4], the overall prognosis of AIH is mostly determined by response to corticosteroid therapy. Overall, long-term survival and average life expectancy are excellent and estimated to be comparable with those of the normal population[5].

IMMUNOPATHOGENESIS

Although the pathogenetic mechanism of the disease is still unknown, an underlying genetic predisposition has been suggested because of the fact that patients are predominantly female and the association of the disease with certain human leucocyte antigens (HLAs). HLA genes reside in the major histocompatibility complex (MHC), which is located on the short arm of chromosome 6. The MHC is a genetic system with extensive polymorphism. Although multiple genes are probably involved, HLA genes appear to play the dominant role in predisposition to AIH[6]. Particularly, HLA B8, DR3 and DR4 are found at a significantly higher frequency

in different populations with AIH[7,8]. The challenge is to investigate whether these findings help to better understand the etiology of AIH, predict its prognosis, or further improve its treatment.

DIAGNOSIS

The presentation of AIH is very heterogeneous, and may be characterized by an undulating course with periods of decreased or increased activity; thus, clinical manifestations are variable, ranging from asymptomatic disease to severe icteric hepatitis, and even fulminant hepatic failure that requires liver transplantation, depending on the intensity of the autoimmune reaction[1].

Patients may present with non-specific symptoms of varying severity, such as fatigue, lethargy, malaise, anorexia, nausea, abdominal pain, and itching. Arthralgia of the small joints is common. Physical examination may be without pathological findings, but may also reveal hepatomegaly, splenomegaly, jaundice, and signs and symptoms of chronic liver disease[1,9]. Many patients with acute presentation have histological evidence of chronic disease upon liver biopsy, which indicates that they probably have had subclinical disease for a long time (acute or chronic disease). Long periods of subclinical disease may also occur after presentation. In addition, diseases with an autoimmune background such as Hashimoto thyroiditis, ulcerative colitis, type 1 diabetes, rheumatoid arthritis, and celiac disease are more frequently found in patients with AIH[10].

In general, hepatitis with elevation of aspartate aminotransferase (AST) and alanine aminotransferase leads to the diagnosis of AIH. In particular, viral and toxic hepatitis must be excluded. Some cases, however, are characterized by cholestasis, with high levels of conjugated bilirubin and alkaline phosphatase. In such circumstances, extrahepatic obstruction and cholestatic forms of viral hepatitis, drug-induced disease, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and variant syndromes must be considered.

One characteristic laboratory feature of AIH is elevation of serum globulins, in particular, gamma globulin, with a selective increase in IgG, which is generally 1.2-3.0 times higher than the upper level of normal. The characteristic circulating autoantibodies seen in AIH include antinuclear antibodies (ANAs), smooth-muscle antibody (SMAs), SLA/LP autoantibodies, and liver-kidney microsome (LKM) autoantibodies. In addition, perinuclear antineutrophil cytoplasmic antibodies and liver-cytosol type 1 antibodies are frequently encountered in patients with AIH. Antimitochondrial antibodies are sometimes present in patients with AIH[11,12]. In these patients, an overlap syndrome of AIH and PBC should be considered[3]. However, it should be noted that autoantibodies are found in various liver diseases, and their presence, by itself, is not diagnostic of AIH. With respect to the pathogenesis of AIH, there is little evidence that autoantibodies play a crucial role.

Since there is no single test proving the diagnosis of AIH (an exception could be SLA/LP autoantibodies), liver histology remains of central importance. As percutaneous liver biopsy frequently suffers from a high rate of sampling error with respect to the staging of fibrosis and cirrhosis[13], we frequently perform (mini)laparoscopy-guided liver biopsy in AIH patients, particularly at initial diagnosis.

The histological appearance of AIH is the same as that of chronic hepatitis of other etiology, and although certain changes are characteristic, no findings are specific for AIH. AIH is generally characterized by a mononuclear-cell infiltrate that invades the limiting plate (periportal infiltrate, also called piecemeal necrosis or interface hepatitis that progresses to lobular hepatitis). There may be an abundance of plasma cells, a finding that in the past has led to the use of the term "plasma-cell hepatitis". Eosinophils are frequently present. The portal lesion generally spares the biliary tree. Fibrosis is present in all but the mildest forms of AIH. In advanced disease, fibrosis is extensive, and with the distortion of the hepatic lobule and the appearance of regenerative nodules, it results in cirrhosis[14].

TREATMENT

If untreated, severe AIH has a very high mortality rate of up to 50% after 3-5 years of diagnosis[15]. Immunosuppressive therapy with corticosteroids, usually in combination with azathioprine is considered the gold standard to induce and maintain remission. Moreover, response to immunosuppressive therapy confirms the diagnosis of AIH[16]. The therapeutic goal should be complete normalization of transaminases because progression to liver cirrhosis may occur in patients with residual inflammatory activity within the liver. However, side effects of therapy must be taken into consideration. The magnitude of aminotransferase and gamma globulin elevations does not necessarily correlate with the histological extent of injury and provides little help with respect to the initiation of treatment.

Under immunosuppression, the vast majority of patients achieve complete remission[17]. In patients not sufficiently responding to immunosuppressive therapy, the diagnosis of AIH should be thoroughly reevaluated. In some patients an overlap syndrome of AIH with PBC or PSC can be a reason for insufficient response to immunosuppression, and addition of ursodeoxycholic acid may further improve laboratory results.

Although some patients remain in remission after drug treatment is withdrawn, most require long-term maintenance therapy. Even though there is only scarce evidence for how long maintenace therapy should be given, it has been proposed that patients should be in stable remission for at least 4 years before withdrawal of immunosuppressive therapy can be considered[17]. However, patients positive for LKM1 antibodies (type 2 AIH) should be treated with life-long immunosuppression, which can only be stopped in patients with ANA- and SMA-positive AIH (type 1 AIH). Since biochemical response and clinical remission do not necessarily mean that there is histological evidence of resolution of AIH, repeated liver biopsy should be performed, particularly if withdrawal of immunosuppressive therapy is planned.

In the very few patients that do not tolerate or have

significant side effects to standard therapy, alternative immunosuppressive therapies have been proposed, mainly on the basis of small series or case reports. Cyclosporine appeared to be effective in a group of adult patients who were corticosteroid-resistant[18]. A regimen of cyclosporine for 6 mo followed by the administration of prednisone and azathioprine was reported as successful in inducing remission in children[19]. Limited data are available concerning the use of tacrolimus[20], methotrexate[21], cyclophosphamide[22], ursodiol[23] and mycophenolate mofetil (MMF)[24]. Data on a novel large study on budenoside in combination with azathiorpine are discussed below[25].

Although AIH primarily shows a chronic course with relapses under therapy (if immunosuppression is rapidly reduced), and particularly after discontinuation of immunosuppressive therapy, long-term prognosis is excellent, assuming close medical surveillance and/or treatment[5]. For some reason, the development of hepatocellular carcinoma is a very rare complication in patients with AIH, even though many patients have established liver cirrhosis at the time of diagnosis and patients are immunosuppressed. This fact might give further insight into the pathogenesis of liver carcinogenesis.

OVERLAP SYNDROMES-UPDATE 2008: SYSTEMATIC REVIEW REVEALS HIGH ASSOCIATION OF AIH WITH AUTOIMMUNE THYROIDITIS

Although the pathogenetic mechanisms of autoimmune diseases in various organs remain unresolved, an accumulation of autoimmune diseases in individual patients has been observed. An overlap of AIH and PBC or PSC has been well documented. However, overlap with autoimmune diseases other than PBC or PSC has not yet been investigated in a large cohort. In a systematic review of our cohort of 278 patients with AIH, 111 (40%) were diagnosed with additional autoimmune diseases. Besides overlap syndromes with PBC and PSC, autoimmune thyroiditis was the most common concurrent disease, and was diagnosed in 28 patients (10%). Other concurrent autoimmune diseases comprised vitiligo (five patients) rheumatoid arthritis (five patients), Sjogren´s syndrome (four patients) ulcerative colitis (four patients), conjunctivitis (four patients), celiac disease (three patients), systemic lupus erythematosus (two patients) type 1 diabetes (two patients), multiple sclerosis (two patients), polymyalgia rheumatica (two patients), and urticaria (two patients). One patient each was diagnosed with Crohn's disease, autoimmune gastritis, collagen colitis, hypophysitis and sarcoidosis. In conclusion, overlap with other autoimmune diseases is common in patients with AIH and mirrors the full range of known autoimmune diseases. Therefore, an extended diagnostic screening for accumulating autoimmune diseases seems reasonable in patients with AIH. In particular, monitoring for autoimmune thyroiditis must be considered mandatory as 10% of our patients with AIH developed such a condition[26].

GENETIC BASIS OF AIH-UPDATE 2008: A GENOME-WIDE DNA MICROSATELLITE STUDY REVEALS MICROSATELLITE ASSCOCIATIONS

Although the pathogenesis of the disease is still unknown, an underlying genetic predisposition has been suggested because of the fact that patients are predominantly female, and the well-documented association of the disease with certain HLAs. It has been suggested that multiple individual genes are involved in the development of AIH, mostly based on the identification of single nucleotide polymorphisms[27-30]. However, until recently, a genome-wide search for the underlying genetic mechanisms has not been performed.

Yokosawa *et al*[31] investigated 400 polymorphic microsatellite markers in 81 patients with type 1 AIH. These markers covered the complete genome, with an average spacing of 10.8 cM. Of these, two markers, on chromosome 11 and 18, D11S902 and D18S464, respectively, were demonstrated to be significantly associated with AIH. An additional seven markers (D2S367, D6S309, D9S273, D11S1320, D16S423, D17S938 and D18S68) were designated as candidate susceptibility regions. Furthermore, a total of 17 markers were suggested to be relevant for resistance towards AIH. To further narrow down the genetic basis to individual genes within these microsatellite regions, a 500-kb perimeter of the D11S902 and D18S464 regions was screeened for genes with a biological function that would fit into a potential pathogenetic mechanism of autoimmune liver disease. Several candidate genes such as PIK3C2A, ABCC8, KCNJ11 and VAPA were named to be involved in diverse cell functions that need to be further evaluated and investigated by means of molecular biology[31]. The genetic data were then integrated with clinical data on the course of the disease. However, no differences were seen in the clinical courses of patients with respect to their genetic background, mirrored by means of the identified microsatellite profile. The authors pointed out, that these differences in microsatellite profile were not observed in HLA-DR4-negative patients, a finding that needs to be further investigated and confirmed[31].

MODELING AIH-UPDATE 2008: A NOVEL MOUSE MODEL OF AIH

Mouse models of human diseases are of significant help in exploring the basic principles of disease development. However, modeling AIH in mice has been challenging. Concanavalin A (Con-A)-induced acute liver injury is considered to be a model of human AIH. Major criticisms of this model are that mice stimulated with Con A do not develop autoantibodies and that a single injection of Con A may induce rapid damage of liver cells, which culminates in lethal fulminant hepatitis, in contrast to the more chronic nature of AIH.

Kido *et al*[32] have now presented a new mouse model

of AIH based on an NTx- and PD-1 double knock out. By influencing the regulatory T cells (Tregs), these mice develop characteristics of AIH. Tregs are a specialized subpopulation of T cells. They function as suppressors of immune system activation, and maintain tolerance to autoantigens and immune system homeostasis. Tregs have been previously at the center of attention, as this T cell population has been demonstrated to be defective numerically and functionally in patients with AIH[33].

A recently described mouse model of AIH provides improved modeling of the disease, as the mice develop ANAs as well as CD4+ and CD8+ T-cell infiltration[32]. Furthermore, on a histological level, these NTx-PD-1 double knock out mice develop a significant mononuclear cell infiltration and massive lobular necrosis, without bile duct destruction and fibrosis. This model certainly needs further evaluation, with respect to the underlying pathogenetic mechanisms, especially as it has been demonstrated that simply deleting Tregs is not sufficient for inducing AIH. Furthermore, PD-1 deficiency alone does not alter the suppressive activity of Tregs. Thus, the details of PD-1/Treg interaction may be of great interest in the further appreciation of this novel mouse model of AIH. Nevertheless, providing this novel mouse model of AIH, Kido *et al*[32] have certainly discovered a powerful tool for genetic research on the development of AIH and additional treatment options.

DIAGNOSIS OF AIH-UPDATE 2008: SIMPLIFIED CRITERIA FOR THE EVALUATION OF AIH

Given the diverse clinical presentation of AIH, its diagnosis often remains challenging. To date, only SLA/LP autoantibodies are highly specific for the diagnosis of AIH[11]. However, they are only present in about 20% of patients. Currently, the diagnosis of AIH is made using the scoring system of The International Autoimmune Hepatitis Group. The scoring system has been demonstrated to be highly sensitive with a range from 97% to 100%, and its specificity for excluding chronic hepatitis C ranges from 66% to 92%[2]. However, handling of these criteria is rather laborious because they were primarily designed for scientific purposes.

Hennes *et al*[34] have now proposed shortened, easier-to-use criteria that consist of a set of only four relevant pieces of information. These clinical criteria are IgG, autoantbodies (ANA, SMA and SLA), histology, and exclusion of viral hepatitis. The novel shortened criteria have been demonstrated to either confirm or exclude the diagnosis of AIH with both positive and negative predictive values well over 90%.

In detail, the authors have proposed the following scoring system. Patients with IgG > 16 g/L, ANA and SMA > 1:40, and a compatible histology are assigned one point for each applicable criterion. Those with IgG > 18 g/L, ANA and SMA > 1:80, the presence of SLA/LP antibodies, histology compatible/typical for AIH, and negative viral markers are assigned two points per applicable criterion. In summary, six or more points made the diagnosis of AIH very likely, and seven or eight points confirmed the diagnosis of definite AIH (Table 1).

Table 1 Simplified criteria for the diagnosis of AIH by Hennes *et al*[35] (2008)

Criteria	1 point	2 points
IgG	> 16 g/L	> 18 g/L
ANAs, SMAs	> 1:40	> 1:80
SLA autoantibodies		Positive
Histology typical for AIH		Positive
Viral hepatitis markers		Negative
6 points: AIH very likely		
8 points: AIH confirmed		

Patients with IgG > 16 g/L, ANA and SMA > 1:40, and a compatible histology are assigned one point for each applicable criteria. Patients with IgG > 18 g/L, ANA and SMA > 1:80, presence of SLA/LP antibodies, histology compatible/typical for AIH, and negative viral markers are assigned two points per applicable criterion. Six or more points made the diagnosis of AIH very likely, and seven or eight points confirmed the diagnosis of definite AIH.

These novel criteria for the diagnosis of AIH provide a valuable simplification for daily clinical practice. However, they should be further investigated and confirmed by independend prospective studies[34].

UPDATE 2008-ALTERNATIVE TREATMENT OF AIH WITH MMF

In most cases, patients with AIH can be treated successfully with prednisone, with or without azathioprine. However, a considerable number of patients with AIH, and in need of immunosuppressive treatment, tolerate azathioprine only poorly, or do not respond efficiently to treatment[1]. A smaller number of patients will even fail to respond to this conventional therapy[1]. As discussed above, several other therapies have been studied for their effciacy in AIH, but these studies were small or single case reports. However, MMF has demonstrated encouraging results in a few studies on small cohorts of patients with AIH. Thus, MMF has increasingly shifted into the center of attention as an alternative treatment option of AIH.

MMF inhibits purine synthesis by acting as an inhibitor of inosine monophosphate dehydrogenase. MMF treatment has been established successfully in many other conditions, such as rheumatoid arthritis or Crohn's disease. In addition, the drug has become routinely used in immunosuppressant regimens in patients who have undergone solid organ transplantation.

Triggered by a first case report in 1998[35], several smaller studies have reported consecutively on successful treatment of AIH with MMF. Richardson *et al*[24] have reported on successful MMF treatment of seven patients. Five of these patients had normal transaminases after 3 mo treatment, as well as a siginificant reduction in steroid dose and hepatic activity index[24]. These findings were

further supported by a series of five Canadian patients who also benefited from transaminase normalization, a steroid sparing effect and histological remission[36]. Lately, Inductivo-Yu *et al*[37] and Chatur *et al*[38] have reported on an additional 31 patients with AIH being successfully treated with MMF. In addition to the benefits observed in the earlier case report series, Inductivo-Yu *et al*[37] have also documented that the inflammatory scores and Ishak fibrosis scores were decreased. These results have been further supported by observations from patients who had received a liver transplantation for AIH and required immunosuppressant therapy after transplantation. In these patients, MMF was also demonstrated to be part of the immunosuppressant regimen[39].

In 2008, Hennes *et al*[40] reported the largest cohort to date of 36 patients treated with MMF. In contrast to earlier studies, they observed a much lower frequency of response to MMF treatment, as only 14 patients (39%) experienced remission, which was defined as AST less than twice the upper limit of normal. Twenty-two patients (61%) did not respond sufficiently to MMF. In a subset analysis, they further demonstrated that the response rate to MMF was dependent on the cause of treatment cessation of azathioprine. Most patients with prior non-response to azathioprine did not respond to MMF treatment either.

MMF certainly provides a valuable therapeutic option in patients with AIH. However, the latest and, so far, largest study by Hennes *et al*[40] suggests that less than half of all patients may benefit from MMF. Thus, MMF may be a valuable alternative to azathioprine but does not seem to be an option for the treatment of AIH after azathioprine non-response.

TREATMENT OF AIH-UPDATE 2008: ESTABLISHING TREATMENT WITH BUDENOSIDE

Budesonide is a corticosteroid with the highest affinity for the glucocorticoid receptor when compared with other steroids. The drug has a high first pass metabolism, which results in a low incidence of systemic glucocorticoid-related adverse effects[41,42]. Budesonide has been demonstrated to be a highly efficient therapeutic option in the treatment of a wide range of diseases. Budesonide has been demonstrated to be a therapeutic option for inducing remission of Crohn's disease. It has been demonstrated to be less effective than conventional steroids, but also exhibited fewer adverse events and lower adrenal suppression[43]. Furthermore, the combination of budesonide and formoterol is an efficient alternative in asthma management in patients not adequately controlled by conventional regimens with short-acting β-adrenoceptor agonists[44].

Given a high efficiency in several inflammatory diseases and fewer adverse effects, treatment of AIH with budenoside may highly beneficial if its efficiency were comparable to the conventional prednisolone. Over the past decade, several smaller studies have suggested the efficacy of budenoside for remission induction[45]. However, one study failed to demonstrate such efficacy in AIH[46].

Recently, Manns *et al*[47] have compared combined budenoside and azathioprine to standard prednisolone treatment of 208 patients with AIH. The primary end point of the study was complete remission without typical steroid adverse effects, defined as facial swelling, diabetes mellitus, acne, hirsuitism, striae and glaucoma.

Budenoside treatment was initiated at 3 mg three times daily and was reduced to 3 mg twice daily when the patients reached clinical remission. Prednisolone was initiated at 40 mg/d and reduced to 10 mg/d in week 9. Azathioprine was administered to both groups at a dose of 1-2 mg/kg per day. In comparison between these two groups, significantly more patients in the budenoside group reached the predefined primary endpoint of biochemical remission without stypical adverse effects of steroids (47% *vs* 18.4%, $P < 0.00001$). Furthermore, for secondary endpoints, especially biochemical remission, budenoside was superior to prednisolone (60% *vs* 38.8%, $P = 0.00128$). However, for the long-term results of normalization of bilirubin and IgG over a 6-mo period, budenoside was not superior to prednisolone, as 83% *versus* 89.3% of patients experienced normalization of bilirubin and 56.0% *vs* 62.1% normalization of IgG. Although these data seemed convincing at first sight, the studies have been discussed controversially with respect to the prednisolone dose. A potentially too-low dose of prednisolone was considered to be responsible for a very low 18.6% remission rate. These results seem poor compared to the approximately 90% remission in previous studies on AIH treatment.

In this first large study, budenoside has been proven to be an efficacious alternative to prednisone, with a highly beneficial adverse effect profile in patients with AIH. However, long-term results of budenoside treatment have yet to be collected and data on longer follow-up are expected soon[47]. Special attention needs to be given to the question of a comparable prednisolone dosage in the (control) conventionally treated patients.

CONCLUSION

Over the past 2 years, substantial progress has been made in evaluating alternative treatment options for AIH. With respect to the pathophysiological changes that lead to the development of AIH, microsatellite studies have identified novel genomic regions that are involved in the development of the disease. Finally, the diagnosis of the disease may become easier if the novel shortened criteria for the diagnosis of AIH prove to be accurate in further studies.

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TOPIC HIGHLIGHT

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Spontaneous bacterial peritonitis

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Abstract

Since its initial description in 1964, research has transformed spontaneous bacterial peritonitis (SBP) from a feared disease (with reported mortality of 90%) to a treatable complication of decompensated cirrhosis, albeit with steady prevalence and a high recurrence rate. Bacterial translocation, the key mechanism in the pathogenesis of SBP, is only possible because of the concurrent failure of defensive mechanisms in cirrhosis. Variants of SBP should be treated. Leucocyte esterase reagent strips have managed to shorten the 'tap-to-shot' time, while future studies should look into their combined use with ascitic fluid pH. Third generation cephalosporins are the antibiotic of choice because they have a number of advantages. Renal dysfunction has been shown to be an independent predictor of mortality in patients with SBP. Albumin is felt to reduce the risk of renal impairment by improving effective intravascular volume, and by helping to bind pro-inflammatory molecules. Following a single episode of SBP, patients should have long-term antibiotic prophylaxis and be considered for liver transplantation.

Key words: Spontaneous bacterial peritonitis; Infection; Ascites; Leucocyte reagent strips; Portal hypertension; Ascites

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INTRODUCTION

It seems that all diseases or syndromes that comprise our routine differential diagnoses were, not so long ago, obscure clinical entities, at least until an astute clinician came across them. It was not any different for spontaneous bacterial peritonitis (SBP).

Although Laënnec's name had been connected with cirrhosis since the early 1800s, it was only much later that SBP was diagnosed as a separate entity. The papers of Kerr *et al*[1] and Conn[2], which were published within a year of each other, describe the infection of ascitic fluid in the absence of a contiguous source of infection or an intra-abdominal inflammatory focus. Although similar reports had been published in the French literature since 1893, Conn[2] was the one who eventually coined the term (SBP) in his 1964 paper.

Since then, further research has made the once-feared disease (early reported mortality of 90%)[2] a treatable complication of decompensated cirrhosis[3], albeit with a steady prevalence and high recurrence rate[4,5]. The plethora of publications has also led to national/international guidelines and recommendations over the last 10 years[5-11].

PATHOGENESIS

The importance of the liver as a bacterial filter is well established. However, it was Conn[2] who hypothesized that intestinal bacteria escaping into the blood stream cause prolonged bacteremia, and in turn, a

greater chance of ascitic fluid invasion[3]. Other early reports have emphasized the possibility of abdominal paracentesis-induced SBP[1,3], and certainly, prior to the use of stringent skin disinfection and protective clothing, the incidence of paracentesis-induced peritonitis would have been higher. The negative impact of this thinking created generations of clinicians who were hesitant and unsure about dealing with infective ascites. The persistence of researchers has helped to assuage concerns and has led to a more liberal and appropriate paracentesis protocol[12-14].

Bacterial translocation (BT), the key mechanism in the pathogenesis of SBP, is only possible because of the concurrent failure of defensive mechanisms in cirrhosis[15-19]. Since the early 1990s, on-going research has confirmed the intensity of BT in cirrhotic rats[15-18,20,21]. Investigators have also demonstrated pronounced impairment of gastrointestinal tract motility in cirrhosis[22-24]. The disturbance of gut flora microecology that follows, in association with changes in the (ultra)structure of the gastrointestinal tract[25-27] and reduced local and humoral immunity paves the way for the relatively free flow of microorganisms and/or endotoxins to the mesenteric lymph nodes[25-27].

CLINICAL MANIFESTATIONS OF SBP

The clinical manifestations of SBP are subtle and require a high index of suspicion (Table 1). Previously, there was often delay in diagnosis, which led to considerable mortality and morbidity[28].

SBP almost always occurs in large volume ascites, in patients with liver cirrhosis. Ascites of other causes or low volume rarely gives rise to SBP. Patients with cirrhosis usually have hypothermia; therefore, any temperature > 37.8℃ should be investigated, unless it is clearly caused by flu-like symptoms. The necessary investigations are full blood count (FBC), urinalysis, ascitic fluid cell count, and ascites, blood and urine culture. Fever caused by SBP is differentiated from that of alcoholic hepatitis, in which the ascitic fluid neutrophil count is normal[28]. Alterations in mental status may be subtle and only apparent to someone close to the patient. A connect-the-number test, e.g. Reitan trail test, is preferable to testing serum ammonia levels[29]. Abdominal pain can be continuous and is different from tense ascites. Tenderness is a common feature. Paralytic ileus, hypotension and hypothermia are seen in advanced illness, where prognosis may be dire. Thirteen percent of patients have no signs or symptoms[28]. A 'diagnostic tap' should be performed in all patients with ascites admitted to hospital. SBP in outpatients with cirrhotic ascites is less frequent, occurs in patients with less advanced liver disease, and may have a better outcome than its counterpart in hospitalized patients[30]. A retrospective review of 916 outpatient AF samples from the United States showed that abnormal AF appearance had a sensitivity of 98.1% [(95% confidence interval (CI): 95.3%-99.5%] and a specificity of 22.7% (95% CI: 19.4%-26.3%) in the detection of SBP[31]. For out- and inpatients, laboratory abnormalities such as leukocytosis, metabolic acidosis and azotemia, should prompt investigations for SBP, even in the absence of other clinical features.

Table 1 Symptoms and signs of ascitic fluid infection

Symptom or sign	Frequency (%)		
	SBP	Bacterascites	CNNA
Fever	68	57	50
Abdominal pain	49	32	72
Abdominal tenderness	39	32	44
Rebound	10	5	0
Altered mental status	54	50	61

TECHNIQUES AND LABORATORY DIAGNOSIS

The process of ascitic fluid analysis has come a long way. Inspections for color and transparency (as first evidence of infection) will probably always be carried out. Practice from this point forward, however, varies between regions and to a lesser extent, between hospitals. Over the last decade, it seems that a selective, possibly common-sense approach has started to prevail over the light-hearted dictum "send it (AF) for everything".

The diagnostic algorithm proposed by Runyon[28] (Figure 1) remains the most logical and cost-effective way to handle an abdominal paracentesis specimen, and we recommend that every gastrointestinal (GI) ward should have a laminated copy readily available in the doctors' office or protocol folder. Diagnostic paracentesis is now regarded as a safe procedure. Undoubtedly, there are complications inherent with the test, but the incidence rate of these is low[32-34]. The reported risks of diagnostic paracentesis include bleeding (hemoperitoneum or abdominal wall hematoma), visceral perforation, local infection at the site of paracentesis, or peritonitis. However, the most common complication is persistent leak. Post-procedural bleeding risk is very low, not only for diagnostic, but also for therapeutic taps[33-36]. Runyon has suggested that the practice of attempting to correct any coagulopathy prior to paracentesis is not cost-effective[28]. The use of trans-abdominal ultrasound (TUS) assists in a more accurate AF tap; therefore, it is an appealing alternative to the blind technique[37-39].

The majority of the inpatient diagnostic AF taps are performed with a blind technique. The accepted area of preference is away from the midline, at the point of maximal dullness, and ideally in the left iliac fossa, two fingerbreadths medial and two ventral to the anterior superior iliac spine ("Runyon's spot")[28]. We advise that after two dry taps, TUS should be used to mark the best insertion spot. Equipment required for the tap comprises: 10-mL syringe; 1.5-inch, 22-gauge metal (or 18-gauge) needle; pack of sterile gloves and a galipot

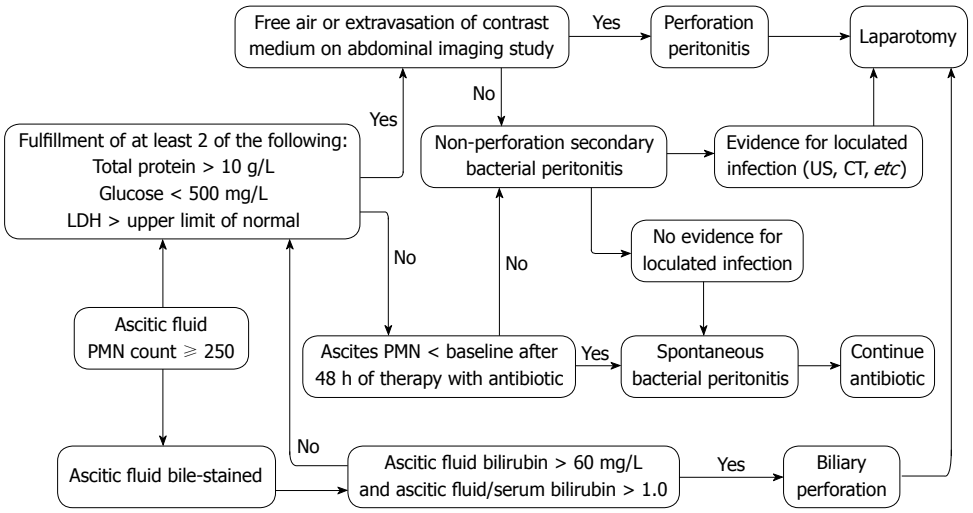

Figure 1 Algorithm for differentiating spontaneous from secondary bacterial peritonitis in patients with neutrocytic ascites (i.e. neutrophil count of 250 cells/mm^3 or greater) in the absence of hemorrhage into ascitic fluid, tuberculosis, peritoneal carcinomatosis, or pancreatitis. CT: Computed tomography; LDH: Lactate dehydrogenase; PMN: Polymorphonuclear neutrophil; US: Ultrasound (Reproduced with permission from Akriviadis EA, Runyon BA: The value of an algorithm in differentiating spontaneous from secondary bacterial peritonitis. Gastroenterology 98: 127, 1990. Copyright 1990 by the American Gastroenterological Association).

with skin disinfectant[34,40]. Thirty milliliters of ascitic fluid should be aspirated and distributed between two blood culture bottles (aerobic and anaerobic, ideally 5-10 mL in each after replacement of the paracentesis green needle by a sterile one), a purple top tube and a brown top one for the necessary biochemistry.

The biochemical tests required for every ascitic fluid sample are for protein, albumin, glucose and lactate dehydrogenase, while other tests are graded between optional and unnecessary. Further expansion on AF biochemistry is beyond the scope of this review and the reader is advised to consult relevant textbooks/reviews[28]. Reference will only be made to AF tests used for the diagnosis of SBP.

A review of the laboratory diagnosis of SBP would not be complete without alluding to the most recent and practical change in protocol. Following aspiration of the AF sample, after inoculating the culture bottles and prior to splitting the rest of the sample into the purple- and brown-topped tubes, a small amount should be poured over a leukocyte esterase reagent strip (LERS) (any urine dipstick has the relevant reagent square), in order to detect any color change in the respective square. The colorimetric scale reference chart can be viewed on the side of the storage container. Results are obtained by direct optical comparison of the LERS with the scale, or, when available, by spectrophotometric analysis. Hepatologists, gastroenterologists and internists have developed an interest in this new addition (at least for AF analysis), especially as satisfactory sensitivity and specificity for SBP detection have been reported in small French and Spanish studies[41-43]. Further studies have been conducted worldwide[44-48]. However, initial enthusiasm and suggestions that LERSs may be used as the sole method of detection of AF infection have been tempered by the latest reports and two systematic reviews[49-51]. It appears that enthusiasm alone replaced structured, evidence-based approaches for LERSs in the presumptive diagnosis of SBP[50,51].

In rural, remote and smaller hospitals and in developing countries, LERSs shrink the 'tap-to-shot' time i.e. the time between paracentesis and first antibiotic dose, to only a few minutes. LERSs bear no resemblance to pH, lactate, lactoferrin or other difficult-to-measure infection indices. However, they are cheap and readily available. Moreover, no diagnosis is made in a clinical vacuum and in the right clinical context, the use of a single 'stat' dose prompted by a positive LERS can potentially lessen the burden of infection[51-53].

Eventually, the AF sample will find its way to the bench of a busy clinical laboratory. It is known that in SBP, the number of polymorphonuclear neutrophils (PMNs) in the ascitic fluid is ⩾ 250/mL[6,28]. Despite numerous publications emphasizing the contrary[13,28,54,55], many AF samples are prioritized inappropriately by clinical laboratories, giving rise to a significant delay in results. The manual count (performed by the traditional hematological method utilizing a microscope and Bucker chamber) is laborious and, in many instances, subjective. Angeloni *et al*[56] have produced clinical evidence that manual and automated PMN counting is equally efficient[57]. Cereto *et al*[58] have confirmed these results. Two years later, Link and colleagues (prompted by the statement of the International Ascites Club consensus document) examined the use of automated counters in detecting the total leucocyte count in ascitic fluid and diagnosing SBP[59]. It is surprising that such a crucial issue in expediting the diagnosis of SBP remained unaddressed for so long by many laboratories, which, despite the above evidence, continued to employ the old-fashioned manual technique over the automated one. At this point, it is necessary to highlight an important caveat when determining AF PMN count: an accurate PMN count may only be determined after non-traumatic paracentesis. If the tap is traumatic or the fluid is a priori hemorrhagic (red cells ⩾ 10 000/mL), the PMN count should be corrected as follows: subtract (from the measured PMN count) 1 PMN for every 250 red cells[7].

Opinion is still divided on the issue of automated *vs* manual testing, but utilization of culture bottles in SBP diagnosis is now the well-established gold standard. SBP is a low-colony-count, monomicrobial infection of the

AF and, in this context, is very similar to bacteremia. The use of blood culture bottles can increase the yield of AF culture from 40% to > 80%[7].

Although initially attractive[60], pH testing of the AF, has now fallen into obscurity[28,34]. This is partly attributable to limited clinical accessibility and partly to increased investigator interest in newer measurements, i.e. procalcitonin and lactoferrin[4,60]. pH was last used in a clinical study in 1995[34]. In their systematic review, Wong *et al*[34] have found that ascitic fluid pH $\leqslant$ 7.35 and blood-ascitic fluid odds ratio (OR) $\geqslant$ 0.10 had the highest diagnostic OR for SBP, and it may be reasonable to suggest a return to pH testing combined with LERSs as an appropriate means to diagnose SBP. The majority of urine dipsticks include a pH reagent square and the latest study on the subject has demonstrated that combination of LERSs with nitrite offers no additional benefit in SBP detection[48]. As far as we are aware, no study has investigated the combination of pH squares with LERSs. We can, however, envisage similar problems to those experienced by investigators in LERS studies occurring in this instance, namely, the lack of specificity of the reagents used for the usual pH values of AF (urine pH reference range is 6.75-7.5).

The use of procalcitonin should also be mentioned. Procalcitonin is the pro-hormone of calcitonin. It is synthesized in many different tissues of infected organs and has been hailed as a novel index of inflammation. Initial interest in its use in SBP[61] was eventually dampened by another study a year later[62]. Lactoferrin seems far more promising to serve as a rapid and reliable screening tool for SBP in patients with cirrhosis, and a recent study has suggested the need to develop an AF-specific dipstick[63].

SBP VARIANTS

Bacterascites (monomicrobial non-neutrocytic bacterascites) is the term used to describe the colonization of ascitic fluid by bacteria, in the absence of an inflammatory reaction in the bacterial fluid. By definition, the PMN count is < 250/mm^3 and bacterial culture is positive, while the patient may present with symptoms and signs of infection. The natural course of bacterascites, if untreated, is variable. Diagnosis of bacterascites can only be made 2-3 d after initial paracentesis (the time necessary for culture growth), and a repeat ascitic tap is recommended on day 3. If the second sample has a PMN count > 250/mm^3, the current recommendation is to treat as for SBP. If the PMN count is < 250/mm^3, but the second set of cultures is positive, treat again as for SBP. If the PMN count is < 250/mm^3 and the second set of cultures is negative, no further action is recommended[7,28].

Culture-negative neutrocytic ascites is the term used to describe the clinical situation in which the ascitic PMN count is > 250/mm^3 but fluid cultures fail to grow any bacteria. It is considered to represent the expected 20% failure rate of culture to isolate microorganisms, and it requires antibiotic treatment as if it were SBP. However, the term is now considered obsolete[28,55].

Table 2 Pathogens in ascitic fluid infection

Micro-organism	Frequency (%)	
	SBP	Bacterascites
Escherichia coli	37	27
Klebsiella pneumoniae	17	11
Pneumococci	12	9
Streptococcus viridans	9	2
Staphylococcus aureus	0	7
Miscellaneous gram-negative	10	14
Miscellaneous gram-positive	14	30

Table 3 Costs of antibiotics used for spontaneous bacterial peritonitis

Route of administration	Antibiotic	Costs (£)[1] including VAT
Intravenous	Ciprofloxacin vial 400 mg	29.60 (per vial)
	Ciprofloxacin vial 200 mg	19.50 (per vial)
	Ofloxacin vial 200 mg	22.63 (per vial)
	Cefotaxime vial 1 g	0.94 (per vial)
	Ceftriaxone vial 1 g	0.91 (per vial)
	Augmentin[2] vial 1.2 g	1.35 (per vial)
Oral	Ciprofloxacin tabl 500 mg (10 tablets pack)	0.40 (4 p per tablet)
	Ciprofloxacin tabl 250 mg (20 tablets pack)	0.36 (1.8 p per tablet)
	Norfloxacin 400 mg (6 tablets pack)	2.30 (40 p per tablet)

VAT: Value added tax; [1]£1 approximately €1.45 and US$2.00; [2]Amoxicillin-clavulanic acid.

MANAGEMENT

Appropriate antibiotic therapy should achieve resolution of infection in most cases of SBP[64]. However, the management of SBP is complex and not just a matter of empirical therapy. Important issues include: (1) identification of the underlying organism; (2) choice of safe and appropriate antibiotics; (3) preservation of renal function and treatment of renal dysfunction; (4) duration of antibiotic therapy; and (5) subsequent antibiotic prophylaxis.

Whilst clarifying the diagnosis of SBP with paracentesis, an attempt should be made at identification of the underlying organism with inoculation of ascitic fluid into blood culture bottles. This vastly improves the identification of the responsible organism and, therefore, allows improved treatment of atypical or resistant organisms. Inoculation into blood culture bottles improves diagnostic yield from 40% to around 80%[65]. Simultaneous blood cultures should be taken as 50% of cases of SBP are associated with bacteremia[66].

The common causative organisms of SBP are Gram-negative bacteria such as Escherichia coli and other coliforms such as Klebsiella spp. These account for at

least 50% of cases. Other causative organisms include pneumococci, streptococci and miscellaneous Gram-positive and -negative organisms[28,55,65,66] (Table 2).

Empirical therapy should not be delayed (beyond the first few minutes needed for LERS reading) while awaiting identification of the exact organism. Third generation cephalosporins are the antibiotic of choice as they have a number of advantages: (1) relatively safe and well tolerated; (2) broad spectrum activity; and (3) effectiveness, with many studies confirming high levels of SBP resolution.

Cefotaxime 2 g every 12 h is often used intravenously for at least 5 d[67-69]. A 5-d course of treatment has been shown to be equally effective as 10 d[70]. Other third generation cephalosporins (e.g. ceftriaxone) are felt to be equally effective[3,71-73]. Alternative antibiotic regimens include amoxycillin/clavulanic acid, fluoroquinolones or piperacillin/tazobactam[74-77] (Table 3). Regional resistance patterns should be accounted for with early communication with a microbiologist if necessary[11,77]. According to the International Ascites Club, it is important to perform a second tap 48 h after the start of therapy. If there is a less than a 25% drop in PMN count from baseline, a change of antibiotic should be considered[4,5].

Renal function

One third of patients with SBP will develop renal failure. The renal dysfunction is thought to occur as a result of a reduced effective circulating volume[7,78]. Renal dysfunction has been shown to be an independent predictor of mortality in patients with SBP[79]. Therefore, close attention to renal function and the avoidance of nephrotoxic medication is paramount. On the other hand, diuretic therapy and large-volume paracentesis should not be necessarily withheld (they potentially exacerbate the reduction in effective circulating volume and contribute to renal deterioration) if albumin is administered[80,81]. The benefit of human albumin solution for treating renal dysfunction has been studied in randomized controlled trials[82,83]. Albumin is thought to reduce the risk of renal impairment by improving effective intravascular volume and by helping to bind pro-inflammatory molecules[7,8,11]. Studies have shown an improvement in short-term survival and a reduction in renal impairment in patients with SBP treated with albumin. Although these studies have been subject to criticism[84,85], most authors agree that infusion of 1.5 g/kg on day 1 and 1 g/kg on day 3 is beneficial in patients that have developed, or are developing renal dysfunction[7,61]. Patients with normal renal function are unlikely to benefit from albumin therapy.

PROPHYLAXIS

Unfortunately, the long-term prognosis of patients with cirrhosis who have had a prior episode of SBP is poor. Mortality rates of 50%-70% have been reported at 1 year follow-up[7,11]. This is largely a result of the advanced stage of liver cirrhosis in these patients, along with the associated complications[86]. The recurrence rate of SBP following a first episode is up to 70% at 1 year[7,86]. Given the high recurrence rate, it seems sensible to recommend prophylaxis to this group of patients and referral for transplant assessment. This therapy is backed up by evidence showing a reduction in recurrence of SBP from 68% to 20% in one study[87].

Norfloxacin 400 mg/d or ciprofloxacin 500 mg/d orally appear to be the most studied and commonly recommended regimes[87-92]. Levofloxacin or antibiotic cycling may be used as an alternative[93-95]. There is debate over the use of antibiotics as primary prophylaxis against SBP. Some studies have shown reduced rates of SBP in selected patients deemed at high risk of developing SBP (those with low ascitic total protein)[79,91,96]. However, there are various criticisms of these studies, and at present, primary prophylaxis is not recommended. Further studies may help clarify this issue.

The last group of patients that are felt to benefit from antibiotic prophylaxis are those with known cirrhosis admitted with GI bleeding. Infection rates are high in this group regardless of whether they have ascites. The infection rates are also higher than those in patient with cirrhosis admitted for other reasons[61]. Several studies have shown a clear benefit from initiating antibiotic prophylaxis in this group[97-100]. Reductions in infection rate and mortality have been noted. Once again, the choice of antibiotic should be broad spectrum and guided by local policy; either oral norfloxacin or ciprofloxacin have been suggested[7,61].

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TOPIC HIGHLIGHT

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Role of upper endoscopy in diagnosing opportunistic infections in human immunodeficiency virus-infected patients

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Abstract

Highly active antiretroviral therapy (HAART) has dramatically decreased opportunistic infections (OIs) in human immunodeficiency virus (HIV)-infected patients. However, gastrointestinal disease continues to account for a high proportion of presenting symptoms in these patients. Gastrointestinal symptoms in treated patients who respond to therapy are more likely to the result of drug-induced complications than OI. Endoscopic evaluation of the gastrointestinal tract remains a cornerstone of diagnosis, especially in patients with advanced immunodeficiency, who are at risk for OI. The peripheral blood CD4 lymphocyte count helps to predict the risk of an OI, with the highest risk seen in HIV-infected patients with low CD4 count (< 200 cells/mm^3). This review provides an update of the role of endoscopy in diagnosing OI in the upper gastrointestinal tract in HIV-infected patients in the era of HAART.

Key words: Human immunodeficiency virus; Opportunistic infections; Upper gastrointestinal tract; Gastrointestinal endoscopy; Highly active antiretroviral therapy

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INTRODUCTION

Highly active antiretroviral therapy (HAART) has dramatically reduced the incidence of opportunistic infections (OIs) in human immunodeficiency virus (HIV) disease. Different factors may be related to the decreasing prevalence of OI in the era of HAART. Besides restoring immune function[1], antiretroviral protease inhibitors have been reported to have a direct inhibitory effect on the proteases of certain pathogens, including the aspartyl proteases of some parasites[2,3].

Nevertheless, the gastrointestinal (GI) tract is still affected by OI in HIV-infected patients undergoing HAART, especially in those with severe immunosuppression[4]. Patients undergoing HAART may not have a sustained CD4 lymphocyte count increase for several reasons, including poor adherence to therapy, drug toxicity or interactions, acquisition of a drug-resistant strain of HIV, and/or the development of a discordant immunological response, which leads to low CD4 cell counts despite optimal suppression of plasma HIV viremia[5]. Whatever the reason, HIV-infected patients with a low CD4 cell count remain at high risk for OIs, including GI infections. Also, these patients may have an atypical presentation of OI, either early after the initiation of therapy or after prolonged treatment[6]. Hence, a CD4 count < 200 cells/mm^3 remains an important marker for those patients in whom OIs should be suspected as a cause of GI symptoms.

Nowadays, HAART-related GI adverse events have been recognized as a frequent cause of GI complaints[7]. When a physician is challenged with an HIV-infected patient with upper GI complaints undergoing HAART, his final diagnosis is usually unrelated to HIV-associated immunodeficiency. Therefore, careful evaluation is needed when considering GI symptoms in these patients, especially in those with advanced immunodeficiency.

The tropism of many pathogens for the squamous mucosa of the esophagus (*Candida*, herpesviruses), as well the appearance of new diseases (idiopathic esophageal ulceration) has established the upper GI tract as a common site for complications[8]. Upper endoscopy with mucosal biopsies is a standard part of the evaluation of upper GI symptoms in these cases, especially because the therapy will depend on the specific pathogen found. In this review, we discuss the role of endoscopy in diagnosing OIs in the upper GI tract, in the HAART era.

ESOPHAGUS

HAART has changed the epidemiology of esophageal infections in HIV disease. Mönkemüller *et al*[9] have evaluated the prevalence of GI OI in symptomatic HIV-infected patients undergoing endoscopic procedures from 1995 to 1998. They observed that the prevalence of OI fell from 69% to 13% coincident with the use of HAART. The number of patients identified with esophageal candidiasis or cytomegalovirus (CMV) infection fell by 80%, while the prevalence of gastroesophageal reflux disease rose eight-fold. In another more recent study[4], this same author observed GI OI in 9% of patients undergoing HAART. Those patients had a significantly decreased CD4 count (mean 23 cells/μL) despite HAART use.

There is not a close relationship between esophageal symptoms and OI. However, patients presenting with odynophagia are more prone to esophageal ulcers[10]. Biopsy is mandatory when finding esophageal ulcers upon endoscopy. Definitive differential diagnosis of esophageal ulcers can only be made by histological examination.

On the other hand, normal-appearing esophageal mucosa at endoscopy has a good correlation with the absence of OI. We have done a prospective study in a large cohort of dyspeptic and immunosuppressed HIV-infected patients (n = 1010), who underwent endoscopy with esophageal biopsies in order to evaluate the presence of OI in a normal-appearing esophagus. A pathogen was found in normal-appearing esophageal mucosa in only one patient (0.09%)[11]. We concluded that it is not necessary to perform biopsies in normal-appearing esophageal mucosa, even in patients with advanced immunosupression.

Candida spp.

Despite the decreasing prevalence of OI, *Candida* spp. continue to be the most common cause of OI in the esophagus, followed by viral infection, especially CMV. Patients often present with dysphagia, but may also develop odynophagia and/or acute retrosternal chest pain. The presence of oral candidiasis (thrush) suggests candidal esophagitis. On the other hand, the absence of thrush *per se* does not exclude it. Some authors recommend empirical antifungal therapy for HIV-infected patients who present with dysphagia, postponing endoscopy for those individuals in whom symptoms persist[12]. This approach seems to be a safe, efficacious, and cost-effective procedure.

At endoscopy, *Candida* esophagitis shows a characteristic superficial mucosal pattern: focal or confluent yellowish-white plaques that overlie an erythematous mucosa. It is seldom related to mucosal ulceration, which in general results from causes other than *Candida* infection[13].

Endoscopy has also been used to grade the severity of *Candida* infection. We have analyzed the relationship between *Candida* esophagitis severity and peripheral blood CD4 cell count in a prospective study of a large cohort of adult HIV-infected patients (n = 261, mean CD4 cell count = 78.8 cells/mm^3). Severity was graded I to IV[14] according to the extent of mucosal lesions. We have shown that the least severe disease (grade I) was related to the highest CD4 cell counts when compared to all others (P = 0.0003). Meanwhile, the progression of disease severity from grade II to IV was not related to a corresponding decrease in CD4 cell counts. These findings suggest that even in immunosuppressed HIV-infected patients, immunological status may play a role in limiting *Candida* disease in initial grades, but seems to be irrelevant in the following progression of the infection[15]. Other mechanisms, such as the local epithelial defenses, may be involved with the development of these OIs in the GI mucosa.

CMV

Patients that present with severe odynophagia or who fail empirical antifungal therapy usually have viral esophagitis or esophageal ulcers. These patients should be promptly referred for upper GI endoscopy, since they exhibit increased morbidity and may rapidly become malnourished[10].

The most common virus detected is CMV, which may cause erosive esophagitis or deep esophageal ulcers. Upon endoscopy, CMV esophagitis appears frequently as small, well-circumscribed ulcerations, with a normal appearance of the intervening mucosa[16]. This appearance is similar to herpes esophagitis, but it is usually distinguishable from esophageal candidiasis. CMV ulcers are usually located in the middle or distal esophagus and are characteristically deep, with a halo of edema. This appearance is identical to the large idiopathic esophageal ulcerations associated with HIV infection[17]. The former ulcers are believed to be secondary to CMV-induced vasculitis, with ischemic injury of the endothelium. The CMV viral cytopathic effect is rarely identified in squamous epithelial cells alone, and thus, biopsies of the ulcer base should be carried out. The diagnostic feature of CMV with hematoxylin-eosin (H&E) staining is a central dense eosinophilic inclusion with a surrounding halo, which leads to an owl's eye nuclear inclusion appearance. It may also show basophilic granular cytoplasmic inclusions[18].

Herpes simplex virus (HSV)

HSV type 1 or 2 infection is associated with small, superficial, scattered or coalescent shallow ulcers with

exudate, which are separated by normal-appearing mucosa[17]. It has also been associated with deep ulcers. Biopsies should be done at the edge of the ulceration, as the HSV viral cytopathic effect is more reliably found in squamous cells. Histological analysis reveals typical Cowdry type-A intranuclear inclusion bodies[19].

Mycobacterium

Esophageal tuberculosis is rare and is associated with direct extension of the disease from adjacent mediastinal lymph nodes or lung foci. The middle third of the esophagus is the typical site of tuberculous involvement. It may exhibit different endoscopic appearances. In the first form, deep single or multiple ulcerations of various sizes form, with shallow smooth edges and a gray-white base, and the surrounding mucosa may contain small nodules or ulcerations. The second form is characterized by a hypertrophic or a granular-appearing lesion. This form may produce granulomatous fibrosis in the esophageal wall with stricture of the lumen. The third form consists of a protruding subepithelial mass. Fistulas may develop, and it may be bronchoesophageal as well as esophagoesophageal[20]. Biopsies should be taken from the edge of the lesions. Histological findings infrequently show acid-fast bacilli and caseating granulomas[17].

Idiopathic ulceration

Idiopathic ulceration may develop at the time of initial HIV infection or may occur long after the initial seroconversion period. Endoscopy usually shows a large single or multiple deep ulcers in the mid or lower esophagus, with transverse ridges visible in the base, which represent circular muscle bundles of the esophageal muscularis propria. The margins show variable degrees of inflammation, are often irregular, and overhang into the central ulceration. Evidence indicates that these ulcers are caused by HIV[20]. These lesions are negative on biopsy for known viral and fungal agents. Electron microscopy can be used to confirm the presence of HIV-like viral particles in these ulcers[21].

STOMACH

Symptoms of dyspepsia, such as epigastric pain, fullness, nausea and vomiting, are frequently reported by HIV-infected patients, mainly those undergoing HAART[7]. These symptoms may have different etiologies, including any adverse drug effects (HAART or others), the HIV disease itself, and GI infections. It is still unknown whether GI OI may cause dyspepsia as the main symptom.

Under normal conditions, most organisms cannot thrive in the acidic gastric environment. A decrease in gastric acidity has been described in HIV-infected patients[22], possibly providing a more suitable environment for pathogen colonization. However, gastric OI seems to be an infrequent event even among HIV-infected patients[8].

We have performed upper GI endoscopy with biopsies of the stomach and duodenum in a large cohort of HIV-infected patients (n = 528) with dyspeptic symptoms undergoing HAART. We have shown a low prevalence (3.66%) of OI in these patients. It is noteworthy that the few cases of observed gastrointestinal OI were seen exclusively in HIV-infected patients with CD4 $\leqslant$ 200 cells/mm^3[23].

In order to look for a correlation between OI and dyspeptic symptoms, we have performed another prospective study in a large cohort of HIV-infected patients (n = 690) with advanced immunodeficiency (CD4 < 300 cells/mm^3; mean = 154.3 cells/mm^3), despite HAART. We have compared the prevalence of GI OI in dyspeptic (n = 500) *versus* non-dyspeptic (n = 190) patients. All patients underwent upper digestive endoscopy with tissue biopsies from stomach and duodenum. Although GI OI was detected exclusively in the dyspeptic patient group, we could not demonstrate a relationship between GI OI and dyspepsia, since it occurred in low numbers (just 1.6% of patients)[24].

Although not frequent, gastritis and/or gastric ulcers have been reported to be associated with some viral, helminthic, protozoan, and fungal pathogens[25-28].

CMV

Gastric CMV is the most common OI of the stomach[29]. It is commonly associated with non-specific symptoms such as epigastric pain, nausea and vomiting. Upon endoscopic examination, gastric CMV is usually associated with ulcerations, erosions and mucosal hemorrhage[25,30], although it may be present in a normal-appearing mucosa[31]. Less commonly seen lesions are thickened edematous folds[32], nodules[33], and masses[34]. CMV targets endothelial cells, and related injuries often induce epithelial and interstitial necrosis that resembles ischemic damage[30]. The presence of cytomegalic cells in tissue biopsies stained by H&E is considered the gold standard for establishing a diagnosis of CMV GI disease. When the diagnosis is uncertain, additional immunohistochemical methods may be useful in confirming the presence of CMV[35]. However, the number of tissue samples appears to be especially important for diagnosing CMV. Goodgame *et al*[31] have reported that even when immunoperoxidase staining was used to make a diagnosis of CMV, after routine histology failed to demonstrate cytomegalic cells, a positive result seemed equally dependent on the number of biopsies as on routine histopathology. When histology involved multiple sections of 8-10 biopsies, the frequency of diagnosing CMV by histology was greater than by culture.

Schistosoma mansoni

Gastric infection from *S. mansoni* is extremely rare, as this helminth usually infects the intestine and liver, which leads to portal hypertension[26]. In a large cohort of dyspeptic HIV-infected patients (n = 690), we have shown gastric *S. mansoni* (gastric ulcer) in just one

patient[24]. Reported endoscopic findings are gastric ulcers[36] and pseudopolypoid lesions[26]. Tissue biopsies reveal ova of *S. mansoni* stained by H&E, accompanied by little or no inflammatory or fibroblastic response.

Cryptosporidium

Cryptosporidium is a coccidian protozoan that more commonly affects the proximal small bowel. Gastric infection is considered a secondary localization. Parasites reach the stomach through duodenal backwash and localize mostly in the antrum because of its proximity[37]. There is no specific pathognomonic endoscopic appearance. Gastric hyperemia, edema and erosions, especially in the antrum, have all been reported[24,27]. Gastric cryptosporidiosis may also occur in normal-appearing mucosa[24]. Histological examination shows *Cryptosporidium* parasites mainly on epithelial cells covering gastric pits and stained by H&E[37]. Rivasi *et al*[38] have demonstrated a close relationship between the intensity of *Cryptosporidium parvum* infection and the degree of histological alterations. They did not find, however, a clear correlation between the endoscopic and histological alteration types found[38].

Strongyloides stercoralis

Str. stercoralis larvae infect the duodenum and the first part of the jejunum; it is considered an opportunistic agent when found in the gastric mucosa. There are remarkably few reports of gastric strongyloidiasis in HIV-infected patients. Among these reports, gastric ulcers[39], and edematous and thickened gastric folds have been reported[40]. Strongyloidiasis in normal-appearing gastric mucosa has also been reported by our group[24]. A true pathognomonic endoscopic finding does not exist, although a brownish mucosal discoloration of the gastric or duodenal mucosa is frequently observed[40]. The diagnosis is easily made by H&E tissue stained sections, identification of *Str. stercoralis* larvae and eggs, infiltration of eosinophilic cells into the lamina propria, and villous blunting.

Leishmania donovani

A few cases of gastric localization of *L. donovani* have been reported in severely immunosupressed HIV-infected patients. Endoscopy has shown gastric ulcers, erosions and a normal-appearing gastric mucosa as well[41,42]. Histological study has shown large macrophages, lymphoid cells and plasma cells infiltrating the lamina propria. Characteristically, macrophages are filled with round or oval nucleated complete microorganisms that contain kinetoplasts. Both the kinetoplasts and nuclei stain bright red with Giemsa staining and H&E[18].

SMALL INTESTINE

Chronic diarrhea is an important clinical problem in HIV-infected patients and is still a cause of morbidity and mortality in the HAART era[43]. Diarrhea in the setting of HIV infection may have many causes; it may be a consequence of HAART[44], the HIV infection itself or may result from any bacterial, viral or parasitic infection[45].

Currently, HAART-induced diarrhea is the primary reason for the continually high prevalence of diarrhea in HIV-infected patients, especially with the use of protease inhibitors[46]. The likelihood of an opportunistic process is linked to the severity of immunodeficiency. Therefore, the search for a typical HIV-associated process should be undertaken based on risk stratification of the patient. Patients with CD4 counts of < 100 cells are most at risk for *Cryptosporidium*, *Microsporidium* and CMV disease[8].

A consensus panel in 1999[47] recommended a stepwise approach to investigate diarrhea in HIV-infected patients at risk for OI: step 1, at least three sets of stool specimens for common enteric bacteria and parasites, including microsporidia and cryptosporidia; and step 2, colonic mucosal biopsies using flexible sigmoidoscopy or colonoscopy. Upper GI endoscopy with biopsy of the duodenum for light-microscopic examination, mycobacterial culture, and electron microscopy is considered the third recommendation if no pathogen is identified after performing steps 1 and 2. Duodenal aspirate seems to be of little value in the workup of these patients[48].

Cryptosporidium

Cryptosporidium is a protozoan that infects the small bowel mucosa and, in immunosuppressed persons, the large bowel and extraintestinal sites. Endoscopy may show fold thickening of the mucosa, with an erythematous and granular appearance that is most prominent in the duodenum[49]. In general, duodenal erosions or ulcers are not found. Histological study of duodenum samples shows a partial villus atrophy with crypt hypertrophy and increased chronic inflammatory cells, particularly eosinophils and plasma cells. The organisms are seen positioned along the brush border of the surface and crypt epithelium[37].

Microsporidia

Intestinal microsporidiosis is caused by *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*. The diagnosis is frequently established by examination of three stool samples with chromotrope and chemofluorescent stains. There is not a typical endoscopic appearance. A small bowel biopsy, especially in the jejunum may show microsporidium organisms in villus enterocytes by different stains such as H&E, Giemsa, Warthin-Starry silver staining, or Chromotrope 2A[50,51].

CMV

Isolated lesions caused by CMV in the duodenum may result in severe GI bleeding[52]. Also, diffuse mucosal involvement of the duodenum and jejunum may lead to malabsorption. Rare manifestations of CMV infection include isolated ulcers that may cause perforation, terminal ileitis mimicking Crohn's disease[53], and ileal

obstruction that results from a large inflammatory mass[54].

Mycobacterium avium complex (MAC)

MAC, commonly seen in the pre-HAART era, is now very rare and is most likely to be found in patients who first present with end-stage HIV infection. The most common site of MAC infection of the GI tract is the small bowel. The endoscopic appearance may mimic Whipple's disease with diffuse, scattered white nodules and plaques that may be yellow, white, yellow-whitish, or pink, located in the second portion of the duodenum. Therefore, it is often described as pseudo-Whipple disease[17]. Although nodular lesions are frequent, other endoscopic findings have also been described in the small bowel such as ulcerations, erythema, edema, friability, reduced mucosa vascular pattern, erosions, strictures and even normal-appearing mucosa[55]. Microscopically, the affected tissue is filled with large numbers of distended histiocytes that are packed with acid-fast organisms. Usually, granuloma formation or associate inflammatory response is minimal[56].

Mycobacterium tuberculosis

M. tuberculosis bowel infection is also rare. It usually involves the small bowel and ileocecal region. It may be associated with granulomatous reactions that lead to ulcers, fistulas and even perforations. Upon endoscopy, ulcers have a cratered appearance with mass-like edges. Upon histology, there are few acid-fast bacilli and usually they do not form well-developed non-caseating granulomas in HIV-infected patients[57].

Histoplasma capsulatum

Uncommonly, fungal organisms may cause disease in the small intestine in HIV-infected patients. H*istoplasma capsulatum* most commonly causes disease in the ileum, but may also cause disease in the jejunum. Ulcers are often found, but nodules, pseudopolyps or plaques caused by collections of infected macrophages have also been described[58]. Microscopic findings include lymphohistiocytic infiltration, infected macrophages within the lamina propria, and less commonly, granulomas[58].

The role of upper GI endoscopy in the diagnosis of OI in HIV-infected patients with GI complaints without diarrhea is still more controversial. We have done endoscopy with duodenal biopsies in a large cohort (n = 690) of HIV-infected patients that were severely immunosupressed (mean CD4 count 154.3 cells/mm^3), who were undergoing HAART and presented with GI complaints but without diarrhea, and we found a very low incidence of OI and non-opportunistic parasites in the tissue specimens (five patients, 1.0%). In 80% of these patients, the duodenum showed a normal-appearing mucosa upon endoscopy, which suggested the relevance of taking biopsies even from a normal-appearing mucosa when an OI diagnosis is suspected[24]. Our results seem to disagree with Olmos *et al*[59]. These authors observed a low prevalence of OI in HIV-infected patients without diarrhea when the duodenal mucosa was normal. They suggested that biopsies should not be taken from normal duodenal mucosa in patients without diarrhea. Pathogens found in biopsies of normal duodenum in our study (*Cryptosporidium* and *Giardia*), however, should also be detectable in stool samples. One possible option is that more stool tests should be performed prior to pursuing endoscopy in these patients.

CONCLUSION

Although there has been a decrease in the incidence of GI OI in the era of HAART, the gastroenterologist evaluating HIV-infected patients with GI symptoms should not discard this possibility. Since adverse events related to HAART are a frequent cause of GI complaints among HIV-infected patients, the approach for HIV-infected patient with CD4 counts > 200 cells/mm^3 and upper GI complaints should parallel those of any other patient when immunodeficiency is not advanced. Patients with CD4 $\leqslant$ 200 cells/mm^3, however, should be referred earlier for upper GI endoscopy, in order to diagnose OI early, especially because many of these infections are now treatable. Different pathogens can result in similar endoscopic findings. To correctly diagnose OIs, multiple biopsy specimens may be necessary even from normal-appearing mucosa.

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Omentum facilitates liver regeneration

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Abstract

AIM: To investigate the mechanism of liver regeneration induced by fusing the omentum to a small traumatic injury created in the liver. We studied three groups of rats. In one group the rats were omentectomized; in another group the omentum was left *in situ* and was not activated, and in the third group the omentum was activated by polydextran particles.

METHODS: We pre-activated the omentum by injecting polydextran particles and then made a small wedge wound in the rat liver to allow the omentum to fuse to the wound. We monitored the regeneration of the liver by determining the ratio of liver weight/body weight, by histological evaluation (including immune staining for cytokeratin-19, an oval cell marker), and by testing for developmental gene activation using reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: There was no liver regeneration in the omentectomized rats, nor was there significant regeneration when the omentum was not activated, even though in this instance the omentum had fused with the liver. In contrast, the liver in the rats with the activated omentum expanded to a size 50% greater than the original, and there was histologically an interlying tissue between the wounded liver and the activated omentum in which bile ducts, containing cytokeratin-19 positive oval cells, extended from the wound edge. In this interlying tissue, oval cells were abundant and appeared to proliferate to form new liver tissue. In rats pre-treated with drugs that inhibited hepatocyte growth, liver proliferation was ongoing, indicating that regeneration of the liver was the result of oval cell expansion.

CONCLUSION: Activated omentum facilitates liver regeneration following injury by a mechanism that depends largely on oval cell proliferation.

Key words: Cytokeratin; Foreign body; Growth factors; Oval cell; Progenitor cells

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INTRODUCTION

The omentum has been called the "policeman of the abdomen" because after traumatic injury it migrates to the injured site, adheres to the wound, and promotes healing[1,2]. These properties have found clinical application where the omentum is surgically brought into contact with injured tissues such as ischemic heart, fractured bones, or injured spinal cord[3-6]. We have recently shown that introducing a foreign body into the peritoneal cavity further enhanced the healing power

of the omentum by causing it to expand, surround the foreign body, and transform itself from mostly fatty tissue to tissue abundant in progenitor cells and rich in growth and angiogenic factors (activated omentum)[7,8].

Because liver regeneration can be brought about by resident stem cells (oval cells) even in the absence of hepatocyte multiplication[9,10], we attempted to use the activated omentum to facilitate liver regeneration. The procedure involved removing a small wedge of tissue (traumatic injury) in rats and allowing the omentum to adhere to the wound in order to supply the liver with stem cells. We also studied two other groups of rats (controls); one in which the omentum was left in its native state (inactivated omentum), and the other in which the omentum was surgically removed (omentectomized), and focused on the cellular and developmental gene activation at the site of injury and omental adhesion.

Figure 1 The traumatic liver injury model used to induce regeneration. The wound was created in one of the lobes of rat liver by removing a wedge of tissue (3-4 mm on each side) with a pair of fine scissors. In rats with activated or unactivated omentum, the wound was filled with new liver tissue by day 7. On the other hand, in omentectomized rats the original wound edges as seen in the picture remained visible for up to 28 d. The horizontal black bar in the picture represents 3 mm.

MATERIALS AND METHODS

Traumatic injury of the liver

Animal experimentation was conducted according to the approval of the Institutional Animal Care and Use Committee (IACUC).

Under general anesthesia, male Sprague-Dawley rats (200-250 g) were laparotomized and the most anterior and prominent of the liver lobes lying in the middle of the abdominal cavity was exposed. Using a pair of fine scissors a small V-shaped cut was made in the lobe (3-4 mm on each side) and the wedge of liver was removed and later used as normal tissue for immunostaining and quantitative reverse transcription polymerase chain reaction (RT-PCR) (Figure 1). The rats were divided into three groups. In the activated omentum group, before the incision was sutured, 5 mL of polydextran particle slurry (Biogel P-60, 120 μmol/L; Biorad Laboratories, Richmond, CA, USA) (1:1 in normal saline) was introduced into the abdominal cavity to activate the omentum. The inactivated omentum group underwent similar hepatic wedge injuries. However, polydextran slurry was not placed in the abdomen and, thus the omentum was not activated (inactivated omentum). The omentectomized group underwent similar hepatic wedge resections, in addition to an omentectomy. An omentectomy was performed by surgically excising the entire omentum from the lower curvature of the stomach.

The animals were maintained on normal rat chow and water *ad libitum* from three to twenty eight days. At the time of sacrifice, the livers were examined, wholly removed, and weighed. Liver mass was expressed conventionally as a percent ratio: liver weight/body weight. Pieces of the re-grown liver from the point of omental fusion, and at 0.5 cm and 1.0 cm away from the wound as well as portions from an uninjured lobe were collected for immunostaining and quantitative RT-PCR.

To test whether liver regeneration by omental intervention depended upon hepatocyte proliferation, rats were injected intraperitoneally daily for four days with 2-acetyl-amino-fluorene, which inhibits hepatocyte proliferation (2-AAF; 30 mg/kg dissolved in M400 polyethylene glycol (Avg. MW = 400); both chemicals were obtained from the Sigma Chemical Company, St Louis, MO, USA), followed by liver wounding and omental activation (at day 5 by intraperitoneal injection of polydextran), and further daily injections of 2-AAF for four days to inhibit expansion of hepatocytes. The rats were sacrificed 14 d after liver wounding and the livers were examined, wholly removed, and weighed.

Histological processing and immunostaining of the regenerated liver

Pieces of normal and regenerated liver (including the omental attachment) were fixed for histology and immunostaining by immersion in Histochoice® (Amersco Inc., Solon, OH, USA). Following dehydration and paraffin embedment, tissues were sectioned (5 μm thick) and stained with hematoxylin-eosin (HE) or Trichrome stain. Immunostaining was carried out by first pressure-cooking the sections for 10 min in a solution of BorgDecloaker® (Biocare Medical; Walnut Creek, CA, USA) for antigen enhancement. For immunofluorescent staining the sections were incubated with monoclonal (mouse) anti-rat cytokeratin-19 (Sigma Chem. Co, St Louis, MO, USA) followed by washing and re-incubating with fluorescein (FITC) labeled anti-mouse IgG antibody (Sigma Chem. Co., St Louis, MO, USA). The slides were washed and wet-mounted in glycerol-PBS. For immunoperoxidase staining, sections were sequentially incubated with monoclonal (mouse) anti-rat cytokeratin-19, anti-mouse IgG-biotin conjugate, avidin-horse radish peroxidase and finally developed with diaminobenzidine-H_2O_2 (brown color) (Vector Laboratories, Inc., Burlingame, CA, USA). The slides were examined either by epifluorescent or light microscopy and digitally photographed (Nikon Inc., New York, NY, USA).

Table 1 Primer sequences of the selected developmental genes that were tested in the regenerating liver tissue by RT-PCR technique

Gene[1]	Primer	Predicted size (bp)	Accession number[2]	Entrez gene ID[2]
β-actin	F (926) 5'-TCATGAAGTGTGACGTTGACATCCGT-3'[3]			
	R (1210) 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'	285	NM_031144	81822
Wnt-4	F (127) 5'-GAAACGTGCGAGAAGCTCAAAG-3'			
	R (513) 5'-AAAGGACTGTGAGAAGGCTACG-3'	387	NM_053402	84426
WT-1	F (1059) 5'-TGAGAAACCATACCAGTGTGAC-3'			
	R (1458) 5'-GTAGGTGAGAGGGAGGAATTTC-3'	400	NM_031534	24883
Nanog	F (541) 5'-ATCCATTGCAGCTATTCTCAGG-3'			
	R (850) 5'-CTTCCAAATTCGCCTCCAAATC-3'	310	XM_575662	414065
AFP	F (1421) 5'-CAGTGAGGAGAAACGGTCCG-3'			
	R (1672) 5'-ATGGTCTGTAGGGCTCGGCC-3'	252	NM_012493	24177
Oct-4	F (633) 5'-GGAGATATGCAAATCGGAGACC-3'			
	R (984) 5'-CGAGTAGAGTGTGGTGAAATGG-3'	352	NM_001009178	294562
HNF-6	F (1698) 5'-AAGACCAGGACCTCAAGATAGC-3'			
	R (2001) 5'-GCAGTGTGGTGGAACAGATAAG-3'	304	NM_022671	25231

[1]Wnt-4: Wingless-type mouse mammary tumor virus integration site family, member 4[21,26,27]; WT-1: Wilm's tumor suppressor gene[16,22]; Nanog: One of the gene markers of pluripotency[24]; AFP: α-fetoprotein[15,23]; Oct-4: Octomer-4[25]; HNF-6: Hepatic nuclear factor-6[16,28]; [2]Accessed from http://www.ncbi.nlm.nih.gov; [3]Numbers in parentheses after forward (F) and reverse primers (R) denote the nucleotide number in the cDNA sequence.

Quantification of mRNA for selected developmental genes in regenerated liver by RT-PCR

Liver tissues at the point of omental attachment or wound edge (in omentectomized rats), at 0.5 and 1.0 cm away from the omental attachment, and from a remote uninjured lobe were tested for expression of developmental genes. The specific genes and their respective forward and reverse primer sequences are listed in Table 1. The liver tissue was cleared of the attached omental tissue and processed for total RNA extraction by Trizol using a RNA purification kit (Invitrogen, CA, USA). The RT-PCR procedure was carried out in one step using 3 μg of total tissue RNA and primers using the Invitrogen RT-PCR system (Invitrogen, CA, USA). The system uses Superscript Ⅱ reverse transcriptase for first strand synthesis and *Taq* DNA polymerase for second strand cDNA synthesis and amplification (30 cycles). β-actin amplification was performed from the total RNA preparations (60 ng) as a control. The RT-PCR products were quantitated as the ratio of gene band density/β-actin band density by image analysis using MIPAV software (JAVA imaging software inspired by the National Institutes of Health).

Statistical method

Quantitative data presented in Figures 2 to 6, which compare the differences between different groups, were analyzed by student's *t* test. The differences were considered significant when $P < 0.05$.

RESULTS

Fusion of the omentum to the wounded liver resulted in new liver growth

In all rats in which the liver was traumatically wounded and the omentum was intact, whether activated ($n = 24$) or inactivated ($n = 12$), there was fusion between the omentum and the wound edge of the liver, and the omentum remained attached to the injury site for up to 28 d. On gross inspection, by day 14 new tissue filled the resected wedge, and the location of the resection site was only identifiable by the omental attachment. In omentectomized rats ($n = 12$), there was an absence of omental attachment and of liver growth at the wound, making the wound edges noticeable until at least day 28 (Figure 1).

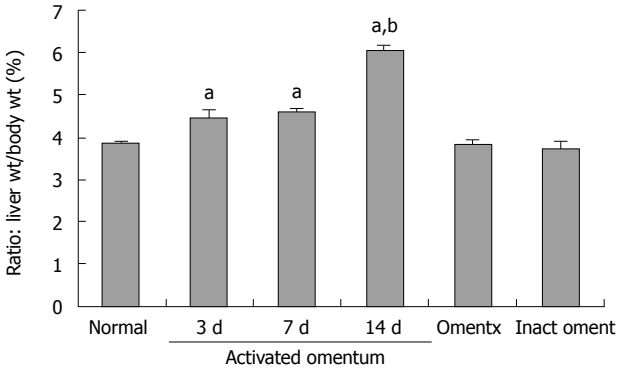

Figure 2 Liver mass (as a ratio of body weight) at different times after injury and fusing of the activated omentum to the wound. The ratio of liver wt/body wt in normal rats was established to be 3.85% ± 0.07%. 'Omentx' are rats in which the omentum was removed before liver injury ($n = 12$) and 'inact oment' are rats in which the liver was injured, but the omentum was inactivated ($n = 12$). Liver regeneration following wounding and fusion of activated omentum was rapid, and by day 3 the liver grew to 110% of the original mass. The liver continued to grow, reaching a maximum size of 150% of the original mass by day 14, after which growth stopped (day 28, data not shown). Normal = 15 rats and there were 6 in each of the 3, 7, 14 and 28 d groups. [a]Denotes statistical difference from normal or 'omentx' or 'inact oment' groups at $P < 0.05$. [b]Denotes statistical difference from day 3 and day 7 groups at $P < 0.05$. With regard to 'omentx' and 'inact oment' groups, no differences were seen at days 3, 7, 14 and 28 compared to Normal (only day 14 data is shown in the figure; $n = 3$).

In rats with activated omentum, there was additional liver growth, especially in the wounded lobe at the point of omental attachment. There was also growth in other lobes which was suggested by alterations in the natural contours of the edges of the uninjured liver lobes. Figure 2 shows the liver mass (as a percent ratio to body

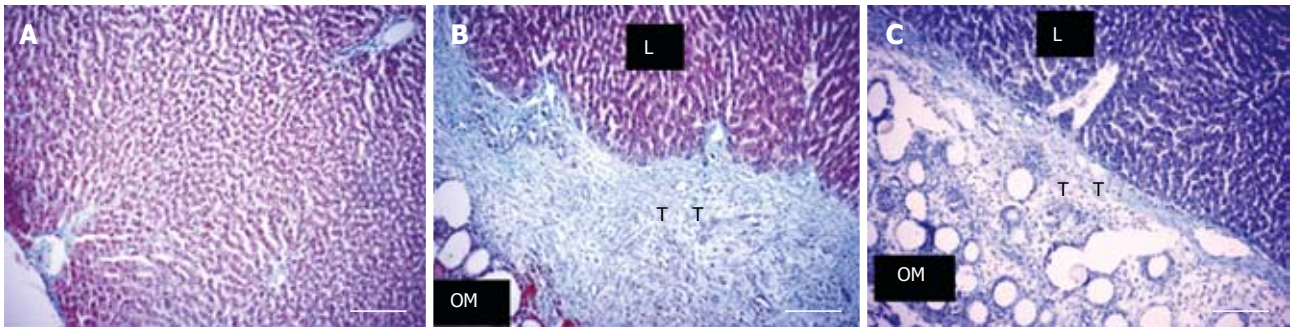

Figure 3 Histology of the boundary between the growing edge of the liver and the activated omentum. A: Normal rat liver; B: 7 d after liver injury the liver and the omentum were separated by a wide and compact interlying tissue (400-600 μm). On one side of the interlying tissue (T) lay the omental tissue (OM) with the embedded polydextran gel particles and on the other side was the liver tissue (L). Occasionally, islands of liver tissue were observed in the interlying tissue (Figure 4G). The compactness and the width of the interlying tissue was maximal between 3 and 7 d after liver injury (B) which became thinner (100-150 μm) and looser by day 14 (C). By 28 d the interlying tissue was barely appreciable and looked like a tissue septum (picture not shown). Trichrome staining. The horizontal white bar in the pictures represents 100 μm.

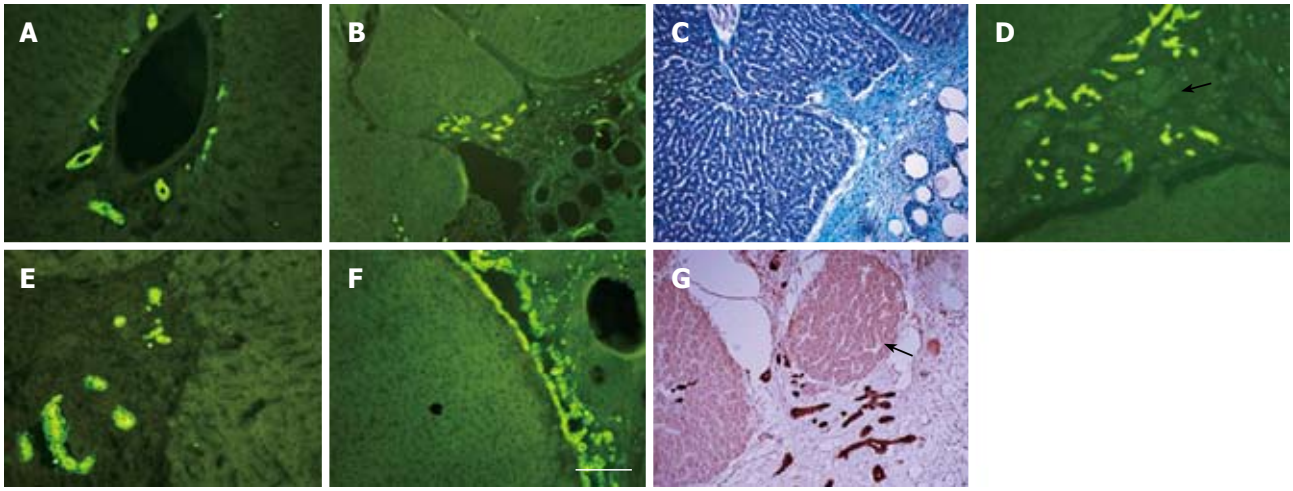

Figure 4 Immunostaining of normal and regenerated rat liver (from activated omentum) for cytokeratin-19, a marker of oval cells. A: Normal or uninjured liver lobe showing widespread presence of oval cells in the lining of bile ducts lying around a central vein; B, D, E, G: Different areas of injured liver showing extensions of cytokeratin-19 positive bile ducts in the interlying tissue between the liver and the activated omentum; C: Tissue section shown in B stained with Trichrome to show the bile ducts lying in the interlying omental tissue; F: Occasionally, the growing edge of the liver lying in the interlying tissue was seen to be entirely covered with cytokeratin-19 positive cells; G: Islands of liver tissue, probably newly formed, were seen in the interlying tissue (white arrows; also seen in D); A, B, D-F were stained by immunofluorescence (green); G was stained by immunoperoxidase (brown). The horizontal white bar in F represents 100 μm for all pictures.

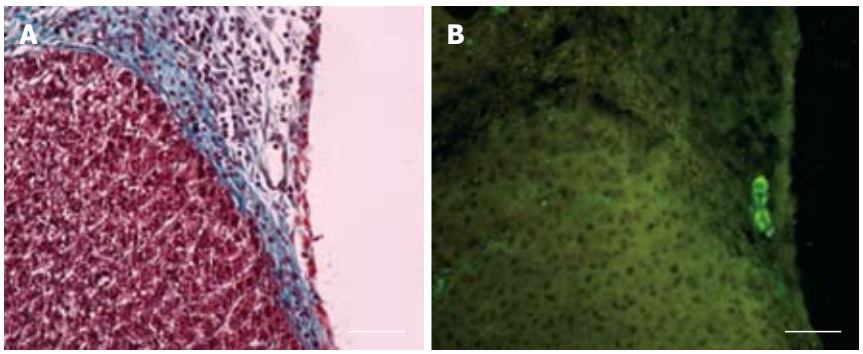

Figure 5 Histology and cytokeratin-19 immune staining of the liver at the boundary between the growing edge of the liver and the inactivated omentum at day 3 or 7 after injury. A: Trichrome stained section showing the adherent omental tissue with a thinner interlying tissue (blue stained) than that seen in the activated omentum group (Figure 3 for comparison); B: Cytokeratin-19 positive bile ducts were seen in the interlying tissue (same section as A) although these were much less frequent than those seen in the activated omentum group (Figure 4 for comparison). The horizontal white bar in pictures represents 100 μm.

weight) at different times after wounding and fusing of the omentum to the wound. The percent ratio in normal rats was established to be 3.85 ± 0.07 [Normal (n = 15); Figure 2]. In the activated omentum group, the liver grew to 110% of its original mass by day 3 (percent ratio: 4.4 ± 0.24) and to a maximum size of 150% by day 14 (percent ratio: 6.0 ± 0.16) (n = 6 at days 3, 7, 14 and 28; Figure 2). From day 14 to day 28, the liver did not grow any further, but remained enlarged (data not shown).

In rats with inactivated omentum, growth was observed at the site of omental fusion and filled the resected site with new tissue, however, the overall liver mass did not increase at any of the time points compared with the established normal liver mass [n = 3

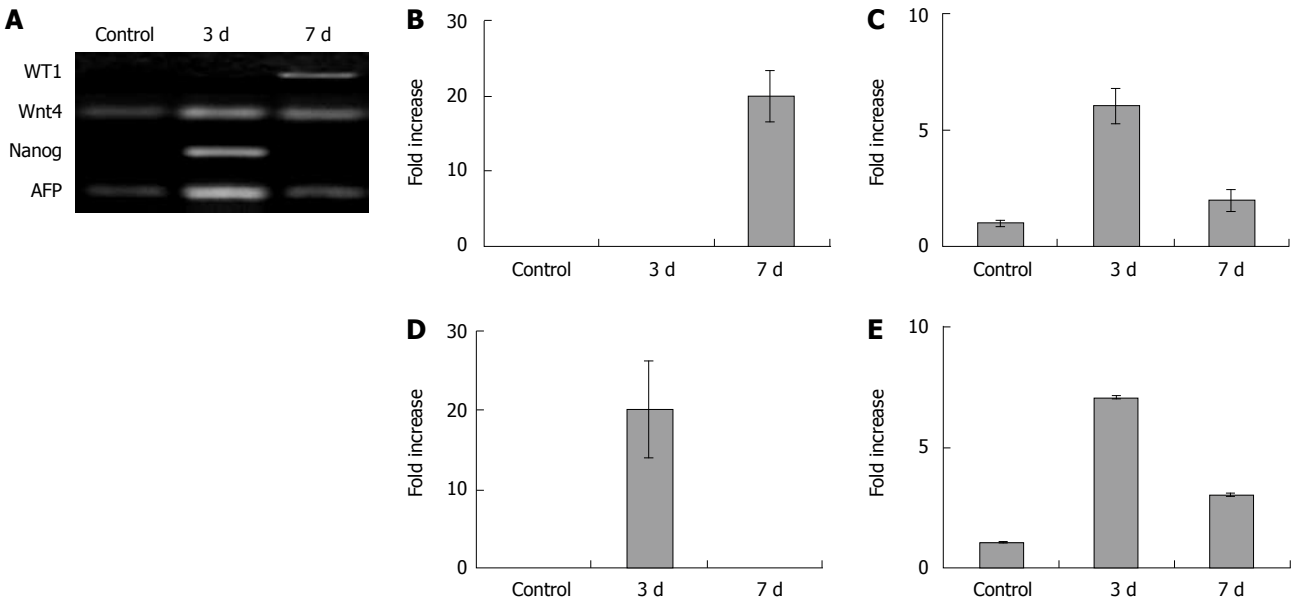

Figure 6 Activation of developmental genes in the regenerated liver at the wound site at days 3 and 7 after injury and fusion of the activated omentum. The regenerating part of the liver (part of liver attached to the omentum) showed high expression levels (7 to 20-fold) of WT-1, Wnt-4, Nanog, AFP by RT-PCR (A) compared with normal rat adult liver tissue (control). Wnt-4 (C), Nanog (D), and AFP (E) were maximally activated at day 3, while WT-1 (B) showed maximal activation at day 7. Tissue from regenerated liver, from sites further away from the wound area (0.5 cm, 1.0 cm further away in the same lobe and from an uninjured lobe), showed reduced activation of *WT-1*, *Wnt-4* and *AFP* genes (although higher in all cases compared with normal adult liver), suggesting that the regeneration stimulus 'rippled' throughout the liver from the wound area (data not shown). $n = 3$ in each bar and the differences amongst the bars within each of the figures B, C, D, and E are statistically significant ($P < 0.05$).

at days 3, 7, 14 and 28 (day 14 data shown in Figure 2)].

In omentectomized rats, the liver did not grow and the resection site remained visible up to day 28. The percent ratio of liver wt/body wt in this group was similar to that of normal rats at all time points [$n = 3$ at days 3, 7, 14 and 28 (day 14 data shown in Figure 2)].

Histology of the regenerated liver

In the activated omentum group, in which liver tissue grew to more than normal size, histological examination at the site of injury revealed normal hepatic architecture up to the point of omental fusion (Figure 3B). At the site of fusion there was a wide and compact band of interlying tissue between the omentum and the growing edge of the liver (Figure 3B). On one side of the interlying tissue lay the liver tissue and on the other side was the omental tissue with the embedded polydextran gel particles. The compactness and the width of the interlying tissue was maximal between 3 and 7 d after liver injury (400-600 μmol/L) (Figure 3B) and became thinner (100-150 μmol/L) and less compact by day 14 (Figure 3C). In the interlying tissue, small islands of liver tissue, probably newly formed, were seen (Figure 4G). By day 28 the interlying tissue was barely visible and appeared like a tissue septum (not shown).

In the inactivated omentum group in which liver growth was minimal, although the adherent omental tissue was clearly visible, a thinner interlying tissue than that seen in the activated omentum group was observed (Figure 5A). In the omentectomized group, the void left by the injury was visible at all time points. The injury site lacked omental attachment, but showed a layer of dead tissue (approx. five cells thick) which sloughed off from the wound edge by day 14 (not shown).

Immunostaining of the regenerated tissue for cytokeratin-19

Immunostaining of normal adult rat liver for cytokeratin-19, a well-known marker for bile ducts as well as oval cells (oval cells are presumed to be liver stem cells), showed the expected widespread presence of oval cells in the lining of bile ducts lying in the liver triads (Figure 4A). In the liver with or without omental attachment, cytokeratin-19 positive bile duct staining in the triads of the uninjured lobes was similar in intensity to that seen in the normal liver (not shown). In the liver tissue with activated omentum, remarkably, cytokeratin-19 positive bile ducts in the regenerated liver (day 3 or 7) extended into the interlying area between the liver and the omentum (Figure 4B-E). Occasionally, the growing edge of the liver at the interlying tissue was seen to be entirely covered with cytokeratin-19 positive cells (Figure 4F). Islands of liver tissue, probably newly formed, were present in the interlying tissue (Figure 4G).

In the inactivated omentum group, the interlying tissue attached to the wound edge also showed extensions of the cytokeratin-19 positive bile ducts (at days 3 and 7) (Figure 5B), although these were much less frequent than those seen in the activated omentum group. In omentectomized rats, the wound edge was devoid of omental attachment, and as expected no cytokeratin-19 positive bile ducts were seen outside the liver tissue (not shown).

Expression of developmental genes in the liver tissue attached to the activated omentum

To further investigate if activated omentum fused to the injured liver triggered developmental events in the adult liver, we determined the expression levels of several important genes associated with (1) pluripotent embryonic stem cell activity (Nanog, Oct-4), (2) liver differentiation (WT1, Wnt-4, HNF-6) and (3) fetal liver synthetic activity (α-fetoprotein; AFP) at days 3 and 7 after wounding using RT-PCR. Comparisons between normal rat adult liver and the regenerated liver attached to the activated omentum showed high expression levels (7-20 fold) of four of these genes (*WT-1*, *Wnt-4*, *Nanog*, *AFP*) in the regenerated liver tissue (Figure 6) (Oct-4 and HNF-6 levels were negative; not shown). Wnt-4, Nanog, and AFP were maximally activated at day 3, while WT-1 showed maximal activation at day 7.

Regenerated liver tissue from sites further away from the injured area (0.5 cm and 1.0 cm away from the activated omentum in the same lobe, and tissue from an uninjured lobe), showed reduced expression of *WT-1*, *Wnt-4*, and *AFP* genes (although higher in all cases compared to normal adult liver (Nanog, Oct-4, AFP did not change), suggesting that the regeneration stimulus 'rippled' from the injured area further into the liver tissue (data not shown).

In contrast, in the inactivated omentum group (compared to normal adult liver) the expression level of WT-1, Wnt-4 and Nanog increased to a much smaller degree than that observed in the activated omentum group at days 3 and 7 (WT-1 by 1.5-fold, Wnt-4 by 1.9-fold and Nanog by 1.2-fold), while AFP decreased by 0.8-fold ($P < 0.05$ in all cases; no detectable changes were seen in Oct-4 and HNF-6).

In the omentectomized group, while the expression levels of WT-1 and Nanog did not change, the levels of Wnt-4, Oct-4, AFP and HNF-6 decreased by 0.78-fold-undetectable levels compared with normal adult liver ($P < 0.05$ in all cases).

Omentum-assisted liver regeneration in rats treated with 2-AAF, a drug that blocks proliferation of hepatocytes

Because liver mass can increase due to either progenitor cell activation or hepatocyte multiplication, we performed the wedge wound experiment in a small group of rats in which hepatocyte multiplication was blocked by treatment with 2-AAF. This was performed to confirm that liver regeneration was *via* progenitor cells and not by hepatocyte expansion in our model. Fourteen days after wounding, 2-AAF treated rats showed complete healing of the wedge wound and increased the liver mass to 135% of the original mass [liver weight/body weight ratios: 5.1 ± 0.2 in 2-AAF treated ($n = 4$) *versus* 3.85 ± 0.07 in normal controls ($n = 15$); $P < 0.05$], confirming that omentum-assisted liver regeneration was not mediated by hepatocyte expansion, but by progenitor cell activation.

DISCUSSION

For many years the omentum was believed to have no specific function. During the course of the 19th century, however, several investigators recognized that it possessed healing properties. These were later exploited in a variety of surgical procedures designed to facilitate the healing of bone fractures, spinal cord injuries, and heart ischemia[1-6]. In previous studies we investigated this process and found that these properties can be enhanced by physically expanding the omentum with foreign particles. Under such conditions, the expanded omentum becomes rich in growth and angiogenic factors, and has abundant progenitor cells[7,8]. In a separate study we used this approach to generate new β-cells from the diabetic pancreas[11].

Here we applied these findings in an attempt to regenerate liver tissue by creating a surgical wound and allowing the omentum to fuse with the wound. We studied groups of rats with (1) no omentum (omentectomized), (2) inactivated omentum, and (3) omentum pre-activated by foreign polydextran particles. We found no liver growth in omentectomized rats. In rats with inactivated omentum, the omentum fused with the injured site and although new growth was noted, this did not result in a significant increase in liver mass. However, in rats with activated omentum, following omental fusion, the liver grew to fill the wound and continued to grow, both at the wound site and globally, to a level 50% greater than the original mass. These findings suggest that the omentum plays an important role in bringing about growth and regeneration of the injured liver. The amount of liver growth induced by the omentum was proportional to the degree of omental activation, consistent with our previous observations that the concentration of growth factors and the number of progenitor cells in the omentum increase with increased activation[7,8].

Clinicians have long known that the liver has the ability to regenerate. Experimentally, a 70% hepatectomy (either surgically or chemically) induces a form of liver regeneration in which growth is largely due to hepatocyte proliferation[9-13]. When hepatectomy is carried out following the administration of drugs which inhibit hepatocyte proliferation, the regeneration is mainly due to the expansion of oval cells[14-17]. In these various models, there is a massive loss of functional liver tissue, which then systemically triggers a cascade of cytokines (such as tumor necrosis factors-α, IL-6 and growth factors). In our model, the injury was so slight that regeneration would not occur unless the omentum was activated, as shown in our omentectomized control rats.

In further studies we attempted to understand the mechanism by which activation of the omentum causes liver regeneration. Histologically, at the fusion site between the activated omentum and the liver, we found a wide and compact interlying band of tissue into which tubular structures resembling bile ducts extended and proliferated. On staining, these structures were strongly positive for cytokeratin-19, a known marker for oval cells, believed to be liver progenitor cells[18-20]. At an early stage of regeneration the oval cells in the interlying tissue were seen near small islands of liver tissue; later these islands became integrated into the native liver, so

that the border between the native and the new liver could no longer be discerned in stained sections. Because proliferation of the progenitor oval cells took place in the omentum rather than in the liver, they may have had more room to proliferate and expand, accounting for the robust, supra normal liver growth.

Because liver tissue can also regenerate by hepatocyte proliferation we repeated our experiment in rats treated with 2-AAF, a drug commonly used to inhibit hepatocyte multiplication. Our finding that the activated omentum continued to exert its regenerative property in 2-AAF-treated rats by increasing the liver mass to 135% of the native mass, further suggested that the proliferation of progenitor oval cells rather than proliferation of hepatocytes was responsible for liver regeneration in our model.

As genes and proteins involved in liver development (Wnt-4, WT-1, HNF-6, AFP) may become reactivated during liver regeneration[15-17,21-23], we tested these developmental genes and also Nanog and Oct-4, markers of early progenitor cells[24,25], to see if they were altered in regenerating liver tissue. We found that many of these genes, silent in the adult liver, were highly up-regulated (7 to 20-fold) after fusion of the activated omentum. Nanog was up-regulated by 20-fold three days after omental fusion and returned to undetectable levels by day seven, suggesting the transient presence of early progenitor cells. Gene expression of Wnt-4 and AFP was highest at day 3 and decreased by day 7, in contrast to WT-1 which was unchanged at day 3 and highest at day 7. These findings are consistent with the transient activation of these transcription factors (WT-1 and Wnt-4) known to occur in liver re-modeling and differentiation[16,26,27]. HNF-6, a marker strongly associated with hepatocyte proliferation[28], was unchanged, as also noted in a previous study of non-hepatocyte mediated (but progenitor cell mediated) liver regeneration[16]. This was not surprising because liver growth by omental fusion was *via* oval cells and not dependent on hepatocyte proliferation. Interestingly, we also found a few selected genes (*WT-1*, *Wnt-4* and *AFP*) to be activated in regions of the native liver 0.5 cm and 1.0 cm from the wound edge. The level of activation decreased as the distance from the wound edge increased, suggesting that a paracrine effect was exerted by the omentum. Importantly, we found lower activation levels of genes in the inactivated omentum group (1.2-1.9-fold), consistent with reduced liver growth seen in these rats. Furthermore, in omentectomized rats where there was no liver growth, a decrease in gene expression levels was observed compared with normal liver.

The present study is the first to demonstrate the unique role of the omentum in traumatic wound healing of the liver. We have shown previously that omental derived factors stimulate wound healing and can be upregulated by pre-activating the omentum. By bringing the omentum into close contact with injured liver we observed a vigorous regeneration of liver tissue. Although the liver is known to regenerate to the original size following a significant loss of hepatic tissue, there are no reports of liver regeneration up to 150% of the original size as noted in our study. As both cytokeratin-19 positive cells and expression of developmental genes were increased, we postulate that both growth factors and stem cells are conveyed to the site of injury by omental fusion. It may be argued that bringing the omentum into contact with damaged organs after controlled deliberate wounding may have immediate clinical applicability. Also, the use of progenitor cells isolated from the activated omentum or of the growth factors secreted by these cells holds further promise of other exciting therapeutic possibilities.

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COMMENTS

Background

Although the liver is a unique tissue that can regenerate after an acute injury, it has been a challenge to induce such regeneration after chronic liver disease. It is, therefore, important to study mechanisms of liver regeneration in order to devise new approaches for regeneration following damage by chronic disease. Although embryonic stem cells have the power to regenerate liver tissue, their use is hampered by ethical, political and safety concerns. In that regard, the use of adult stem cells derived from the patient's own tissue to regenerate the liver is free of such concerns and, therefore an alternative approach.

Research frontiers

Stem cells have been derived from several adult organs such as bone marrow, skin, hair, kidney and dental pulp. Although these cells express stem cell markers and differentiate to other cell phenotypes in culture they seem to lack the potency to regenerate an organ *in vivo*. Identifying a source of adult stem cells that could regenerate liver or other organs would be an immense advantage.

Innovations and breakthroughs

Singh and his colleagues devised a methodology to harness adult stem cells to regenerate the liver by first activating the omentum using a foreign body to increase its content of stem cells and growth factors. They then cut and removed a small piece of the liver tissue and let the activated omentum adhere to the wound in order to supply stem cells to the injured liver. They found that the liver of these rats with an activated omentum expanded to a size 50% greater than the original, an outcome never reported before. This approach represents an application of adult stem cells to regenerate an organ *in vivo*.

Applications

This method of liver regeneration is novel and could be attempted in patients with liver failure in order to regenerate new liver tissue.

Terminology

Activated omentum, which is central to this methodology of liver regeneration, was created by injecting polydextran particles (foreign body) into the abdominal cavity. As the omentum naturally grows to encapsulate the particles individually it expands 20-30 times its original size and has abundant stem cells and growth factors, which appear to be the basis of the regenerating power of the omentum.

Peer review

Reviewers considered the use of the omentum to regenerate the liver as meritorious and interesting. Further they thought the paper was well written, results were clear, and the data supported the conclusions reached by the authors.

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Aminoguanidine impedes human pancreatic tumor growth and metastasis development in nude mice

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Abstract

AIM: To study the action of aminoguanidine on pancreatic cancer xenografts in relation to cell proliferation, apoptosis, redox status and vascularization.

METHODS: Xenografts of PANC-1 cells were developed in nude mice. The animals were separated into two groups: control and aminoguanidine treated. Tumor growth, survival and appearance of metastases were determined *in vivo* in both groups. Tumors were excised and *ex vivo* histochemical studies were performed. Cell growth was assessed by Ki-67 expression. Apoptosis was studied by intratumoral expression of B cell lymphoma-2 protein (Bcl-2) family proteins and Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling (Tunel). Redox status was evaluated by the expression of endothelial nitric oxide synthase (eNOS), catalase, copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx). Finally, vascularization was determined by Massons trichromic staining, and by VEGF and CD34 expression.

RESULTS: Tumor volumes after 32 d of treatment by aminoguanidine (AG) were significantly lower than in control mice ($P < 0.01$). Median survival of AG mice was significantly greater than control animals ($P < 0.01$). The appearance of both homolateral and contralateral palpable metastases was significantly delayed in AG group. Apoptotic cells, intratumoral vascularization (trichromic stain) and the expression of Ki-67, Bax, eNOS, CD34, VEGF, catalase, CuZnSOD and MnSOD were diminished in AG treated mice ($P < 0.01$), while the expression of Bcl-2 and GPx did not change.

CONCLUSION: The antitumoral action of aminoguanidine is associated with decreased cell proliferation, reduced angiogenesis, and reduced expression of antioxidant enzymes.

Key words: Aminoguanidine; Pancreatic ductal carcinoma; Tumor growth; Metastasis; Apoptosis

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INTRODUCTION

Pancreatic cancer is an aggressive carcinoma usually diagnosed at an advanced stage and shows a median

survival time of only three months. Approximately half of the cases are metastatic at the time of diagnosis, while the remainder have locally advanced unresectable disease. To date, the only effective treatment is surgical therapy. Adjuvant chemo- and radiotherapy have not led to significant improvements in outcome[1].

Nitric oxide synthase (NOS) produce nitric oxide (NO) by the oxidation of L-arginine. There are three known isoenzymes of NOS: two constitutive forms [neuronal NOS (nNOS) and endothelial NOS (eNOS)] and one inducible form, inducible NOS (iNOS). Constitutive isoforms nNOS and eNOS respond to a calcium influx with a transient release of NO[2]. On the other hand, iNOS always generates high quantities of NO over a prolonged period[3]. This isoform is not only expressed in activated macrophages, usually infiltrating tumors, but also in various types of malignant cells.

Nitric oxide is a highly diffusible, lipophilic free radical. Under certain pathological conditions, NO can combine with the superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), a potent reactive nitric oxide species that nitrates tyrosine residues in proteins and induces DNA damage and lipid peroxidation, leading to cell damage and often cell death. NO in cancer exhibits both a cytotoxic and a cytoprotective effect according to its concentration within the tumor microenvironment. Low levels of NO produced by tumor cells themselves aid tumor progression, while the high level of NO produced by tumor adjacent macrophages function as a tumor suppressor agent through the induction of apoptosis[4-7].

Aminoguanidine, a nucleophilic hydrazine compound first synthesized more than 100 years ago, is an irreversible inhibitor of iNOS, which also inhibits eNOS and nNOS at higher concentrations[8]. It has been shown *in vivo* to prevent disease states characterized by the pathological overproduction of NO, such as diabetic complications[9], age-related arterial stiffening, cardiac hypertrophy[10] and also tumors (including cholangiocarcinoma[11] and gastric cancer[12]). These effects of AG are exerted by modulating proliferation[12], apoptosis[12], angiogenesis[12], by the production of free radicals[12] and by preventing the formation of advanced glycation end products (AGEs)[13,14].

The human pancreatic ductal carcinoma, PANC-1, cells express constitutive eNOS. However, though it is the most commonly tumor associated synthase isoform, PANC-1 cells do not express iNOS[15].

Although the *in vitro* action of NOS inhibitors has been extensively studied, little research has been undertaken as regards their *in vivo* effects on cancer growth. Therefore, the aim of this work was to study the action of the NOS inhibitor AG in PANC-1 human pancreatic cancer xenografts in nude mice in relation to tumor growth, angiogenesis and the expression of antioxidant enzymes.

MATERIALS AND METHODS

Xenografts in nude mice

PANC-1 cells (7×10^6) were collected by centrifugation and resuspended in 100 μL RPMI-1640 (GIBCO, Grand Island, New York, USA) to be inoculated in the dorsal flank of each nude mouse (cepa N:NIH(S)-nu). When the tumors that developed reached a volume of 500 mm^3 they were excised, cut into 27 mm^3 pieces and grafted into the dorsal flank of another nude mouse. Xenografted mice were separated in four groups (n = 7) and received daily doses of AG (aminoguanidine hydrochloride, Sigma, Saint Louis, Missouri, USA) of 1, 2 or 4 mg/mL in drinking water. *In vivo* treatments began when the graft volumes reached 100-150 mm^3. The control group was left without treatment. Tumor size was measured with a caliper three times a week and volume was calculated as $[(\text{mayor diameter} + \text{minor diameter})/4]^3 \times \pi 4/3$. Treatments lasted 32 d. Two-way ANOVA, Bonferroni post test and non linear fit of tumor growth data were carried out by GraphPad Prism version 5.00™. All the experiments using mice were performed according to the NRC [National Research Council] Guide for the Care and Use of Laboratory Animals, 7th ed, Washington DC, National Academy Press, 1996.

Survival

Mice bearing xenografts were divided into two groups (n = 7), AG (2 mg/mL in drinking water) and control, and followed until spontaneous death. Kaplan-Meier survival curves, median survival time of each group and P value were obtained by GraphPad Prism™. Development of palpable metastases was checked three times a week in both groups and the metastases were distinguished according to their location as either homolateral (those that appeared in the same flank as the xenograft) or contralateral (those that appeared in the opposite flank).

Histochemistry

At the end of treatments, tumors were excised, fixed in 40 g/L formaldehyde in PBS (formalin), paraffin embedded and sliced into 3-μm thick sections for: (1) Tunel, using TdT-FragEL DNA Fragmentation Detection Kit (Calbiochem, a brand of EMD Biosciences, La Jolla, California, USA) according to the manufacturer's instructions. 3,3'-Diaminobenzidine tablets (DAB; Sigma) were used for staining and methyl green for counterstaining; (2) Immunohistochemical detection using antibodies against Ki-67 (1/50, Dako Cytomation, Carpinteria, California, USA), PCNA (1/100, Dako Cytomation), Bcl-2 (1/50, Santa Cruz Biotechnology, Santa Cruz, California, USA), Bax (1/50, Santa Cruz Biotechnology), eNOS (1/30 Sigma), VEGF (1/20, R&D Systems, USA), catalase (1/50, Sigma), CuZnSOD (1/50, Calbiochem), MnSOD (1/50, Calbiochem) and GPx (1/125, Stressgen, Ann Arbor, Michigan, USA). The appropriate secondary HRP-conjugated antiserum was employed in each case. DAB tablets were used for staining and hematoxylin for counterstaining; (3) Tumor vascularization was assayed by means of Masson trichrome and CD34 (1/50, Santa Cruz Biotechnology) staining; and (4) hematoxylin and eosin staining of tumors and metastatic lymph nodes.

Microscopic observations were performed using an Axiolab Karl Zeiss microscope by two independent observers. Photographs were taken with a Canon Power

Table 1 AG enlarges tumor volume doubling time (mean ± SD)

Treatment	Doubling time (d)
Control	7.8 ± 0.3
AG 1 mg/mL	7.8 ± 0.3
AG 2 mg/mL	10.0 ± 0.5[a]
AG 4 mg/mL	11.1 ± 0.7[a]

Tumor volume doubling times were obtained by non linear fit of tumor growth rate (shown in Figure 1) to an exponential growth equation. [a]$P < 0.01$ *vs* control and AG 1 mg/mL, ANOVA one way with Bonferroni post test.

Shot G5 digital camera and processed with Remote Capture 2.7 software. Metastatic lymph nodes, Tunel, trichrome stain and CD34 of control mice were observed at 400 × and the remaining determinations at 630 ×. Microscopic observations were done in ten random fields and graded as percent of positive cells. Positive nuclei were considered as positive cells for Tunel, Ki-67 and PCNA, whereas diffuse positive cytoplasms were considered as positive cells for cytoplasmic proteins. Non parametric Mann Whitney tests on the percent of positive cells in control and AG groups were performed by GraphPad PrismTM. Peritumoral vascularity was assessed by screening trichrome stained sections at 50 × magnification to identify the largest vascular areas around the tumor. In these hot spots, individual vessel count was evaluated at 400 × magnification using an ocular grid. Intratumoral vascularity was evaluated on trichrome and CD34 stained slices, counting vessels inside the tumor at 400 × magnification in ten random fields (in the identified hot spots).

RESULTS

In vivo studies

Tumor growth: Animals were separated into four groups (control and 1, 2 and 4 mg/mL AG) to evaluate the effect of AG on tumor growth. Mice were treated for 32 d when grafts volume reached 100-150 mm^3. Tumor volumes were determined three times a week and finally referred to the initial volume for each treatment (Figure 1). AG (2 and 4 mg/mL) treatment significantly diminished the tumor growth rate. Tumor volumes of mice treated with AG 2 and 4 mg/mL from day 28 of treatment on were significantly different from tumors of control animals ($P < 0.01$ for AG 2 and 4 mg/mL *vs* control, two way ANOVA and Bonferroni post test).

In the same way, tumor volume doubling time of mice treated with AG 2 or 4 mg/mL was significantly greater than the other groups (Table 1; $P < 0.01$ *vs* control and AG 1 mg/mL, Anova one way with Bonferroni post test).

Tumor histology: After excision of tumors, xenografts usually showed macroscopic infiltration of the dermis and abdominal muscular wall. Under microscopic observation, tumors presented undifferentiated adenocarcinoma cells, with high grade of atypia, marked anisokaryosis and anisocytosis. Multinucleated cells and atypical mitoses (often tri- and tetrapolar mitoses) were frequently observed.

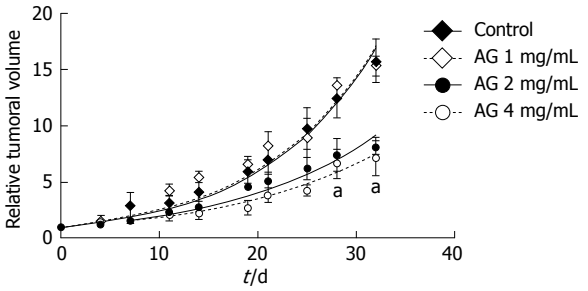

Figure 1 Antitumor effect of AG in PANC-1 xenografted mice. Tumor volumes were determined and referred to the initial volume for each treatment. [a]$P < 0.01$ for AG 2 and 4 mg/mL *vs* control, two way ANOVA and Bonferroni post test.

Survival and metastases: Two extra groups of PANC-1 xenografted animals (control and AG 2 mg/mL) were followed three times a week until spontaneous death. Kaplan-Meier survival curves (Figure 2A) were obtained for AG 2 mg/mL (dotted line) and control (solid line) groups. Median survival, i.e. the time at which survival percent equals 50%, of AG treated animals (95 d) was significantly greater than that of control mice (74 d; $P < 0.01$ for AG *vs* control, log-rank test).

The same groups of animals were checked for the development of palpable metastases (concomitantly with survival studies) until the death of the animals. The total number of both homolateral and contralateral palpable metastases developed in each group ($n = 7$ each group) was determined (Figure 2B). Appearance of metastases was significantly delayed in AG treated mice. The medians of appearance were 59 d for control homolateral metastases, 78 d for AG homolateral metastases and 92 d for AG contralateral metastases. The median for control contralateral metastases could not be determined since only two metastases appeared by the time of death. Curves were significantly different.

Also, the number of homolateral metastases per animal was lower in the AG group. The first control mouse died at 64 d; therefore, the mean number of metastases per animal at 63 d was obtained and compared for each of the four groups. Means, expressed as mean ± SD, were significantly different. The number of homolateral metastases in the control (1.8 ± 0.4 per mouse) was significantly greater than in the other groups (0.3 ± 0.3 per mouse for control contralateral, 0.1 ± 0.1 for AG homolateral and 0 for AG contralateral).

The metastases that developed were analyzed by microscopic observation after the mice died (Figure 3A). Both homolateral and contralateral metastases appeared mainly in the lymph nodes. The node architecture was only preserved in a few peripheral sections due to the considerable extent of tumor infiltration. Infiltrating tumor cells showed the same histological characteristics as xenograft neoplastic cells (Figure 3B). This finding confirms the metastatic character of the multiple masses found.

Ex vivo studies

Apoptosis and cell proliferation: Cell proliferation

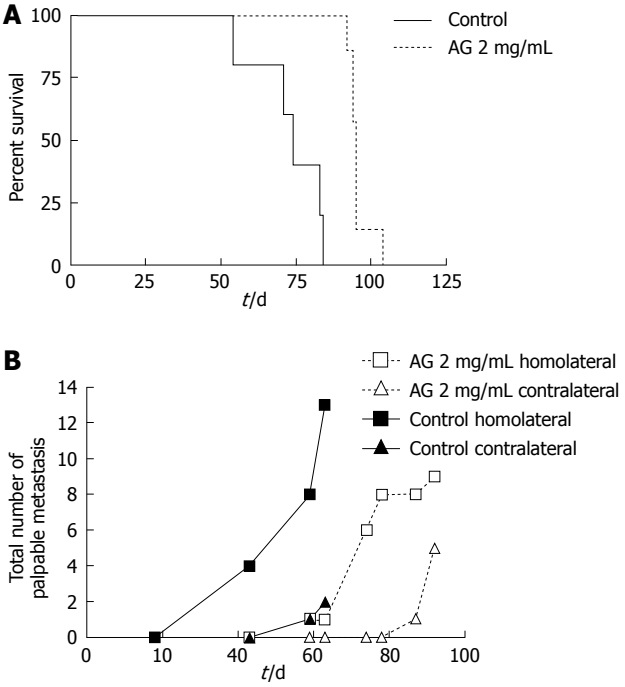

Figure 2 Action of AG on survival and metastases in PANC-1 xenografted mice. A: Time of spontaneous death of mice was determined, Kaplan-Meier survival curves were plotted and median survival was calculated. $P < 0.01$ for AG *vs* control, log-rank test; B: The development of palpable homolateral and contralateral metastases, which appeared mainly in lymph nodes, was checked until the death of the animals. Total number of metastases developed in both groups ($n = 7$ each group) and medians of appearance were determined. Curves were compared by logrank test, $P < 0.01$.

in xenografts was evaluated through the expression of Ki-67[16] (Figure 3C and F). Apoptosis was studied in tumors by the Tunel assay (Figure 3D and G) and by the expression of antiapoptotic protein Bcl-2 and proapoptotic Bax (Figure 3E and H).

Ki-67 expression, evaluated as percent of positive cells (mean ± SD), was lower in tumors of AG treated mice than in the control group (19 ± 11 *vs* 78 ± 10, $P < 0.01$, Mann Whitney test). Similar results were obtained by PCNA immunodetection, another proliferation marker.

The Tunel test showed a lower proportion of apoptotic cells in grafts of animals which had received AG than the control group, (1 ± 1 *vs* 5 ± 1, $P < 0.01$, Mann Whitney test), evaluated as percent of positive cells (mean ± SD). In the same way, the expression of Bax, evaluated as percent of positive cells (mean ± SD), was significantly lower in tumors of AG treated animals than in control animals (17 ± 13 *vs* 88 ± 11, $P < 0.01$, Mann Whitney test). Lastly, Bcl-2 expression was not altered by AG treatment (20 ± 10 *vs* 15 ± 9, AG *vs* control). These results, therefore, indicate an antiapoptotic effect of AG in xenografts.

Angiogenesis: Angiogenesis is essential for the growth, invasion and metastasis of a tumor. eNOS has been shown to participate in tumor progression by promoting angiogenesis[17,18]. Vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen and vascular permeability factor, is mainly implicated in tumor growth *via* the stimulation of NO production[6,18]. To evaluate tumor angiogenesis in PANC-1 xenografts, eNOS and VEGF expressions were assayed, as well as intra and peritumoral vascularity (by the trichrome stain and CD34 expression; a marker of endothelial cells).

The expression of VEGF (Figure 3I and L) and eNOS (Figure 3J and M) was determined as percent of positive cells (mean ± SD). Grafts of AG treated animals expressed lower levels of eNOS and VEGF than tumors of control mice (9 ± 9 *vs* 75 ± 9, $P < 0.01$ and 25 ± 17 *vs* 84 ± 9, $P < 0.01$, Mann Whitney test, respectively).

Vascularity evaluated by the trichromic stain (Figure 3N) in xenografts of untreated mice showed capillaries and medium size vessels in intratumoral areas, while medium and large size vessels were observed in peritumoral tissues. AG treatment did not modify peritumoral vascularity; however, a diminished level of intratumoral vascularity was detected. As shown in Figure 3K and N, the intratumoral observations were confirmed by CD34 staining (6 ± 3 *vs* 21 ± 6, AG *vs* control, $P < 0.05$, Mann Whitney test), indicating an antiangiogenic effect of AG.

Expression of antioxidant enzymes: Reactive oxygen species (ROS), reactive nitrogen species (RNS) and redox state modulate cell proliferation, apoptosis and angiogenesis. Cells defend themselves against ROS mainly by antioxidant enzymes. In this way, superoxide dismutases convert superoxide radicals into hydrogen peroxide, which is in turn scavenged by catalase and GPx. According to their intracellular distribution, cytosolic CuZnSOD protects against cytosolic superoxide, while mitochondrial MnSOD decomposes mitochondrial-generated superoxide[19]. The four antioxidant enzymes were assessed in PANC-1 xenografts. Tumors of AG treated mice showed a lower expression (evaluated as percent of positive cells; mean ± SD) than grafts of control animals, for CuZnSOD (14 ± 10 *vs* 80 ± 15), MnSOD (21 ± 12 *vs* 77 ± 17) and catalase (9 ± 6 *vs* 49 ± 12) ($P < 0.01$, Mann Whitney test). Meanwhile, GPx expression remained unchanged (70 ± 11 *vs* 81 ± 9, AG *vs* control).

DISCUSSION

The effect of the NOS inhibitor aminoguanidine was studied in xenografts obtained by inoculation of PANC-1 human pancreatic ductal carcinoma cells into nude mice.

In vivo results showed an antitumor effect of AG, including suppression of tumor growth, enhanced survival, delayed appearance of metastases and a lower number of homolateral metastases per animal. Similarly, tumor development diminished in a hamster model of cholangiocarcinoma[11]. However, AG displayed an antimetastatic action on a model of inflammation-based murine fibrosarcoma progression, altering neither tumor incidence nor tumor growth[20]. Metastatic cell behavior could be positively or negatively regulated by nitric oxide, accordingly to iNOS and eNOS expression in endothelial cells, macrophages, stromal fibroblasts and cancer cells[21]. In an attempt to explain the observed

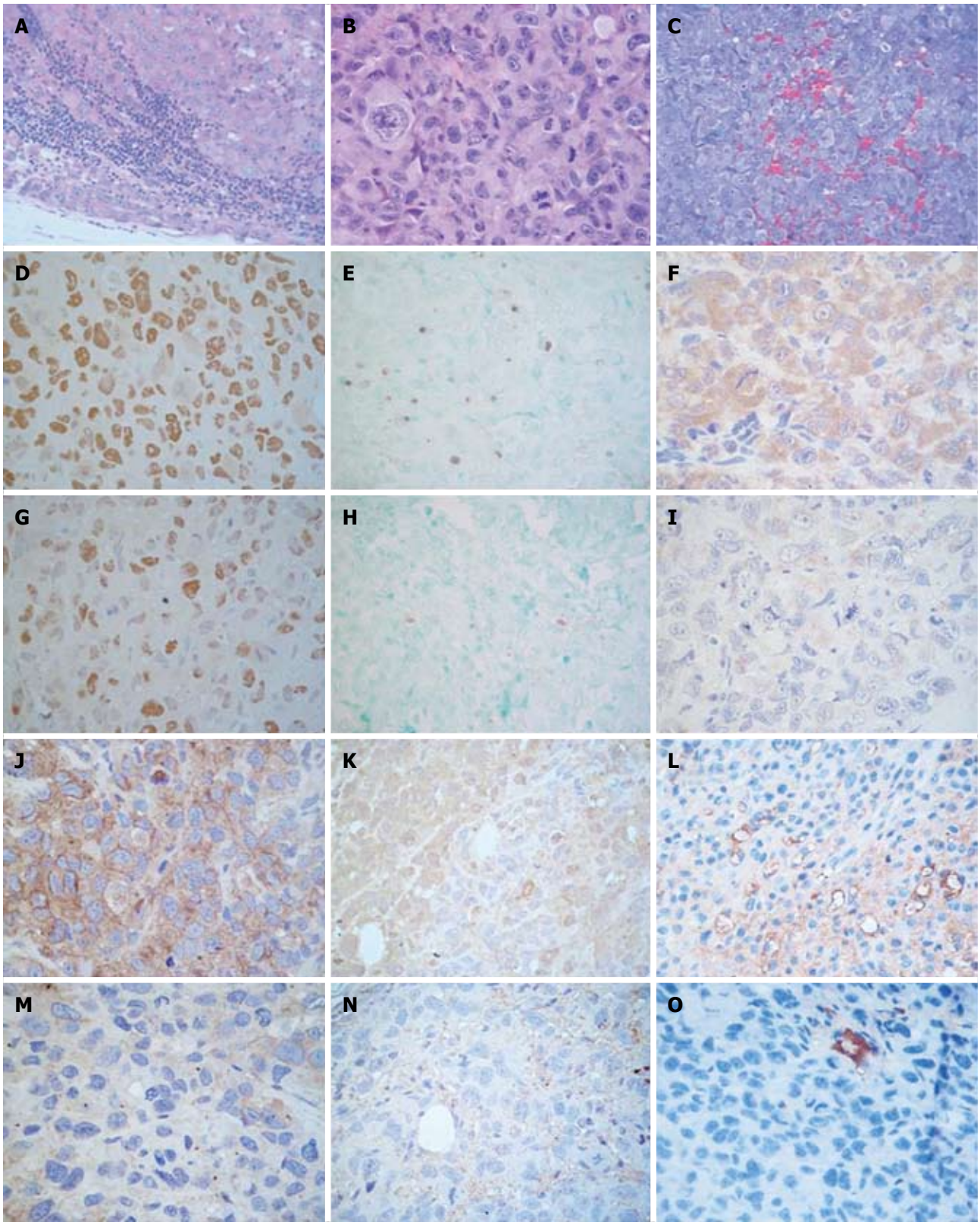

Figure 3 Histopathology and immunohistochemistry of PANC-1 xenografted mice. Formalin fixed paraffin embedded tissue sections of control mice were stained with HE. A: Metastatic lymph node (× 400); B: Xenografts (× 630). Immunohistochemistry in control and AG treated mice. Tissue sections were stained with DAB and counterstained with hematoxylin; C: Formalin fixed paraffin embedded tissue sections of control mice were stained with trichromic solution (× 400); D, G: Ki-67 (× 630); E, H: Tunel (× 400); F, I: Bax (× 630); J, M: VEGF (× 630); K, N: eNOS (× 630); L, O: CD34 in control (× 400) and AG treated (× 630) mice. Formalin fixed paraffin embedded tissue sections of control mice were stained with trichrome solution.

in vivo antitumor action of AG, multiple *ex vivo* experiments were performed.

Tissues, both normal or abnormal, grow mostly by increasing the number of cells. In turn, the cell number of a given population is regulated by the balance between proliferation and death[22]. Altered indices of proliferation and/or apoptosis could explain the diminished tumor growth observed in AG treated mice xenografts. The

proliferation index, assessed by Ki-67 and PCNA expression, indicated that AG was antiproliferative in PANC-1 xenografts. Moreover, the apoptotic pathway state (evaluated by Bcl-2/Bax expression ratio) and apoptotic death (assayed by the Tunel assay) revealed an antiapoptotic action of AG in pancreatic tumors. It has been hypothesized that Bcl-2 binds proapoptotic Bax to counteract its effects. Thus, the relative expression of Bcl-2 and Bax are involved in the regulation of the cell death program. In this sense, AG treatment enhanced the Bcl-2/Bax ratio while diminishing the rate of apoptosis. Therefore, AG did not lead to increased tumor cells apoptosis, but caused them not to proliferate.

Many articles support the proapoptotic action of NO associated with iNOS induction and high NO levels[5], while others report a positive association between iNOS induction and tumor cell growth[11,23]. In a recent report[24], the *in vivo* and *in vitro* inhibition of hepatocellular carcinoma growth by AG was associated with a proapoptotic effect of this drug. The cross-talk between NO and RAS/ERK and IKK/NF-κB pathways was determined to be crucial to this action. Conversely, our data show that the inhibition of PANC-1 xenografts' growth induced by AG is coupled to an antiapoptotic effect. It is well known that NO levels are quite different depending on the NOS isoform (eNOS or iNOS) involved in NO production. Although different NO concentrations regulate pathways related to either survival or cell death, the final effect on cell fate also depends on such factors as cell type, signaling pathways involved, genetic background and NO concentration in the microenvironment. Our *in vitro* experiments using PANC-1 cell line showed that the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) prevents cell proliferation (unpublished results). We have also demonstrated that activated ERK 1/2 down-modulation is related to the *in vitro* inhibition of the proliferation of PANC-1 cells[25]. Thus, even though a direct action of the low levels of NO produced by eNOS on this signal transduction pathway was not assessed in our work, it cannot be ruled out.

Another potential contribution to *in vivo* tumor growth might be determined by interactions with stroma. Fibroblasts and inflammatory cells express iNOS and NO is related to cytokines and growth factors secretion. The iNOS inhibition in stromal cells by AG treatment might modulate neoplastic cells' survival and death[26,27]. In addition, vascularity modulation is also involved in tumor growth. Locally, new blood vessel formation is essential for supplying oxygen and other nutrients to tumor cells and is one of the altered manifestations that dictate metastatic tumor growth. It is well known that the VEGF-mediated angiogenesis requires NO production *via* eNOS in endothelial cells. Growing evidence supports the hypothesis that reciprocal relations between NO and VEGF might contribute to drive angiogenesis in pathophysiological conditions, depending on the amount of produced NO[28]. Our results showed that AG reduced tumor vascularization and VEGF and eNOS expression in PANC-1 grafts. Small amounts of NO synthesized by eNOS are required for VEGF up-regulation[28]. The low level of NO produced by the AG-inhibited eNOS enzyme might induce the down-modulation of VEGF. In turn, VEGF down-regulation might hinder eNOS up-regulation, consistent with the reduced eNOS expression detected in PANC-1 grafts. Therefore, the antiangiogenic action of AG could account for the reduced tumor growth rate, the lower rate of metastasis and the delay in its appearance.

Lastly, the expression of CuZnSOD, MnSOD and catalase antioxidant enzymes was diminished by AG. The same effect on these enzymes was observed in diabetic rats[29] and on superoxide dismutases in doxorubicin treated rats[30]. GPx activity was slightly decreased after AG treatment in a model of liver injury in rat[31]. Chronic treatment with AG might cause a disruption of the cellular redox balance due to its capacity to scavenge oxidant reactive species[32] and to inhibit NO synthesis. This could explain the reduced expression of antioxidant enzymes as a compensatory mechanism. Accordingly, our preliminary *in vitro* experiments in PANC-1 cells showed that hydrogen peroxide and NO intracellular levels were modified by AG, while cell proliferation was reduced. Cell cycle progression is modified by cellular redox status and the magnitude of its imbalance might lead to cell proliferation, differentiation, growth arrest, apoptosis or necrosis[33]. Different cell lines require the pro-oxidant status to persist beyond the G1-S restriction point in the cell cycle[34,35]. The AG driven impaired production of NO could hinder that oxidant level and, in turn, could prevent proliferation.

In conclusion, aminoguanidine, by exerting an antiapoptotic effect, shows an antitumoral action evidenced by a lower tumor volume at the end of treatment, a delayed appearance of palpable metastases and an extended life span. The antiproliferative action is associated with a lower intratumoral Ki-67 content, an antiangiogenic effect, a reduced NO production and reduced expression of antioxidant enzymes.

COMMENTS

Background

Pancreatic cancer is a devastating disease because of its high mortality rate. Chemotherapy alone or combined with radiotherapy are poor attempts to overcome the illness. In cancer, the free radical nitric oxide exhibits both a cytotoxic and a cytoprotective effect according to its concentration within the tumor microenvironment. Although the *in vitro* action of nitric oxide synthase inhibitors (such as aminoguanidine) has been extensively studied in tumor cells, little research has been undertaken as regards their *in vivo* effects on cancer growth.

Research frontiers

Nitric oxide synthases catalyze the production of nitric oxide (NO), which, depending on its concentration, could act both as tumor promoter or suppressor. Compounds that modify expression and/or activity of these enzymes may be considered useful tools in cancer research.

Innovations and breakthroughs

The nitric oxide synthase (NOS) inhibitor aminoguanidine, by exerting an antiapoptotic effect, shows an antitumoral action evidenced by a lower tumor volume at the end of treatment, a delayed appearance of palpable metastases and an extended life span. The antiproliferative action is associated with a lower expression of endothelial NOS (eNOS) and antioxidant enzymes.

Applications

Chemoresistance is still the major problem of anticancer drug treatment of malignant diseases such as pancreatic carcinoma. Aminoguanidine could

provide an attractive line of investigation as a multi-modal avenue due to its different effects on tumor biology.

Terminology

Nitric oxide is synthesized by a group of enzymes: the nitric oxide synthases. At least three isoforms have been described: eNOS, neuronal NOS (nNOS) and inducible NOS (iNOS). Most cancer cells express these enzymes and the NO produced is involved in biological processes associated with both survival and cell death.

Peer review

In this work, the authors have studied the action of the nitric oxide synthase inhibitor aminoguanidine (AG) in PANC-1 cells xenografts in relation to cell proliferation, apoptosis, angiogenesis and redox status. Interestingly, authors indicate that AG has strong effects on tumor progression, through inhibiting growth, apoptosis, vascularization and metastasis. This study will be of great interest to the carcinogenesis field, particularly in the design of new therapeutic drugs for pancreatic cancer.

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ORIGINAL ARTICLES

Suppression of matrix metalloproteinase-2 *via* RNA interference inhibits pancreatic carcinoma cell invasiveness and adhesion

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Abstract

AIM: To investigate the inhibitory effects of RNA interference (RNAi) on expression of *matrix metalloproteinase-2* (*MMP-2*) gene and invasiveness and adhesion of human pancreatic cancer cell line, BxPC-3.

METHODS: RNAi was performed using the vector (pGPU6)-based small interference RNA (siRNA) plasmid gene silence system to specifically knock down MMP-2 expression in pancreatic cancer cell line, BxPC-3. Four groups of different specific target sequence in coding region of MMP-2 and one non-specific sequence were chosen to construct four experimental siRNA plasmids of pGPU6-1, pGPU6-2, pGPU6-3 and pGPU6-4, and one negative control siRNA plasmid of pGPU6 (-). MMP-2 expression was measured by reverse transcription polymerase chain reaction (RT-PCR) and Western blot. Cell proliferation and apoptosis were examined by methyl thiazolyl tetrazolium (MTT) and flow cytometry, respectively. The abilities of adhesion and invasion were detected by cell adhesion assay and cell invasion assay using Transwell chambers.

RESULTS: The expression of MMP-2 was inhibited and the inhibitory effects of different sequence varied. pGPU6-1 group had the most efficient inhibitory effect, followed by pGPU6-2 and pGPU6-3 groups. Invasiveness and adhesion were more significantly reduced in pGPU6-1, pGPU6-2 and pGPU6-3 groups as compared with pGPU6 (-) and blank control groups. However, no difference concerning cell proliferation and apoptosis was observed after transfection between experiment groups and control groups.

CONCLUSION: RNAi against MMP-2 successfully inhibited the mRNA and protein expression of MMP-2 in the pancreatic cancer cell line, BxPC-3, leading to a potent suppression of tumor cell adhesion and invasion without affecting cell proliferation and apoptosis. These findings suggest that the RNAi approach towards MMP-2 may be an effective therapeutic strategy for the clinical management of pancreatic tumor.

Key words: Pancreatic neoplasm; Tumor metastasis; Matrix metalloproteinase-2; Small interfering RNA; Tumor invasiveness

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INTRODUCTION

Pancreatic cancer is one of the most aggressive common tumors, most patients die within months as a result of rapid local spread of the tumor or metastatic dissemination[1]. The very poor prognosis may in part be attributed to the high invasive potential of this malignancy, and the invasion or metastasis of pancreatic cancer has been known to be a complex process involving many molecular mechanisms, of which proteolytic degradation of extracellular matrix (ECM) exerted by matrix metalloproteinases (MMPs) was

considered to be an essential step[2]. Some data suggest that MMP-2 is involved in pancreatic cancer invasion and metastasis, and a high level of MMP-2 has been found to correlate with poor prognosis in patients with pancreatic cancer[3]. Therefore, inhibition of MMP-2 may be of great value in both preventing pancreatic cancer and blocking metastasis of established tumors.

RNA interference (RNAi) is a conserved biologic response to double-stranded RNA that results in the sequence-specific silencing of target gene expression. As a kind of highly efficient, specific and relatively stable tool, RNAi technology has already been used to silence specific target gene expression[4].

In this report, we used a vector-based MMP-2 siRNA expression system to suppress the expression of MMP-2 in pancreatic cancer cell line BxPC-3 and to evaluate its efficacy in the adhesion and invasion of pancreatic cancer cell. We found that specific down-regulation of MMP-2 by RNAi successfully inhibited the mRNA and protein expression of MMP-2, leading to significant inhibition of adhesion and invasion of pancreatic cancer cells without affecting cell proliferation and apoptosis. Thus, RNAi towards MMP-2 may be an effective therapeutic strategy for the treatment of patients with pancreatic cancer.

MATERIALS AND METHODS

Cell culture

Human pancreatic cancer cell line BxPC-3, obtained from Shanghai Institute of Biochemistry and Cell Biology, were maintained in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS; Hyclone Co., Ltd.) and were incubated in a humidified (37℃, 5% CO_2) incubator, grown in 75-cm^2 culture flasks and passaged upon reaching 80% confluence.

Construction of siRNA plasmids expression vector against MMP-2

We used the pGPU6 siRNA plasmid vector-based gene silence system to produce stable transfection, plasmid vector pGPU6 was purchased from Shanghai GenePharma Co., Ltd. Aiming at the sequence of MMP-2, four DNA chains with the following sense and antisense sequences were synthesized: no. 1, 5'-GGA GAGCTGCAACCTGTTTGT-3' (sense) and 5'-ACA AACAGGTTGCAGCTCTCC-3' (antisense); MMP-2 siRNA no. 2, 5'-GCTCCACCACCTACAACTTTG-3' (sense) and 5'-CAAAGTTGTAGGTGGTGGAGC-3' (antisense); MMP-2 siRNA no. 3, 5'-GCAAACAGGA CATTGTATTTG-3' (sense) and 5'-CAAATACAAT GTCCTGTTTGC-3' (antisense); MMP-2 siRNA no. 4, 5'-GGAGATACAATGAGGTGAAGA-3' (sense) and 5'-TCTTCACCTCATTGTATCTCC-3' (antisense). The target sequence of negative control group which is named pGPU6 (-) is 5'-GTTCTCCGAACGTGTCACG T-3' (sense) and 5'-ACGTGACACGTTCGGAGAAT-3' (antisense), which has no homology with that of human beings or mice. The cancer cells without any plasmid were defined as blank control group. All DNA chains were designed and synthesized by Shanghai GenePharma Co., Ltd., Shanghai, China. We contrived the structure of the DNA chains to be *Bam*HI + sense chain + loop + antisense chain + termination signal + *Eco*RI + *Hin*dIII. The four DNA chains were annealed and ligated into (*Bam*HI/*Eco*RI) sites of pGPU6 to generate the plasmid pGPU6/ MMP-2. The negative control plasmid pGPU6 (-) was constructed using the same procedure. As a result, four experimental groups of plasmids pGPU6-1, pGPU6-2, pGPU6-3, pGPU6-4, and negative control group plasmid pGPU6 (-) were generated. The plasmids were extracted and the accuracy of the constructs was confirmed by sequencing.

Transfection of siRNA

BxPC-3 cells were seeded in a 24-well culture plate and divided into blank control group, pGPU6 (-) group and positive experimental groups (pGPU6-1, pGPU6-2, pGPU6-3 pGPU6-4). Each group contained 3 culture wells. 2×10^5 cells were plated into each culture well 24 h before transfection, and cultured in a humidified (37℃, 5% CO_2) incubator. BxPC-3 cells were stably transfected with pGPU6-1, pGPU6-2, pGPU6-3, pGPU6-4, or pGPU6 (-) in the presence of Lipofectamine 2000 (Invitrogen Co., Ltd.) following the manufacturer's instructions. No plasmid was introduced in the blank control plates; only Lipofectamine 2000 was used for the transfection in the blank control group. The cells were transfected with plasmid DNA (2 μg) and transfection reagent (4 μL) at a DNA: reagent ratio of 1:2, and then incubated at 37℃ in a CO_2 incubator for 24 h prior to testing for gene expression.

Expression of MMP-2 mRNA detected by reverse transcription polymerase chain reaction (RT-PCR)

Twenty-four hours after the transfection, 5×10^5 cells were collected and total RNA was extracted using the Trizol reagent (Invitrogen Co., Ltd.) following the manufacturer' s instructions. The concentration and purity of the total RNA were detected with ultraviolet spectrophotometer. Glutaraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The primer sequences for the genes and expected product sizes were as follows: 5'-TG ATCTTGACCAGAATACCA-3' (sense), 5'-TGCCATAC TTCTTGTCGCGGT-3' (antisense) for MMP-2 (731 bp), 5'-CCATGGAGAAGGCCGGGG-3' (sense), 5'-CAAA GTTGTCATGGATGACC-3' (antisense) for GAPDH (200 bp). The RT reaction was performed at 25℃ for 10 min, then 37℃ for 60 min. PCR amplification was performed under the following reaction conditions: 94℃ for 30 s, 55℃ (MMP-2) or 53℃ (GAPDH) for 30 s, 72℃ for 1 min, and a final extension at 72℃ for 7 min. The amplification used 28 cycles for MMP-2, and 26 cycles for GAPDH. PCR products were analyzed by electrophoresis on 1% agarose gel and were visualized by ethidium bromide staining under ultraviolet light. The expression intensity of MMP-2 was denoted with the ratio of the photodensity of the RT-PCR products of MMP-2 to GAPDH. The inhibition ratio of MMP-2 expression was calculated with the following formula: inhibition ratio of MMP-2 expression = (1-the expression

intensity of MMP-2 in the experiment group/the expression intensity of MMP-2 in the blank or negative control group) × 100%.

Cell lysis and Western blotting

Cells were collected 24 h after transfection, washed twice with phosphate buffered solution (PBS), and the supernatant was scraped off. Cell pellets were then lysed in iced bath for 30 min. The lysates were transferred to new tubes and centrifuged at 12000 r/min for 30 min at 4℃. Proteins were separated by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmacia Biotech, Germany). The membranes were blocked for 1 h in 3% bovine serum albumin (BSA) in PBS and then incubated with monoclonal antibodies recognizing MMP-2 or actin (Santa Cruz, USA). Second antibody incubations were carried out using peroxidase-conjugated goat anti-rabbit antibody, and reactive bands were detected by chemiluminescence. The expression levels of MMP-2 and actin protein were quantified by densitometry. The signal strength of each MMP-2 signal was normalized against the corresponding actin control. The inhibition ratio of MMP-2 expression was calculated with the following formula: inhibition ratio of MMP-2 expression = (1-the relative intensity of MMP-2 expression in the experiment group/the relative intensity of MMP-2 in the blank or negative control group) × 100%.

Methyl thiazolyl tetrazolium (MTT) assay

Cell proliferation was assessed by using the MTT assay. Cells were collected 24 h after transfection, pancreatic cancer cells were plated at 1×10^4 cells/well in 96-well plates in DMEM containing 10% FBS. Five duplicate wells were set up for each group. Blank control cells served as control. After 24, 48 and 72 h of incubation, 200 μL of 5 mg/μL MTT solution (Sigma Co., Ltd.) in PBS was added to each well for 4 h. Absorbance of each well was measured on a microplate reader at a wave length of 492 nm.

Flow cytometry

Flow cytometry was used to estimate the apoptosis,. The harvested cells were washed with PBS, fixed with cold 75% ethanol at -20℃ for 24 h, treated with 0.1 mL RNase A (Sigma Co., Ltd.) and then stained with propidium iodide (Sigma Co., Ltd.). Finally, cell cycle analysis was carried out using flow cytometer.

Adhesion assay

Cells were collected 24 h after transfection. The cancer cells were trypsinized and seeded at 1×10^4 cells/well in 96-well plates. Five duplicate wells were set up for each group. The cells were cultured for 60 min, and washed twice with PBS to remove the cells not adherent. The MTT assay as above was performed to assess A value of the adhesive cells. The inhibition ratio was calculated with the following formula: inhibition ratio = (1-A value of the experiment group/A value of the control group) × 100%.

Table 1 Down-regulation of MMP-2 mRNA expression detected by RT-PCR

Groups	Ratio of MMP-2 to GAPDH
Blank control	0.93 ± 0.02
pGPU6 (-)	0.91 ± 0.03
pGPU6-1	0.29 ± 0.02[b,d]
pGPU6-2	0.33 ± 0.04[b,d]
pGPU6-3	0.45 ± 0.03[b,d]
pGPU6-4	0.88 ± 0.03[a,c]

[a]$P > 0.05$ *vs* blank control, $P = 0.057$; [b]$P < 0.05$ *vs* blank control; [c]$P > 0.05$ *vs* pGPU6 (-), $P = 0.288$; [d]$P < 0.05$ *vs* pGPU6 (-).

Cell invasion assay

Cell invasion assay was performed using Transwell chambers (Corning Co., Ltd.). After 24 h transfection, 5×10^5 cells were suspended in 100 μL serum-free medium and placed into the upper compartment of the Transwell chambers. The lower compartment of the chambers was filled with 200 μL serum-containing medium and the cells were allowed to migrate for 24 h. After a 24-h incubation, cells on the lower surface of the filter were fixed in cold ethanol and stained with 0.5% crystal violet (CV) for 30 min, and 5 random fields were counted at 200 × magnification. Data represent the average cells of 5 fields were compared between the experimental groups and control group.

Statistical analysis

All results were expressed as mean ± SD. All statistical analyses were performed using one-way ANOVA. $P < 0.05$ was considered significant.

RESULTS

Suppression of MMP-2 mRNA in human pancreatic cell by siRNA

Four MMP-2 siRNA–expressing plasmids (pGPU6-1, pGPU6-2, pGPU6-3 and pGPU6-4) and one negative plasmid pGPU6 (-) were constructed using the pGPU6 vectors. Twenty-four hours after the transfection, we observed significant inhibition of MMP-2 mRNA expression in the experimental groups (pGPU6-1, pGPU6-2 and pGPU6-3) compared with blank control and pGPU6 (-) group ($P < 0.05$) whereas the slight inhibition was observed in pGPU6-4 cells ($P > 0.05$) (Table 1). The inhibition ratio was 75.3% (pGPU6-1), 64.5% (pGPU6-2), 51.6% (pGPU6-3) with respect to blank control and 74.7% (pGPU6-1), 63.7% (pGPU6-2), 50.5% (pGPU6-3) compared to pGPU6 (-). The transfection with pGPU6 (-) had no significant inhibitory effect on the expression of MMP-2 mRNA compared to the blank group ($P > 0.05$) (Figure 1).

Significant down-regulation of MMP-2 protein expression by siRNA

The levels of MMP-2 protein in the total cell lysates were assessed by Western blot. Western blot analyses using the anti-MMP-2 antibody revealed significant

Table 2 Down-regulation of MMP-2 protein expression detected by Western blot

Groups	Relative density
Blank control	0.86 ± 0.03
pGPU6 (-)	0.82 ± 0.02
pGPU6-1	0.18 ± 0.02[b,d]
pGPU6-2	0.31 ± 0.04[b,d]
pGPU6-3	0.41 ± 0.02[b,d]
pGPU6-4	0.82 ± 0.03[a,c]

[a]$P > 0.05$ *vs* blank control, $P = 0.102$; [b]$P < 0.05$ *vs* blank control; [c]$P > 0.05$ *vs* pGPU6 (-), $P = 0.885$; [d]$P < 0.05$ *vs* pGPU6 (-).

Table 3 Effects of MMP-2 siRNA on tumor cell adhesion

Groups	A value
Blank control	0.35 ± 0.03
pGPU6 (-)	0.34 ± 0.04
pGPU6-1	0.13 ± 0.02[b,d]
pGPU6-2	0.20 ± 0.01[b,d]
pGPU6-3	0.26 ± 0.01[b,d]
pGPU6-4	0.33 ± 0.02[a,c]

[a]$P > 0.05$ *vs* blank control, $P = 0.119$; [b]$P < 0.05$ *vs* blank control; [c]$P > 0.05$ *vs* pGPU6 (-), $P = 0.274$; [d]$P < 0.05$ *vs* pGPU6 (-).

decreases in MMP-2 expression after transfection with siRNA in positive experimental groups (pGPU6-1, 2, 3) compared with blank control and pGPU6 (-) group ($P < 0.05$). However, no change in MMP-2 expression was observed after transfection with siRNA in pGPU6-4 and pGPU6 (-) groups when compared with the blank control ($P > 0.05$) (Table 2). Quantitative analysis of MMP-2 protein by densitometry revealed a decrease in protein expression with pGPU6-1 by 79.1%, pGPU6-2 by 64%, pGPU6-3 by 52.3% compared with blank control group and pGPU6-1 by 78%, pGPU6-2 by 62.2% and pGPU6-3 by 50.5% compared with pGPU6 (-) group (Figure 2).

Effects of MMP-2 siRNA on tumor cell proliferation

To address whether siRNA directed against MMP-2 has an inhibitory effect on pancreatic cancer cell proliferation, cell proliferation was assessed using the MTT assay. We found that treatment of pancreatic cancer with MMP-2 siRNA did not cause any significant inhibitory effect in tumor cell proliferation. And the pGPU6 (-) group did not significantly decrease tumor cell proliferation compared with blank control group ($P > 0.05$) (Figure 3).

Effects of MMP-2 siRNA on tumor cell apoptosis

The effects of MMP-2 siRNA molecules on the induction of apoptosis in pancreatic cancer cells were inspected by flow cytometry. However, no discrepancy was observed after transfection with siRNA in positive experimental groups (pGPU6-1, 2, 3, 4) compared with blank control and pGPU6 (-) group ($P > 0.05$). Besides, the pGPU6 (-) group did not significantly increase cell apoptosis compared with blank control group ($P > 0.05$) (Figure 4).

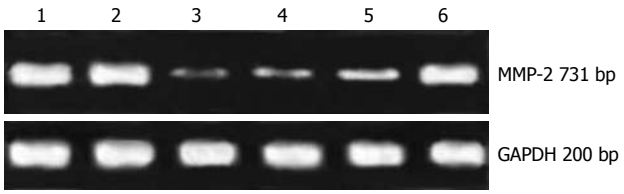

Figure 1 Down-regulation of MMP-2 mRNA expression detected by RT-PCR. Lane 1: Blank control; Lane 2: pGPU6 (-); Lane 3: pGPU6-1; Lane 4: pGPU6-2; Lane 5: pGPU6-3; Lane 6 : pGPU6-4.

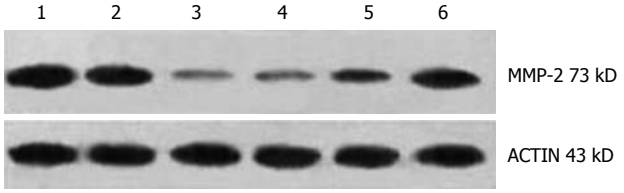

Figure 2 Down-regulation of MMP-2 protein expression detected by Western blot. Lane 1: Blank control; Lane 2: pGPU6 (-); Lane 3: pGPU6-1; Lane 4: pGPU6-2; Lane 5: pGPU6-3; Lane 6: pGPU6-4.

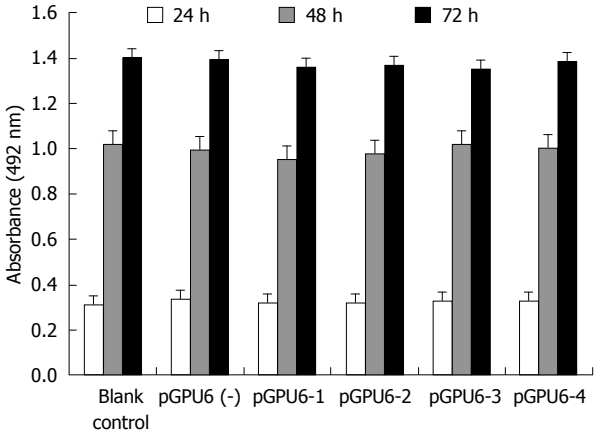

Figure 3 Effects of MMP-2 siRNA on tumor cell proliferation.

Effects of MMP-2 siRNA on tumor cell adhesion

Cell adhesion assay revealed significant decreases in cancer cell adhesion after transfection with siRNA in experimental groups (pGPU6-1, 2, 3) compared with blank control and pGPU6 (-) group ($P < 0.05$). However, no change was observed after transfection with siRNA in pGPU6-4 and pGPU6 (-) groups when compared with the blank control ($P > 0.05$) (Table 3). The inhibition ratio were 63.1% (pGPU6-1), 42.9% (pGPU6-2) and 25.6% (pGPU6-3) compared with the blank control, and 62.2% (pGPU6-1), 41.6% (pGPU6-2) and 23.9% (pGPU6-3) compared with pGPU6 (-) group.

Effects of MMP-2 siRNA on tumor cell invasion

Cell invasion was assessed using Transwell chambers. As shown in Table 4, for each 200 × field under microscope, the average migrated cell number of 5 fields of experimental groups (pGPU6-1, 2, 3) were observed to be significantly lower than the number of blank control and the pGPU6 (-) groups ($P < 0.05$), which was not found in the pGPU6-4 group. In addition, there was

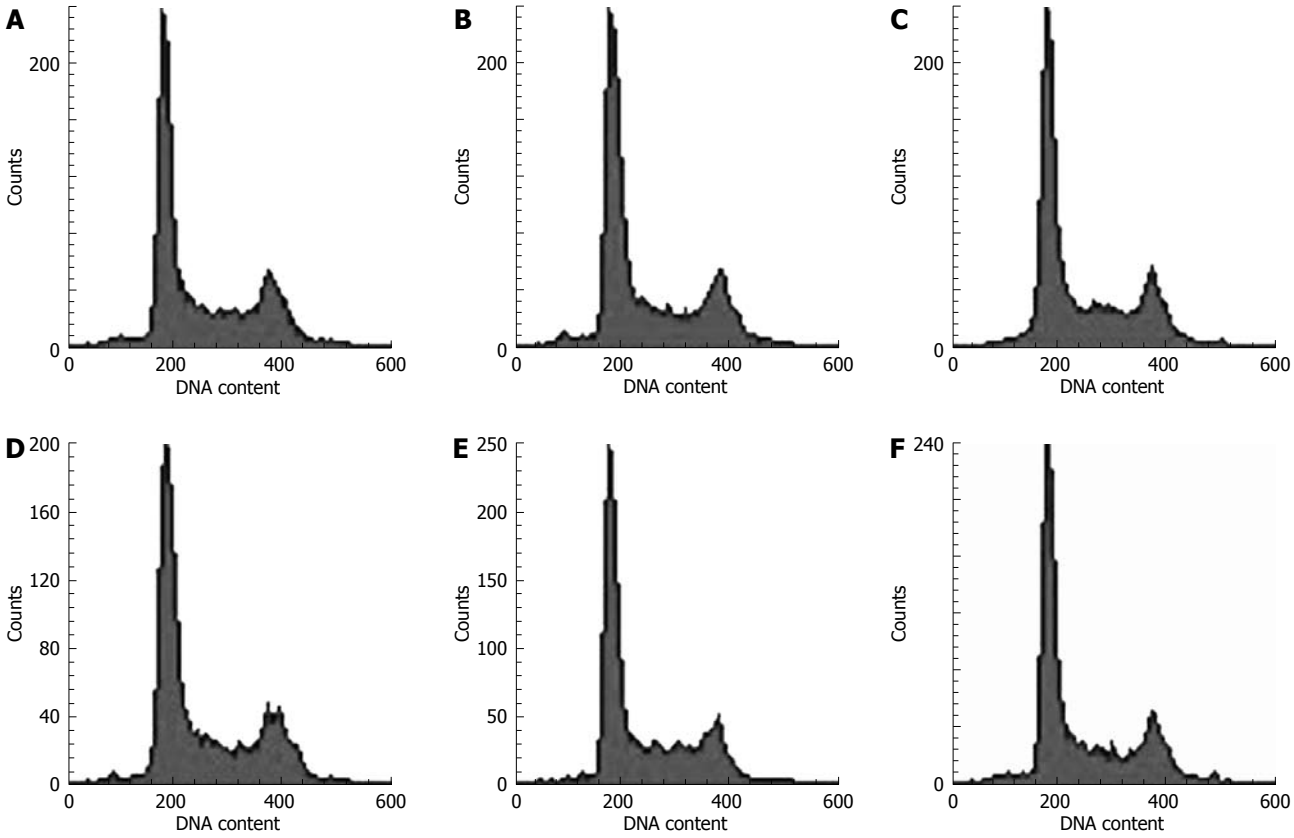

Figure 4 Effects of MMP-2 siRNA on tumor cell apoptosis. A: pGPU6-1; B: pGPU6-2; C: pGPU6-3; D: pGPU6-4; E: pGPU6 (-); F: Blank control.

Table 4 Effects of MMP-2 siRNA on tumor cell invasion

Groups	Invasive cell number
Blank control	180 ± 12
pGPU6 (-)	176 ± 8
pGPU6-1	42 ± 7[b,d]
pGPU6-2	49 ± 9[b,d]
pGPU6-3	58 ± 6[b,d]
pGPU6-4	171 ± 10[a,c]

[a]$P > 0.05$ *vs* blank control, $P = 0.090$; [b]$P < 0.05$ *vs* blank control; [c]$P > 0.05$ *vs* pGPU6 (-), $P = 0.328$; [d]$P < 0.05$ *vs* pGPU6 (-).

little difference between blank control and the pGPU6 (-) groups ($P > 0.05$) (Figure 5).

DISCUSSION

MMPs are a group of enzymes, which degrade the macromolecules of connective tissue, ECM and basement membrane. These enzymes are believed to play important roles in tumor metastasis, invasion and angiogenesis[5-7]. As a subgroup of MMPs, MMP-2 seems to play an important role in the progression of pancreatic cancer. Bramhall *et al*[8] found MMP-2 messenger RNA was the most commonly expressed MMP in pancreatic tumor specimens (93%), but was not seen in normal pancreas. Apparently, MMPs, particularly MMP-2 play an important role in the pathogenesis of pancreatic cancer. Consequently, a tumor therapy targeting MMP-2 would be particularly efficacious in the treatment of pancreatic cancer. As a very potent tool, RNAi technology can generate a cellular knockdown of a desired gene utilizing a plasmid-based system that stably expresses siRNA molecules to target specific mRNAs for degradation[9,10]. In this study, we developed a siRNA sequence, when stably integrated into cellular DNA, which can selectively target MMP-2 expression. Our study demonstrated that RNAi against MMP-2 successfully inhibited the mRNA and protein expression of MMP-2 in the pancreatic cancer cell line BxPC-3. In contrast, MMP-2 expression was unchanged in the control groups. By using a stably integrating plasmid to express our siRNA molecule, we obtained at the most a 75% mRNA reduction and a 79% protein reduction in MMP-2 expression. Some studies are similar to our findings, which also draw a conclusion that MMP-2 mRNA and protein levels can be significantly inhibited by RNAi in solid tumors[11,12].

On the basis of significant inhibitory effects of MMP-2 mRNA and protein, we investigated the effects of MMP-2 siRNA on tumor adhesion and invasion. We found that the MMP-2 siRNA not only suppressed the adhesion of the cancer cell, but also their ability to migrate and invade. Similar to our findings, Sun *et al*[13] also found that MMP-2 silencing by RNAi could inhibit invasion and growth of laryngeal cancer, and MMP-2 might be a potential target for gene therapy in laryngeal cancer. Moreover, a study *in vivo* showed that tumors implanted in MMP-2-deficient mice had decreased invasive properties, which further suggest that MMP-2 is important for pancreatic cancer invasion[14].

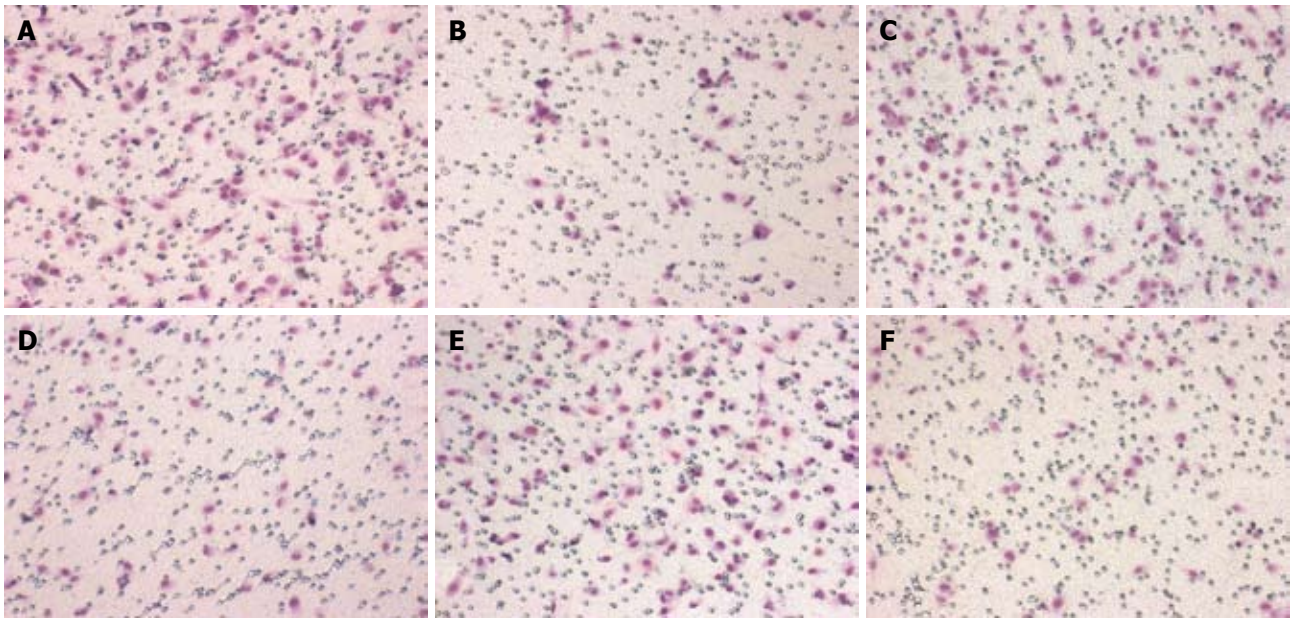

Figure 5 Cell invasion observed by CV stained (× 200). A: Blank control; B: pGPU6-1; C: pGPU6 (-); D: pGPU6-2; E: pGPU6-4; F: pGPU6-3.

The mechanism of MMP-2 involvement in the tumor invasion and metastasis is complex, some studies suggest that MMP-2 is associated with the degradation of type Ⅳ collagen which is the main component of basement membranes. Furthermore, Kim *et al*[15] found that one additional mechanism by which MMP-2 was proposed to increase the local invasiveness was to facilitate angiogenesis. Interestingly, we got the suppressing effects of tumor cell adhesion and invasion by RNAi targeting the MMP-2 gene, but we did not find any significant change in tumor cell proliferation and apoptosis between the experimental groups and the control group. Some studies support our data showing that down-regulation of MMP-2 can inhibit cell invasion without affecting cell proliferation[16,17]. Based on these findings, we hypothesize that the ability of the pancreatic tumor cell adhesion and migration, but not the quantity of tumor cell proliferation, is the crucial factor for MMP-2 involved in pancreatic tumor cell invasion and metastasis. Some findings agreed with our hypothesis, which also observed a decrease in pancreatic cancer cell invasion through a reconstituted matrix in a dose dependent fashion without affecting cell proliferation *in vitro*[18,19]. Matrix metalloproteinases are enzymes responsible for extracellular matrix degradation, a critical component influencing metastatic potential of cancer. When endothelial cell MMP-2 gene is silenced, cell growth cycle will change correspondingly, which will be blocked in G1 stage. This may explain why MMP-2 gene affects only the ability of pancreatic cancer cells to modify the extracellular matrix to facilitate invasion and growth without affecting cell proliferation.

At time of diagnosis, 75%-85% of patients with pancreatic cancer can not accept resectable operation and conventional therapies which virtually are ineffective[20]. Therefore, there is clearly a need for new approaches to the treatment of this cancer. The over-expression of MMP-2 in pancreatic tumor is a mark of poor prognosis with respect to disease progression as well as survival[21]. Our data has provided evidence that RNAi against MMP-2 successfully inhibited the mRNA and protein expression of MMP-2 in the pancreatic cancer cell line BxPC-3, leading to a potent suppression of tumor cell adhesion and invasion without affecting cell proliferation and apoptosis. These findings suggest that the RNAi approach towards MMP-2 may be an effective therapeutic strategy for the clinical management of pancreatic tumor. Although the leap to clinical practice remains elusive, gene therapy targeting MMP-2 is attractive and warrants further investigations.

COMMENTS

Background

Pancreatic carcinoma is an aggressive malignancy with an extremely poor prognosis. Most patients with pancreatic carcinoma have extremely poor prognosis, and the reason may in part be attributed to the high invasive potential of this malignancy leading to early metastasis. However, either invasion or metastasis of pancreatic carcinoma has been known to be a complex process involving molecular mechanisms. Activation of matrix metalloproteinase-2 (MMP-2) has been implicated in the progression, invasion, and metastasis of various cancers, but little information is available with regard to its role in pancreatic carcinoma with poor prognosis.

Research frontiers

MMP-2 has an activity to degrade type Ⅳ collagen and is associated with invasion angiogenesis of malignant tumors. It seems that MMP-2 plays an important role in the progression of pancreatic carcinoma. In the area of pancreatic carcinoma gene therapy, one of the research hotspots is how to down-regulate MMP-2. As a kind of highly efficient, specific and relatively stable tool, RNA interference technology has already been used to silence specific target gene expression. Thus, RNA interference towards MMP-2 may be an effective therapeutic strategy for the treatment of patients with pancreatic cancer.

Innovations and breakthroughs

A gene silencing system using the vector (pGPU6)-based small interference RNA (siRNA) plasmid has been established to specifically knock down MMP-2 expression in pancreatic cancer cells. MMP-2 expression was measured by

reverse transcription polymerase chain reaction (RT-PCR) and Western blot. Cell proliferation and apoptosis were examined by MTT and flow cytometry, respectively. The abilities of adhesion and invasion were detected by cell adhesion assay and cell invasion assay using Transwell chambers. RNA interference against MMP-2 successfully inhibited the mRNA and protein expression of MMP-2 in the pancreatic cancer cell line, BxPC-3, leading to a potent suppression of tumor cell adhesion and invasion without affecting cell proliferation and apoptosis.

Applications

RNA interference towards MMP-2 may be an effective therapeutic strategy for the clinical management of pancreatic tumors. Although the leap to clinical practice remains elusive, gene therapy targeting MMP-2 is attractive and warrants further investigations.

Terminology

The MMPs are a family of zinc-dependent endopeptidases. Their primary function is degradation of proteins in the extracellular matrix. RNA interference is a process of post-transcriptional gene silencing in which double-stranded RNA inhibits gene expression in a sequence dependent manner *via* degradation of the corresponding mRNA.

Peer review

This is an interesting study. The invasiveness is usually related to cell growth. The manuscript is well written, but it needs explanation of the discrepancy in the study.

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Docosahexaenoic acid suppresses arachidonic acid-induced proliferation of LS-174T human colon carcinoma cells

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Abstract

AIM: To investigate the impact of arachidonic acid (AA) and docosahexaenoic acid (DHA) and their combination on colon cancer cell growth.

METHODS: The LS-174T colon cancer cell line was used to study the role of the prostaglandin precursor AA and the omega-3 polyunsaturated fatty acid DHA on cell growth. Cell viability was assessed in XTT assays. For analysis of cell cycle and cell death, flow cytometry and DAPI staining were applied. Expression of cyclooxygenase-2 (COX-2), p21 and bcl-2 in cells incubated with AA or DHA was examined by real-time RT-PCR. Prostaglandin E_2 (PGE_2) generation in the presence of AA and DHA was measured using a PGE_2-ELISA.

RESULTS: AA increased cell growth, whereas DHA reduced viability of LS 174T cells in a time- and dose-dependent manner. Furthermore, DHA down- regulated mRNA of bcl-2 and up-regulated p21. Interestingly, DHA was able to suppress AA-induced cell proliferation and significantly lowered AA-derived PGE_2 formation. DHA also down-regulated COX-2 expression. In addition to the effect on PGE_2 formation, DHA directly reduced PGE_2-induced cell proliferation in a dose-dependent manner.

CONCLUSION: These results suggest that DHA can inhibit the pro-proliferative effect of abundant AA or PGE_2.

Key words: Colorectal carcinoma; Colon cancer; Omega-3; Omega-6; Polyunsaturated fatty acids; Arachidonic acid; Docosahexaenoic acid; Prostaglandin E_2; Cyclooxygenase-2; Apoptosis

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INTRODUCTION

Colon cancer is one of the leading causes of death in Western countries[1]. Increased levels of cyclooxygenase-2 (COX-2) were detected in 50% of colorectal adenomas and in up to 85% of colorectal cancers[2-4]. Prostaglandin E_2 (PGE_2) is generated from the omega-6 polyunsaturated fatty acid (n-6 PUFA) arachidonic acid (AA) *via* action of the COX-1 and -2. Several studies have established PGE_2 as an important factor for proliferation of colon cancer cells *in vitro*[5-7].

Regular Western diets are highly abundant in n-6 PUFAs[8]. Since AA is the precursor of PGE_2, this may contribute to the high prevalence of colon cancer in the Western world[9]. In contrast, diets rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are mainly found in fish oil, might reduce the risk of colon cancer development, and an inverse association of consumption of fish and colon cancer has been observed epidemiologically[10-12]. EPA was found to inhibit colon crypt cell proliferation *in vivo*[13]. A recent study has demonstrated an inverse association of the n-6/n-3 ratio with colon adenoma formation[14]. In an animal model system with increased amounts of endogenously synthesized n-3 PUFA (the fat-1 mouse), two studies have shown a protective effect against colon tumor development[15,16].

In vitro studies in Caco-2 colon cancer cells with the n-3 PUFA DHA have demonstrated growth-inhibitory effects by induction of apoptosis[17-20]. Other results have shown that PGE_2 formation and vascular endothelial growth factor expression are suppressed, while apoptosis is induced by DHA and EPA in the HT-29 colon cancer cell line[21]. In contrast, several other studies in colon cancer cell lines have demonstrated that AA as well as DHA or EPA suppresses growth[22,23]. Other studies have found a pro-apoptotic effect of DHA and AA in HT-29 colon cancer cells[24,25] and in the A549 lung cancer cell line[26]. These *in vitro* observations have led to uncertainty regarding a differential role of n-3 and n-6 PUFA for growth of tumor cells. Furthermore, they do not address the effect of a changed n-3/n-6 ratio on cell proliferation.

In the present study, we used the LS-174T colon cancer cell line, for which a potent PGE_2-triggered activation of proliferation has been demonstrated previously[6,27-29], to test the effect of DHA co-incubation with AA or PGE_2 on cell growth, thereby mimicking a change in the ratio of n-3/n-6 fatty acids. We show that DHA suppressed cell growth, while AA increased proliferation, and that DHA co-incubation suppressed AA- and PGE_2-induced cell growth.

MATERIALS AND METHODS

Cell culture

Cells were cultured in a saturated atmosphere of 5% CO_2 and 95% air at 37℃. LS-174T cells were grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) without phenol red, which contained 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mmol/L glutamine and 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA). Medium that contained PUFAs (NuchekPrep, Elysian, MN, USA) or PGE_2 (Caymanchem, Ann Arbor, MI, USA) was prepared with 2% FBS and 1 mg/mL fatty-acid-free bovine serum albumin (BSA). All chemicals used were bought from Sigma (St. Louis, MO, USA) except where stated otherwise.

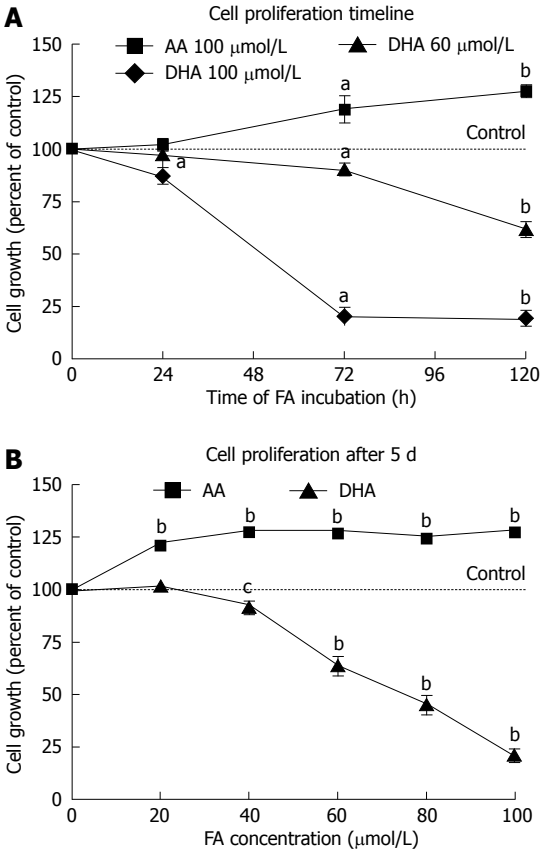

Figure 1 Effects of fatty acids on cellular viability. A: Growth of LS-174T cells during incubation with different concentrations of fatty acids in the medium with 1 mg/mL BSA. Data points represent at least five independent experiments. $^aP < 0.05$ *versus* control, $^bP < 0.001$ *versus* control. B: Concentration-dependent effect of DHA and AA on growth of LS-174T colon cancer cells after 5 d incubation. Data points represent at least 13 independent experiments. $^cP < 0.01$ *versus* control, $^bP < 0.001$ *versus* control.

Cell proliferation assay

Cell viability was determined by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Briefly, 2500 LS-174T cells per well were seeded into a 96-well plate. After 24 h, medium was removed and replaced by medium that contained the appropriate concentration of respective PUFAs. In order to avoid unspecific toxic effects of free long-chain fatty acids, the maximum total fatty acid concentration used in the long-term incubation cell viability experiments was 100 μmol/L. Cell proliferation was assessed photometrically in dual wave length measurements at different time points after addition of activated XTT assay solution.

Flow cytometry assays

For cell cycle analysis 5×10^5 cells were plated in 10-cm dishes. After 24 h, medium was removed and replaced by 10 mL medium that contained PUFAs. Cells were harvested for flow cytometry after 72 h. For detection of the sub-G1 DNA fraction, cells were stained with 0.1 mg/mL propidium iodide, which contained 0.5 mg/mL RNase and 0.1% NP40 detergent. Afterwards, cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA,

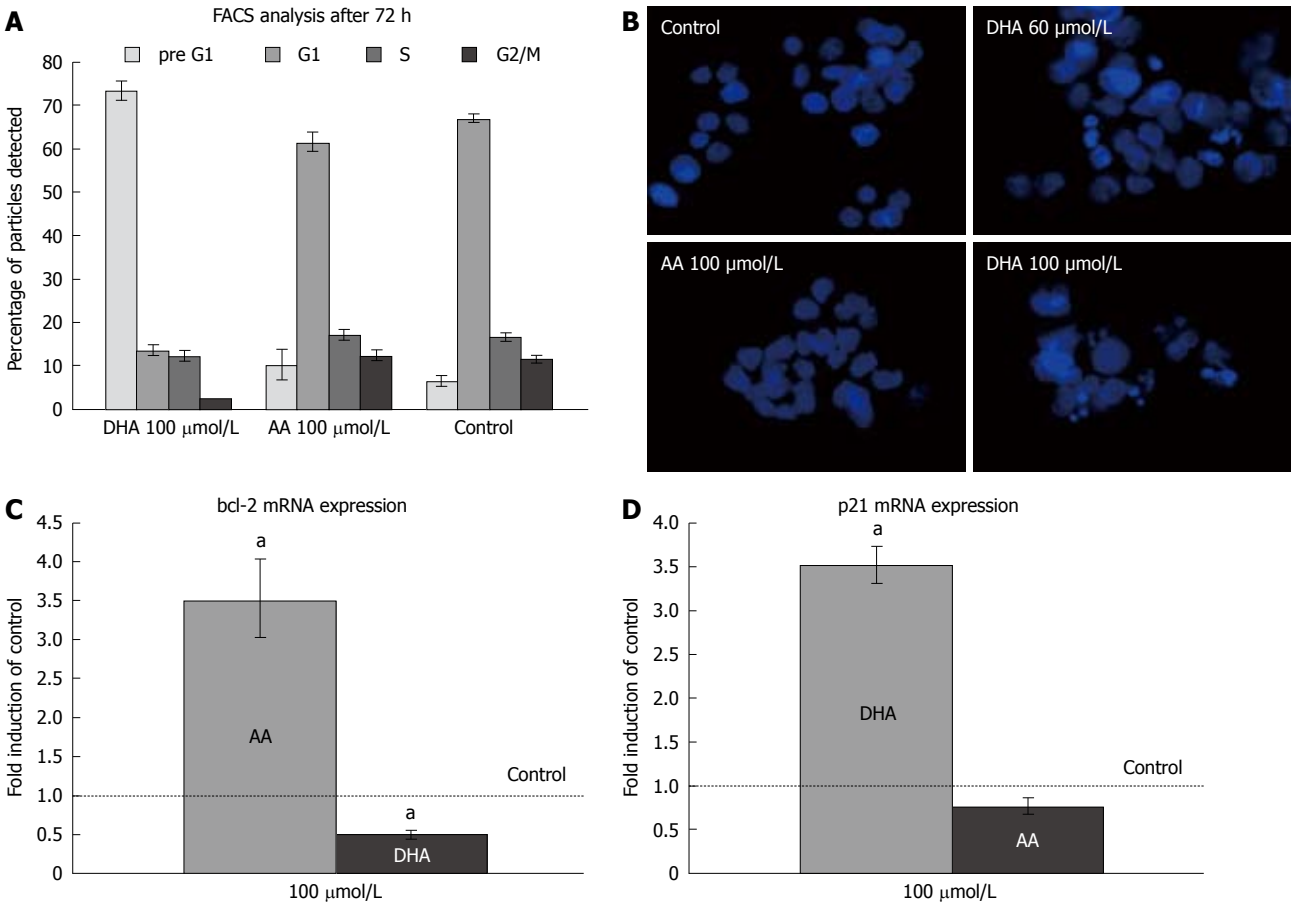

Figure 2 Effect of DHA and AA on cell cycle and apoptosis. A: Cell cycle analysis by flow cytometry. Induction of apoptosis is indicated by an increased pre-G1 fraction. Results represent five independent experiments. B: DAPI staining of LS-174T cells incubated with different concentrations of AA and DHA showed a clear increase of apoptotic bodies in cells incubated with DHA. C: RT-PCR demonstrated induction of bcl-2 expression by AA, while DHA suppressed bcl-2 mRNA expression. [a]$P < 0.01$ *versus* control. $n = 3$ for each group. D: DHA induced transcription of p21, while AA did not alter p21 mRNA formation. [a]$P < 0.01$ *versus* control. $n = 3$ for each group.

USA) flow cytometer.

4',6'-diamidino-2-phenylindole (DAPI) staining

Cells were fixed using 2% paraformaldehyde and permeabilized with 0.1% Triton X 100. Cells were stained with DAPI solution and assessed for cell morphology and apoptotic bodies.

Semi-quantitative real-time RT-PCR

Total RNA was isolated from LS-174T cells using the RNAeasy mini kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. Reverse transcription of mRNA was performed using random primers (Promega, Madison, WI, USA) to generate cDNA. Real-time RT-PCR was carried out using Absolute QPCR SYBR Green Mix (ABgene, Rockford, IL, USA) in an ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. Primers were designed with Primer Select 5.00 Software (DNASTAR Inc., Madison, WI, USA). Primer sequences were: COX-2for CGCTCAGCCATACAGCAAATCCTT, COX-2rev AATCCTGTCCGGGTACAATCGCA; p21for GTGGGGGCATCATCAAAAACTT, p21rev ACCCCACCTTCCCCCTGCCTTCAC; bcl-2for CATGCCAAGGGGGAAACACCAGAA, bcl-2rev CACGGCCCCCAGAGAAAGAAGAGG; GAPDHfor GGTGAAGGTCGGAGTCAAC, GAPDHrev CCATGGGTGGAATCATATTG.

PGE_2-ELISA

For PGE_2 analysis, cells were treated with PUFAs, as described above. The PGE_2-ELISA was then performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All results are presented as mean ± SE, except where stated otherwise. Student's *t* test was used to evaluate the difference between two groups. RT-PCR was analyzed by using the 2ΔCt method. Statistical significance was accepted at the level of $P < 0.05$, and Prism 4 for Windows Software (GraphPad, La Jolla, CA, USA) was used for calculations.

RESULTS

Opposing effects of AA and n-3 PUFA on colon cancer cell proliferation

LS-174T cells were treated with different concentrations

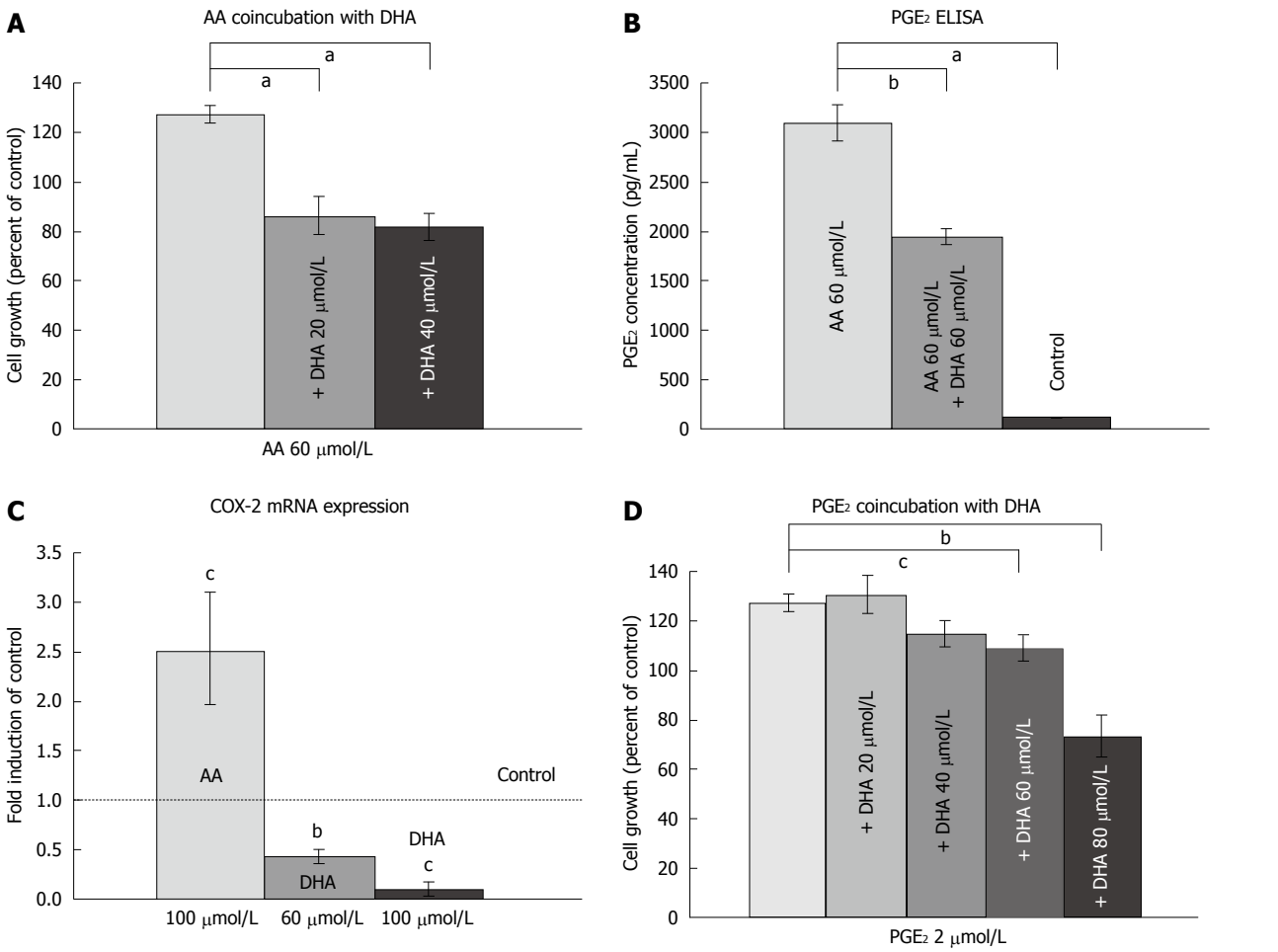

Figure 3 DHA suppresses AA- and PGE2-induced proliferation. A: DHA inhibited AA-induced proliferation, $^{a}P < 0.001$. Results represent six independent experiments. B: PGE_2 formation was induced by AA treatment and was suppressed by concomitant DHA incubation, $^{a}P < 0.001$, $^{b}P < 0.01$. Results represent the mean of PGE_2 measurements from three independent samples. C: COX-2 transcription was activated by AA, but suppressed by DHA. $^{c}P < 0.05$ *versus* control, $^{b}P < 0.01$ *versus* control. Bars represent at least three experiments. D: DHA suppressed PGE_2-induced cell proliferation, $^{c}P < 0.05$, $^{b}P < 0.01$. Results represent five independent experiments.

of fatty acids bound to BSA. In XTT assays, DHA significantly diminished cell growth and viability in a time- and dose-dependent manner. At the same time, AA at identical concentrations was found to increase proliferation (Figure 1A and B).

In order to further explain the suppression of cell proliferation by DHA, we studied apoptosis by flow cytometry and DAPI staining. DHA increased the pre-G1 fraction (an indicator of apoptosis) in LS-174T cells, while the same concentrations of AA did not significantly alter the pre-G1 fraction compared to untreated cells (Figure 2A). DHA-induced apoptosis was further confirmed by DAPI staining (Figure 2B). We then investigated differential cellular gene expression by means of semi-quantitative RT-PCR. DHA significantly down-regulated anti-apoptotic bcl-2 mRNA, while in contrast, AA up-regulated bcl-2 (Figure 2C). In addition, DHA up-regulated the expression of p21, while AA did not alter the amount of p21 mRNA (Figure 2D).

Inhibition of AA- and PGE_2-induced cell growth by DHA

LS-174T cells were treated with combinations of fatty acids bound to BSA and proliferation was assessed in XTT assays. DHA co-incubation was able to reverse the proliferation associated with AA (Figure 3A). The anti-proliferative effect of DHA in the context of high AA concentrations was associated with a significant reduction of PGE_2 formation from AA (Figure 3B). This may have been caused by decreased presence of COX-2, as DHA incubation significantly reduced COX-2 gene expression in a dose-dependent manner (Figure 3C). However, the effect of DHA-associated growth inhibition was in part independent from a pure blocking effect on PGE_2-formation, as co-incubation experiments with DHA and PGE_2 revealed that DHA also suppressed the PGE_2-induced induction of proliferation (Figure 3D).

DISCUSSION

As far as we are aware, our results demonstrate for the first time that DHA can directly suppress AA-induced colon cancer cell growth. Our data confirm that AA is a potent proliferative agent for colon cancer cells that are responsive to PGE_2. In contrast, the n-3 PUFA DHA down-regulates anti-apoptotic factors, induces

apoptosis and decreases PGE_2 formation. This leads to a potent suppression of tumor cell growth by DHA. Our results confirm several previous studies that have shown that DHA is a potent suppressor of colon cancer cell proliferation and stimulates apoptosis[17-19,21,30]. However, previous studies have failed to address the differential effects of n-3 PUFA and n-6 PUFA, and of their combination, on cancer cell growth. In light of several studies that have demonstrated cancer cell growth inhibition by n-3 PUFA or n-6 PUFA[22,23,26], our data help to clarify the issue of differential effects of n-6 and n-3 PUFAs on apoptosis and cell growth.

The most important result presented here is that the proliferation-stimulating effect of high concentrations of AA as a precursor of proliferation-stimulating lipid mediators (most notably PGE_2) can be suppressed by increasing the DHA content of the cells. Indeed, DHA can also directly inhibit PGE_2-induced proliferation in this context. Although our results are limited to an *in vitro* setup, they add evidence to the argument that the ratio of n-6/n-3 PUFA (and in particular the ratio of AA *versus* DHA) may be a critical determinant of proliferation and tumor growth in the colon, and that DHA supplementation can suppress tumor cell growth, even in the presence of high AA- and PGE_2-levels.

COMMENTS

Background

Colon cancer is one of the leading causes of death in Western countries. It is known, that prostaglandin E_2 (PGE_2), generated from the omega-6 polyunsaturated fatty acid (n-6 PUFA) arachidonic acid (AA) is important in the tumorigenesis of colon cancer.

Research frontiers

Several studies with the LS-174T colon cancer cell line have shown an important role of PGE_2 for tumor cell growth, but the effect of n-3 and n-6 PUFA has not been examined. Here, we used the LS-174T colon cancer cell line to study the role of the prostaglandin precursor AA and the omega-3 polyunsaturated fatty acid (n-3 PUFA) docosahexaenoic acid (DHA) on cell growth.

Innovations and breakthroughs

The results presented here demonstrate that the n-3 PUFA DHA can directly suppress AA- as well as PGE_2-induced colon cancer cell growth. The data add evidence to the argument that the ratio of n-6/n-3 PUFA (and in particular the ratio of AA *versus* DHA) may be a critical determinant of proliferation and tumor growth in the colon, and that DHA supplementation can suppress tumor cell growth even in the presence of high AA- and PGE_2-levels.

Applications

The results suggest that supplementation of DHA may be a powerful tool to counteract AA- and PGE_2-promoted colon cancer cell growth that might be associated with the predominant Western diet.

Terminology

PGE_2 is generated from the n-6 PUFA AA *via* action of cyclooxygenases 1 and 2. PGE_2 is important for proliferation of colon cancer cells *in vitro*. In contrast, diets rich in n-3 PUFAs, such as DHA and eicosapentaenoic acid, which are mainly found in fish oil, might reduce the risk of colon cancer development.

Peer review

The authors show that addition of DHA to cell cultures decreased cell proliferation in a dose- and time-dependent manner. Overall, these studies establish the importance of the ratio of n-3 to n-6 PUFA and the beneficial effect of fish oil in neoplastic growth. Although this is a limited *in vitro* study, its implications are significant.

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Colonoscopic yield of colorectal neoplasia in daily clinical practice

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Abstract

AIM: To assess the prevalence and location of advanced neoplasia in patients undergoing colonoscopy, and to compare the yield per indication.

METHODS: In a multicenter colonoscopy survey (n = 18 hospitals) in the Amsterdam area (Northern Holland), data of all colonoscopies performed during a three month period in 2005 were analyzed. The location and the histological features of all colonic neoplasia were recorded. The prevalence and the distribution of advanced colorectal neoplasia and differences in yield between indication clusters were evaluated. Advanced neoplasm was defined as adenoma > 10 mm in size, with > 25% villous features or with high-grade dysplasia or cancer.

RESULTS: A total of 4623 eligible patients underwent a total colonoscopy. The prevalence of advanced neoplasia was 13%, with 281 (6%) adenocarcinomas and 342 (7%) advanced adenomas. Sixty-seven percent and 33% of advanced neoplasia were located in the distal and proximal colon, respectively. Of all patients with right-sided advanced neoplasia (n = 228), 51% had a normal distal colon, whereas 27% had a synchronous distal adenoma. Ten percent of all colonoscopies were performed in asymptomatic patients, 7% of whom had advanced neoplasia. In the respective procedure indication clusters, the prevalence of right-sided advanced neoplasia ranged from 11%-57%.

CONCLUSION: One out of every 7-8 colonoscopies yielded an advanced colorectal neoplasm. Colonoscopy is warranted for the evaluation of both symptomatic and asymptomatic patients.

Key words: Colorectal cancer; Screening; Advanced neoplasia; Colonoscopy; Adenoma

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INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of

cancer-related death in the Western world and the incidence in Asia is also rising[1,2]. In The Netherlands, 63 cases per 100 000 inhabitants were found in 2003, whereas the incidence in the United States was 52 cases per 100 000[3,4]. While several countries have already started nation-wide screening programs for colorectal cancer, in The Netherlands, the scale and mode of CRC screening are still being debated[5-8]. One issue is whether sigmoidoscopy or colonoscopy should be performed with particular emphasis on the potential differences in yield and spatial distribution of colorectal carcinomas and advanced adenomas. Before embarking on gradual implementation of any kind of endoscopic screening in The Netherlands, we need to understand the distribution of CRC as well as the high risk precursors within the colorectum. In recent advice to the government, the Health Council of The Netherlands acknowledged the importance of this issue, and indicated that additional research is required before commitment to a national screening program[6]. Although colonoscopy and sigmoidoscopy constitute a significant proportion of the endoscopic workload in daily clinical practice, the yield of pathology, except for highly selected populations, has not well been described[9]. In particular, international data are lacking real life incidence figures of both advanced and non-advanced colorectal neoplasia found in routine endoscopy programs. As a result, accurate data on CRC and its precursors obtained in this study could inform decisions in choosing a future screening modality and could facilitate future national research initiatives in the field of CRC.

Furthermore, in view of the relatively fixed endoscopic resources and the potential increase in endoscopic procedures related to a future CRC screening program, a clear insight into endoscopic utilization in daily clinical practice is mandatory. Colonoscopy is considered the gold standard for the evaluation of the symptomatic patient. However, indication clusters might predict the presence of advanced neoplasia located in the proximal or distal colon and, thus, might indicate whether a colonoscopy or a sigmoidoscopy is warranted. This insight might not only lead to changes in future manpower planning, but it might also lead to changes in endoscopic utilization depending on initial clinical indication, thereby potentially alleviating future endoscopic workload[10].

In the present study, we evaluated the diagnostic yield in terms of advanced and non-advanced neoplasia in a large cohort of Dutch patients referred for lower gastrointestinal (GI) endoscopy. Within this study, our primary objective was to assess the prevalence and location of advanced colorectal neoplasia in all patients clinically referred for colonoscopy. A secondary objective was to compare the yield of proximally located advanced neoplasia *versus* distally located advanced neoplasia in several indication clusters in total colonoscopies.

MATERIALS AND METHODS

Study design

In this multicenter study, daily endoscopic clinical practice was prospectively monitored during a three month period in 2005 in the province Northern Holland (Amsterdam area). All colonoscopies and sigmoidoscopies performed in this time interval were evaluated. The province Northern Holland, serving a total community of 2 599 103 inhabitants (www.cbs.nl) has 18 hospitals (2 academic hospitals and 16 general/teaching hospitals). All 18 hospitals participated in this study. The study protocol was approved by the central medical ethics review board of the VU University Medical Centre in Amsterdam.

Age, gender, procedure indications, and endoscopic findings were obtained from all patients referred for lower GI endoscopy. All hospitals were visited every two weeks and all lower GI endoscopy reports between September 1st 2005 and December 1st 2005 were evaluated.

Study procedure and definitions

All examinations were performed by gastroenterologists, GI fellows, internists or colorectal surgeons. For the purpose of our analysis, the distal colon was defined as the rectum, sigmoid, and descending colon including the splenic flexure. The proximal colon was defined as the transverse colon, the ascending colon and the cecum, as assessed by the endoscopist. The percentage of complete colonoscopies was scored. Cecal intubation was considered a complete colonoscopy.

Indications for procedures were clustered in categories. In total, twelve clusters were defined as shown in Table 1. In many cases, more than one procedure indication was present. There was considerable overlap among the indications of abdominal pain (Ⅰ), change in bowel habits (Ⅱ), bloating (Ⅲ), diarrhea (Ⅳ) and constipation (Ⅴ). We defined an irritable bowel syndrome (IBS) cluster as including one or more of the above-mentioned symptoms (Ⅰ-Ⅴ), as has been described previously[7]. The IBS cluster excluded patients who underwent colonoscopy or sigmoidoscopy for surveillance of inflammatory bowel disease (IBD) or established IBD, weight loss, or GI bleeding [anemia/iron deficiency, positive fecal occult blood test (FOBT), hematochezia or melena].

All pathological and clinicopathological findings were categorized as indicating non-neoplastic mucosa (no polyps), hyperplastic polyps, adenomas with low-grade dysplasia, or advanced neoplasia. An advanced neoplasm was defined as an adenoma ⩾ 1.0 cm, an adenoma with villous or tubulovillous architecture (⩾ 25% villous component), an adenoma with high-grade dysplasia, or cancer. Advanced adenoma was defined as an adenoma ⩾ 1.0 cm, an adenoma with villous or tubulovillous architecture (⩾ 25% villous component) or an adenoma with high-grade dysplasia. Subsequently, the term advanced neoplasm was defined as comprising advanced adenomas and cancer, as has been described previously. A non-advanced neoplasm was defined as a hyperplastic polyp, an adenoma ⩽ 1.0 cm with low-grade dysplasia or an adenoma ⩽ 1.0 cm with ⩽ 25% villous component of the architecture. Findings such as lipomas, lymphoid aggregates and inflammatory or juvenile polyps were categorized as indicating non-neoplastic mucosa. In the case of patients with more than one polyp in either the proximal or distal segment of the colon, the most

Table 1 Indication cluster definition

Indication cluster (*n*; %)	Consists of the following indications
G-I bleeding (696; 15)	Hematochezia and/or melena
Anemia (356; 8)	Any kind of anemia
CRC suspicion (204; 4)	Clinical and/or radiological suspicion CRC
Weight loss (101; 2)	Weight loss
Family history CRC[1] (447; 10)	Any family history of CRC or screening
IBS (969; 21)	Abdominal pain, change in bowel habits, bloating, diarrhea, constipation
IBD exacerbation (256; 6)	Clinical suspicion IBD and/or endoscopic evaluation of IBD exacerbation
CRC surveillance (454; 10)	Follow up after CRC
Polyp surveillance (583; 13)	Follow up after polypectomy
IBD surveillance (142; 3)	Surveillance for dysplasia in IBD
FAP/HNPCC surveillance (84; 2)	Screening and/or surveillance in FAP/HNPCC families
Other/non-specified (331; 7)	No indication mentioned, ileus and desufflation therapy, fecal incontinence, monitoring diverticulitis after treatment, tenesmus, endoscopic treatment radiation enteritis

[1]Known hereditary CRC syndromes like HNPCC, FAP or MYH-polyposis are excluded. Note 1: The number of patients included in both study objectives was 4623 patients; Note 2: Within the indication cluster, the numbers and percentages of patients are placed between brackets.

Table 2 Yield of advanced neoplasia per indication in all complete colonoscopies *n* (%)

Indication cluster	Right-sided advanced neoplasia	Left-sided advanced neoplasia	Synchronous left- and right-sided advanced neoplasia	Total number of patients with advanced neoplasia
G-I bleeding (*n* = 696)	19 (11)	146 (83)	11 (6)	176 (25)
Anemia (*n* = 356)	35 (57)	21 (34)	5 (8)	61 (17)
CRC suspicion (*n* = 204)	29 (33)	54 (61)	6 (7)	89 (44)
Weight loss (*n* = 101)	2 (22)	7 (78)	0	9 (9)
Family history CRC[1] (*n* = 447)	10 (30)	19 (58)	4 (12)	33 (7)
IBS (*n* = 969)	22 (26)	57 (66)	7 (8)	86 (9)
IBD exacerbation (*n* = 256)	1 (33)	2 (67)	0	3 (1)
CRC surveillance (*n* = 454)	11 (29)	23 (60)	4 (11)	38 (8)
Polyp surveillance (*n* = 583)	29 (41)	36 (51)	6 (8)	71 (12)
IBD surveillance (*n* = 142)	3 (43)	4 (57)	0	7 (5)
FAP/HNPCC surveillance (*n* = 84)	2 (25)	5 (63)	1 (13)	8 (10)
Other/Non-specified[2] (*n* = 331)	20 (48)	21 (50)	1 (2)	42 (13)
Total (*n* = 4623)	183 (29)	395 (63)	45 (7)	623 (13)

[1]Known hereditary CRC syndromes like HNPCC, FAP or MYH-polyposis are excluded; [2]Including no indication mentioned, ileus and desufflation therapy, fecal incontinence, monitoring diverticulitis after treatment, tenesmus and endoscopic treatment radiation enteritis. NB: Within the indication cluster, the numbers of patients are placed between brackets.

advanced lesion in this particular segment was included in the analysis. The size of the polyp was estimated either with the use of open-biopsy forceps or on the basis of clinical judgement.

Pathology specimens were evaluated by local pathologists, who classified polyps according to the criteria established by the World Health Organization[11]. Pathology reports were accessible through the national pathology data system (PALGA)[12]. The prevalence and location of advanced neoplasia were assessed for all colonoscopies and for each indication cluster separately. All sigmoidoscopies and incomplete colonoscopies were excluded, except for incomplete colonoscopies due to an obstructing CRC. Other exclusion criteria were colonoscopies with insufficient bowel cleansing and colonoscopies in patients with a known advanced neoplasm *in situ* (procedure indication is polypectomy or endoscopic re-evaluation of the anatomic position of the tumor). In case a patient had undergone multiple colonoscopies, we only analyzed the examination in which the most advanced neoplastic lesion was found. In case a patient had a synchronous right-sided and left-sided advanced neoplasm, we only analyzed the most proximal lesion or we analyzed both synchronous lesions separately (Table 2).

Statistical analysis

Primary objective: In all successful total colonoscopies, the prevalence and location of advanced colorectal neoplasia and the age and gender of patients were assessed.

Secondary objective: For each indication cluster separately, the prevalence and location of advanced colorectal neoplasia were assessed in all successful total colonoscopies.

For comparison of proportions, the Fisher's exact

Table 3 Prevalence and distribution of advanced colorectal neoplasia in 4623 patients *n* (%)

Localization in the colo-rectum	CRC	Advanced adenoma	Advanced neoplasia[1]
Rectum	86 (31)	74 (22)	160 (26)
Sigmoid	77 (27)	131 (38)	208 (33)
Descending colon	19 (7)	29 (8)	48 (8)
Transverse colon	23 (8)	19 (6)	42 (7)
Ascending colon	37 (13)	46 (13)	83 (13)
Caecum	39 (14)	43 (13)	82 (13)
Total	281 (100)	342 (100)	623 (100)

[1]Advanced neoplasia was defined as an adenoma > 1.0 cm and/or > 25% of villous architecture and/or high-grade dysplasia or cancer; In two patients, two CRC's were found. In one case, both were located in the distal colon. In the other case, a distal and a proximal tumor was found; In 45 patients, both proximally and distally located advanced neoplasia were found.

test or chi-square test with Yates correction were used. Analyses were performed with SPSS for Windows software, version 12.0 (SPSS Inc., Chicago, Illinois).

RESULTS

General results

In total, 5652 colonoscopies and 3444 sigmoidoscopies were performed in 8636 patients during a three month period. Figure 1 shows an overview of the inclusion and exclusion criteria. After excluding all sigmoidoscopies (n = 3444), incomplete colonoscopies (n = 817), patients with insufficient bowel cleansing (n = 36), patients < 16 years (n = 2) and patients with a known advanced neoplasm *in situ* [procedure indication is polypectomy or endoscopic re-evaluation of anatomic position of the tumor (n = 85)], the total study cohort consisted of 4623 patients (mean age ± SD 58.8 ± 16 years, range 16-100 years). In 4 patients (0.1%), the age was not mentioned in the endoscopy report. Forty-seven percent and 53% of the patients were male and female, respectively (mean age for males 59.3 ± 15 years, mean age for females 58.4 ± 16 years, P = NS). In 0.1% of the patients (n = 4) gender was not mentioned in the endoscopy report. In 16% and 84% of the patients, endoscopies were performed in an academic hospital and general/teaching hospital, respectively. In all patients undergoing colonoscopy, the cecal intubation rate was 83%. In 14% of the cases, the cecum was not visualized and in 3% of the cases the issue was not accounted for in the colonoscopy report.

Prevalence and location of advanced neoplasia

The prevalence and distribution of CRCs, advanced adenomas and advanced neoplasia are listed in Table 3. Furthermore, in all complete colonoscopies, three incident cases of carcinoid tumors, one anal carcinoma and three metastatic lesions of other primary tumors were detected. In patients with CRC (n = 281), 52% were males (mean age ± SD 68.0 ± 11 years) and 48% were females (mean age ± SD 70.6 ± 12 years) (P = NS). In patients with CRC, the tumor was located in the distal colon and

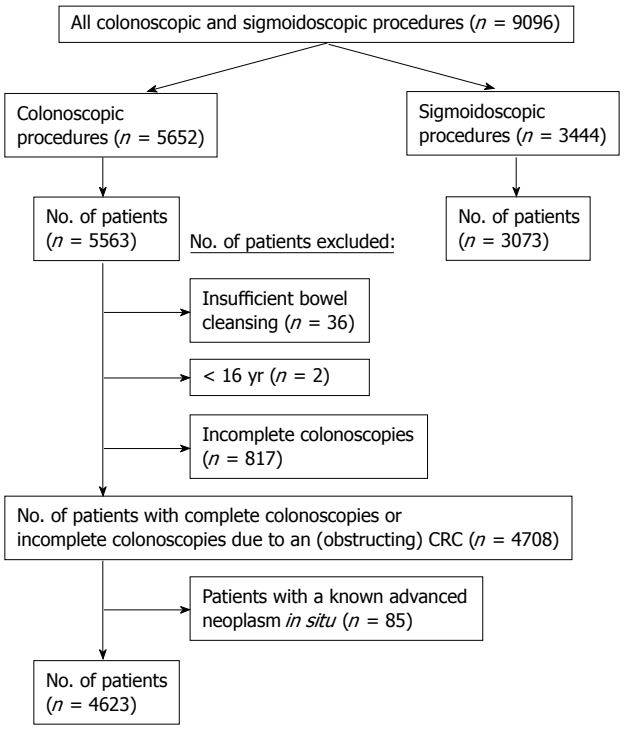

Figure 1 Number of patients included in the primary and secondary study objectives (n = 4623).

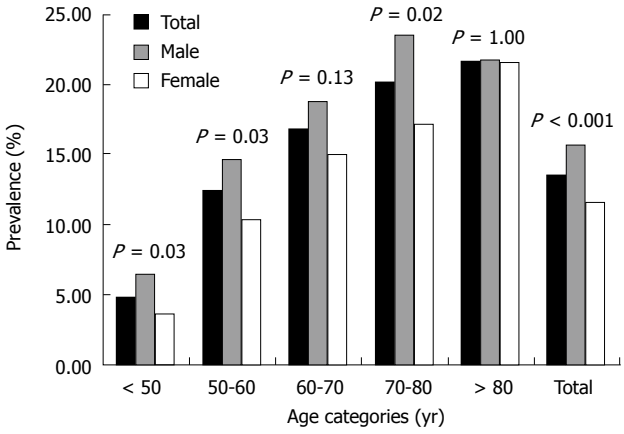

Figure 2 Yield of advanced neoplasia in different age categories (n = 4623). *P*-values for males compared to females.

proximal colon in 65% and 35% of cases, respectively. Of all patients with right-sided advanced neoplasia (i.e. advanced adenomas and/or cancer, n = 228), 51% had a normal appearing distal colon, whereas 49% had a synchronous distal polyp (41% advanced neoplasm, 14% small adenoma, 12% hyperplastic polyp and 33% non-specified polyp). Overall, 2.5% of the total study cohort had a proximally located advanced neoplasm without a synchronous distal polyp. Figure 2 illustrates the prevalence of advanced neoplasia in different age categories for males and females. Advanced neoplasia became more prevalent with increasing age. In 22% of the patients over 80 years an advanced neoplasm was found, compared to 5% of the patients under 50 years (P < 0.0001) Men were more likely than women to have advanced

neoplasia (15.6% for men *versus* 11.6% for women, odds ratio (OR) corrected for age is 1.4; 95% confidence interval (CI) 1.18 to 1.67; $P < 0.0001$).

Yield of advanced neoplasia per indication

The yield and distribution of advanced neoplasia are summarized per indication cluster in Table 2. Advanced neoplasia were found in 25% of patients who presented with GI bleeding. Moreover, in cases of a clinical or radiological suspicion of CRC, the yield of advanced neoplasia was 44%. In all of the procedure indication clusters, the prevalence of right-sided advanced neoplasia ranged from 11%-57%. In patients who presented with GI bleeding, predominantly left sided advanced neoplasia were found (83%). In contrast, in patients who presented with anemia mostly right-sided advanced neoplasias were encountered (57%, $P < 0.001$). Advanced neoplasia were found in 7% of asymptomatic patients (10% of the total study cohort), who presented with a family history of CRC or with a CRC screening request. Finally, both left- and right-sided advanced neoplasias were found in 7% of all patients.

DISCUSSION

This study included all procedures from patients clinically referred for colonoscopy in a three month period. In The Netherlands, all colonoscopies are performed in a hospital setting (academic, teaching or general hospitals). No other institutions, like private practices or doctor's offices, perform endoscopies. Our study includes all colonoscopies performed in Northern Holland, representing a large unselected sample of the population of The Netherlands. Therefore, our data accurately represent the entire lower GI endoscopic practice in Northern Holland which we regard to be representative for the whole of The Netherlands. This study cohort yielded 281 CRCs. However, all CRCs found using sigmoidoscopy ($n = 95$) or during abdominal surgery without prior endoscopy ($n = 38$), were excluded (data not shown in results section). Extrapolation of the total number of CRCs found to annual incidence figures would show a substantial increase in incidence of CRC compared to national/regional cancer registries (67 cases per 100 000 inhabitants compared to 63 cases per 100 000 inhabitants in 2003)[3]. In line with international data on the rising incidence of CRC, this finding would emphasize the importance of implementing a CRC screening program in The Netherlands to improve survival by diagnosing CRC or its precursors at an earlier stage[4,13].

In this referral population, more than 13% of colonoscopies performed yielded an advanced neoplasm (CRC/advanced adenoma). Of all advanced neoplasia found, 33% were located in the proximal colon and 67% were located in the distal colon. Similar to other studies, we identified male sex and increasing age as independent risk factors of advanced neoplasia, either distally located or proximally located[14-17]. However, male sex adjusted for age and distal findings did not significantly increase the risk of advanced proximal neoplasia. As shown in Figure 2, at least a 4-fold increase in prevalence of advanced neoplasia was observed in patients > 70 years compared with those of < 50 years. This age-related increase in prevalence of advanced neoplasia is in keeping with previous Western and Asian reports[18-20]. Unfortunately, the presence of a right-sided advanced neoplasm can not be adequately predicted by distal colonoscopic findings since 51% of proximally located advanced neoplasia had no distal polyps. If distal adenomas are considered sentinel lesions that warrant a complete colonoscopy, the percentage of detected proximally located advanced neoplasms would have been 27% if only sigmoidoscopy had been performed. However, in this study, a substantial proportion of distal polyps was not specified, which could be an important confounder (33% of all distal polyps in patients with proximally located advanced neoplasia). In accordance with other studies in which patients were referred for colonoscopy, no significant differences in the prevalence of proximally located advanced neoplasia with a normal appearing distal colon were found [prevalence of isolated, proximal advanced neoplasia in the United States (2.7%), Asia (2.2%) and The Netherlands (2.5%)][17,21]. Moreover, compared to the Asian situation, no significantly different percentages were observed in terms of missed proximally located advanced neoplasia if only sigmoidoscopy had been carried out[17].

When taking into account the different procedure indication clusters, the prevalence of proximally located advanced neoplasia ranged from 11%-57%. In patients who presented with GI bleeding, only 11% of advanced neoplasia were located in the proximal colon, while 83% were located in the distal colon ($P < 0.001$). Six percent of the patients with GI bleeding had synchronous advanced neoplasia in both the distal and the proximal colon. Consequently, the majority of advanced neoplasia in patients with GI bleeding are found within reach of the sigmoidoscope. However, apart from GI bleeding, which is one of the most frequent procedure indications, right-sided advanced neoplasia is a common finding which cannot be ignored when considering the proper endoscopic procedure in clinical practice. Even in the IBS indication cluster, which has a low pretest likelihood ratio for advanced neoplasia[22,23], similar percentages of right-sided advanced neoplasia were found compared to indications such as weight loss, family history of CRC, CRC surveillance and FAP/HNPCC surveillance (P = N.S.). A change in bowel habits was included in the IBS indication cluster (Table 1) which may be responsible for the high yield of advanced neoplasia, particularly in patients > 50 years. Surprisingly, there were hardly any referrals for colonoscopy based on a positive FOBT result ($n < 10$), which is a frequent procedure indication in other studies[7]. In all probability, this is due to a lack of confidence in the FOBT as a diagnostic test in The Netherlands. We hypothesize that this finding might also reflect the Dutch lagging behind in CRC awareness and pre-screening activities compared to other European countries[24,25].

In this accurate regional representation of Dutch

endoscopic practice, 10% of all colonoscopies in routine endoscopy programs were performed in asymptomatic patients. This ranged from an individual screening request without family history of CRC to a request because of a history of CRC in a 1st-3rd degree family relative. The diagnostic yield in terms of advanced neoplasia in this indication cluster was substantial (7%), and right-sided advanced neoplasms were frequently found (30%). Taking into account the yield of right-sided advanced neoplasia in each indication cluster, and in asymptomatic patients in particular, it can be argued whether these findings would be truly different in a CRC screening setting. To further elaborate on this conclusion, the majority of advanced colorectal adenomas and a proportion of early cancers are asymptomatic. These neoplasias are detected by chance during colonoscopy. Therefore, the topographic distribution and epidemiology of colorectal neoplasia, particularly advanced adenomas, found in this study should largely reflect the actual situation in The Netherlands where screening colonoscopy is non existent. This also means that this study could not have been performed in a screening population only. However, because of the increasing attention to CRC screening, in both policy makers, medical doctors and the general population, a substantial number of endoscopies are performed in daily clinical practice in asymptomatic patients. To a certain extent, our asymptomatic patients are comparable to a screening population. Therefore, this study contains an informative mix of symptomatic and asymptomatic patients with a comparable distribution rate of advanced neoplasia.

Our findings should be interpreted taking into account several potential caveats in case of extrapolation to a screening setting. Firstly, in this study the majority of patients were symptomatic or in a surveillance program which may be accompanied by a higher likelihood of having colorectal neoplasia. In contrast to screening colonoscopy, in which age limits are restricted, the wide age range of our study population may have influenced the rate of advanced colorectal neoplasia. The Dutch Health Council, however, asked for such routine endoscopy data before implementing a CRC screening program. Secondly, histology reports were generated by local pathologists meaning that there was an inherent risk of inter-observer variability in characterization of the histological types and degrees of dysplasia of polyps[26,27]. Furthermore, in the total study cohort 331 patients had a non-specified polyp (7% of all patients and 18% of all colorectal neoplasms). Non-specification was mainly due to insufficient retrieval of snared polyps, lack of biopsies and poor quality of biopsy specimens. Although the percentage of advanced neoplasia that are missed because of non-specification remains elusive, the high number of non-specified polyps is rather worrisome for routine practice. Thirdly, polyp size is frequently misjudged by endoscopists[28]. In our study, no systematic size estimate was used and, therefore, an arbitrary cut-off value of 10 mm was used for discrimination of small and large polyps, leaving judgement of sizes to each endoscopist individually. Thus, due to the lack of predefined standardization, the proportion of truly advanced neoplasia may not be accurately reflected in this cohort.

Surprisingly, colonoscopy in daily clinical practice was incomplete in 17% of cases. Whether an incomplete colonoscopy was followed by a double-contrast barium enema or CT colonography to visualize the total colon is not known. Major contributors to cecal intubation failure were inflammation due to IBD or diverticulitis, extensive diverticular disease, stenosis/adhesions after abdominal surgery and large advanced adenomas. Undoubtedly, the miss rate of advanced neoplasia due to incomplete colonoscopies needs further clarification. Such low cecal intubation rates may frustrate future colonoscopy-based screening programs. Recently, simple measures have been proposed to optimise quality in colonoscopy[29,30]. These studies and this low cecal intubation rate underscore the importance of continuous quality control in terms of reporting and appropriate training.

In conclusion, this study is an exact representation of daily clinical practice, and as such provides relevant data on the performance of colonoscopy with respect to the detection of advanced neoplasia. Our data are mandatory for the future planning of CRC screening in The Netherlands. Although this referral population may have a higher pre-test likelihood for colorectal neoplasia, the distribution of these lesions throughout the colorectum may be the same. At present, 10% of all colonoscopies in routine endoscopy programs are performed in asymptomatic patients with a substantial yield of advanced neoplasia. Based on clinical indication, no significant changes in endoscopic utilization can be realized to alleviate endoscopic workload since substantial numbers of right-sided advanced neoplasia are found in each indication cluster. Extrapolation of our data indicates that sigmoidoscopy would miss 33% of advanced neoplasia. Hence, our data show that colonoscopy is warranted for the evaluation of both symptomatic and asymptomatic patients.

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COMMENTS

Background

Colorectal cancer (CRC) awareness accounts for an increasing number of colonoscopies performed in asymptomatic patients with a screening request or family history of CRC in The Netherlands. Before embarking on endoscopic screening, we need to understand the distribution of CRC as well as the high risk precursor lesions within the colorectum. International data are scarce regarding real life incidence figures of colorectal neoplasia found in routine endoscopy programs, evaluating both symptomatic and asymptomatic patients.

Research frontiers

Knowledge of the incidence and distribution of CRC and high-risk precursor lesions in the colo-rectum in both symptomatic and asymptomatic patients, could tailor endoscopic utilization. Furthermore, it could facilitate making informed decisions in choosing a future screening modality and future national research initiatives in the field of CRC.

Innovations and breakthroughs

The overall yield in advanced neoplasia was significantly higher in this study than in the Asian situation (13.5% *vs* 9.4%). In accordance with Unite States and Asian studies, in which patients were referred for colonoscopy, high percentages of proximally located advanced neoplasia with a normal appearing distal colon were found. Extrapolation of our data indicates that sigmoidoscopy would miss 33% of advanced neoplasia. The yield of advanced neoplasia in asymptomatic patients is substantial (7%). Although this referral population may have a higher pre-test likelihood of colorectal neoplasia compared to a screening population, the distribution of these lesions throughout the colorectum may be the same. In The Netherlands, where screening colonoscopy is non existent, 10% of all colonoscopies in routine endoscopy programs are performed in asymptomatic patients.

Applications

This study shows that colonoscopy has a high yield in detecting advanced colorectal neoplasia in daily clinical practice. Colonoscopy is warranted for the evaluation of both symptomatic and asymptomatic patients, since substantial numbers of right-sided advanced neoplasia are found in both patient groups. These data are mandatory for the future planning of CRC screening in The Netherlands.

Terminology

Advanced colorectal adenoma is defined as an adenoma $\geqslant$ 1.0 cm, an adenoma with villous or tubulovillous architecture ($\geqslant$ 25% villous component) or an adenoma with high-grade dysplasia. Advanced colorectal neoplasm is defined as an adenoma $\geqslant$ 1.0 cm, an adenoma with tubulovillous or villous architecture ($\geqslant$ 25% villous component), an adenoma with high-grade dysplasia, or cancer. Subsequently, the term advanced colorectal neoplasm was defined as comprising advanced adenomas and cancer.

Peer review

This is a useful study reporting the prevalence of colonic lesions in symptomatic and asymptomatic patients in a Dutch province containing 18 hospitals. The sample size is large, and it is fairly well written.

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Liver histology according to the presence of metabolic syndrome in nonalcoholic fatty liver disease cases

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Abstract

AIM: To investigate the histologic features of the liver in nonalcoholic fatty liver disease (NAFLD) cases according to the presence of metabolic syndrome or its individual components.

METHODS: We enrolled 81 patients (40 male, 41 female) who were diagnosed with fatty liver by ultrasonographic scan and fulfilled the inclusion criteria. First anamnesis, anthropometric, clinical, laboratory and imaging features of all participants were recorded and then liver biopsy was performed after gaining consent from patients. Diagnosis of metabolic syndrome was dependent on patients having 3 or more out of 5 risk criteria defined by the WHO. Biopsy specimens were assessed according to Brunt *et al*'s classification.

RESULTS: Sixty-nine of the 81 patients had nonalcoholic steatohepatitis (NASH), 11 had simple fatty liver and 1 had cirrhosis according to histologic evaluation. Comparisons were made between two groups of NASH patients, those with and without metabolic syndrome. We did not detect statistically significant differences in liver histology between NASH patients with and without metabolic syndrome.

CONCLUSION: NASH can progress without metabolic risk factors or the presence of metabolic syndrome.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is common and has a spectrum of liver pathologies beginning with simple fatty liver and progressing to steatohepatitis, cirrhosis and liver failure[1,2]. NAFLD is frequently present along with the components of metabolic syndrome and, hence, is generally regarded as a manifestation of metabolic syndrome[3]. As insulin resistance (IR) is a main mechanism in the pathogenesis of metabolic syndrome, it is also thought to be an initiating factor in the process of NAFLD[4,5]. Nevertheless, some NAFLD cases did not fulfill all criteria of metabolic syndrome and did not display IR at the onset of disease according to the literature[6]. Certain recent studies revealed that all patients with NAFLD did not also have metabolic syndrome or its separate symptoms, including IR[6].

In the present study, differences in liver histology according to the presence of metabolic syndrome or its individual components were investigated. We also explored the effect of IR on the development of NAFLD. The features of patients with an NAFLD-like clinical course, accompanying diseases, laboratory findings and histologic aspects, are able to provide remarkable clues into the etiopathogenesis of the disease. Although there were many common points and reported issues supporting the presence of metabolic disorder and its components in the etiology of NAFLD, some studies revealed that NAFLD could also progress in lean people, nondiabetics, males, adolescents and children[7,8].

Certain articles in the literature have disclosed striking findings; for example, the frequency of IR in NAFLD patients varies from 47%-98% without diabetes also being present. Likewise the prevalence of metabolic syndrome in NAFLD patients was as low as 36% in some studies[6]. Furthermore, in different populations the prevalence of metabolic syndrome is about 22% and in NAFLD patients there was a subgroup who did not have IR[6]. We aimed to reveal whether there is a group of NAFLD patients without metabolic syndrome and IR or not. Recently, increasing number of studies on this topic are being presented. But more investigations are needed to attain convincing outcomes.

MATERIALS AND METHODS

Patients

This study consisted of 81 patients who were referred to Uludag University Gastroenterology Division. All 81 patients were diagnosed with fatty liver by ultrasonographic scan. After this complete clinical, anthropometric and laboratory assessments and liver biopsy were performed. Exclusion criteria included: alcohol consumption of > 20 g/d, pregnancy, positive tests indicating the presence of hepatitis B or C virus, autoimmune liver disease, hemochromatosis, Wilson's disease, α-1 antitrypsin deficiency, primary biliary cirrhosis, primary sclerosing cholangitis and toxic liver disease.

Laboratory studies

After taking a medical history, all cases underwent liver examination by ultrasonography and then clinical, anthropometric, complete blood count and biochemical assessments were performed. Biochemical evaluation consisted of assessment of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), bilirubin, albumin, high density lipoprotein (HDL)-cholesterol, triglycerides, glucose, and insulin levels and an oral glucose tolerance test (OGTT). Anthropometric parameters measured were height, weight, body mass index (BMI), waist and hip circumferences and waist/hip ratio values. Assessment of obesity was dependent on WHO criteria[9]. American Diabetes Association (ADA) criteria were used to define type 2 diabetes, impaired glucose intolerance, and impaired fasting glycemia[10]. Patients receiving oral antidiabetics or insulin therapy were accepted as diabetics. Hypertension was considered to be present when resting blood pressure was ⩾ 140/90 mmHg or patients were receiving antihypertensive drug therapy. The homeostasis model assessment of IR (HOMA-IR) method was used to measure IR and patients were classified as 'insulin resistant' when HOMA-IR value was > 2.70. ALT levels 1.5 or more times higher than upper normal values indicated an elevation in ALT. The diagnosis of metabolic syndrome was made according to WHO criteria[10,11] (BMI ⩾ 30 kg/m^2, waist/hip circumference ratio > 0.90 in men and > 0.85 in women, fasting blood glucose ⩾ 1100 mg/L, overt diabetes, presence of impaired glucose tolerance and/or IR, triglycerides ⩾ 1500 mg/L, HDL-cholesterol < 400 mg/L in men and < 500 mg/L in women, arterial blood pressure ⩾ 140/90 mmHg and presence of microalbuminuria). Patients should have at least three of these criteria to be diagnosed with metabolic syndrome. The study was approved by the hospital ethics committee.

Pathology

Liver biopsies were performed in 81 patients according to the severity of the clinical disease after the patients had given consent. All liver biopsy specimens were examined by a liver pathologist. Scoring of necroinflammmation and fibrosis was performed using criteria devised by Brunt *et al*[12,13]. Nonalcoholic steatohepatitis (NASH) was diagnosed according to liver histology indicating steatosis (mild: < 33% of lobules, moderate: 33%-66% of lobules and severe: > 66% of lobules) with (1) ballooning degeneration of hepatocytes/mallory bodies; (2) necroinflammation (lobular or portal); (3) fibrosis (perisinusoidal, periportal and/or bridging) or cirrhosis.

Statistical analysis

Due to the number of patients being small, statistical evaluation and *P* values were not available, as shown in all tables. Hence, features of patients were evaluated according to their percentage values.

RESULTS

Anthropometric, clinical and laboratory results

Eighty-one patients (40 male, 41 female) who were diagnosed as having fatty liver by ultrasonographic examination participated in this study at the Uludag University Gastroenterology Division. Only 8% of patients had slight and dull abdominal pain. The prevalence of hepatomegaly was 16% and 4% in NASH and simple fatty liver groups, respectively. All 81 patients underwent liver biopsy; 69 (35 male, 34 female) were diagnosed with NASH, 11 (4 male, 7 female) were diagnosed with simple fatty liver and 1 (male) was diagnosed with cirrhosis. First, we compared all cases with NASH and simple fatty liver to each other according to anthropometrical, clinical and laboratory data, including presence of IR and metabolic syndrome, but we did not find any significant difference between the 2 groups. For instance, numbers and proportions of IR and metabolic syndrome in NASH patients were 30 (43.4%) and 46 (66.7%) respectively and in simple fatty liver patients were 6 (54.5%), and 9 (81.8%) respectively. Then, the features of liver histology were examined in detail with regard to indivudial components of metabolic syndrome. As shown in Table 1, liver steatosis and necro-inflammation were evaluated with respect to individual parameters of metabolic syndrome. Because the numbers of cases in each section of Table 1 were too small, statistical assessments were not available and data analysis and interpretation were performed using percentage values. It seemed that the presence of individual risk factors did not affect the severity of steatosis and necroinflammation. Similarly, in Table 2 progression of liver fibrosis was

Table 1 Presence of metabolic risk factors and liver histology (steatosis/necroinflammation) in NASH cases

	NASH patients (n = 69)					
	Fatty infiltration			Necroinflammation		
	Mild (%)	Moderate (%)	Severe (%)	Mild (%)	Moderate (%)	Severe (%)
Gender						
Male	37.1	40.0	22.9	31.4	62.9	5.7
Female	41.2	35.3	23.5	20.6	64.7	14.7
Hepatomegaly						
(+)[1]	31.3	37.5	31.2	25.0	62.5	12.5
(-)[1]	38.5	38.5	23.0	27.0	61.5	11.5
Body mass index						
18.5-24.9	0	66.6	33.4	33.4	33.3	33.3
25-29.9	35.3	41.2	23.5	26.5	64.7	8.8
30-39.9	46.5	25.0	28.5	28.6	57.1	14.3
> 40	25.0	75.0	0	25.0	75.0	0
Central obesity						
(+)	38.8	40.8	20.4	26.5	63.3	10.2
(-)	35.0	30.0	35.0	30.0	55.0	15.0
Hypertension						
(+)	33.4	33.3	33.3	19.0	62.0	19.0
(-)	39.6	39.6	20.8	33.3	58.3	8.4
Diabetes						
(+)	40.0	35.0	25.0	33.4	57.1	9.5
(-)	38.7	38.7	22.6	30.7	59.1	10.2
Hypertriglyceridemia						
(+)	36.2	34.0	29.8	21.4	63.8	14.8
(-)	41.0	54.5	4.5	41.0	54.5	4.5
Insulin resistance						
(+)	33.3	36.7	30.0	13.3	73.4	13.3
(-)	43.5	34.7	21.8	47.8	43.5	8.7

[1](+): Present; (-): Absent. Presence of hypertriglyceridemia and insulin resistance seemed to increase the severity of steatosis and necroinflammation but these findings were not significant.

Table 2 Presence of metabolic risk factors and liver histology (stage) in NASH cases

	NASH patients (n = 70)[1]				
	Fibrosis				Cirrhosis (%)
	Absent (%)	Perisinusoidal/ Pericellular (%)	Periportal (%)	Bridging (%)	
Gender					
Male	51.4	23.0	14.3	8.60	2.70
Female	38.2	47.0	5.9	8.90	-
Hepatomegaly					
(+)[2]	44.1	31.2	6.3	12.5	5.90
(-)[2]	44.3	36.5	13.5	7.70	-
Body mass index					
18.5-24.9	33.3	0	33.3	33.3	-
25-29.9	38.2	44.1	11.7	5.8	-
30-39.9	46.4	25.0	14.2	10.7	3.12
> 40	25.0	75.0	0	0	-
Central obesity					
(+)	46.9	30.6	16.3	6.1	2.04
(-)	65.0	20.0	10.0	5	-
Hypertension					
(+)	47.8	42.8	4.7	4.7	-
(-)	58.4	24.4	12.7	4.5	2.04
Diabetes					
(+)	28.5	38.0	14.2	14.2	4.76
(-)	53.1	25.5	12.7	8.5	-
Hypertrigly-ceridemia					
(+)	50.0	25.0	15.6	9.3	-
(-)	59.3	31.2	6.2	3.1	4.34
Insulin resistance					
(+)	53.3	36.6	13.3	3.3	3.33
(-)	65.7	18.4	10.5	2.6	-

[1]69 patients with NASH and 1 cirrhotic patient; [2](+): Present; (-): Absent. Presence of diabetes and insulin resistance seemed to increase the severity of fibrosis, but these findings were not significant.

evaluated with respect to individual parameters of metabolic syndrome and again it seemed that individual metabolic risk factors did not initiate or advance liver fibrosis. In Table 3, dual combinations of risk factors were compared to grading and staging values of liver histology and there was no remarkable outcome. Finally, in Table 4 detailed histological parameters were evaluated according to the presence of metabolic syndrome. However, we did not determine any correlation between histological severity and the presence of metabolic syndrome.

When the distribution of risk factors and metabolic syndrome was examined in 11 simple fatty liver patients, the following results were found: central obesity 57%, hypertension 53%, diabetes 18.1%, hypertriglyceridemia 58%, low HDL level 57%. While 9 of these 11 patients had metabolic syndrome, the remaining 2 patients had only 2 risk factors for metabolic syndrome. The single cirrhotic patient was a 55-year-old male with metabolic syndrome who had obesity (also central obesity), diabetes and a low-HDL level.

Histopathology

The important highlights of liver histology belonging to our 81 cases were investigated. Since the numbers of patients in each of the subgroups were too small, statistical assessments were not available and interpretations of histological findings in all tables were dependent on percentage values. As shown in Table 1, liver steatosis and necroinflammation were evaluated in detail according to the individual presence of metabolic risk factors and there was no significant difference in the two groups. Similarly Table 2 showed that when liver fibrosis was studied with respect to the presence of individual risk factors there was no significant difference. In Tables 3 and 4, the presence of dual combinations of risk factors and the presence of defined metabolic syndrome, respectively, were compared to liver histology. Neither the presence of a dual combination of risk factors nor the presence of defined metabolic syndrome were found to be closely related with the severity of steatosis, necroinflammation and fibrosis. İnterestingly, among 11 patients with simple fatty liver each patient had at least two metabolic risk factors. In the simple fatty liver group, the prevalence of defined metabolic syndrome was 81.8% which was higher than that in the NASH group. Finally, 1 patient who was diagnosed with cirrhosis according to liver histology had metabolic syndrome.

DISCUSSION

The relationship between NAFLD and metabolic

Table 3 Dual combinations of risk factors and liver histology in NASH cases

Liver histology	NASH cases		
	Ob + DM (n = 6)	Ob + Htg (n = 8)	DM + Htg (n = 2)
Grade			
1	3	2	0
2	2	6	1
3	1	0	1
Stage			
0	1	3	0
1	4	4	1
2	1	0	0
3	0	1	1
4	0	0	0

Ob: Obesity; DM: Diabetes mellitus; Htg: Hypertriglyceridemia. Dual combination of risk factors did not seem to effect liver histology.

Table 4 Liver histology according to the presence of metabolic syndrome in NASH cases (%)

Liver histology	Patients with NASH (n = 69)	
	With metabolic syndrome (n = 46, 66.6%)	Without metabolic syndrome (n = 23, 33.4%)
Fatty infiltration		
Mild	20 (43.4)	11 (47.8)
Moderate	19 (41.3)	6 (26.1)
Severe	7 (15.3)	6 (26.1)
Necroinflammation		
Absent	0 (0)	0 (0)
Mild	13 (28.3)	9 (39.1)
Moderate	28 (60.9)	13 (56.5)
Severe	5 (10.8)	1 (4.40)
Fibrosis		
Absent	20 (43.4)	9 (39.1)
Perisinusoidal/pericellular	16 (34.7)	9 (39.1)
Periportal	7 (15.4)	2 (8.60)
Bridging	3 (6.50)	3 (13.2)
Cirrhosis	1 (2.12)	0 (0)

Evaluations were performed using percentage values. Presence of metabolic syndrome seemed to increase the severity of steatosis, necroinflammation and fibrosis in liver, but these results were not significant as well.

syndrome is well known. Certain metabolic disorders like obesity, diabetes, hypertriglyceridemia and hypertension frequently associate with NAFLD and are also components of metabolic syndrome[3,4,14]. Insulin resistance was thought to be a shared and basic metabolic disturbance in both these groups of diseases[15]. In the general population, the prevalence of NAFLD is 10%-24% while the prevalence of NASH is about 1%-5%[16].

The assocation between NAFLD and metabolic syndrome gave rise to many studies on this subject. The prevalence of metabolic syndrome in NASH and simple fatty liver cases is 22.8%-88% according to the literature[14,17-20]. This suggests the relationship between NAFLD and metabolic syndrome is not a stable and constant feature. Moreover, the presence of IR was suggested to be a common and frequent finding in both NAFLD and metabolic syndrome in various studies[5,14,15,21]. Marchesini *et al*[17] revealed the prevalence of IR in NAFLD was 61%; but in certain recent studies, a low prevalence of IR in NAFLD was found[6,22,23].

The influence of individual risk factors and defined metabolic syndrome on liver histology have become considerable and have inspired comprehensive studies. Marchesini *et al*[17] and Angelico *et al*[24] found a correlation between various degrees of liver steatosis (mild, moderate and severe) and BMI. According to studies by Willner *et al*[21], Angulo *et al*[25] and Ratziu *et al*[26] advanced obesity may be a risk factor for the development of liver fibrosis. But Xanthakos *et al*[27] stressed that in morbidly obese adolescents, severe NASH was uncommon and the presence of metabolic syndrome did not distinguish NASH from steatosis. We did not observe any connection between increased BMI and liver histology (steatosis and necroinflammation/fibrosis) in our NASH cases (Tables 1 and 2). Camilo Boza *et al*[28] did not find a significant association between BMI and histological changes; but in their study, high HOMA-IR values and ALT levels were the only independent predictors of NASH. Among our 69 cases with NASH, only 3 (4.34%) had normal body weight and among our simple fatty liver group (n = 11) only 1 (9.09%) patient had normal body weight; there was no significant difference between these two groups. Diabetes and dyslipidemia (especially hypertriglyceridemia and low HDL level) were also considered to affect liver histology[29-31]. Risk factors for metabolic syndrome and defined metabolic syndrome was strongly considered to affect liver histology according to Marceau *et al*[32].

But, still there are important and controversial points in the natural course of NAFLD. Which one has a precedence: liver steatosis or IR? Recently it was noticed that NAFLD could occur in nonobese, nondiabetic persons and even in infants and adolescents[7]. Some patients with NAFLD may not have metabolic risk factors initially and the components of metabolic syndrome may emerge during the course of the disease[24]. In these patients, after diagnosis of NAFLD the required time for genesis of metabolic disorders like hyperglycemia, hypertension and hyperlipidemia is not well known. Furthermore, not all NAFLD patients fulfill the criteria of metabolic syndrome according to the literature. Recently, certain studies showed that there have been lower prevalances of metabolic syndrome among NAFLD patients. For instance Moon *et al*[33] performed research to identify metabolic risk factors and clinical features for each stage of liver fibrosis in NAFLD patients and in their 25 study cases with NAFLD, only 14 patients (56%) had metabolic syndrome. They found no difference in the prevalence of metabolic syndrome between the simple steatosis and the NASH subgroups (5/10, 50% *vs* 9/15, 60%). In addition, there were no significant differences in the histological features of two separate NASH groups which were constituted according to the presence or absence of metabolic syndrome. Similarly, we detected some cases which did not have metabolic syndrome,

but had NASH (23 cases = 33.4% of all NASH cases). Conversely, some cases had metabolic syndrome, but were not diagnosed with NASH. The latter only had simple fatty liver (9 cases = 81.8% of all simply fatty liver cases). In our study, approximetely 2/3 of the 69 NASH cases (66.6%) fit the criteria of metabolic syndrome and the remaining patients (33.4%) did not fit the full criteria of metabolic syndrome. These results suggest different causes of NASH other than metabolic syndrome should be searched for or that these NASH cases may represent patients in the early stages of metabolic syndrome. However**,** Kang *et al*[34] stated that a low proportion, 34% (31 of 91 patients), of NAFLD patients had metabolic syndrome, but these patients also had higher scores for steatosis and NASH activity.

Recent studies claimed that not only metabolic risk factors, but IR also could influence liver histology. Dixon *et al*[22] reported that HOMA-IR, ALT and arterial hypertension were independent predictors for NASH; but, they also found that 7.8% of their study patients had NASH even though they had normal AST and HOMA-IR values. Bahrami *et al*[35] found the rate of IR was only 54.7% in 53 patients with NASH. Similarly, Guidorizzi de Siqueira *et al*[23] determined the frequency of IR among NAFLD patients and described IR according to metabolic risk factors and histological findings. In their study, IR was detected in only 33% of NAFLD patients; but, there was a high frequency of IR in patients with advanced fibrosis, suggesting that IR may influence the prognosis of NAFLD. Sakurai *et al*[36] found that only steatosis was significantly and independently associated with elevated HOMA values; but there was no similar association with the grade or stage of NASH. However, we did not detect any connection between the presence of IR and liver histology. An interesting observation was expressed by Machado *et al*[6] who found that rates of IR in NAFLD patients ranged from 47% to 98% and only 36% of patients with NAFLD fulfilled three criteria of metabolic syndrome. The authors of this study designed it so that certain patients did not have IR at the onset of the study. The results of the study have been attributed to different factors. For instance, liver disease may precede IR or there may be a lack in sensitivity in the HOMA method.

In our study, NAFLD did not change histologically according to the presence of metabolic syndrome and its individual components. At the onset of NAFLD, metabolic disturbances may not be present, so patients with simple fatty liver should be followed for progression of metabolic disorders in the future.

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COMMENTS

Background

Obesity, diabetes and hyperlipidemia are components of metabolic syndrome and are frequently associated with nonalcoholic fatty liver disease (NAFLD). NAFLD consists of simple fatty liver and nonalcoholic steatohepatitis (NASH). The prognosis of NAFLD may worsen when there is an association with risk factors or metabolic syndrome. Insulin resistance (IR) is considered the common pathogenetic factor in both metabolic syndrome and NAFLD. We aimed to emphasize that NAFLD and NASH could progress not only in patients with metabolic risk factors, but also in nonobese healthy persons. Hence we investigated the histologic features of liver in NAFLD cases according to the presence of metabolic syndrome or its individual components.

Research frontiers

In certain patients with NAFLD and NASH, the prevalence of metabolic syndrome was low and the influence of risk factors or metabolic syndrome on liver histology was not significant when compared to those without metabolic syndrome.

Innovations and breakthroughs

It is important to be aware that it is not just NAFLD or NASH patients with metabolic syndrome who are at risk of advanced liver disease, but other NAFLD and NASH cases without metabolic syndrome may have severe liver disease. A new approach for these patients should be designed.

Applications

For general public health, individuials diagnosed by ultrasonography scan as having fatty liver with or without risk factors and metabolic syndrome should be followed up closely for further serious complications and outcomes.

Terminology

NAFLD and NASH may progress and worsen without metabolic syndrome (obesity, diabetes and hyperlipidemia) being present. In contrast to general opinion, the approach to individuals diagnosed with fatty liver by ultrasonographic examination should not be limited to the presence of metabolic syndrome. All patients with fatty liver should be advised about the hazardous outcomes of NAFLD.

Peer review

This article is consistent and factual, and meets the aims of introducing NAFLD and NASH, advising patients to avoid their likely noxious outcomes and recommending clinical staff make the requisite inspections if there has been a diagnosis of NAFLD or NASH.

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Upper gastrointestinal bleeding etiology score for predicting variceal and non-variceal bleeding

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Abstract

AIM: To identify clinical parameters, and develop an Upper Gastrointesinal Bleeding (UGIB) Etiology Score for predicting the types of UGIB and validate the score.

METHODS: Patients with UGIB who underwent endoscopy within 72 h were enrolled. Clinical and basic laboratory parameters were prospectively collected. Predictive factors for the types of UGIB were identified by univariate and multivariate analyses and were used to generate the UGIB Etiology Score. The best cutoff of the score was defined from the receiver operating curve and prospectively validated in another set of patients with UGIB.

RESULTS: Among 261 patients with UGIB, 47 (18%) had variceal and 214 (82%) had non-variceal bleeding. Univariate analysis identified 27 distinct parameters significantly associated with the types of UGIB. Logistic regression analysis identified only 3 independent factors for predicting variceal bleeding; previous diagnosis of cirrhosis or signs of chronic liver disease (OR 22.4, 95% CI 8.3-60.4, $P < 0.001$), red vomitus (OR 4.6, 95% CI 1.8-11.9, $P = 0.02$), and red nasogastric (NG) aspirate (OR 3.3, 95% CI 1.3-8.3, $P = 0.011$). The UGIB Etiology Score was calculated from (3.1 × previous diagnosis of cirrhosis or signs of chronic liver disease) + (1.5 × red vomitus) + (1.2 × red NG aspirate), when 1 and 0 are used for the presence and absence of each factor, respectively. Using a cutoff $\geqslant$ 3.1, the sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) in predicting variceal bleeding were 85%, 81%, 82%, 50%, and 96%, respectively. The score was prospectively validated in another set of 195 UGIB cases (46 variceal and 149 non-variceal bleeding). The PPV and NPV of a score $\geqslant$ 3.1 for variceal bleeding were 79% and 97%, respectively.

CONCLUSION: The UGIB Etiology Score, composed of 3 parameters, using a cutoff $\geqslant$ 3.1 accurately predicted variceal bleeding and may help to guide the choice of initial therapy for UGIB before endoscopy.

Key words: Non-variceal bleeding; Predictor; Score; Upper gastrointestinal bleeding; Upper gastrointestinal hemorrhage; Variceal bleeding

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INTRODUCTION

Upper Gastrointesinal Bleeding (UGIB) is a common gastrointestinal emergency and carries a mortality rate of 5%-14%[1]. The causes of UGIB have been classified into variceal bleeding (esophageal and gastric varices) and non-variceal bleeding (peptic ulcer, erosive gastroduodenitis, reflux esophagitis, tumor, vascular ectasia, *etc*). Currently, emergency esophagogastrodu odenoscopy (EGD) is the standard investigation of choice for active UGIB since it provides both diagnosis and treatment of UGIB[2-11]. However, in the real life situation, emergency EGD is seldom available in most hospitals due to the difficulty of setting up emergency services in non-official time, an insufficiency of well-

trained endoscopists and medical teams and lack of equipment. Thus, most patients are usually treated medically for a period of time before being referred for EGD at the centers with available facilities.

Some practice guidelines on non-variceal bleeding[5,6], variceal bleeding[12,13] including Thai guidelines in 2004[14] recommend giving empirical treatments to patients with UGIB while waiting for EGD. If variceal bleeding is suspected, empirical treatment with vasoactive agents (e.g. somatostatin, octreotide, terlipressin, *etc*) is strongly recommended, since they can stop bleeding in up to 70%-80% of cases and a decrease in mortality has been shown with some agents (i.e. terlipressin)[12,13]. In contrast, for suspected non-variceal bleeding, empirical treatment with a high-dose proton pump inhibitor is recommended since it reduces the stigmata of recent hemorrhage[5,15,16].

From a clinical viewpoint, to diagnose variceal bleeding precisely and to promptly administer vasoactive drugs to these patients, is crucial because variceal bleeding has a very high early mortality rate of up to 30% and up to 47%-74% of patients will have recurrent bleeding[12,13]. To predict which patients have variceal bleeding is not always easy. Some authors have suggested that the clinical signs of cirrhosis or portal hypertension[17-19], painless hematemesis and bleeding with significant change in hemodynamics may indicate variceal bleeding. In contrast, nonsteroidal anti-inflammatory drug (NSAID) users, the presence of dyspepsia or coffee-ground NG aspirate have been suggested to favor non-variceal bleeding[18,19]. These suggestions are often expert opinions and have never been formally validated.

The aims of this study are to assess the clinical and basic laboratory parameters which may help differentiate variceal and non-variceal bleeding before performing EGD, to develop a model of the UGIB Etiology Score for predicting the cause of UGIB based on clinical parameters and to validate the accuracy of this suggested score.

MATERIALS AND METHODS

All consecutive patients who presented with acute UGIB at Siriraj Hospital from June 2006 to December 2007 were prospectively enrolled into the study. Patients who presented in the initial period during June 2006 to December 2006 were included for score derivation purposes and patients who presented in the later period during May 2007 to December 2007 were included for score validation. The inclusion criteria were: 1. UGIB, defined by the presence of hematemesis, melena or hematochezia, and a positive NG tube aspiration for coffee-ground, black or bloody contents 2. EGD within 72 h after the onset of UGIB 3. Patients aged $\geqslant$ 18 years. An exclusion criterion was patients whose definite cause of UGIB was undetermined or inconclusive during EGD.

Data collection

Data were collected by gastroenterology fellows at the time of the patients' presentation. Patients' history included age, gender, appearance of vomitus, (red bloody, coffee-ground, clear), appearance of stool (red or maroon stool, melena, brown or yellow stool), presence of dyspepsia or abdominal pain, underlying cirrhosis, history of previous variceal or non-variceal bleeding within 1 year), comorbid diseases (e.g. acute or chronic kidney diseases, diabetes, hypertension, cardiac diseases, chronic lung diseases, and cerebrovascular diseases, *etc*), history of medications used within 4 wk (i.e. NSAIDS, aspirin, anticoagulants, corticosteroids and alcohol).

Physical examinations included blood pressure at presentation (presence of shock or BP < 90/60 mmHg), heart rate at presentation (presence of tachycardia, HR > 100 beats/min), degree of pallor (marked, mild/moderate, none), findings on NG tube aspiration (red blood, coffee-ground, clear), findings on rectal examination (red or maroon stool, melena, brownish to yellowish stool), the presence of any sign of chronic liver disease (spider angioma, palmar erythema, gynecomastia, testicular atrophy or parotid gland enlargement), epigastric tenderness, ascites, splenomegaly, and hepatic encephalopathy.

Laboratory data included hemoglobin, hematocrit, white blood cell count, platelet count, BUN, creatinine, prothrombin time, and a panel of liver chemistry tests.

Esophagogastroduodenoscopy

EGD was performed within 72 h of admission in all cases. Causes of bleeding were classified into variceal (esophageal or gastric varices) and non-variceal (e.g. peptic ulcer, erosive gastroduodenitis, reflux esophagitis, tumor, vascular ectasia, *etc*).

Statistical analysis

Statistical analysis was performed by using SPSS Program version 13.0. Univariate analysis for the associations between clinical parameters and the types of UGIB was carried out using the χ^2 test or Fisher's exact test for categorical variables and Student's *t*-test for continuous variable data. $P < 0.05$ was considered statistically significant. Logistic regression analysis to identify independent parameters was performed and is presented with odds ratio and 95% confidence interval.

The UGIB Etiology Score was developed from the parameters derived from the multivariate analysis. The best cutoff of the score was chosen from the receiver operating curve (ROC) and the sensitivity and specificity for predicting the types of UGIB were calculated. The score was then tested in the validation group and the positive (PPV) and negative predictive value (NPV) were calculated.

The present study was approved by the Ethics Committee of Siriraj Hospital.

RESULTS

There were 261 patients enrolled into the score derivation group, of which 214 patients (82%) had non-variceal

Table 1 Univariate analysis of clinical parameters of patients with variceal and nonvariceal bleeding *n* (%)

Clinical parameter	Cause of UGIB		*P*
	Variceal (*n* = 47)	Nonvariceal (*n* = 214)	
Age, mean ± SD (yr)	53 ± 15	61 ± 15	0.001
Male	41 (87)	151 (71)	0.030
Character of vomitus			< 0.001
Red	28 (60)	39 (18)	
Coffee-ground or clear	19 (40)	175 (82)	
Stool appearance			0.220
Red or maroon	6 (13)	14 (6)	
Melena, brown or yellow	41 (87)	200 (93)	
Dyspepsia or abdominal pain	3 (6)	45 (21)	0.032
NSAID, ASA, anticoagulant use	10 (21)	114 (53)	< 0.001
Previously diagnosed cirrhosis	17 (36)	41 (19)	< 0.001
History of variceal bleeding	13 (28)	8 (4)	< 0.001
History of non-variceal bleeding	0 (0)	21 (10)	0.018
Comorbid illness	13 (28)	132 (62)	< 0.001
Alcohol drinking	14 (30)	43 (20)	0.207
Hypotension	13 (28)	39 (18)	0.206
Tachycardia	26 (55)	93 (44)	0.188
Epigastric tenderness	2 (4)	25 (12)	0.212
Signs of chronic liver disease	30 (64)	32 (15)	< 0.001
Splenomegaly	15 (32)	14 (6)	< 0.001
Ascites	20 (43)	20 (9)	< 0.001
Hepatic encephalopathy	7 (15)	10 (5)	0.018
Character of NG aspirate			< 0.001
Red	28 (60)	38 (18)	
Coffee-ground or clear	19 (40)	176 (82)	

Table 2 Univariate analysis of laboratory findings of patients with variceal and nonvariceal UGIB *n* (%)

Laboratory findings	Causes of UGIB		*P*
	Variceal (*n* = 47)	Nonvariceal (*n* = 214)	
Hemoglobin, (g/dL)	8.6 ± 2.2	8.5 ± 2.6	0.731
Hematocrit, (%)	25.8 ± 6.3	25.9 ± 7.3	0.965
WBC (× 10^3/ mm^3)	12.2 ± 8.7	14.3 ± 13.5	0.319
Platelets (× 10^3/mm^3)	165.0 ± 115.8	248.6 ± 129.9	< 0.001
< 100 × 10^3/mm^3	16 (34)	23 (11)	< 0.001
BUN (mg/dL)	31 ± 18	44 ± 29	0.003
Creatinine (mg/dL)	1.3 ± 0.7	1.6 ± 1.8	0.190
Albumin (g/L)	2.8 ± 0.7	3.2 ± 0.7	0.001
Globulin (g/L)	3.7 ± 0.9	3.2 ± 0.8	< 0.001
Albumin/globulin ratio < 1	38 (81)	83 (45)	< 0.001
Total bilirubin (mg/dL)	4.1 ± 5.8	2.3 ± 5.5	0.054
SGOT (U/L)	133 ± 187	62 ± 107	0.001
> 2 × UNL	25 (53)	36 (20)	< 0.001
SGPT (U/L)	62 ± 76	36 ± 50	0.003
> 2 × UNL	8 (21)	21 (12)	0.359
SGOT/SGPT > 1	43 (92)	132 (75)	0.025
Alkaline phosphatase (U/L)	158 ± 112	115 ± 105	0.015
Prothrombin time (s)	21 ± 11	16 ± 8	0.002
> 12.5 s	44 (94)	58 (29)	< 0.001

UNL: Upper normal limit.

and 47 (18%) had variceal bleeding according to EGD findings. None had negative or inconclusive causes of UGIB by EGD. The causes of non-variceal bleeding were gastric ulcer (39%), duodenal ulcer (22%), both gastric and duodenal ulcer (9%), erosive gastroduodenitis (12%), GI malignancies (5%), Mallory-Weiss syndrome (3%), reflux esophagitis (2%) and miscellaneous (8%). The causes of variceal bleeding were esophageal varices (89%) and gastric varices (11%). Clinical characteristics and laboratory data of the 2 groups together with the univariate analysis of the associations between these factors and the causes of UGIB are shown in Tables 1 and 2.

Clinical characteristics

Variceal bleeding occurred significantly more often than non-variceal bleeding in younger patients (mean age 52.7 *vs* 60.8 years). Patients with variceal bleeding commonly presented with red bloody vomitus (60% *vs* 18%), red NG aspirate (60% *vs* 18%), were often previously diagnosed with cirrhosis (36% *vs* 19%), often had signs of chronic liver disease (64% *vs* 15%), splenomegaly (32% *vs* 6%) and hepatic encephalopathy (15% *vs* 5%). Patients with non-variceal UGIB more commonly had comorbid diseases (62% *vs* 28%), a history of ulcerogenic drug use (53% *vs* 21%) and dyspeptic symptoms (21% *vs* 6%) as compared to those with variceal bleeding. Hemodynamic changes (hypotension or tachycardia) at presentation were not significantly different between patients with variceal and non-variceal bleeding.

Eighty-two patients were either previously diagnosed with cirrhosis or had signs of chronic liver disease; however, only 40 (49%) of these patients had variceal bleeding.

Laboratory findings

Patients with variceal bleeding had lower platelet counts, and albumin level, but more commonly had reverse albumin/globulin ratio (81% *vs* 45%), and higher mean AST and ALT levels (133 *vs* 62 U/L and 62 *vs* 36 U/L, respectively). Prolonged prothrombin time was found in 94% of patients with variceal bleeding as compared to 29% of patients with non-variceal bleeding.

Multivariate analysis

Multivariate analysis was performed by a stepwise logistic regression analysis. Three factors were found to be independently associated with variceal bleeding; previous diagnosis of cirrhosis or signs of chronic liver disease (OR 22.4, 95% CI 8.3-60.4, $P < 0.001$), red vomitus (OR 4.6, 95% CI 1.8-11.9, $P = 0.020$) and red NG aspirate (OR 3.3, 95% CI 1.3-8.3, $P = 0.011$) as shown in Table 3.

UGIB Etiology Score

Using the 3 independent factors, the formulation for calculating the UGIB Etiology Score was constructed for the prediction of variceal bleeding. The formulation was as follow:

UGIB Score = (3.1 × previous diagnosis of cirrhosis or the presence of signs of chronic liver disease) + (1.5 × presence of red vomitus) + (1.2 × presence of red NG aspirate).

To calculate the score, a previous diagnosis of cirrhosis or the presence of signs of chronic liver diseases was scored 1 if present and 0 if absent. Red vomitus was

Table 3 Multivariate analysis showing independent factors associated with variceal bleeding

Parameter	Odds ratio	95% CI	*P*
Previous diagnosis of cirrhosis or signs of chronic liver disease	22.4	8.3-60.4	< 0.001
Red vomitus	4.6	1.8-11.9	0.020
Red NG aspirate	3.3	1.3-8.3	0.011

Table 4 Clinical information of patients with variceal and nonvariceal bleeding in the validation group (mean ± SD) *n* (%)

Clinical parameter	Cause of UGIB	
	Variceal (*n* = 46)	Non-variceal (*n* = 149)
Age (yr)	64.51 ± 16	56.43 ± 14
Male	31 (67)	76 (51)
Previous diagnosis of cirrhosis or signs of chronic liver disease	35 (76)	12 (8)
Red vomitus	33 (72)	27 (18)
Red NG aspirate	23 (50)	21 (14)

scored 1 if present and 0 if absent. Similarly, red NG aspirate was scored 1 if present and 0 if absent.

Using the receiver operating curve (ROC) in Figure 1, a cutoff ⩾ 3.1 was chosen as the best cutoff for predicting variceal bleeding. The sensitivity, specificity, accuracy, PPV and NPV for variceal bleeding with this cutoff were 85%, 81%, 82%, 50% and 96%, respectively.

Validation of the UGIB Etiology Score

The UGIB Etiology score was prospectively validated in another set of 195 patients with UGIB. Forty-six patients had variceal and 149 had non-variceal bleeding. None had negative or inconclusive etiologies of UGIB by EGD. The 3 clinical parameters are shown in Table 4. The PPV and NPV of the UGIB Etiology Score for predicting variceal bleeding in the validation group using the same cutoff of ⩾ 3.1 were 79% and 97%, respectively.

DISCUSSION

In the present study, the value of clinical and basic laboratory parameters for predicting the types of UGIB (variceal or non-variceal bleeding) was assessed before endoscopy. The present study differs considerably from other previously published studies on the use of clinical predictors in patients with UGIB, as most studies were aimed at predicting the risk of worst outcome or mortality from UGIB in order to triage patients for appropriate care. These studies similarly demonstrated that clinical parameters (e.g. hemodynamics[17,20-23], comorbid illnesses[17,20-23], NG aspirate[17,24,25]), endoscopic findings (stigmata of recent hemorrhage[17,21-23,26], and the presence of varices[17,21-23]) were strongly associated with the worst outcome, the need for hospitalization or interventions. Multiple scoring systems in UGIB,

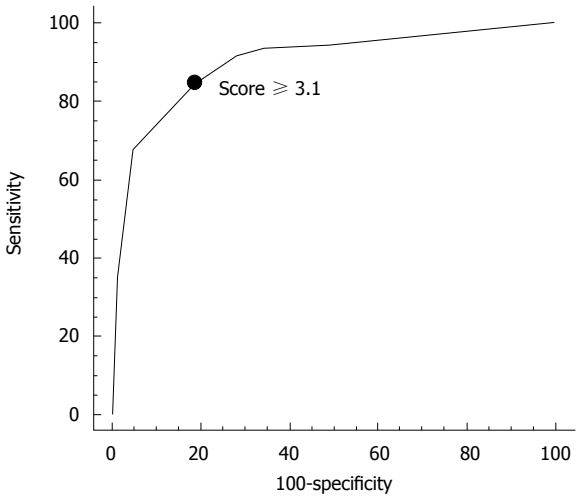

Figure 1 Receiver operating curve of the UGIB Etiology Score. The best cutoff point is the score ⩾ 3.1.

e.g. the Rockall score[21], Baylor bleeding score[22], Blatchford score[20], Cedars-Sinai score[23] and other scoring systems[27] including a scoring system in Thai patients[28] were also developed for these purposes. In contrast, the present study aimed to determine clinical parameters for use in a scoring system to predict the types of UGIB. Results of the present study may help physicians, particularly those in general practice, where emergency EGD is often unavailable, to decide on the type of empiric treatment more accurately, i.e. the use of pharmacological treatments and in some situations, the use of balloon tamponade in cases with a very high likelihood of severe variceal bleeding.

The present study demonstrated that variceal and non-variceal bleeding have many significant distinct features; but only 3 independent factors were able to predict variceal bleeding, i.e. previous diagnosis of cirrhosis or the signs of chronic liver disease, red vomitus, and red NG aspirate. Although these 3 factors are not new findings, our study clearly strengthened and demonstrated the power of these factors. Furthermore, some previously believed predictors, e.g. splenomegaly or thrombocytopenia (for variceal bleeding)[18,19] or the presence of dyspepsia (for non-variceal bleeding)[18,19] were found not to be useful due to rarity or weak associations. Other factors, particularly the severity of hemodynamic changes at presentation were also found to be an indistinguishable factor.

In the present study, the UGIB Etiology Score was developed from these 3 clinical parameters. Using a cutoff of ⩾ 3.1, the UGIB Score was shown to be accurate in predicting variceal bleeding with a sensitivity of 85% and a specificity of 81%. The strength of this study is the accuracy of this score which was validated in another set of patients and consistently gave good results. Although a PPV of 79% is not very high, a NPV of 97% for variceal bleeding is very appropriate in the setting of UGIB where variceal bleeding should never be missed. Therefore, a score < 3.1 will help rule out variceal bleeding with confidence.

Since the cutoff of ⩾ 3.1 reflected the presence of only one parameter, i.e. previous diagnosis of cirrhosis or the presence of signs of chronic liver disease this may be sufficient to predict variceal bleeding, it may be argued that considering only this parameter might be as accurate as calculating the UGIB Etiology Score. This is probably true; but, considering the other two parameters is also helpful because it increases the PPV of variceal bleeding to 86%-89% with the presence of another parameter and to 96% if all three parameters are present. The latter setting may help physicians to consider balloon tamponade[12,13,29] (which carries significant risks) in cases with severe unstable variceal bleeding when emergency EGD is unavailable or vasoactive agents fail.

Although there have been a few studies on UGIB in Thailand[28,30], the present study is the largest prospective study on UGIB in Thailand. The prevalence of variceal bleeding in both study periods was 23%, which was slightly higher than the rate of 6%-14% in the literature[1] and might reflect the tertiary care setting of the present study. However, the present study demonstrated that patients with a history of previously diagnosed cirrhosis or the presence of signs of chronic liver disease had an approximately 50% chance of bleeding from varices. These findings are comparable to those from other studies which showed that 50%-60% of cirrhotic patients with UGIB would bleed from varices[1,31-33]. Nevertheless, the result of this study and the accuracy of the UGIB Etiology Score should be further validated in other hospitals, where the setting may be different.

In conclusion, the UGIB Etiology Score derived from 3 parameters, using a cutoff ⩾ 3.1, may be accurate enough to predict variceal causes of UGIB and may help in guiding the choice of initial therapy for UGIB before endoscopy.

COMMENTS

Background

Upper Gastrointesinal Bleeding (UGIB) is classified by etiology into variceal and non-variceal bleeding based on esophagogastroduodenoscopy (EGD) findings. Although emergency EGD is the standard investigation and treatment of UGIB, it is seldom available in most hospitals, particularly in the developing world. Patients are usually treated empirically for some time, while waiting for EGD, with vasoactive agents or acid suppressants based on the clinical suspicion of variceal or non-variceal bleeding, respectively. Therefore, the clinical prediction of which patients have variceal or non-variceal bleeding is critical.

Research frontiers

Clinical prediction between variceal and non-variceal bleeding has not been extensively studied. Most suggestions have been based on opinions rather than evidence.

Innovations and breakthroughs

The present study prospectively analyzed the clinical and basic laboratory data which were able to differentiate between variceal and non-variceal bleeding in a group of patients with UGIB. Only 3 independent factors were identified; previous diagnosis of cirrhosis or the presence of signs of chronic liver disease, red vomitus and red NG lavage. The UGIB Etiology Score was constructed and a score cut-off of 3.1 had a fair to good positive predictive value (PPV) but excellent negative predictive value (NPV) to rule out variceal bleeding. The accuracy of the score was also confirmed in another set of patients. The present study differs considerably from most other studies on scoring systems in UGIB which mostly aimed to identify high-risk patients with a poor outcome.

Applications

The UGIB Etiology Score ⩾ 3.1 had fair to good PPV for variceal bleeding; thus, it can allow physicians to initiate vasoactive agents for variceal bleeding, while a score < 3.1 helped to rule out variceal bleeding with confidence. The presence of all 3 factors or a score of 5.8 indicated variceal bleeding and may be enough for physicians to consider balloon tamponade if bleeding is severe, EGD is unavailable or vasoactive agents fail.

Terminology

Variceal bleeding is UGIB caused by esophageal or gastric varices. Non-variceal bleeding is caused by any etiology of UGIB other than varices.

Peer review

This is a nicely performed study aiming to develop and validate a scoring system for predicting variceal *vs* non-variceal bleeding. The steadily high NPV of the score makes the developed scoring system accurate in excluding variceal bleeding.

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Indistinguishable cellular changes in gastric mucosa between *Helicobacter pylori* infected asymptomatic tribal and duodenal ulcer patients

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Abstract

AIM: To investigate the changing pattern of different histological parameters occurring in the stomach tissue of *Helicobacter pylori* (*H pylori*) infected tribal populations and duodenal ulcer patients among ethnic Bengalis and correlation of the genotypes of *H pylori* with different histological parameters.

METHODS: One hundred and twelve adult individuals were enrolled into this study between 2002 and 2004. Among them, 72 had clinical features of duodenal ulcer (DU) from ethnic Bengali population and 40 were asymptomatic ethnic tribals. Endoscopic gastric biopsy samples were processed for histology, genotyping and rapid urease test. Histologically, haematoxylin and eosin staining was applied to assess the pathomorphological changes and a modified Giemsa staining was used for better detection of *H pylori*. For intestinal metaplasia, special stainings, i.e. Alcian blue periodic acid-Schiff and high iron diamine-Alcian blue staining, were performed. PCR was performed on bacterial DNA to characterize the presence or absence of virulence-associated genes, like *cagA*, and distribution of different alleles of *vacA* and *iceA*.

RESULTS: Intraglandular neutrophil infiltration, a hallmark of activity of gastritis, was present in 34 (94%) of tribals (TRs) and 42 (84%) of DU individuals infected with *H pylori*. Lymphoid follicles and aggregates, which are important landmarks in *H pylori* infection, were positive amongst 15 (41%) of TRs and 20 (40%) of DU subjects. Atrophic changes were observed in 60% and 27.7%, respectively, among DU cases and tribals ($P > 0.003$). Metaplastic changes were detected in low numbers in both groups. Moderate to severe density distribution of *H pylori* in the gastric mucosa was 63% among TRs, whereas it was 62% in DU subjects. There were no significant differences in the distribution of virulence-associated genes like *cagA*, *vacA* and *iceA* of *H pylori* strains carried by these two populations.

CONCLUSION: Our study showed almost similar distribution of inflammatory cells among asymptomatic tribals and DU Bengali patients. Interestingly, the tribal population are free from any clinical symptoms despite evidence of active histologic gastritis and infection with *H pylori* strains carrying similar virulence markers as of strains isolated from patients with DU. There was an increased cellular response, especially in terms of neutrophil infiltration, but much lower risk of developing atrophy and metaplastic changes among the tribal population.

Key words: *Helicobacter pylori*; Tribal; Neutrophil; Mononuclear cells infiltration; Lymphoid follicles

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INTRODUCTION

Helicobacter pylori (*H pylori*) is of growing concern today because of its crucial role in the pathogenesis of chronic gastritis, peptic ulcer diseases and in the multi-step carcinogenic process of gastric cancer[1]. In developing countries, 70%-90% of the population carries *H pylori* and develop persistent inflammation in their stomachs, which lasts for decades unless treated with antibiotics[2]. About 60%-95% of peptic ulcer diseases are thought to be idiopathic and it is now well established that *H pylori* is the causative agent of nearly all of these cases in adults. In addition, almost all *H pylori* infected individuals develop gastritis[3]. Severe gastritis is believed to be the denominator of peptic ulcer diseases and atrophic gastritis, which may lead to gastric cancer[4,5]. However, it is not clear why a few strains are associated with ulcer formation with relevant clinical symptoms, while others are not associated with any disease manifestation.

H pylori is one of the most genetically diverse of bacterial species, with any given isolate easily distinguished from most others by DNA fingerprinting. Only one-half to two-thirds of US and European strains carry the *cag* pathogenicity island (*cag* PAI) and such strains are recovered preferentially from persons with overt disease. In contrast, nearly all-Asian strains carry the *cag* PAI, independent of disease status. Potentially more significant in terms of host interaction and evolution were the findings that East Asian and Western strains differ markedly in DNA sequence motifs in the *vacA* and *cagA* genes., We have previously reported that sequences of the *cagA* gene in Indian strains show a close match with ethnic European strains, but are distinct from East Asian strains. On the other hand, DNA sequence motifs of an informative middle region of *vacA* gene from Indian *H pylori* strains are distinct both from European and East Asian strains. So, there are strong indications of significant geographic differences among strains[6,7].

Moreover, gastric cancer is more prevalent in Japan and China than any other parts of the world and duodenal ulcer is more common in India as compared to gastric ulcer. The distribution and the nature of gastritis are, thus, major determinants of clinical outcome of *H pylori* infection. It is, therefore, important to understand the dynamics of gastritis associated with this infection in developing countries like India, where *H pylori* infection is highly prevalent and *H pylori* is acquired early in life. Surprisingly, even the healthy individuals in India carry the toxigenic *vacA* s1, *vacA* m1 alleles and *cag*-PAI[8]. However, previous reports do not indicate whether this lack of disease association with putative virulent *H pylori* strains is due to a lesser bacterial load in gastric mucosa leading to insignificant level of epithelial injury.

Santhals and Oroans are two distinct ethnic tribal groups that had settled in the Birbhum district of West Bengal centuries ago[7]. They constitute less than 5% of the overall population of West Bengal (Census of India, 2001). Ethnically, Santhals are proto-Australoids and speak the Santhali dialect of the Austro-Asiatic language family, while the Oroans are ethnically Dravidian and speak the Khurukh dialect of the Dravidian linguistic family. That is, the two lineages to which they belong have been distinct for millennia. In contrast, the ethnic Bengalis have an Indo-European ancestry, and their Bengali language is derived from Sanskrit. Traditionally, both Santhals and Oroans have been hunters-gatherers, but most have now become settled as agriculturists. Nevertheless, they remain culturally and linguistically distinct from most of Indian society and rarely intermarry with people of other ethnicities. Their separation from mainstream Bengalis and other Indians during much of human history is reflected in genetic differences in autosomal and mitochondrial DNA markers. Our previous study[7] showed that the majority of these tribal communities are infected with *H pylori*; but, interestingly, none of them shows any symptoms. On the other hand, duodenal ulcer, which is *H pylori*-associated, is of particular importance in ethnic Bengali populations and is far more common than in most other geographic regions[9].

These considerations and our interest in the dynamics of gastritis associated with this infection motivated the present study. We wanted to investigate the changing pattern of different histological parameters occurring in the stomach tissue of *H pylori*-infected tribal populations and duodenal ulcer patients among ethnic Bengalis. Our aim was to get insights of the cause for the near absence of *H pylori*-associated overt disease in these tribal populations and to correlate the *H pylori* genotypes with the different histological findings.

MATERIALS AND METHODS

A total of 112 adult mainstream Bengali and ethnic tribal individuals (72 Bengalis and 40 tribals) of both sexes (aged between 20-65 years) underwent a non-sedated upper gastrointestinal endoscopy (GIF XQ 30, Olympus optical company, Japan) under topical lignocaine anesthesia at the hospital of the Institute of Post Graduate Medical Education and Research,

Table 1 Primers used in this study

Region(s) amplified	Primer	Nucleotide sequence	References
vacA s1 or *vacA* s2	VA1-F	5'-ATGGAAATACAACAAACACAC	[7]
	VA1-R	5'-CTGCTTGAATGCGCCAAAC	
vacA m1 or *vacA* m2	VAG-F	5'-CAATCTGTCCAATCAAGCGAG	[28]
	VAG-R	5'-GCGTCAAAATAATTCCAAGG	
cagA (5' end)	cag5c-F	5'-GTTGATAACGCTGTCGCTTC	[28]
	cag3c-R	5'-GGGTTGTATGATATTTTCCATAA	
cag-PAI empty site	Luni 1	5'-ACATTTTGGCTAAATAAACGCTG	[7]
	R5280	5'-GGTTGCACGCATTTTCCCTTAATC	
iceA1	IceA1F	5'-TATTTCTGGAACTTGCGCAACCTGAT	[7]
	M.Hpy1R	5'-GGCCTACAACCGCATGGATAT	
IceA2	cycSF	5'-CGGCTGTAGGCACTAAAGCTA	[7]
	IceA2R	5'-TCAATCCTATGTGAAACAATGATCGTT	

Kolkata, India, throughout the years 2002-2004. Out of 72 suspected duodenal ulcer (DU) cases, the mean age of 43 males and 29 females was 45 ± 11.72 and 42.7 ± 9.16, respectively. Among 40 tribals, the mean age of 24 males and 16 females was 31.4 ± 6.22 and 32.13 ± 6.44, respectively. Seventy-two Bengali patients were chosen for endoscopy from individuals with abdominal pain seeking care at outpatient department as possible DU patients and for comparative analysis, 40 asymptomatic individuals from tribal (TR) population (Santhals and Orans) were recruited. A detailed history was taken, and a physical examination of each subject was carried out prior to endoscopy. The objectives of the study were explained to all. Informed consents were obtained from each individual under protocols approved by the institutional ethical committees of the Post-Graduate Medical Education and Research and National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India. None of these asymptomatic individuals reported to have any gastro-duodenal discomfort. Exclusion criteria were: use of antibiotics, antihistamines and proton pump inhibitors during the three months prior to this study. From each participant, four biopsies were obtained, three from the antrum and one from the fundus. Of the three antral biopsy specimens, one was used for an in-house rapid urease test (RUT), one for culture and the third one, along with one biopsy from the fundus, was processed for histologic examination.

Culture of H pylori

Biopsies for culture were taken in 1 mL of brucella broth (Difco) containing 15% glycerol and transported to the National Institute of Cholera and enteric Diseases in ice-cold condition. Biopsy samples in transport medium were vortexed vigorously for 2 min and 200 μL of the broth were streaked on brain heart infusion (BHI) agar (Difco) enriched with 7% sheep blood, 0.4% IsovitaleX and *H pylori* selective supplement-Dent (Oxoid, Basingstoke, Hampshire, England). Plates were incubated at 37℃ in double-gassed incubator, which maintains 10% CO_2, 5% O_2 and 85% N_2 for 3-6 d. The organisms were identified by their typical colony morphology, appearance on Gram staining and positive reactions in urease, catalase and oxidase tests.

Characterization of H pylori strains by PCR

A modification of the method of Murray and Thompson[10] was used for *H pylori* genomic DNA extraction. In brief, cells from a confluent lawn of bacterial culture on BHI agar plate were collected and resuspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), treated with 10% SDS and freshly prepared proteinase K and incubated at 37℃ for 1 h. After incubation, CTAB/NaCl (10% cetyl trimethyl ammonium bromide in 0.7 mol/L NaCl) was added and incubated at 65℃ for 10 min. The aqueous phase was then treated with phenol-chloroform and DNA pellet was washed with 70% ethanol. The nucleic acid was suspended in TE and treated with RNAse at 37℃ for 30 min. Specific PCR was carried out in 20 mL volumes using 10 ng of DNA, 1 U of *Taq* polymerase (Promega, Madison, Wis.), 10 pmol of each primer per reaction, 0.25 mmol/L (each) deoxynucleoside triphosphate, and 2 to 3 mmol/L $MgCl_2$ in standard PCR buffer for 30 cycles generally under the following conditions: 94℃ for 40 s, 55℃ for 40 s, and 72℃ for a time chosen based on the size of the expected fragment (1 min/kb). The primers are listed in Table 1.

Histology

One biopsy from antrum and one from fundus of the stomach were fixed in 10% buffered formalin overnight, dehydrated in graded series of alcohol and xylene and were processed for paraffin embedding. Serial thin (3-4 μm) sections were cut by Rotary microtome (Leica 2145, Germany) and stained with haematoxylin and eosin (H&E) stain to see the morphological changes. For better visualization of *H pylori*, modified Giemsa stain was done in all the cases along with H&E stain, as the sensitivity and specificity of this added stain exceeds 90%[11].

The histologic changes and grading were done according to updated Sydney system[12]. All the biopsy specimens were number-coded and examined by a single pathologist who was unaware of the result of the other tests while examining the slides. *H pylori* in the biopsy specimens was looked for carefully and then the bacterial density was measured as it may have an impact on disease association and epidemiologic importance.

Table 2 Histological changes of gastric mucosa among *H pylori* associated symptomatic duodenal ulcer (DU) and asymptomatic tribal (TR) individuals *n* (%)

	Symptomatic DU (*n* = 50)	Asymptomatic TR (*n* = 36)	*P*-values	OR (95% CI)
Neutrophil infiltration	42 (84)	34 (94.4)	0.124	3.24 (0.57-23.76)
Lymphoid follicle/aggregates	20 (40)	15 (41.7)	0.876	1.07 (0.41-2.80)
Atrophy	30 (60)	10 (27.7)	0.003[1]	0.26 (0.10-2.67)
Metaplasia	7 (14)	3 (8.3)	0.325	0.56 (0.10-2.67)

[1]Indicates statistically significant *P*-value.

H pylori was measured in the modified Giemsa stained sections by counting the *H pylori* like organisms on the mucosal surface and in the foveolae[13]. In brief, bacterial density was measured by comparing the histologic presence of bacteria on the gastric surface epithelium using the visual analogue scale. Severe colonization was defined as the presence of large groups of organisms on the surface and upper pits of more than 2/3rd of the mucosal surface examined. Mild colonization was defined as individual organisms or small groups covering les than 1/3rd of the mucosal surface. Moderate colonization was between these two.

Chronic gastritis, activity, atrophy and *H pylori* density were scored as 0 (absent), 1 (mild), 2 (moderate) and 3 (severe). Intestinal metaplasia and lymphoid follicles/aggregates were graded as 0 (absent) or 1 (present). If antral and fundal biopsy sites showed different grades for any variable, the higher score was used. Intestinal metaplasia was classified as type 1, 11 or 111 by Alcian blue periodic acid-Schiff (AB-PAS) and high iron diamine-Alcian blue (HID-AB) staining.

RESULTS

Among 112 individuals included in the study, 72 had clinical features of DU from ethnic Bengali population and 40 were asymptomatic ethnic tribals. In 50 out of 72 (69%) DU and 36 out of 40 (90%) TR subjects, evidence of *H pylori* infection was evaluated by three methods (RUT, histology and culture) and was included in further study. All of these subjects had evidence of chronic gastritis. A grade from 0 (absent) to 3 (severe) was assigned for five histological parameters, i.e. inflammation (chronic inflammatory cells), activity (neutrophils), glandular atrophy, intestinal metaplasia and *H pylori* density, and the grading was done according to the Sydney system[12].

Histology

Histologically active chronic gastritis was detected in 34 (94%) TRs and 42 (84%) DU patients (all from antral sites), whereas 30 TRs and 38 DU subjects showed evidence of chronic active gastritis from fundic sites. Intestinal metaplasia was detected in 3 (8.3%) TRs and 7 (14%) DU cases-all from antral sites of biopsy specimens (Table 2). No metaplastic changes were detected at fundic sites. For ease of the correlative analysis of the histological findings with genotypes, only antral biopsy specimens were evaluated further.

Cellular infiltration and epithelial changes in gastric mucosa

Gastritis is characterized by lymphocytes, plasma cells and scattered polymorphonuclear leucocytes (PMNs) in gastric mucosa. Chronicity or persistent infection was a common feature in all of our study subjects. Chronic gastritis was evident histologically by the presence of inflammatory infiltrates, which was essentially made up of mononuclear cells like macrophages, lymphocytes and plasma cells. Intraglandular neutrophil infiltration, a hallmark of activity of gastritis was present in 34 (94%) of TRs and 42 (84%) of DU individuals infected with single strain of *H pylori*. Neutrophil infiltration inside glandular epithelium and in the lamina propria of a DU and a TR case are shown in Figure 1A and B, respectively.

Mucus depletion, derangement of normal cellular architecture and foveolar hyperplasia with erosion were noted among TRs; but, frank ulceration in the gastric surface mucosa was not detected. Among most of the DU cases, ulceration, haemorrhage with exudation, loss of surface epithelium at places and atrophic changes were the frequent findings.

Lymphoid follicle/aggregates

Lymphoid follicles and aggregates were examined carefully as the positivity of this parameter is almost pathognomic of *H pylori* infection and the follicles/aggregates were positive amongst 15 (41%) of TRs and 20 (40%) of DU subjects. An almost comparable distribution pattern of lymphoid follicles/aggregates was present among DU patients and the tribal population. Mononuclear cells infiltration in the lamina propria with lymphoid follicle in a DU and lymphoid aggregate in a TR case has been represented in Figure 2A-B.

Atrophy and metaplasia

Atrophic changes among 50 DU patients were detected in 30 (60%) cases, where mild form of atrophy was present in 18 (36%) and a moderate form of atrophy was found in 12 (24%). Among 36 asymptomatic TRs, atrophic changes were identified in 10 (27.7%), where 7 (19.4%) showed mild form of atrophy and 3 (8.3%) showed moderate form of atrophy. Although the incidence of active gastritis was quite high, especially among tribals, atrophy and metaplastic changes in the gastric mucosa were much rarer than in the urban DU patients. Metaplasia was recognized morphologically by the presence of goblet cells, absorptive cells and cells resembling colonocytes in the surface epithelium and

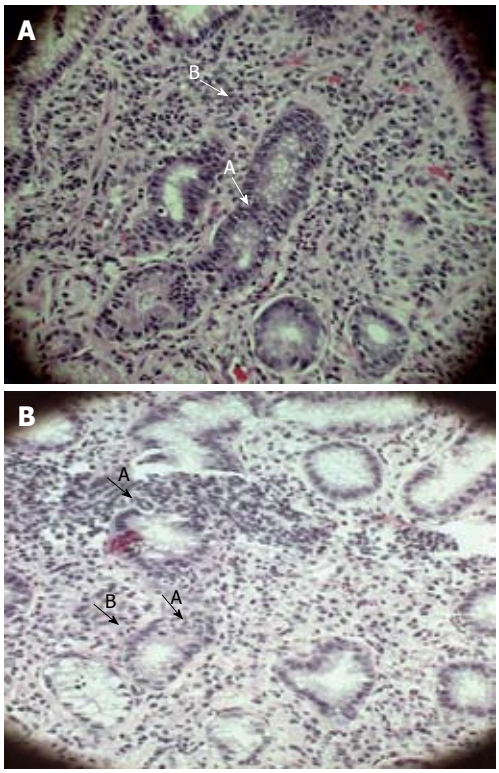

Figure 1 The histopathological features of active gastritis in DU and TR. A: Polymorphonuclear cells infiltration inside glands (HE, × 400) (A) and in lamina propria (B)-DU (HE, × 400); B: Neutrophils in glands (A) (HE, × 400) and in lamina propria (B)-TR (HE, × 400).

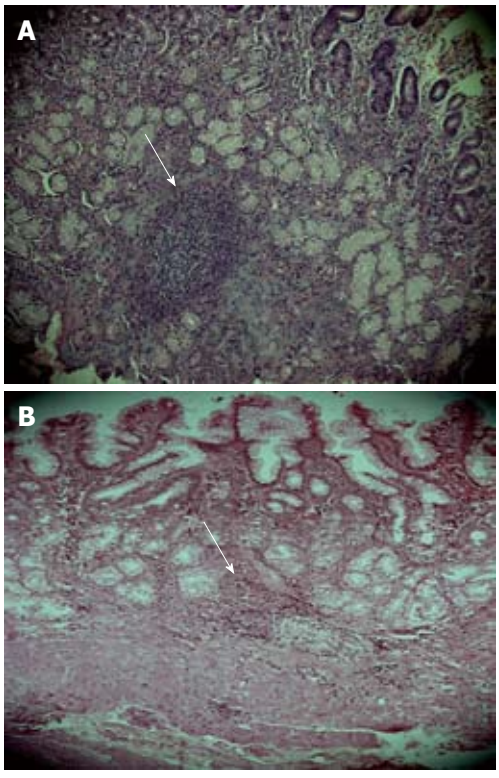

Figure 2 Mononuclear cells, lymphoid follicles and aggregates in DU and TR. A: Arrow indicates mononuclear cells in lamina propria with a lymphoid follicle in DU (HE, × 200); B: Arrow indicates mononuclear cells in lamina propria with lymphoid aggregate in TR (HE, × 200).

glands of the gastric mucosa and (Type 1) or complete

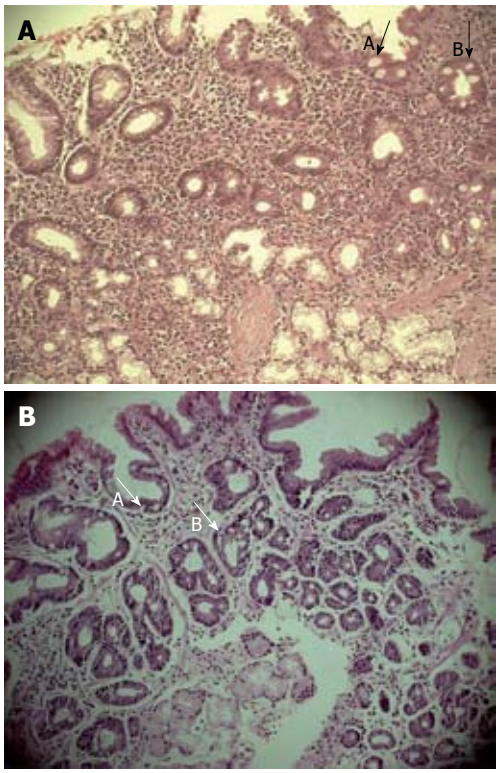

Figure 3 Intestinal metaplastic changes in DU and TR. A: Metaplastic changes with goblet cells in glands (B) and surface epithelium of gastric mucosa (A)-DU (HE, × 200); B: Metaplastic cells replacing the normal gastric epithelium in glands (B) and surface mucosal epithelium (A)-TR (HE, × 200).

type of intestinal metaplasia (Figure 3A-B) were detected in 3 TRs (8.3%) and 7 DU (14%) subjects (Table 2).

H pylori density

Moderate to severe density distribution of *H pylori* at the surface and in the pits of the gastric mucosa was found in 63% of TRs and in 62% of DU subjects.

Genotyping of H pylori strains

The presence or absence of the *cag* PAI was scored by PCR with specific primers using DNA extracted from cultured strains. As shown in Table 3, a 350-bp product indicative of the *cag* PAI was obtained with primers specific for the *cagA* gene from each of the 36 strains in tribal population. None yielded a 550-bp product expected of a *cag* empty site, which would indicate complete absence of the *cag* PAI. On the other hand, in the symptomatic Bengali population, all yielded band specific for *cagA* gene and three cases also produced a 550-bp product for *cag* PAI empty site indicating that these patients had mixed infections with both *cagA* positive and negative strains. The presence of potentially toxigenic *vacA*s1 *versus* nontoxigenic *vacA*s2 alleles at the 5' end of *vacA* was determined based on sizes of PCR products (259 bp *versus* 286 bp, respectively) generated with *vacAs* region-specific primers. All 36 tribal strains yielded a 259-bp fragment, indicating that they carried s1 alleles; no s2 alleles were found; but, in the Bengali population, three strains produced both s1 and s2 fragment whereas the rest produced a s1 fragment. The alleles of the

Table 3 Genotypes of *H pylori* strains from duodenal ulcer (DU) and tribal (TR) subjects

Genotype	No. (%) of strain from DU patients (n = 50)	TRs (n = 36)
cagA positive only	47 (94)	36 (100)
cagA negative only	1 (2)	0 (0)
Both $cagA^+$ and $cagA^-$	2 (4)	0 (0)
vacA s1 only	47 (94)	36 (100)
vacA s2 only	1 (2)	0 (0)
vacA s1 and *s2* mixed	2 (4)	0 (0)
vacA m1 only	31 (62)	26 (72.2)
vacA m2 only	17 (34)	7 (19.4)
vacA m1 and *m2* mixed	2 (4)	3 (8.3)
ice A1 only	29 (58)	15 (41.7)
ice A2 only	18 (36)	14 (38.9)
ice A1 and *ice A2* mixed	3 (6)	7 (19.4)

vacA middle(m) region, which determines the cell type specificity of the vacuolating cytotoxin action, were also studied by PCR. Products were obtained only with *vacA* m1 primers in 26 of 36 tribal strains, only with *vacA* m2 primers in seven strains and with both m1 and m2 primers in three strains, indicating a mixed infection. Among the 50 Bengali strains, 31 were positive for m1 while 17 strains had the m2 allele alone and the remaining two had both alleles. PCR was used to test for *iceA1*, which is virulence-associated in some populations, and the completely unrelated *iceA2* gene, which occupies the same chromosomal locus in strains lacking *iceA1*. The *iceA1* gene was found alone in 15 of 36 tribal cultures, *iceA2* was found alone in 14 strains, and a mixture of *iceA1* and *iceA2* alleles (again, indicating mixed infection) was found in seven tribal cultures (Table 3). Among strains isolated from Bengali DU patients, *iceA1* and *iceA2* were found in 29 and 18 cases, respectively whereas *iceA1-iceA2* mixed infections were found in 3 cases. Genotyping results from this study and sequence-based analysis from a previous study[7] clearly indicated that tribal strains are closely matched to those of mainstream Bengalis.

Statistical analysis

χ^2 test was employed to compare the histological parameters between symptomatic and asymptomatic subjects to know the status of few important histological parameters like neutrophil infiltration, lymphoid follicle/aggregates formation and atrophy and metaplastic changes. Neutrophil infiltration was almost three times higher in the asymptomatic tribal population compared to urban DU cases, although the difference was not statistically significant. Regarding the atrophic and metaplastic changes, DU subjects were 4 and 2 times at higher risk of developing further disease process than TRs. Atrophic changes among DU cases were statistically significant (Table 2). Genotyping of *H pylori* strains between DU and TRs were not found statistically significant (Table 3).

DISCUSSION

H pylori infection is common in the Santhal and Oroan ethnic minorities of West Bengal, whereas symptomatic individuals with *H pylori*-associated disease are rare in these populations, even though the genotypes of the strains they carry are similar to those for mainstream Bengalis. The near-universality of *H pylori* infection can be ascribed to relatively low levels of sanitation, hygiene and education, conditions that contribute to a high risk of infection and superinfection, even in adulthood. Hence, although both the tribal communities and Bengali urban population are infected with *H pylori* with similar genetic make-up, there is a distinct difference regarding the manifestation of the disease. This enigma was previously explained, mostly in Western countries, by reporting the association of certain virulence alleles (*vacAs1*, *cagA* and *iceA1*) with the *H pylori*-related disease where around 50% of the *H pylori* strains lack the *cagPAI*. However, this view needs to be reexamined since in the Asian context an overt disease association with these alleles does not exist[6-8]. The present study investigated the histologic findings observed in gastric mucosa of *H pylori*-infected asymptomatic TRs and urban DU subjects (which prevail only in Bengali population, but not in the tribal population) to understand the cause for near absence of *H pylori* associated overt disease in these tribal populations and correlation of the genotypes of *H pylori* with different histological parameters.

Bacterial density is directly related to inflammation in terms of neutrophil, lymphocytes and plasma cell infiltration of the gastric mucosa and we also noticed moderate to severe degree infiltration of the bacteria in the gastric mucosa[14-16], where inflammatory cells were a marked feature. *H pylori*-positive biopsy samples were mostly inflamed with chronic superficial gastritis and the inflammatory cells were mononuclear cells with neutrophil infiltration in the epithelium. The amount of inflammation was highly variable, ranging from minimal infiltration in the lamina propria with intact glandular architecture to severe dense inflammation. Mononuclear cells, consisting mainly of macrophages, lymphocytes and plasma cells, were present to a variable degree in all the study cases, indicating a chronic infection among asymptomatic TRs as well as in DU patients. The presence of PMNs signifying 'a sign of activity' was more pronounced among the tribal population (94%) than DU patients (84%). It may be due to the host immune response against bacteria among tribals, which are stronger. Tribals are less exposed to environmental pollution and hazardous agents and as PMNs are the first line of defense against bacteria, increased cellularity and more active gastritis is well reflected in our study in *H pylori* infection.

A *H pylori* infection with a longer duration leads to loss of gastric glands and development of multifocal atrophic gastritis, which is often accompanied by intestinal metaplasia. In our study population, although the *H pylori*-associated gastritis as well as its activity is quite high among TRs, atrophic changes and the incidence of metaplasia were lower than in DU patients. One of the characteristics of *H pylori* infection is the growth of lymphoid follicles/aggregates. Here, 41% of

the biopsy specimens were positive for lymphoid follicles and aggregates in TRs and a comparable proportion (40%) was found among DU subjects, although we did not encounter any case associated with lymphoma. In a study conducted by Eidt and Stolte, lymphoid follicles or aggregates were detected in 54% of *H pylori* infected cases[17], which is higher compared to our data.

It is now well accepted that peptic ulcer diseases have an etiologic link with *H pylori*; but, not every individual infected by this micro-organism develops the disease clinically[18,19]. The actual element responsible for the pathogenesis of *H pylori* is yet to be determined. The cytotoxin associated gene *cagA* has been related to ulcerogenicity[20]. The genes in the *cag* pathogenicity island are supposed to induce epithelial cells to release interleukin-8 production. This, together with other interleukins, attracts neutrophils, which migrate from capillaries through the lamina propria, and emerge between the epithelial cells. However, in our study, *cagA* was present in TRs as frequently as in DU. Consistent to this finding, polymorphonuclear activity was detected in 97% cases *cagA*-positive TRs, and 95% *cagA*-positive DU subjects, which was quite a high proportion. Correlating the histologic findings of gastritis with the genotypes, like *iceA*, or combination of *iceA*, *vacA* and *cagA*, no particular allelic mosaicism could be identified as responsible for neutrophil and mononuclear cell infiltration, formation of lymphoid follicles and aggregates, atrophic and metaplastic changes and the successive disease outcome. This is in consistent with the findings of few earlier reports[21,22].

An apparent correlation has still to be detected between the different genetic features of *H pylori* strains and the histologic findings of the disease outcome. When the histological changes, such as the presence of neutrophil infiltration, the activity of gastritis, the mononuclear and lymphoid follicle/aggregate formation, the atrophy and the metaplastic changes, were evaluated with respect to the genotypes of the strains of *H pylori*, the differences between the Bengali ethnic duodenal ulcer patients and the tribal population were not not statistically significant, except for the atrophy. However, a few observations in our study are interesting: (a) the almost similar distribution of inflammatory cells among asymptomatic TRs and DU cases; (b) the fact that, in spite of the evidence of active histologic gastritis, tribal groups were free from any clinical symptoms; (c) the increased cellular response, especially in terms of neutrophil infiltration, but much lower risk of developing atrophy and metaplastic changes among the tribal population.

Finally, the present study suggests that, on average, *H pylori* infections are less virulent in these ethnic minorities than in mainstream Indians. Such lack of virulence might be due to subtle features of bacterial strains or aspects of the human host environment. Thus, the bacterial genotype, host genetic factors and environmental factors, all may have important influence in the disease outcome of *H pylori* infected people. At least two reports of decreased virulence apparently being selected *in vivo* have appeared: one during human infection[23] and another during adaptation of human strains to mice[24]. Lack of virulence might also reflect features of the host. One possibility entails concurrent infection with particular parasites that may down-regulate inflammatory responses to infection, as it has been documented in a mouse infection model[25]. Resistance to pathogenic effects of putatively virulent *H pylori* strains might also be determined by features of human host genotype[26-27]. It will be interesting to study why tribal groups are free from any clinical symptoms in spite of evidence of active histologic gastritis and also to identify the host factors that may provide immunity against the pathogenic effects of putatively virulent *H pylori* strains. Such kind of studies may uncover new genetic factor/factors that affect human infection, increase our understanding of bacterium-host interactions in colonization and disease, and provide new insights into the evolution of this diverse and globally distributed human pathogen.

COMMENTS

Background

Helicobacter pylori (*H pylori*) infection and duodenal ulcer disease is common among ethnic Bengali population in West Bengal, India. In contrast, although *H pylori* infection is equally or more common in the ethnic tribal minorities (Santhals and Orans) of West Bengal, symptomatic disease is extremely rare. This study addresses different histological parameters occurring in the stomach tissue of *H pylori*-infected tribal populations and duodenal ulcer patients among ethnic Bengalis for getting insights of the cause for the near-absence of *H pylori*-associated overt disease in these tribal populations and correlate the *H pylori* genotypes with different histological parameters.

Research frontiers

Although both the tribal communities and Bengali urban population are infected with *H pylori* with similar genetic make-up, there is a distinct difference regarding the manifestation of the disease. When the histological changes, such as the presence of neutrophil infiltration, the activity of gastritis, the mononuclear and lymphoid follicle/aggregate formation, the atrophy and the metaplastic changes, were evaluated with respect to the genotypes of the strains of *H pylori*, the differences between the Bengali ethnic duodenal ulcer patients and the tribal population were not statistically significant, except for the atrophy.

Innovations and breakthroughs

The most interesting observations in the study are: (1) the almost similar distribution of inflammatory cells among asymptomatic tribals and duodenal ulcer patients from Bengali population in Kolkata, (2) the fact that, in spite of evidence of active histologic gastritis, tribal groups were free from any clinical symptoms; and (3) the increased cellular response, especially in terms of neutrophil infiltration, but much lower risk of developing atrophy and metaplastic changes among the tribal population.

Applications

The apparent lack of virulence in the tribal group might reflect features of the host. The study raised two important issues: (1) why are tribal groups free from any clinical symptoms, in spite of evidence of active histologic gastritis, and (2) the need to identify the host factors (in tribal patients) that may provide immunity against the pathogenic effects of putatively virulent *H pylori* strains. Such kind of studies may uncover new genetic factor/factors that affect human infection, increase our understanding of bacterium-host interactions in colonization and disease.

Peer review

This article investigated the changing pattern of different histological parameters in the stomach tissue of *H pylori* infected populations. The paper is well written and their results are reliable.

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BRIEF ARTICLES

Effects of different periods of renal ischemia on liver as a remote organ

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Abstract

AIM: To assess the hepatic changes after induction of different periods of renal ischemia.

METHODS: Rats were subjected to either sham operation or ischemia (30, 45 and 60 min) followed by 60 min reperfusion. Liver and renal functional indices were measured. Hepatic glutathione (GSH) and ferric reducing antioxidant power levels and the concentration of interleukin (IL)-10 and tumor necrosis factor (TNF-α) were evaluated. Portions of liver and kidney tissues were fixed for histological evaluation.

RESULTS: Forty-five minutes renal ischemia followed by 60 min reperfusion caused significant changes in liver structure and a significant reduction in renal function. These rats showed a significant decrease in liver GSH, as well as a significant increase in TNF-α and IL-10 concentrations. These results demonstrated that renal ischemia caused changes in liver histology, function, oxidative stress and inflammatory status, which led to a reduction in hepatic antioxidant capacity. With 30 min ischemia, the magnitude of these changes was less than those with 45 or 60 min ischemia.

CONCLUSION: A minimum of 45 min ischemia is needed to study the effects of renal injury on the liver as a remote organ.

Key words: Renal ischemia; Liver; Remote organ; Oxidative stress; Inflammation

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INTRODUCTION

Liver and kidney are both involved in the regulation of body homeostatic responses, metabolism and excretion of drugs and toxic products. Recent studies have suggested cross-talk between the liver and kidneys. Ischemia/reperfusion (I/R)-induced local response in kidney tissue has been well documented in a number of studies[1,2]. However, remote effects of renal I/R injury on the liver need further investigation. Renal injury associated with liver disease is an extensively encountered clinical problem of varied etiology and high mortality. I/R injury induces an inflammatory response, which results in the formation of reactive oxygen species (ROS) that augments local tissue damage or affects organs remote from the site of I/R. An important function of ROS is the regulation of cytokine gene expression[3].

In 2002, Miyazawa *et al*[4] showed an influx of neutrophils and lymphocytes, not only in the clamped kidney, but also in the hepatic sinusoids concomitantly with liver dysfunction. These findings indicate that a systemic cellular immune response, including intermediate T cells, affects multiple organs during ischemic acute renal failure (ARF), which may play an important role in the development of multi-organ failure. Since the liver tissue represents one of the vascular beds into which ROS are delivered, it would be likely to manifest a number of toxic effects of these molecules. It has been suggested that dehydroepiandrosterone treatment has a beneficial effect on antioxidant defenses against hepatic injury after renal I/R in rabbits, possibly by augmenting glutathione (GSH) levels and lowering malondialdehyde (MDA) production[5].

Kielar *et al*[6] have evaluated the extrarenal regulation of ARF. This regulation may be as a result of increased production of cytokines such as tumor necrosis factor (TNF)-α and growth factors such as hepatocyte growth factor (HGF) produced by extrarenal organs[7]. In addition, there is an inflammatory response to renal ischemia that results in secondary injury[8]. Further more, renal ischemia results in increased interleukin (IL)-6 mRNA expression[9], renal production of IL-6, and expression of IL-10 receptors. IL-6 stimulates the production of IL-10 by the liver, which might ameliorate renal injury.

The question remaining is to what extent the renal damage affects the liver, although the liver function itself may enhance or reduce the extent of renal damage. It has been suggested that, while liver disease may alter the course of renal injury, exogenous administration of regulatory factors produced by extrarenal organs may play a therapeutic role in ARF[6,10]. Thus, the aim of the present study was to examine the effects of different periods of renal ischemia on rat liver function, histology, cytokine levels and antioxidant status.

MATERIALS AND METHODS

Surgical procedure

Twenty male Sprague-Dawley rats weighing 250–300 g were included in this study and randomly assigned into one of the four experimental groups (n = 5): (1) sham-operated; (2) 30 min ischemia, 60 min reperfusion; (3) 45 min ischemia, 60 min reperfusion; and (4) 60 min ischemia, 60 min reperfusion.

Rats were placed on a warming pad and anesthetized with pentobarbital sodium (60 mg/kg ip, followed by 6 mg/kg per hour iv). Body temperature was maintained at 37 ± 1℃. A tracheotomy was performed to facilitate free breathing. The right femoral artery was cannulated and connected to a pressure transducer for mean arterial pressure measurement. The tail vein was cannulated for infusion of 0.9% (9 g/L) NaCl solution. A midline laparatomy was performed and the renal arteries were carefully separated from around the tissues. After completion of the surgery, rats were allowed to stabilize for 30 min.

In the I/R groups, renal arteries were occluded by a non-traumatic micro-vascular clips for 30, 45 and 60 min, followed by 1 h reperfusion. Occlusion was approved visually by color change of the kidney to a paler shade and reperfusion by blushing. Sham-operated animals underwent identical surgical treatment, including isolation of both renal arteries. However, artery occlusion was not performed. At the end of the experimental procedure, serum was collected for determination of blood urea nitrogen (BUN) and creatinine. Kidney and liver tissues were removed and prepared for future analysis.

Measurement of arterial blood pressure

Arterial blood pressure and heart rate were continuously monitored *via* a femoral artery cannula that was connected to a pressure transducer device. The transducer was connected to a PowerLab/4SP data acquisition system (AD Instruments).

Biochemical assay

Blood concentration of creatinine, BUN, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by commercially available kits.

Histological procedures

After formalin fixation (10% phosphate-buffered) and dehydration, paraffin-embedded renal and hepatic sections (4 μm) were stained by hematoxylin and eosin. Histopathology for all tissues was evaluated per section in at least 10 randomly selected non-overlapping fields at × 400 magnification of the sections. Kidney tissues were evaluated for the presence of congestion. Tubules were evaluated for the presence of degenerative changes (vacuolization), tubular dilatation, luminal debris and cast formation, and loss of brush borders from proximal tubules. Liver sections were evaluated for the presence of congestion, cellular degenerative changes, cytoplasmic vacuolization and leukocyte infiltration.

Tissue homogenization

A portion of each liver was homogenized in KCl buffer (pH 7.4) for ferric reducing antioxidant power (FRAP) assay and in TCA for GSH assay. After centrifugation for 30 min, the supernatants were removed and subjected to analysis.

GSH assay

Liver GSH was assayed according to the Tietz method. 5, 5'-Dithiobis 2-nitrobenzoic acid was used as a chromogen and the absorbance of the reduced chromogen was measured at 412 nm. The value for each sample was extracted from the standard curve.

Measurement of ferric reducing ability of liver (FRAP)

FRAP assay was performed according to the method of Benzie and Strain (1996)[11]. Briefly, 50 μL supernatant was added to 1.5 mL freshly prepared FRAP solution, and the absorbance was measured at 593 nm.

Measurement of cytokine concentrations in liver tissues

TNF-α and IL-10 concentrations were measured in the liver of animals using an ELISA (Rat IL-10 and TNF-α ELISA kit, Diaclone A; Tepnel, Besancon, France). One hundred milligrams of tissue were homogenized in 1 mL PBS that contained antiproteases (0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA, 20 KI aprotinin A and 0.05% Tween 20). The samples were then centrifuged for 10 min at 3000 r/min and the supernatant was used for ELISA.

Enzyme activities and Northern blot analysis

Total cellular RNA was extracted and hybridization was performed according to Church and Gilbert[12]. The expression of spermidine/spermine N-acetyl-transferase (SSAT) and collaborates with alternate reading frame (CARF), two mediators of tissue injury, in normal and experimental groups was evaluated.

Statistical analysis

The results are given as mean ± SE. Statistical analysis was performed by analysis of variance using a post-hoc Duncan test. The null hypothesis was rejected at the 0.05 level of significance. SPSS 11.0 software (Chicago, IL, USA) was used for data analysis.

RESULTS

In all groups, the mean arterial pressure (MAP) during the experimental period was not significantly different from the basal value (110 ± 9.8 mmHg and 109 ± 10.3, respectively). Data on liver function tests are presented in Table 1.

Effect of renal ischemia on serum biochemical parameters

BUN did not change after 30 min renal artery occlusion followed by 1 h reperfusion, but serum creatinine increased significantly compared to sham-operated animals (9.8 ± 1.1 mg/L *vs* 5.5 ± 1.5 mg/L, $P < 0.05$). Both 45 and 60 min ischemia followed by 1 h reperfusion resulted in significant increases in plasma creatinine (11.1 ± 1.7 mg/L and 12.4 ± 0.7 mg/L *vs* 5.5 ± 1.5 mg/L, $P < 0.05$) and BUN (340 ± 38.5 mg/L and 350 ± 28.1 mg/L *vs* 237.5 ± 11.0 mg/L, $P < 0.05$) compared to the sham-operated group (Figure 1).

Effect of renal ischemia on liver oxidative parameters

The level of liver GSH did not change significantly after 30 min renal ischemia followed by 1 h reperfusion (Figure 2). Both 45 (19.5 ± 2.7 μmol/g, $P < 0.05$) and 60 min (25.86 ± 1.71 μmol/g, $P < 0.05$) ischemia followed by 1 h reperfusion caused a significant reduction in liver GSH compared to the sham-operated group (36.2 ± 2.07 μmol/g, $P < 0.05$). Figure 2 shows the level of FRAP in liver tissues. There were no significant differences between the groups.

Table 1 Data on liver function tests in different groups

Groups	AST (U/L)	ALT (U/L)
Sham-operated	263.7 ± 25.7	128 ± 27.5
30 min ischemia + 1 h reperfusion	275 ± 52.6	119 ± 20.8
45 min ischemia + 1 h reperfusion	459.7 ± 17.7[a]	128 ± 15.5
60 min ischemia + 1 h reperfusion	491.5 ± 74.8[a]	444.6 ± 198.6[a]

[a]$P < 0.05$ compared to sham-operated group. The data are presented as mean ± SE ($n = 5$ in each group).

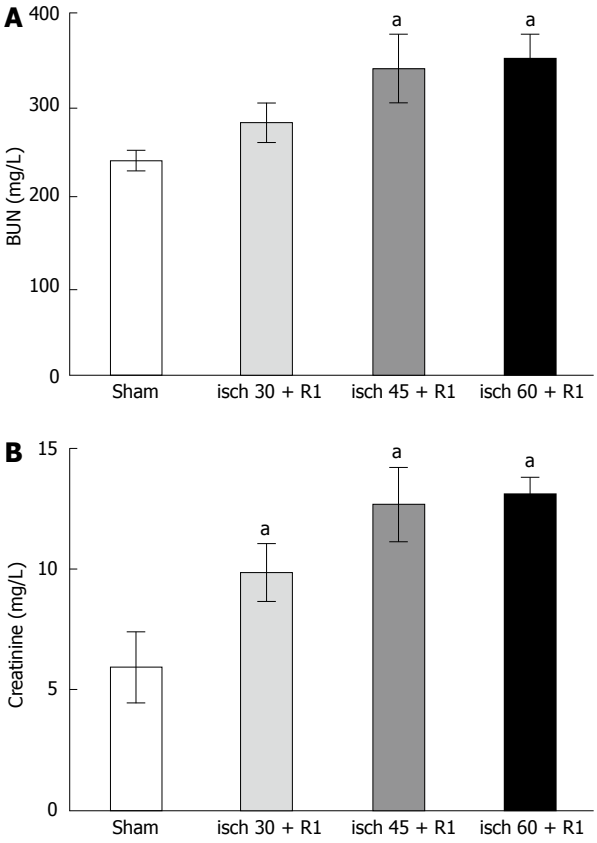


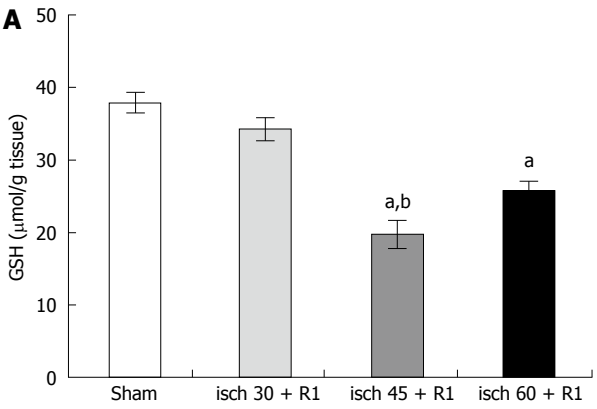
Figure 1 Alterations in renal function during different renal I/R periods. A: BUN; B: Plasma creatinine. The data are presented as mean ± SE. [a]$P < 0.05$ *vs* sham-operated group. All ischemic periods were followed by 1 h reperfusion.

Effect of renal ischemia on liver TNF-α

The level of liver TNF-α was increased significantly after 30 (466.9 ± 71.7 pg/100 mg, $P < 0.05$), 45 (507.2 ± 71.7 pg/100 mg, $P < 0.05$) and 60 min (718.6 ± 68.8 pg/100 mg, $P < 0.05$) renal ischemia followed by 1 h reperfusion compared to the sham-operated group (210 ± 16.6 pg/100 mg). The increase in liver TNF-α following the induction of 60 min ischemia was significantly higher than the other ischemic periods.

Effect of renal ischemia on liver IL-10

Renal ischemia (30, 45 and 60 min) followed by 1 h reperfusion resulted in a significant increase in liver IL-10 compared to that in the sham-operated group (121 ± 34.5 pg/100 mg, $P < 0.05$). Induction of 60 min ischemia followed by 1 h reperfusion (667.8 ± 34.5 pg/100 mg,



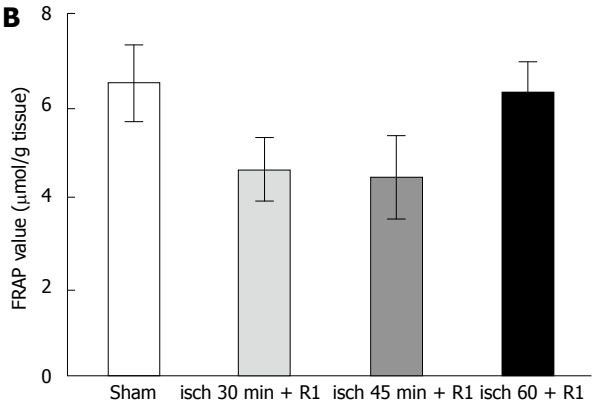
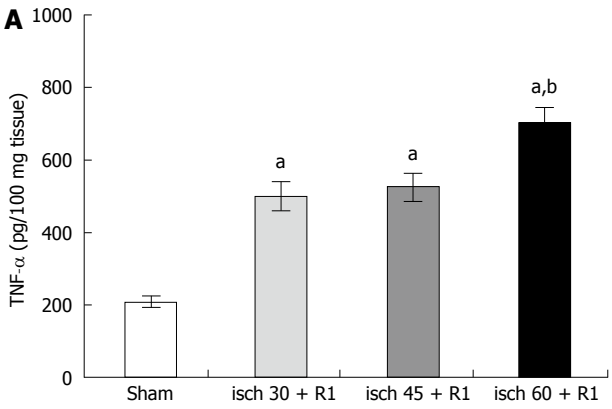


Figure 2 Alterations in liver GSH (A) and FRAP (B) during different renal I/R periods. The data are presented as mean ± SE. $^{a}P < 0.05$ *vs* sham-operated group; $^{b}P < 0.05$ *vs* 30-min ischemia group. All ischemic periods were followed by 1 h reperfusion.



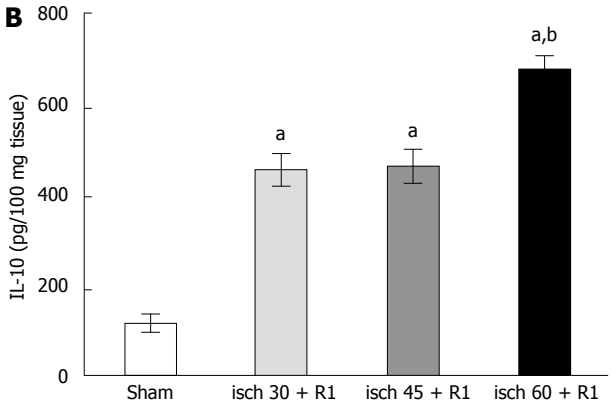


Figure 3 Alterations in liver TNF-α (A) and IL-10 (B) during different renal I/R periods. The data are presented as mean ± SE. $^{a}P < 0.05$ *vs* sham-operated group; $^{b}P < 0.05$ *vs* other groups. All ischemic periods were followed by 1 h reperfusion.

$P < 0.05$) showed a significant difference from the other groups (Figure 3).

Effect of renal ischemia on liver histology

Liver sections were evaluated for the presence of congestion, cellular degenerative changes, cytoplasmic vacuolization and leukocyte infiltration. The sections from the sham-operated rats displayed minimal/no changes. In the 30-min ischemia group, congestion was present, but much less than for the 45- or 60-min ischemic-reperfused group. There was no apparent evidence of cellular degenerative changes including cytoplasmic vacuolization. Leukocyte infiltration was almost absent in this group. Vacuolization was frequent in the 45-min ischemic-reperfused tissues. Irregularity, pale (atypical) nuclei, disintegrated cytoplasm, and infiltration of leukocytes were seen. Similar changes were observed in the 60-min ischemic-reperfused group (Figure 4).

Effect of renal ischemia on SSAT and CARF

RNA isolation and Northern hybridization were performed on some liver samples and the expression of SSAT was examined (Figure 5A). There was an increase in the 60-min renal ischemia compared with the control group, albeit by only about 35%, which was significant. The expression of CARF remained unchanged (Figure 5B).

DISCUSSION

Acute renal ischemic injury continues to be associated with a high mortality rate. Renal I/R injury occurs in many clinical situations, such as transplantation, partial nephrectomy, sepsis, hydronephrosis, or elective urological operations. Although most research in this area has focused on the renal response to this injury, recent work has suggested that renal injury affects and is also regulated by the extra-renal organs including the liver[13]. In the present study, the changes in hepatic function, histology, cytokine levels and antioxidant status were examined after induction of various periods of rat renal ischemic injury.

In 2002, Serteser *et al*[14] demonstrated some changes in hepatic TNF-α levels and oxidation products after renal I/R injury in mice. They have suggested that 30 min ischemia and 60 min reperfusion is sufficient to elicit remote effects of I/R injury. Their study was performed in mice, while we used rats in our study. This may explain why 30 min ischemia was less injurious compared to 45 and 60 min. In 2003, it was suggested that hepatic production of IL-10 and IL-1 receptor antagonists, in response to acute bile duct ligation, ameliorates ischemic ARF[15]. Hoke *et al*[16] in 2007 demonstrated that acute absence of kidney function results in pulmonary injury independent of renal ischemia, and highlighted the critical role of the kidney in the maintenance of serum

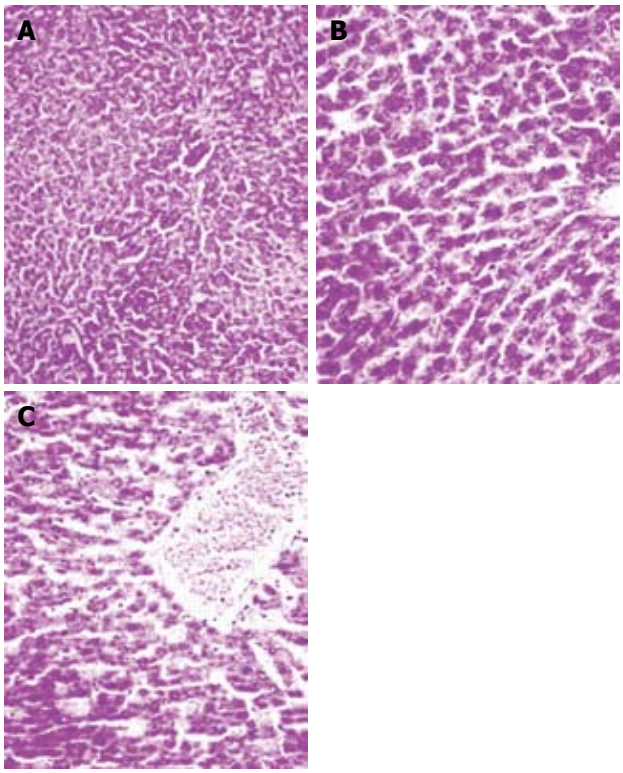


Figure 4 Hematoxylin and eosin-stained sections of rat liver. A: Sham-operated group. B: I/R group, vacuolization was frequent in the 45 min ischemia-reperfused tissues. Irregularity, pale (atypical) nuclei, disintegrated cytoplasm, and infiltration of leukocytes were seen. C: Similar changes were observed in the 60 min ischemia-reperfused group (× 400).

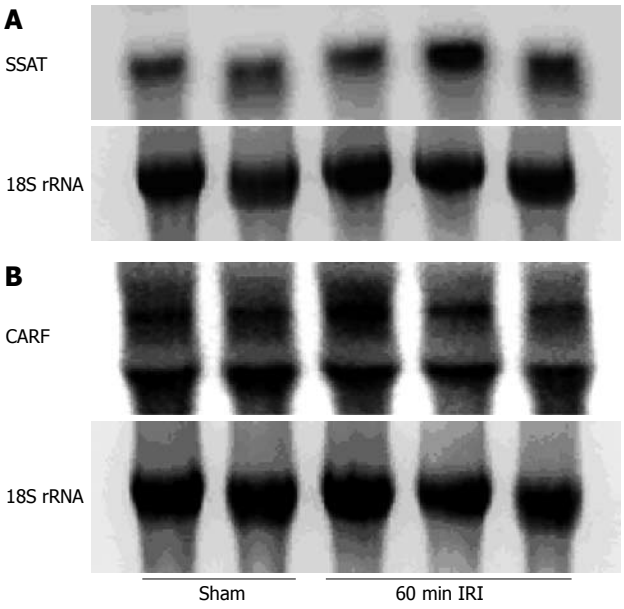


Figure 5 Alterations in liver SSAT and CARF expression during 60 min renal ischemia followed by 1 h reperfusion. A: SSAT mRNA levels in the liver of renal ischemic samples were 139 ± 7% *vs* the sham-operated group, after adjustment for RNA loading as determined by 18S rRNA. $P < 0.05$ *vs* sham-operated group. B: CARF mRNA levels in the liver of renal ischemic samples were 98% ± 8% of levels in sham-operated group, after adjustment for RNA loading as determined by 18S rRNA.

cytokine balance and pulmonary homeostasis. In the present study, all three periods of ischemia caused an increase in hepatic TNF-α levels; but, the increase after 60 min was significantly higher than after 30 and 45 min ischemia. This means that, after 45 min ischemia, there are no irreversible changes, namely severe necrotic alterations.

In our study, as expected, I/R caused a reduction in renal function and structural alteration in an ischemia-time-dependent manner. Liver function was almost preserved after 30 min ischemia, partially reduced after 45 min, but showed a significant reduction in the 60-min ischemia group. This indicated that the liver underwent more prominent and severe damage in the 60-min group. Liver histology showed that, after 30 min ischemia, there was no apparent injury; but, 45 and 60 min ischemia elicited histological changes. The cytokines in the liver tissue were significantly increased after 30 and 45 ischemia; but, the increase after 60 min was very high and showed significant differences from the control and other ischemia groups. Although GSH showed a reduction in all of the experimental groups, the reduction in the 45- and 60-min groups was much higher than that in the 30-min ischemia group. The changes in GSH concentration suggested that ROS mediated the biomolecular alterations. On the other hand, FRAP showed a reduction after ischemia (although not significant). The rise in the 60-min group may have resulted from the contribution of uric acid to the overall concentration of FRAP, as reported previously[2].

RNA isolation and Northern hybridization were also conducted on some liver samples. Total cellular RNA was extracted and hybridization was performed according to Church and Gilbert[12]. The expression of SSAT and CARF, two mediators of tissue injury, in normal and experimental groups was evaluated. The expression of SSAT was increased in the 60-min renal ischemia group compared with the controls. The expression of CARF remained unchanged. It is likely that SSAT is a more sensitive marker of injury than CARF.

These data clearly demonstrate that renal ischemia causes detrimental changes in liver histology, function, oxidative stress and inflammatory status, which leads to a reduction in hepatic antioxidant capacity. After 30 min ischemia, the magnitude of these changes is much less than after 45 or 60 min ischemia. A minimum of 45 min ischemia is needed to study the effects of renal injury on liver as a remote organ. Care should be taken to protect other organs remote from I/R sites, especially during renal surgery.

COMMENTS

Background

The effect of locally applied ischemia/reperfusion (I/R) injury to the kidney has been under investigation for many years. However, little is known about the changes in liver function and oxidative stress in renal I/R injury.

Research frontiers

Renal injury associated with liver disease is an extensively encountered clinical problem of varied etiology and high mortality. Recent studies have suggested crosstalk between the liver and kidneys. I/R-induced local response in kidney tissue has been well documented in a number of studies. However, remote

effects of renal I/R injury on the liver need further investigation. The aim of the present study was to assess the hepatic changes after induction of different periods of renal ischemia.

Innovations and breakthroughs

Rats were subjected to sham operation or ischemia (30, 45 and 60 min) followed by 60 min reperfusion. This study compared different ischemia times by the use of the following indices: hepatic glutathione and ferric reducing antioxidant power levels, and concentration of interleukin (IL)-10 and tumor necrosis factor-α.

Applications

To study the effects of renal injury on liver as a remote organ, a minimum of 45 min ischemia is needed. Care should be taken to protect organs remote from I/R sites especially during renal surgery.

Peer review

The authors describe the effects of different periods of rat renal ischemia on the liver in a rat model. They observed significant hepatic damage after at least 45-60 min of renal ischemia. This manuscript is well written and provides new information concerning the consequences of renal I/R injury on the liver.

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Dietary and socio-economic factors in relation to *Helicobacter pylori* re-infection

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Abstract

AIM: To examine if dietary and socio-economic factors contribute to *Helicobacter pylori* (*H pylori*) re-infection.

METHODS: The population of patients consisted of subjects in whom *H pylori* infection had been successfully treated in the past. Patients were divided into two groups: Ⅰ-examined group (111 persons with *H pylori* re-infection) and Ⅱ-control group (175 persons who had not been re-infected). The respondents were interviewed retrospectively on their dietary habits and socio-economic factors.

RESULTS: A statistically significant lower frequency of fermented dairy products ($P < 0.0001$), vegetables ($P = 0.02$), and fruit ($P = 0.008$) consumption was noted among patients with *H pylori* re-infection as compared to those who had not been re-infected.

CONCLUSION: High dietary intake of probiotic bacteria, mainly *Lactobacillus*, and antioxidants, mainly vitamin C (contained in fruit and vegetables), might decrease the risk of *H pylori* re-infection.

Key words: Nutrition; Probiotics; *Helicobacter pylori* re-infection; Vitamin C; Lifestyle factors

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection exerts a decisive role in the pathogenesis of peptic ulcer disease and gastric cancer[1-3]. Epidemiological studies have shown that it is probably one of the most common bacterial infections throughout the world, involving 30% of the population living in developed countries and up to 80%-90% of the population in developing regions[4]. Poland, like most of the Eastern European countries, has an overall infection rate of 73% and an infection rate for the subjects over 25 years of age of 85%-95%[5]. Infection usually takes place in early childhood and youth; but, a proportion of the population becomes infected as adults[6-9]. Vectors of the bacteria are humans, by whom the infection is transmitted by oral-oral and faecal-oral routes[3,5]. The risk of infection is related mainly to socio-economic status[10-12].

The important role of nutritional factors that might facilitate infection, such as low intake of antioxidants, mainly vitamin C, and high salt consumption, is also stressed[13,14]. Moreover, some research has shown that, in *in vitro* conditions, probiotic bacteria (especially *Lactobacillus*) might reduce the risk of *H pylori* infection[15-17].

Owing to the wide implementation of eradication therapy, the recurrence of peptic ulcers has decreased significantly[18,19]. However, in some patients re-infection occurs and ulcers reappear. Re-infection affects ca. 1%-13% of patients annually, depending on the population studied[20-24]. It is believed that dietary and

socio-economic factors may contribute to the *H pylori* re-infection.

The aim of this study was to evaluate whether there are differences in dietary habits and lifestyle between subjects after effective eradication who were re-infected and patients who were not re-infected.

MATERIALS AND METHODS

The study was carried out in 2002-2007 in a group of patients from the Provincial Gastroenterological Clinic of the Brodnowski Hospital in Warsaw who had ulcer disease or functional dyspepsia, and had been referred for endoscopic examination of the upper digestive tract. All the patients had been successfully treated for *H pylori* infection in the past and successful eradication after at least 6 wk after completion of the treatment was confirmed. The effectiveness of treatment was diagnosed by histology and a urease test (both negative) or urea breath test. Patients with neoplastic diseases were not included, along with persons with no confirmed eradication by the above mentioned methods and those who did not agree to take part in the research.

Patients were divided into two groups by *H pylori* status. One hundred and eleven patients were classified in group I (examined): persons with *H pylori* re-infection, and 175 patients were included in group Ⅱ (control): persons not re-infected. The period following *H pylori* eradication ranged from 3 to 8 years. The mean time after the eradication treatment was similar for both groups: 5.2 years for group I and 5.6 years for group Ⅱ.

Characteristics of both groups are presented in Table 1. Group I consisted of 47 women and 64 men aged 24-88. The control group included more women (n = 103) than men (n = 72). The range of age of the patients in this group was from 17 to 87 years old. No statistically significant differences between mean age and mean BMI between the groups were observed.

The status of *H pylori* was evaluated using the histological method and urease test (both positive) or urea breath test.

For all patients, a BMI value was calculated. An interview on dietary habits and socio-economic factors was performed by a dietician. The patients were interviewed retrospectively. A specially designed questionnaire was used. The first part of the questionnaire contained questions regarding the usual dietary habits during last year, while the second part referred to selected features relating to the patients' lifestyle. The questionnaire contained questions providing information, inter alia, on the applied diet, amounts, regularity and type of meals, frequency of consumption of products from various food groups, with particular attention paid to dairy products and fat, as well as salty products and dishes, along with additional salting. Consumption of products and dishes at least five times a week was regarded as frequent. For some products and dishes, the analysis covered moderate consumption with moderate frequency, i.e. twice to four times a week, and rare consumption, i.e. once a week or more seldom. For other products and dishes consumption frequency of up to four times a week was regarded rare. The second part of the questionnaire contained questions referring to the patients' job and additional employment, stress exposure and smoking. Among the examined factors only those that had an impact on the occurrence of *H pylori* infection were selected and discussed. In the statistical analysis of the differences between studied groups, a χ^2 test was applied, assuming differences of statistical significance for $P < 0.05$.

Table 1 Characteristics of the examined groups

	Group Ⅰ HP (+)			Group Ⅱ HP (-)		
	n	Mean	Range	*n*	Mean	Range
Age (yr)						
Women	47	63	24-88	103	62	17-87
Men	64	57	27-79	72	54	18-80
Total	111	60	24-88	175	58	17-87
BMI						
Women	47	24.8	16.3-32.5	103	24.8	15.6-48.9
Men	64	26.1	15.5-36.1	72	24.9	16.2-39.7
Total	111	25.5	15.5-36.1	175	24.9	15.6-48.9

RESULTS

Dietary factors that are likely to have an impact on *H pylori* infection are presented in Table 2.

Most of the dietary factors analysed in both groups showed no significant differences. Both the patients who had been re-infected and patients from the control group said they ate meals regularly.

Statistically significant differences were noted in case of the frequency of eating dairy products ($P < 0.0001$). The percentage of persons who often ate dairy products among patients with *H pylori* re-infection was much lower (41%) than in the control group (89%), and a higher proportion of the re-infected patients (32%) admitted to eating dairy products rarely, while in the control group this percentage was much lower (6%). A significant difference was also observed in the case of fermented milk drinks ($P < 0.0001$). Less than half (43%) of the re-infected patients consumed these products frequently, while among non-infected persons-almost all (95%) did.

Most patients from both groups ate vegetables frequently (74% in the group re-infected and 87% in the control group); but, the differences in the frequency of the consumption of these products were statistically significant ($P = 0.02$). The frequency of fruit consumption also showed differences; frequent consumption of these products was declared by fewer persons re-infected (58%) in comparison to the patients who were not re-infected (76%) ($P = 0.008$).

Selected aspects relating to the lifestyle of examined patients are presented in Table 3.

Patients with *H pylori* re-infection did not vary significantly from the control group in terms of the analysed lifestyle factors. Most of the patients did not work professionally, but declared frequent tiredness and high stress exposure. In both groups, the majority did not smoke.

Table 2 Comparison of selected dietary factors in the examined groups *n* (%)

Factors	Responses	Group Ⅰ HP (+) *n* = 111[1]	Group Ⅱ HP (-) *n* = 175[1]	Statistical significance (*P*)
Regularity of eating meals (3-5)	Yes	55 (52)	97 (56)	NS
	No	51 (48)	76 (44)	
Meals prepared on their own	Yes	53 (48)	105 (60)	0.02
	Sometimes	18 (16)	33 (19)	
	No	40 (36)	37 (21)	
Adding fat to stewed, fried and baked dishes	Yes	75 (68)	133 (77)	NS
	Sometimes	3 (2)	1 (1)	
	No	33 (30)	38 (22)	
Adding fat or dressing to salads	Yes	67 (61)	121 (69)	NS
	Sometimes	3 (3)	3 (2)	
	No	39 (36)	51 (29)	
Using fats to spread on bread	Yes	94 (85)	157 (90)	NS
	Sometimes	2 (2)	5 (3)	
	No	15 (13)	13 (7)	
Eating dairy products	Frequently	45 (41)	154 (89)	< 0.0001
	With moderate frequency	30 (27)	9 (5)	
	Rarely	36 (32)	11 (6)	
Eating fermented milk drinks (yoghurts, kefirs)	Frequently	48 (43)	166 (95)	< 0.0001
	Rarely	63 (57)	9 (5)	
Eating meat products and dishes	Frequently	82 (75)	120 (69)	NS
	With moderate frequency	19 (17)	40 (23)	
	Rarely	8 (7)	14 (8)	
Types of meat products and dishes eaten	Fatty	3 (3)	2 (1)	NS
	Medium-fatty	8 (8)	5 (3)	
	Lean	63 (60)	110 (66)	
	Varying	30 (29)	50 (30)	
Eating fish	Frequently	31 (28)	53 (30)	NS
	With moderate frequency	33 (30)	58 (33)	
	Rarely	47 (42)	64 (37)	
Eating vegetables	Frequently	82 (74)	152 (87)	0.02
	With moderate frequency	21 (19)	17 (10)	
	Rarely	8 (7)	6 (3)	
Eating fruit	Frequently	65 (58)	133 (76)	0.008
	With moderate frequency	24 (22)	23 (13)	
	Rarely	22 (20)	19 (11)	
Eating sweets	Frequently	26 (24)	47 (27)	NS
	With moderate frequency	28 (25)	46 (26)	
	Rarely	57 (51)	82 (47)	
Sweetening of drinks (coffee, tea)	Yes	79 (71)	115 (66)	NS
	Sometimes	2 (2)	5 (3)	
	No	30 (27)	55 (31)	
Alcoholic drinks consumption	Frequently	14 (13)	9 (5)	NS
	With moderate frequency	18 (16)	29 (17)	
	Rarely	79 (71)	136 (78)	
Eating salty dishes	Yes	35 (32)	52 (30)	NS
	Sometimes	15 (13)	20 (11)	
	No	61 (55)	103 (59)	
Additional salting of products and dishes eaten	Yes	21 (20)	39 (24)	NS
	Sometimes	7 (6)	9 (6)	
	No	79 (74)	113 (70)	

[1]Number of persons changed between 104-111 persons in group Ⅰ and 167-175 persons in group Ⅱ, which results from the fact that some patients did not provide an answer to some questions; NS-value statistically insignificant.

Table 3 Comparison of selected lifestyle factors in examined groups *n* (%)

Factors	Responses	Group Ⅰ HP (+) *n* = 111[1]	Group Ⅱ HP (-) *n* = 175[1]
Working	Yes	28 (25)	39 (23)
	No	83 (75)	134 (77)
Working overtime or on weekends	Yes	18 (78)	22 (60)
	Sometimes	1 (4)	3 (8)
	No	4 (18)	12 (32)
Additional work outside the main job	Yes	6 (8)	8 (7)
	No	65 (92)	110 (93)
Feeling tired	Very often	58 (53)	96 (55)
	Rather often	16 (14)	27 (16)
	Rather rarely	26 (24)	40 (23)
	Hardly ever	10 (9)	11 (6)
Self-assessed stress exposure	Very often	47 (43)	89 (51)
	Rather often	15 (13)	27 (16)
	Rather rarely	37 (34)	42 (24)
	Hardly ever	11 (10)	15 (9)
Smoking	Yes	36 (32)	54 (31)
	No	75 (68)	120 (69)

[1]Number of persons changed between 23-111 persons in group Ⅰ and 37-175 persons in group Ⅱ, which results from the fact that some patients did not provide an answer to some questions.

DISCUSSION

The question of how to lower the risk of *H pylori* re-infection is very important. This bacterium is the main cause of peptic ulcer disease (70%-90% of cases) and in 1% of infected persons, this leads to the development of gastric cancer[25]. Moreover, the treatment of *H pylori* is difficult, requires a two-week application of at least three medicines (proton pump inhibitors and two antibiotics) simultaneously, proves successful in only 80%-90% of cases and is connected with the risk of adverse effects of therapy with antibiotics (15%-30% of the treated)[26,27]. In some patients, *H pylori* re-infection occurs after eradication; but, factors responsible for this phenomenon have not yet been identified. It is presumed that these may be at least partly related to poor sanitary conditions and improper lifestyle, especially diet[12,28,29].

In the present research, the dietary and some socio-economic factors after successful eradication of *H pylori* infection were evaluated. The goal of this retrospective study was to point out potential differences in the dietary patterns of patients with *H pylori* re-infection (group Ⅰ) and in the control not-re-infected group (group Ⅱ).

We showed a significant difference in the frequency of consumption of fermented dairy products containing probiotic bacteria, mainly *Lactobacillus*, between the group with *H pylori* re-infection and the group without re-infection. This indicates that regular consumption of products containing probiotic bacteria might reduce the risk of *H pylori* re-infection.

There is some evidence from *in vitro* and clinical research that can support this hypothesis. Numerous probiotic strains inhibit the growth or adhesion of *H pylori* to epithelium cells in *in vitro* conditions. In

studies on animals infected with *H pylori*, it was also observed that probiotic bacteria lowered the intensity of inflammatory conditions in the stomach mucosa. Michetti *et al*[15] showed that the supernatant of a culture of *Lactobacillus* johnsoni La1 strain inhibited the growth of *H pylori* bacteria whether or not they were connected with epithelial cells. The supernatant was administered for 14 d to 20 volunteers infected with *H pylori* in a double-blind randomised study. The results of urea breath tests at the beginning and in the 6th week after the completion of the treatment were significantly lower than the initial results, which is most probably related to lowering the density of *H pylori* colonies. In a biopsy taken from the mucosa of the stomach, *H pylori* infection was still present[15].

Aiba *et al*[30] showed that *L. salivarius* inhibited the growth of *H pylori in vitro*, and, in an animal model, reduced the inflammatory process in the mucosa of infected mice. No such phenomena were observed in the case of *L. casei* and *L. acidophilus*.

Coconnier *et al*[16] observed that supernatant from the *L. acidophilus* LB culture contains anti-bacterial substances produced by this strain, which reduced the viability of *H pylori* bacteria and inhibited its adhesion to human cells *in vitro* and *in vivo*. Sgouras *et al*[31] used *L. casei* Shirota cells *in vitro* and *in vivo* and noted that the cells (not the supernatant) lowered the activity of *H pylori* urease. In research carried out on mice, after the application of the above strain, the density of *H pylori* colonies decreased, along with the intensity of the inflammation of the mucosa of the stomach[31].

Similar results were obtained in animal; for example, the density of colonisation of stomach mucosa by *H pylori* became lower, and inflammatory changes became smaller, after the administration of *L. rhamnosus*, *L. acidophilus* and *L. gasseri*[32,33]. Kabir *et al*[34] stated that administration of *L. salivarius* to mice infected with *H pylori* decreased the adhesion of pathogens to stomach mucosa cells.

So far, clinical tests have not been able to prove that use of probiotics leads to *H pylori* eradication[35,36]. Wendakoon *et al*[37] made an attempt to prove it in their study of patients with asymptomatic *H pylori* infection. The patients were given *L. acidophilus* and *L. casei* strains for 30 d, which inhibited *H pylori* growth *in vitro*; but, no eradication in any of the patients was observed.

Several clinical surveys showed that some strains of probiotic bacteria might increase the effectiveness of *H pylori* eradication. Canducci *et al*[38] noted higher *H pylori* eradication rate in patients who, in addition to triple therapy based on rabeprazole, clarithromycin and amoxicillin, were given a lyophilized and inactivated culture of *Lactobacillus* acidophilus. In a study by Sýkora *et al*[39] *H pylori*-positive children received the control treatment of omeprazole, amoxicillin and clarithromycin or the treatment consisted of the same antibiotics supplemented with fermented milk (trade name-Actimel) containing *L. casei* DN-114 001. Eradication success was significantly higher in the test group compared with the control group.

Application of probiotics during *H pylori* treatment might not only increase the eradication rate, but it might also decrease the adverse effects of antibiotic therapy. Park *et al*[40] showed that supplements containing probiotic bacteria strains, composed of *Bacillus subtilis* and *Streptococcus faecium*, enhanced the intention-to-treat eradication rate of *H pylori*, improved drug compliance and reduced side effects. Diarrhoea and overall side effects were more common in the group treated with antibiotics only in comparison to the group treated with antibiotics plus probiotics. De Bortoli *et al*[41] examined whether adding bovine lactoferrin and probiotics to the standard triple therapy for *H pylori* infection could improve the eradication rate and reduce side effects. The eradication rate was higher in more patients who underwent standard triple eradication therapy plus bovine lactoferrin and probiotics than in those who underwent standard therapy only. Moreover, fewer patients taking probiotics reported side effects. Improvement of the results of eradication therapy followed by the application of probiotics was also noted in Polish studies covering children with dyspeptic symptoms and confirmed *H pylori* infection[42]. In the group of children who were given probiotics (*L. acidophilus* and *L. rhamnosus*) in addition to standard therapy, not only was significantly higher eradication effectiveness demonstrated, but also a lower intensity of inflammation of the mucosa of the stomach and a lower rate of adverse effects of the therapy were noted.

The results of some studies do not confirm the positive impact of the use of probiotics on the eradication treatment ratio. No difference in eradication rate was observed in *H pylori*-positive patients receiving *L. reuteri* and a placebo[43]. Also Goldman *et al*[44], in their study of children in Buenos Aires, found no significant differences in *H pylori* eradication rates between the group treated with triple therapy plus probiotic food (yogurt containing *Bifidobacterium animalis* and *Lactobacillus casei*) and the control group.

Although not all papers confirm the improvement of treatment results for *H pylori* infection upon simultaneous treatment with antibiotics and probiotics, the meta-analysis performed by Tong *et al*[45], covering 14 randomized trials, suggests that supplementation with probiotics could be effective in increasing eradication rates of anti-*H pylori* therapy, and could be considered helpful for patients with previous eradication failure. Pooled *H pylori* eradication rates were 83.6% and 74.8% for patients with or without probiotics by intention-to-treat analysis. Furthermore, probiotics showed a positive impact on *H pylori* therapy-related side effects. The occurrence of total side effects was 24.7% and 38.5% for groups with or without probiotics.

Results found in most of the studies showed that the use of probiotics during eradication treatment was of benefit to patients. However, more large and well-designed studies of the use of probiotics in *H pylori* eradication treatment are necessary, including comparative and dose-ranging trials[46].

We also demonstrated a significantly higher

consumption of fruit and vegetables among persons who were not re-infected. This is probably related to the consumption of a higher number of anti-oxidants, especially vitamin C. Vitamin C, which is highly concentrated in stomach mucosa and gastric juice and probably lowers the risk of gastric cancer and influences the course of *H pylori* infection through a number of mechanisms[13,47]. It has a positive impact on the stimulation and activity of granulocytes, macrophages and lymphocytes and the production of immunoglobulins. The direct inhibitory impact of this vitamin on the growth of *H pylori* is now being examined.

Jarosz *et al*[13] showed that four weeks treatment of *H pylori* infected patients with chronic gastritis with a high dose of vitamin C caused *H pylori* eradication in 30% of cases. In those patients, a highly significant rise in gastric juice total vitamin C concentration was demonstrated, which persisted for at least four weeks after treatment. However, the mechanism whereby vitamin C treatment results in *H pylori* eradication is unclear.

Ruiz *et al*[48] found a causal association between *H pylori* infection and low ascorbic acid levels in the gastric juice. Their findings supported two hypotheses that explain this phenomenon: increased oxidation and a decreased secretion of ascorbic acid.

The results obtained from the Third National Health and Nutrition Examination Survey showed that ascorbic acid might affect the risk of *H pylori* infection[49]. In that survey, higher serum levels of ascorbic acid were associated with a decreased seroprevalence of *H pylori* and of the presence of pathogenic cagA-positive strain of *H pylori*.

The data of Park *et al*[50] demonstrated that vitamin C levels in whole blood, plasma, and gastric juice and the gastric juice pH in Korean children were closely related to the severity of *H pylori* infection and the histologic changes in the stomach. These data suggest that vitamin C may play a role in determining *H pylori* infection and its progression. Thus, vitamin C supplementation might be an important tool for the management of *H pylori* infection.

There were no differences between analysed lifestyle factors between patients with *H pylori* re-infection and the control group. However, the results of some surveys indicate the influence of socioeconomic status on *H pylori* infection[10-12]. Authors of a Polish study in Lodz observed a much higher prevalence of *H pylori* infection in children from poor living conditions[10]. In adults from Lublin, the *H pylori* infection was strongly affected by the lack of basic personal hygiene[12]. In the Czech Republic, the highest risk of *H pylori* infection was found in children of mothers with basic or lower education, living in crowded accommodations, without access to running warm water, and residing in smaller towns[11]. Low education and heavy smoking were most strongly associated with prevalence of *H pylori* infection in adults and adolescents. Smoking might also influence *H pylori* eradication rates. For example, a Colombian study in patients who smoked found that *H pylori* treatment was less effective[51]. Whereas data from Turkey supported the finding that personal and environmental conditions in adults did not affect *H pylori* infectivity[52]. Such factors as family income, living conditions, smoking, alcohol consumption and hygiene did not differ statistically between the *H pylori* positive and negative subjects. Smoking, alcohol consumption, number of children and pets in the household were also not associated with *H pylori* positivity among adolescents from Novosibirsk[53].

We studied only a few lifestyle factors without taking into account living conditions, personal hygiene and educational level that could influence *H pylori* re-infection. The lack of any relation between working, tiredness, stress exposure and *H pylori* re-infection could be caused by the fact that the majority of studied patients were retired. In our study, smoking did not influence of *H pylori* status, but not all surveys agree with our finding.

To summarise the results of some of the reviewed studies, the regular consumption of fermented milk products and fruit and vegetables might significantly reduce the risk of *H pylori* re-infection and this effect could be used in the prevention of the infection among persons in whom *H pylori* infection had been previously eradicated.

COMMENTS

Background

Helicobacter pylori (*H pylori*) infection is the main cause of peptic ulcer disease (70%-90% of cases) and in 1% of infected persons, leads to the development of gastric cancer. The treatment of *H pylori* infection is difficult and requires a two-week application of at least three medicines simultaneously. In some patients, *H pylori* re-infection occurs after eradication; but, factors responsible for this phenomenon have not yet been identified. It is presumed that these might be at least partly related to poor sanitary conditions and improper lifestyle, especially diet.

Research frontiers

H pylori re-infection affects ca. 1%-13% of patients annually; therefore, it is very important to find out how to lower the risk of *H pylori* re-infection. In this study, the dietary and some socio-economic factors after successful eradication of *H pylori* infection were evaluated. The goal of this retrospective study was to point out potential differences in the dietary patterns of patients with *H pylori* re-infection and in the control not-re-infected group.

Innovations and breakthroughs

The majority of the studies concerned the influence of dietary patterns on *H pylori* infection. The present research used a specially designed questionnaire to find out which factors lower the risk of *H pylori* re-infection.

Applications

The results suggest that the regular consumption of fermented milk products and fruit and vegetables might significantly reduce the risk of *H pylori* re-infection and this effect could be used in the prevention of re-infection among persons in whom the infection had been previously eradicated. The results could also be helpful in preparation of dietary guidelines for patients after *H pylori* eradication.

Terminology

Antioxidant: An antioxidant is a molecule (especially vitamins and microelements) capable of neutralizing free radicals which damage cells; Eradication: Eradication is the elimination or destruction of a thing or group (in this article it is bacteria-*H pylori*); Probiotics: Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host; Supernatant: Supernatant is a liquid remaining above the solid after chemical reaction.

Peer review

The main aspects of the paper are adequate. The discussion is complete and deals with different thoughts that are currently controversial. In summary, this is a good retrospective analysis of factors probably related with *H pylori* re-infection.

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CASE REPORT

Intrahepatic cholestasis of pregnancy: When should you look further?

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Abstract

Pruritis with abnormal liver function tests is the classical presentation of intrahepatic cholestasis of pregnancy (ICP), a condition associated with significant fetal complications. Although the etiology of ICP is unclear in many cases, certain features of the clinical presentation should alert the practitioner to the possibility of an underlying metabolic defect, which may not only affect subsequent pregnancies, but may be an indicator of more serious subsequent liver disease. We report a kindred of Anglo-Celtic descent, among whom many members present with ICP, gallstones or cholestasis related to use of oral contraception. Genetic studies revealed a novel mutation in the *ABCB4* gene, which codes for a phospholipid transport protein. The clinical significance of this mutation and the importance of identifying such patients are discussed.

Key words: *ABCB4* gene; *ABCB4* transporter; Phospholipids; Cholestasis of pregnancy; Gallstones

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) is characterized by pruritis, jaundice and raised serum bile salts, which typically starting in the third trimester of pregnancy, resolve after delivery and recur in subsequent pregnancies[1]. It is associated with an increased incidence of fetal distress, premature delivery and stillbirth[2].

ICP is thought to be caused by abnormal biliary transport, which may result from a number of factors, including hormonal, environmental and genetic. Recently it has been recognized that up to 15% of ICP may be associated with mutations in the MDR3 (*ABCB4*) gene[1]. The ATP binding cassette subfamily B, member 4 (*ABCB4*) gene codes for a protein responsible for the translocation of phosphatidylcholine (PC) from the inner to the outer leaflet of the canalicular membrane of the hepatocyte[3]. It is now becoming increasingly clear that disruption in the *ABCB4* gene can present a spectrum of clinical disorders ranging from ICP and low-phospholipid-associated cholestasis (LPAC), to progressive familial intrahepatic cholestasis type Ⅲ (PFIC Ⅲ), depending on the location of the new mutation[3].

LPAC is a condition characterized by gallstones, high serum gamma glutamyl transferase (GGT), intrahepatic microlithiasis and recurrent biliary symptoms despite cholecystectomy[4], while PFIC type Ⅲ is characterized by chronic cholestasis that presents early in life, which often progresses to end-stage liver disease that requires liver transplantation[3].

CASE REPORT

We report a kindred of Anglo-Celtic descent, who present with features of ICP and gallstones (LPAC), and have been found to have a novel mutation in the *MDR3*

Table 1 Clinical features in various family members of a family with MDR3 mutations

	Index case	Case Ⅱ	Case Ⅲ	Case Ⅳ	Case Ⅴ	Mother
Age of start of cholestasis	30	30	17	28	28	NA
Cholestasis with contraceptive pill	_	+	-	-	-	NA
Cholestasis with pregnancy 1	+	NA	+	-	+	+
Pregnancy 2	+	NA	+	-	+	+
Pregnancy 3	NA	NA	NA	NA	NA	+
Pregnancy 4	NA	NA	NA	NA	NA	+
Pregnancy 5	NA	NA	NA	NA	NA	+
Pregnancy 6	NA	NA	NA	NA	NA	+
Gallstones	+	+	+	+	-	+
Cholecystectomy	+	+	+	+	-	+
Abnormal LFTs	+	+	+	+	+	+
Improvement with ursodeoxycholic acid	+	NA	NA	NA	NA	NA

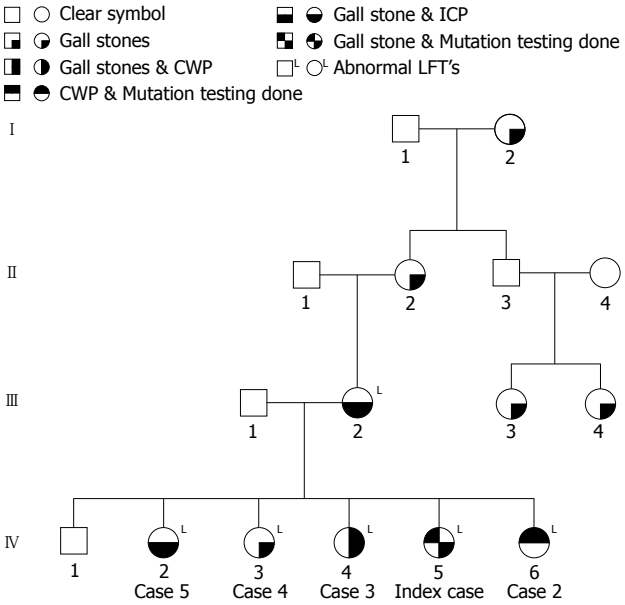

Figure 1 Pedigree showing affected family members with ICP, gallstones, and cholestasis with the oral contraceptive (CWP).

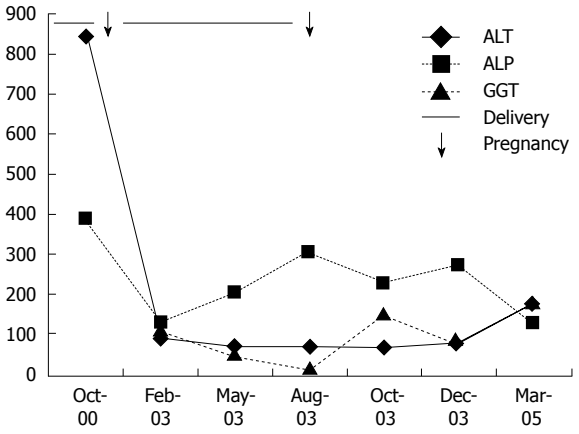

Figure 2 Liver function tests of the index case with an MDR3 mutation.

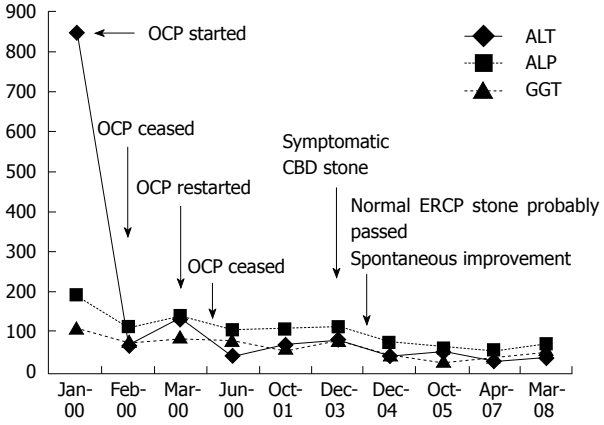

Figure 3 Liver function tests of case 2 with an MDR3 mutation.

gene. The clinical aspects of the presentation that prompted further investigation are discussed.

The family (Figure 1) came to medical attention when the index case (case 1) was referred with persistently abnormal liver function tests following her second pregnancy. She was a 33-year-old healthy G2P2 who had pruritus, but no jaundice during her first pregnancy, and recovered. During the second pregnancy, she again developed pruritus at 32 wk gestation, associated with abnormal liver function tests (Figure 2). She received ursodeoxycholic acid supplements, but with only mild relief, and abnormalities in her liver function tests persisted. She delivered at 37 wk through induced labor because of worsening jaundice and gestational diabetes. Various investigations including serum ceruloplasmin, serum bile salts, alpha-1 antitrypsin assays and viral serology for hepatitis viruses were performed, and were all normal. Further questioning revealed several members of the family with similar symptoms (Table 1).

Case Ⅱ was a 30-year-old, single, healthy, woman who developed pruritus and abnormal liver function tests after she commenced taking oral contraceptives (Figure 3). These symptoms improved after discontinuation of contraceptives; however, they recurred when she re-commenced the contraceptives. She had undergone a cholecystectomy at the age of 26 years following a prolonged history of episodic abdominal pain. Extensive investigations including viral serology, serum ceruloplasmin and copper levels, bile salt and alpha 1 antitrypsin assays for other causes of hepatitis were all normal. She was found to have a stone in her common bile duct upon ultrasound examination; but, subsequent endoscopic retrograde cholangiopancreatography was

normal. Her liver function tests started improving spontaneously, but never normalized.

Subsequently, a third sister (case Ⅲ) was referred for persistently abnormal liver biochemistry that was detected incidentally during routine screening of liver biochemistry while she was taking anti-convulsant drugs for post-traumatic epilepsy. She had a cholecystectomy at the age of 17 years for gallstones. However, 4 years later, she had a severe head injury following which she developed post- traumatic epilepsy. She was treated with sodium valproate and her abnormal liver function tests were initially attributed to this drug. However, drug level monitoring revealed her anti-convulsants to be well within the therapeutic range. Further investigations including viral serology, copper studies and abdominal ultrasound were negative. She also had a liver biopsy that showed very mild chronic lobular inflammation.

Case Ⅳ had a cholecystectomy for gallstones associated with persistently abnormal liver function tests, and case Ⅴ had cholestasis during both her pregnancies. Their mother had cholecystectomy for gallstones and also cholestasis during all her pregnancies.

Interestingly, their brother had normal liver function tests and was asymptomatic. A maternal grandmother had gallstones, but had not undergone cholecystectomy, and a maternal great grandmother died of a secondary liver cancer, the nature of which was uncertain. Two maternal cousins also had gallstones. There was no history suggestive of cholestatic disorders on their father' s side. There was also a history of miscarriages in the family; the mother having had two and the index case and one of the sisters having had one each. The children of all the sisters are below 10 years of age and have not yet shown any features suggestive of cholestatic liver disease.

DNA analysis

DNA of the index cases Ⅰ and Ⅱ was analyzed at the Academic Medical Centre, Amsterdam. Both the sisters were found to be positive for an 1102T > A mutation that converts phenylalanine at position 368 into isoleucine. This phenylalanine is highly conserved and the mutation has not been found in 150 control alleles. The mutation is located between the sixth transmembrane helix and the ATP-binding cassette, and is, therefore, likely to be of functional significance.

DISCUSSION

Pruritus associated with abnormal liver function tests and raised bile acids during pregnancy are the classical presentation of ICP. As this syndrome carries a risk of premature delivery and sudden intrauterine fetal death, most practitioners commence ursodeoxycholic acid, which reduces pruritus, transaminases and probably prematurity, without adverse side effects[1,5]. If the pregnancy proceeds uneventfully, many practitioners do not pursue further follow-up or investigation to look for a cause of the ICP.

However, it is now apparent that, a significant minority of patients with ICP will have underlying MDR3 mutations, which may predispose to further high-risk pregnancies and possibly serious liver disease later in life[3]. The percentage of patients with ICP caused by MDR3 mutations varies in different populations. In a recent Italian study, 7/96 (7.2%) women with ICP had MDR3 mutations identified while other studies have reported up to 15%[1].

The current report highlights a number features in the history of a patient with presumed ICP, which should have alerted the practitioner to the possibility of an underlying mutation and the need for further investigation. Clues that could be readily obtained from the history included a cholestatic reaction to oral contraception, ICP in previous pregnancies and a strong family history of gallstones under the age of 40 years, and recurrence of symptoms after cholecystectomy[4].

In our pedigree, the index case presented with typical ICP symptoms. However, it soon became apparent from the family history that most members had a history consistent with ICP during one or more pregnancies, and that all but one had gallstones at a young age. Whilst a high GGT level is observed in 30% patients with ICP, its presence increases the likelihood of an underlying MDR3 mutation[6]. Importantly, the first clue to the diagnosis and the reason for referral of our index case was that her obstetrician was concerned when GGT levels did not return to normal in the post-partum period.

The MDR3 (*ABCB4* transporter) is responsible for the translocation of PC from the inner to the outer leaflet of the canalicular membrane of hepatocytes. PC is extracted from the membrane by bile salts and then mixes with bile salts to form mixed micelles. These mixed micelles are known to solubilize cholesterol more efficiently than simple bile-salt micelles. On the other hand, the mixed micelles extract phospholipids less well from the membrane than simple bile-salt micelles and this protects the cells lining the biliary tree from membrane solubilization[3]. The rate of phospholipid secretion is an important factor in the prevention of gallstone formation and partial defects in phospholipid secretion may predispose to gallstone formation[4].

Mutations in MDR3 in ICP have been described in multiple exons[7]. The mutation described in this report adds to an expanding group of mutations that have been shown to cause familial cholestatic syndromes including ICP. However, there is still little understanding of why and how various mutations in the *MDR3* gene produce different clinical syndromes.

Factors that occur during pregnancy, which might lead to the development of cholestasis in previously asymptomatic individuals with these disorders, include generalized impairment of bile formation in the third trimester[8] and the effects of sex hormones that are known to promote cholestasis, possibly by inhibition of the bile salt export pump. The administration of exogenous progesterone in the third trimester may also precipitate ICP.

This kindred is especially interesting as different members have presented with various features of the clinical spectrum of *ABCB4* transporter defects over

many years, before the significance of the family history was finally recognized. This highlights the importance of obtaining a family history in all patients with ICP. Even if a positive family history is not obtained, patients who present with abnormal liver tests during pregnancy should have follow-up testing to ensure the abnormality resolves post-partum. The possibility of a bile acid transport defect should be considered in all patients who have a family history of ICP or other symptoms suggestive of a familial cholestatic disorder. Of note, patients presenting with gallstones in young adulthood (under the age of 40 years) should be evaluated further, particularly if there is a suspicious family history. Early detection of such patients should ensure that there is adequate monitoring of subsequent pregnancies, early treatment with ursodeoxycholic acid, and that there is ongoing follow-up to detect and prevent the development of significant liver disease.

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CASE REPORT

Endoclipping treatment of life-threatening rectal bleeding after prostate biopsy

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Abstract

Rectal bleeding is frequently seen in patients undergoing transrectal ultrasound (TRUS)-guided multiple biopsy of the prostate, but is usually mild and stops spontaneously. We report what is believed to be the first case of life-threatening rectal bleeding following this procedure, which was successfully treated by endoscopic intervention through placement of three clips on the sites of bleeding. This case emphasizes endoscopic intervention associated with endoclipping as a safe and effective method to achieve hemostasis in massive rectal bleeding after prostate biopsy. Additionally, current data on the complications of the TRUS-guided multiple biopsy of the prostate and the options for treating fulminant rectal bleeding, a consequence of this procedure, are described.

Key words: Prostate biopsy; Complications; Massive rectal bleeding; Endoscopic treatment; Endoclipping

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INTRODUCTION

Screening for prostate cancer has become an important issue in recent years. Of all procedures used to diagnose prostate cancer, the gold standard is transrectal ultrasound (TRUS)-guided multiple biopsy of the prostate[1,2]. Complications from TRUS-guided prostate needle biopsy are occasionally encountered in the daily practice of urologists; the traditional spring-loaded device with a small-caliber needle used for the prostate biopsy is fast, safe, effective and associated with minimal complications, including self-limiting hematuria, hematospermia and pain[3-5]. Rare major complications include acute prostatitis, acute urinary retention, epididymitis, severe hematuria, sepsis, abscess formation, urinary tract infection, tumor tracking, vasovagal syncope, and significant rectal bleeding[3-7]. Most often, major and especially minor complications resolve with traditional conservative therapy[3,8]. Severe rectal bleeding is traditionally managed by the urologist, with rectum tamponade as the initial and simplest conservative method, or, when necessary, balloon compression by means of a transrectally inserted catheter[8]. Endoscopic intervention with injection of adrenaline and sclerosing solutions, thermocoagulation and band ligation have also been used successfully in some cases[9-13]. We describe, possibly for the first time, the use of endoclipping for the treatment of severe rectal bleeding following TRUS-guided prostate multiple biopsy.

CASE REPORT

A healthy 59-year-old internist was found to have

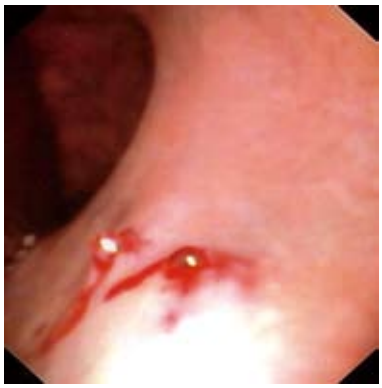

Figure 1 Endoscopic view showing oozing from biopsy sites in the anterior rectal wall.

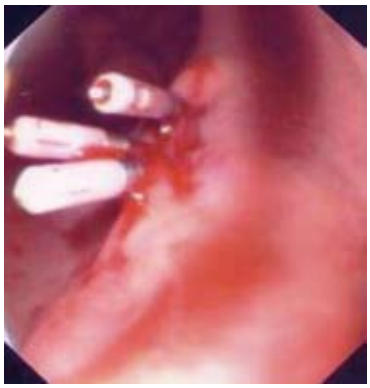

Figure 2 Hemostasis achieved after application of three clips.

prostate-specific antigen (PSA) at 5.8 ng/mL (normal < 3.5 ng/mL) during a screening test for prostate cancer. Laboratory data including platelet count, and prothrombin and bleeding times were normal. He underwent TRUS guided prostate multiple biopsy (18 cores) with a needle. Two hours later, he noticed rectal bleeding and thereafter he continued to pass a large volume of bright red blood through the rectum every 30 min. Manual compression and rectal tamponade with inflation of the balloon of an inserted urine catheter in the rectal cavity by his urologist failed to stop the bleeding. As a result of massive rectal bleeding that caused his hematocrit to drop from 45% to 28% and concomitant hemodynamic instability, he required hospitalization. Two packed red blood cell units were transfused and endoscopic consultation was requested. When transferred to our department, he was diaphoretic, with a pulse rate of 124 bpm and blood pressure of 100/70 mmHg. There was no history of hemorrhoidal disease. Urgent colonoscopy was performed without bowel preparation and revealed a rectal cavity full of fresh blood and clots, without a visible bleeding source. Vigorous washing and suction of the rectal cavity revealed two adjacent bleeding points in the anterior rectal wall, which corresponded to the sites of rectal wall injury caused by prostate multiple biopsy (Figure 1). Three endoclips (MH-858; Olympus, Tokyo, Japan) *via* an HX-6UR-1 applicator (Olympus) were applied to the bleeding lesions (Figure 2) and immediate hemostasis was achieved. The patient's condition was stabilized and 2 d later, he was discharged with an uneventful recovery.

DISCUSSION

To the best of our knowledge, we report the first known severe rectal bleeding following TRUS-guided prostate biopsy, which was effectively managed by endoclipping.

There are two established techniques of prostate biopsy, including the more widely used transrectal technique, and the transperineal technique. Both techniques appear to be equally safe, although the transrectal technique is faster[14]. Currently, the preferred option for initial prostate biopsy is the transrectal procedure[15]. Nevertheless, concerns about the accuracy of the standard sextant prostate biopsy for detecting prostate cancer have led to more cores being taken in each patient. This is not surprising, as mathematical models have shown that sextant biopsy misses 27% of tumors, and the probability of identifying a fixed volume of prostate cancer increases by taking more cores[16]. Results from clinical studies have shown that the sextant protocol for TRUS-guided prostate biopsy can miss cancer in 19%-31% of cases[17,18]. To overcome these diagnostic shortcomings, several extended biopsy policies have been advocated. Increasing the number of cores from six to eight, with extra cores targeted along the post-lateral margins of the gland, identifies up to 20% more tumors[19], but even an eight-core biopsy may miss cancer, and others have advocated[16,18] more biopsies per gland[20-22]. However, trying to improve the diagnostic accuracy should not be at the expense of the increased complication rate that may accompany more core biopsies, particularly bleeding, as occurred in our patient, especially when the prostate and surrounding rectal tissue are supplied by a rich vascular bed that consists of branches of the inferior vesicular artery and the middle and inferior rectal arteries. Moreover, the venous plexus is also dense in the submucosal space of the region, particularly in patients with hemorrhoids. The total incidence of rectal bleeding is listed as 1.3%-58.6%, with a statistically significant positive correlation to the number of core samples obtained. In most cases, the rectal bleeding is slight without necessitating further therapeutic intervention[3,5].

To overcome further the aforementioned diagnostic shortcomings, evaluation of the accuracy of TRUS-guided biopsies, by using combined magnetic resonance imaging (MRI) and magnetic resonance spectroscopic imaging (MRSI) in patients with persistently high PSA levels and negative TRUS-guided biopsy results, has revealed that MRI/MRSI have the potential to guide biopsies to tumor foci in these patients[23]. Overall, MRI and MRSI have accuracy similar to biopsy for intraprostatic localization of tumor and they are more accurate than biopsy in the prostate apex. Therefore, these imaging modalities may supplement biopsy results by increasing physician confidence when evaluating intraprostatic tumor location, which may be essential for planning disease-targeted therapy[24]. Our patient

did not accept further evaluation by these two imaging approaches.

In an extensive research of Medline using the key words rectal bleeding, prostate biopsy, hematochezia and rectal hemorrhage, we found seven publications that describe massive rectal bleeding occurring after transrectal biopsy, which required blood transfusion. In most of the cases, hemostasis was achieved with rectal tamponade by means of fleece tamponing, by urine balloon catheter inserted and inflated in the rectum by a condom filled with fluid in the rectal cavity, or after endoscopic intervention with injection of adrenaline or sclerosing solutions (polidocanol or pure ethanol), thermocoagulation and band ligation[9-13,25]. In our case, neither rectal tamponade nor manual compression of bleeding sites by a urologist succeeded in achieving hemostasis. Since the patient presented with hemodynamic instability (diaphoresis, tachycardia with drop of blood pressure), endoscopic consultation was requested. Having significant experience of endoclips for treatment of upper and lower gastrointestinal bleeding[26,27], we proceeded with urgent endoscopy combined with placement of three clips at the sites of bleeding, which led to immediate hemostasis. We preferred endoclips instead of sclerosing solutions, despite the fact that the latter have been successfully used to achieve hemostasis in post-biopsy prostate bleeding[28,29], because we were concerned about their risk of subsequent formation of deep ulceration. In contrast, the use of endoclipping has been widely reported in gastrointestinal endoscopy, without complications[26,27].

Argon plasma coagulation (APC) is a safe, well-tolerated treatment option in prostatic cancer patients with radiation-proctitis-induced hemorrhage, and historically, has been superior to Nd: YAG laser ablation[30]. Regarding the endoscopic treatment for initial hemostasis in upper and lower gastrointestinal bleeding, apart from the endoscopic hemostatic devices used, APC is an alternative hemostatic method[31,32]. Its potential therapeutic application in patients with severe rectal bleeding following TRUS-guided prostate biopsy remains to be elucidated.

In conclusion, our case emphasizes that urgent endoscopy allows accurate diagnosis and endoclipping is a safe and effective therapy of massive rectal bleeding followed prostate biopsy.

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CASE REPORT

Successful *en bloc* resection of primary hepatocellular carcinoma directly invading the stomach and pancreas

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Abstract

Multivisceral surgical resection for cure was successfully performed in a 70-year-old man suffering from a primary hepatocellular carcinoma (HCC) associated with direct invasion to the stomach and pancreas. The patient presented with gastric outlet obstruction, upper abdominal pain and a history of chronic liver disease due to hepatitis B virus (HBV) infection. Upper gastrointestinal (GI) endoscopy revealed an infiltrating tumor protruding through the gastric wall and obliterating the lumen. Computer tomograghy (CT) and magnetic resonance imaging (MRI) scan demonstrated a 15-cm tumor in the left lateral segment of the liver with invasion to the stomach and pancreas. Alpha-foetoprotein (AFP) levels and liver function tests were normal. The patient underwent an *en bloc* left hepatectomy, total gastrectomy, distal pancreatectomy with splenectomy and radical lymphadenectomy. Pathology revealed a poorly differentiated, giant cell HCC involving the stomach and pancreas. Disease-free margins of resection were achieved. The patient's postoperative course was uneventful. Sixteen months after surgery, he has no recurrence or distal metastasis. Direct invasion of HCC into the GI tract is rarely encountered. Complete surgical resection should be considered in selected patients with an appropriate hepatic functional reserve.

Key words: Hepatocellular carcinoma; Surgery; Stomach; Pancreas; Multivisceral resection

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Korkolis DP, Aggeli C, Plataniotis GD, Gontikakis E, Zerbinis H, Papantoniou N, Xinopoulos D, Apostolikas N, Vassilopoulos PP. Successful *en bloc* resection of primary hepatocellular carcinoma directly invading the stomach and pancreas. *World J Gastroenterol* 2009; 15(9): 1134-1137 Available from: URL: http://www.wjgnet.com/1007-9327/15/1134.asp DOI: http://dx.doi.org/10.3748/wjg.15.1134

INTRODUCTION

Hepatocellular carcinoma (HCC) is characterized by a soft consistency and extensive growth. These specific features of HCC mean that it rarely infiltrates the gastrointestinal (GI) tract directly. The incidence is reported to be 0.5% to 2% of clinical HCC cases[1,2]. Whether such an invasion causes massive hemorrhage or obstruction, a complete *en bloc* resection of these extensive HCCs can be safely performed using modern surgical techniques and sophisticated perioperative management. In this report, we describe a patient suffering from a giant, extrahepatically growing HCC associated with direct invasion to the stomach and pancreas. The tumor was successfully extirpated with a multivisceral oncologic resection. A thorough review of the literature is also presented.

CASE REPORT

A 70-year-old Caucasian male presented with signs of gastric outlet obstruction, upper abdominal pain and

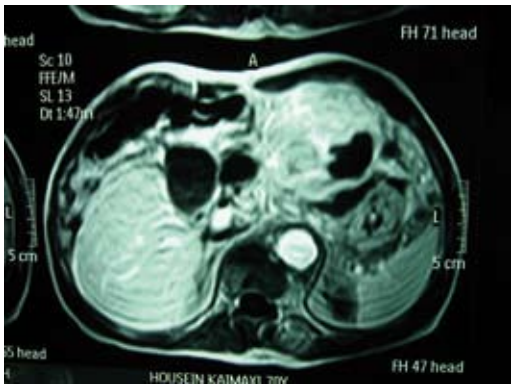

Figure 1 MRI scan demonstrates the presence of a large tumor, 15 cm in diameter, originating from the inferior surface of liver segments Ⅱ and Ⅲ. It is mainly solid with areas of tissue necrosis and shows direct invasion of stomach and pancreas.

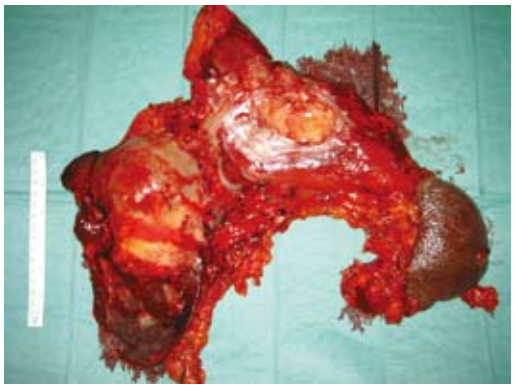

Figure 2 Surgical specimens demonstrating the *en bloc* left hepatectomy, total gastrectomy, distal pancreatectomy with splenectomy, and regional lymphadenectomy.

a chronic hepatitis B virus (HBV) infection. Upper GI endoscopy revealed a large, infiltrating tumor protruding through the anterior wall of the body of the stomach and almost completely obliterating the gastric lumen. No esophageal varices were found. Total colonoscopy showed no abnormality. The computed tomography (CT) scan demonstrated the presence of chronic liver disease and a giant tumor of the left lobe. The magnetic resonance imaging (MRI) revealed a space-occupying lesion, 15 cm × 12 cm × 9.5 cm in size, originating from the inferior surface of segments Ⅱ and Ⅲ. It was mainly solid with areas of tissue necrosis, hemorrhage and cystic degeneration. A smooth fibrous capsule was covering part of its outer surface. The lesion showed extensive extrahepatic growth with invasion to the body and fundus of the stomach, as well as direct contact with the upper surface of the pancreas. A low grade HCC was suggested (Figure 1). Biochemical analysis on admission indicated that alpha-foetoprotein (AFP) level was 2.1 ng/mL (normal value, < 10 ng/mL) and CA19.9 level was 33.2 ng/mL (normal value < 34 ng/mL). Liver function test results were normal. The patient underwent an *en bloc* radiofrequency-assisted left hepatectomy using the RF Cooltip needle (Radionics, Valleylab, MA, USA) and vascular staplers (EndoGIA, Covidien Healthcare, USA), total gastrectomy, distal pancreatectomy with splenectomy, radical hepaticoduodenal, perigastric and celiac trunk lymphadenectomy, as well as cholecystectomy (Figure 2). Total operative time was 160 min and estimated blood loss was less than 200 mL. GI continuity was restored with a Roux-en-Y end-to-side esophagojejunal reconstruction.

Pathology confirmed the presence of a poorly differentiated giant cell HCC developed in a liver cirrhosis. It was characterized by multinucleated giant cells and extensive areas of tissue necrosis (Figure 3). The tumor was invading the resected stomach and capsule of the body of pancreas and it was metastatic to 7 out of 54 resected lymph nodes. Disease-free margins of resection were achieved.

The patient had an uneventful course and was discharged on the 9th postoperative day. No adjuvant treatment was given but close follow-up was suggested. Sixteen months after surgery, he is doing well with no evidence of locoregional recurrence or distant metastasis.

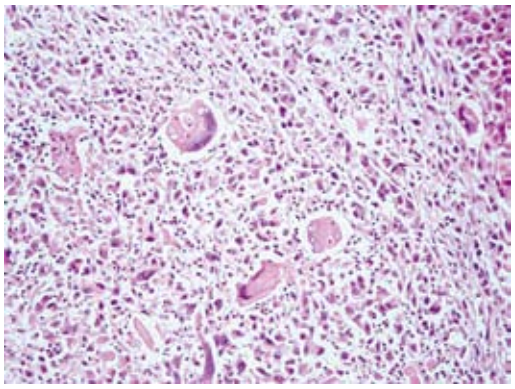

Figure 3 Microscopic appearance of a poorly differentiated HCC developed in liver cirrhosis. It was characterized by pleomorphic, multinucleated giant cells and extensive areas of tissue necrosis (HE, × 100).

DISCUSSION

Involvement of the GI tract by HCC is uncommon. In clinical HCC cases, the prevalence is 0.5% to 2% and in postmortem examination is discovered in about 10% of patients with HCC[2-4]. Chen *et al*[2], who found GI tract invasion by HCC in 8 of 396 patients (2%), were the first to report GI tract involvement by HCC during the course of the disease. The mode of metastasis was presumed to be hematogenous spread in 2 patients (to the stomach in one and the jejunum in the other) and direct invasion in 5 patients (invasion to the stomach in one and of the duodenum in four), but was undetermined in the remaining patient, in whom the stomach was involved.

In the English language literature, 30 patients with direct GI tract invasion from HCC, including the present one, have been found in 12 case reports and 4 retrospective studies[5-10] (Table 1).

The most frequent initial symptom was melena, which was documented in 18 patients[11,12]. Hematemesis was recognized in only 6 of 23 patients in whom tumors

Table 1 Characteristics of patients with HCC invading the GI tract (n = 30)

Characteristics	Data
Sex	
Male	29
Female	1
Age (yr)	
Mean	65.0 (32-73)
Clinical manifestations	
Melena	18
Hematemesis	6
Epigastric pain	4
Nausea/vomiting	4
Abdominal mass	1
Positive FOBT	6
Organs involved	
Duodenum	12
Stomach	9
Colon	7
Duodenum and colon	1
Stomach and pancreas	1
Size of tumor (cm)	
Mean	13.7
Range	4-22
Esophageal varices	
Present	6
Absent	15
Unknown	9
Etiology	
HBV	9
HCV	6
Alcoholic	6
Unknown	9
Treatment	
Surgical therapy	
Curative surgery	8
Palliative surgery	1
Nonsurgical therapy	11
Supportive therapy	10

FOBT: Fecal occult blood test.

involved the upper GI tract. The history of hematemesis points out the site of tissue invasion and the amount of bleeding from the tumor. In those cases, massive varicose hemorrhage from portal hypertension should be considered as an alternative source of hematemesis. Endoscopic studies are essential to determine the source of bleeding. In 19 of the 29 patients, in whom HCC involved directly the upper GI tract; initial endoscopic assessment revealed an ulcerative hemorrhagic tumor protruding into the lumen of the stomach or duodenum.

The most common site of invasion was the duodenum, followed by the stomach and colon[13,14]. This is the first reported case of a direct invasion of both the stomach and pancreas from extrahepatically growing HCC that causes upper GI tract obstruction rather than hemorrhage.

The presumed mode of direct involvement to the GI tract is initiated by the adhesion of the serosal side of the adjacent organ with a bulky, exophytic tumor[2,6]. Some authors[6,8] have noted that in several patients with direct HCC invasion of the GI tract, the patient had received some form of regional therapy [transarterial chemoembolization (TACE), intra-arterial chemotherapy (IA), either alone or in combination] because of unresectability and/or had experienced abdominal surgery. Accordingly, it was postulated that possible mechanisms underlying the direct GI tract involvement by HCC, other than as part of its natural course, may be TACE or IA chemotherapy-induced tumor necrosis, resulting in the promotion of subcapsular tumor adhesion to the GI serosa. Postoperative intraabdominal adhesions and scarring may account for the proximity of the GI tract to the tumor. Although no history of abdominal surgery or regional treatment was encountered, extensive necrosis found in the resected tumor specimen might partly explain its invading behavior in the presented case.

The median survival of patients who received curative surgery, nonsurgical treatment, and supportive therapy were 9.7, 3.0, and 1.2 mo, respectively[10,15]. The patients who had undergone oncologic surgery for cure survived for significantly longer compared to those receiving nonsurgical or supportive treatment, as strongly supported by the long disease-free survival of our patient. In light of the difficulty achieving relief of either bleeding or obstruction, surgical removal of such a tumor, together with involved structures, should be strongly considered.

Direct invasion of extrahepatically growing HCC to the GI tract is an unusual finding. Complete *en bloc* surgical resection of the tumor with negative margins may be the treatment of choice in order to control symptoms and to obtain oncologic cure in selected patients with an appropriate hepatic functional reserve.

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CASE REPORT

Endoscopic papillectomy of minor papillar adenoma associated with pancreas divisum

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Abstract

Tumors of the minor papilla of the duodenum are quite rare. We successfully and safely treated an 18-mm adenoma of the minor papilla associated with pancreas divisum using endoscopic papillectomy. A 64-year-old man was admitted to our hospital for treatment of an asymptomatic mass in the minor papilla detected by upper gastrointestinal endoscopy. Endscopic analysis showed an 18-mm, whitish, sessile mass, located in the duodenum proximal to a normal-appearing major papilla. Endoscopic retrograde pancreatography did not reveal the pancreatic duct. Magnetic resonance cholangiopancreatography showed a lack of the ventral pancreatic duct. We suspected this case was associated with pancreatic divisum; therefore, we performed endoscopic papillectomy of the minor papilla tumor. Subsequently, endoscopic pancreatic stent placement in the minor papilla was done to prevent drainage disturbance. The patient has been asymptomatic without recurrence of tumor or stenosis of the Santorini orifice upon endoscopic examination for the past 2 years.

Key words: Endscopic papillectomy; Minor papillar adenoma; Pancreas divisum; Endoscopic pancreatic stent; Endoscopic retrograde pancreatography

Peer reviewer: Yusuf Bayraktar, Professor, Department of Gastroenterology, School of Medicine, Hacettepe University, Ankara 06100, Turkey

Kanamori A, Kumada T, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Toyoda H, Kawashima H, Itoh A, Hirooka Y, Goto H. Endoscopic papillectomy of minor papillar adenoma associated with pancreas divisum. *World J Gastroenterol* 2009; 15(9): 1138-1140 Available from: URL: http://www.wjgnet.com/1007-9327/15/1138.asp DOI: http://dx.doi.org/10.3748/wjg.15.1138

INTRODUCTION

Tumors arising in the region of the major duodenal papilla account for 5% of gastrointestinal (GI) neoplasms and 36% of resectable pancreaticoduodenal tumors[1]. Adenoma is a particularly common finding in patients with familial adenomatous polyposis (FAP). However, currently, adenomas that involve the papilla have been recognized increasingly often, even in the absence of FAP. With the development of endoscopic tools, the safety and the efficacy of endoscopic papillectomy has improved, and indications for endoscopic papillectomy have recently been expanded[2-5]. Recently, endoscopic papillectomy has been accepted as a viable alternative therapy to surgery in sporadic ampullary adenoma and has yielded high success and low recurrence rates[5,6]. However adenoma of the minor papilla has been reported in only a few cases[7-9]. We report a case of endoscopic treatment of sporadic adenoma of the minor papilla associated with pancreas divisum.

CASE REPORT

A 64 year-old man was admitted to Ogaki Municipal Hospital for evaluation of an asymptomatic duodenal tumor that was found incidentally by X-ray examination of the stomach during a periodic health examination. The patient's medical history was otherwise unremarkable, and he had no family history of FAP. The laboratory findings were within normal limits, including tumor markers. Endoscopic analysis showed an 18-mm, whitish, elevated, slightly rough-surfaced mass, located in the descending duodenum proximal to

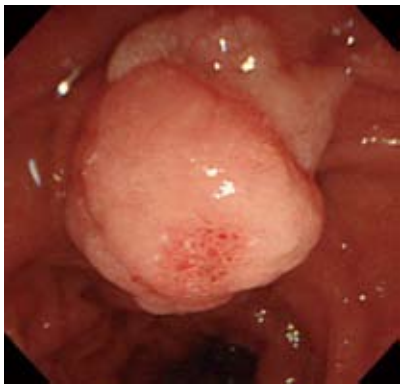

Figure 1 Endoscopy showing an 18-mm, whitish, elevated, slightly rough-surfaced mass, located proximal to the major papilla.

Figure 2 Hypotonic duodenography demonstrating a mass situated 15 mm proximal to the major papilla, which was raised highly from the duodenum.

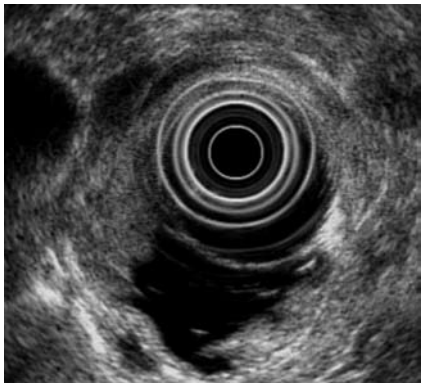

Figure 3 EUS detected an 18 mm × 12 mm homogeneous, hypoechoic mass in the submucosal layer.

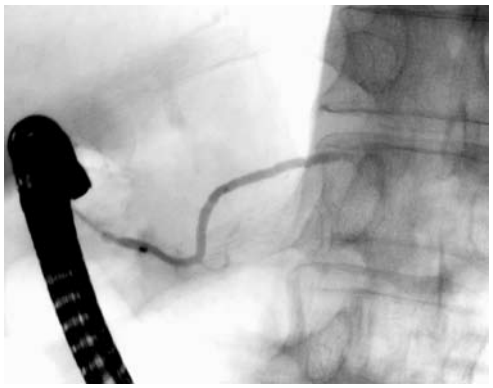

Figure 4 ERP was immediately performed *via* the minor papilla and it showed that the entire dorsal pancreatic ductal system was without communication with the ventral pancreatic duct.

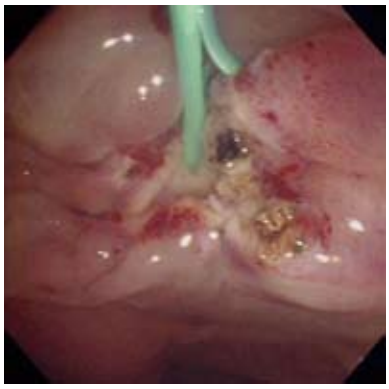

Figure 5 A pancreatic 5Fr stent was placed immediately after endoscopic papillectomy and coagulated the margin of the minor papilla tumor.

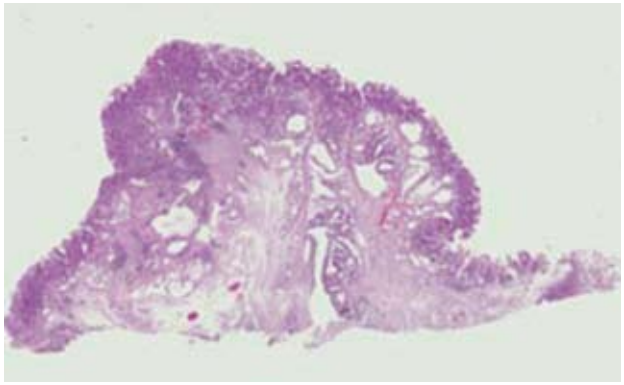

Figure 6 Histopathological findings of the specimen showed tubular adenoma and the margin of the tumor was negative; however, a slight infiltration of the pancreatic duct system was revealed (HE stain, × 4).

a normal-appearing major papilla (Figure 1). Histological examination of forceps biopsy specimens from the mass revealed a tubular adenoma with moderate epithelial atypia. Hypotonic duodenography (double contrast radiographic study) demonstrated a mass situated 15 mm proximal to the major papilla, which was raised highly from the duodenum (Figure 2). Endoscopic retrograde pancreatography (ERP) (JF-230; Olympus, Tokyo, Japan) did not reveal the pancreatic duct upon initial examination.

Magnetic resonance cholangiopancreatography (MRCP) showed the entire dorsal pancreatic duct and the lack of a ventral pancreatic duct. We suspected this case was associated with pancreas divisum and that the tumor had arisen from the minor duodenal papilla. Endoscopic ultrasonography (EUS) (GF-UM240; Olympus) detected an 18 mm × 12 mm homogeneous, hypoechoic mass in the submucosal layer (Figure 3). We further determined that the tumor was not invasive to the muscle layer. Endoscopic papillectomy of the minor papilla was performed after obtaining appropriate written informed consent. Submucosal injection of hypertonic saline-epinephrine (HSE) was carried out, and subsequently, snare excision was performed with polypectomy snare forceps. Following this, ERP was immediately performed *via* the minor papilla and it showed that the entire dorsal pancreatic ductal system was without communication with the ventral pancreatic duct (Figure 4). We placed a prophylactic pancreatic 5Fr stent (Figure 5) and endoscopic papillectomy was

performed successfully without any procedure-related complications. The resected specimen showed tubular adenoma with moderate epithelial atypia in the mucosal layer. However, the margin of the tumor was negative, with a slight infiltration of the pancreatic duct system (Figure 6). One week later, duodenoscopy was performed and no evidence of remaining tumor was seen, and the pancreatic stent was withdrawn. For the last 2 years, the patient has been asymptomatic without evidence of tumor recurrence or stenosis of the pancreatic duct orifice, based on endoscopic examinations performed every 6 mo.

DISCUSSION

Endoscopic papillectomy of minor papilla tumors has been reported in only a few cases. Adenoma of the minor papilla associated with pancreas divisum is particularly rare and has been reported previously only once by Nakamura *et al*[8]. We think that duodenal and periampullary tumors occur in the general population, although patients with FAP invariably develop duodenal adenomas and have a risk of papillary carcinoma[10]. However, with the development of endoscopic tools and techniques, papillectomy has been accepted as a safe and feasible treatment for adenoma of the major papilla. It is important to diagnose tumor ductal infiltration correctly to determine endoscopic respectability by using intraductal ultrasonography (IDUS)[11]. In the present case, because ERP *via* the major and minor papilla was unsuccessful before the treatment of minor papillary tumors, we performed EUS before treatment and diagnosed the tumor as non-invasive to the muscle layer. EUS is a highly accurate and non-invasive modality for staging ampullary neoplasms and for evaluating ductal involvement by a tumor[12]. However, it is also essential to accurately diagnose tumors, adenoma or early cancer as not infiltrating Oddi's muscle layer. Therefore, we think it necessary to undertake IDUS before treatment of minor tumors of the papilla as frequently as possible.

In the present case, we injected HSE into the submucosal layer upon endoscopic papillectomy to reduce the risk of perforation. It is uncertain whether submucosal injections reduce the risk of perforation upon endoscopic papillectomy of a tumor of the major papilla. We think it safer to inject HSE into the submucosal layer upon endoscopic papillectomy of the tumor of the minor papilla. Many authors have reported the efficacy of pancreatic stents for decreasing both post-procedure pancreatitis and stenosis[13,14]. In the present case, the minor papilla drained all of the pancreatic juice flow from the dorsal pancreas. Therefore, we considered the placement of a pancreatic stent to be essential after endoscopic papillectomy of the minor papillary tumor.

We performed follow-up duodenoscopy and computed tomography at 3, 6, 12 and 18 mo later, and there was no evidence of tumor recurrence or stenosis of the orifice of the Santorini duct for more than 2 years. Some authors have reported that long-standing pancreatic duct obstructions caused by relative stenosis of the minor duodenal papilla might be a factor promoting oncogenesis[15]. We will be performing a follow-up study of recurrence of minor papillary tumors and careful surveillance of the duodenum and pancreaticobiliary system.

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CASE REPORT

Intrapancreatic accessory spleen: A case report and review of the literature

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Author contributions: Guo W and Han W contributed equally to this work; Liu J, Jin L and Li JS performed the operation; Zhang ZT and Wang Y contributed to therapeutic decision.

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Abstract

Here, we report a case of intrapancreatic accessory spleen confirmed by pathologic diagnosis and discuss its differential diagnosis and surgical management with a review of the literature.

Key words: Accessory spleen; Pancreas; Differential diagnosis; Surgical management; Congenital defect

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Guo W, Han W, Liu J, Jin L, Li JS, Zhang ZT, Wang Y. Intrapancreatic accessory spleen: A case report and review of the literature. *World J Gastroenterol* 2009; 15(9): 1141-1143 Available from: URL: http://www.wjgnet.com/1007-9327/15/1141.asp DOI: http://dx.doi.org/10.3748/wjg.15.1141

INTRODUCTION

Accessory spleen, a relatively common congenital defect, found in 10%-30% of patients at autopsy, is due to the fusion failure of the splenic anlage, which is located in the dorsal mesogastrium[1-3]. The splenic hilus is the most common site of an accessory spleen followed by pancreatic tail. When an accessory spleen is located in the pancreas, it may mimic a hypervascular pancreatic tumor. Because an accessory spleen does not usually require treatment, accurate preoperative diagnosis is important. Here, we report an intrapancreatic accessory spleen and discuss its differential diagnosis and surgical management.

CASE REPORT

A 51-year-old man without any abdominal discomfort or complaints had a health examination in our hospital in January 2008. Physical examination and laboratory data including peripheral blood counts, blood sugar and liver function tests were all unremarkable. Tumor markers, including CA19-9, CA125, carcino-embryonic antigen (CEA) and alpha-fetoprotein (AFP), were within the normal range. Abdominal sonography was performed, and a well-defined 4.2 cm × 5.2 cm focal lesion was noted in pancreatic tail. The echogenicity of the lesion was homogeneous and lower than that of the pancreatic parenchyma. On color Doppler sonography images, blood echo was observed in the lesion (Figure 1). Computed tomography (CT) confirmed the 4.0 cm × 5.1 cm mass in pancreatic tail (Figure 2). A nonfunctioning islet cell tumor or a solid pseudopapillary neoplasm of pancreatic tail was suspected on January 14, 2008, a laparotomic exploration was arranged to treat the lesion. After anesthesia, a left subcostal incision was performed and the well-defined mass was palpated in pancreatic tail. Because it was very close to the splenic hilus, excision of pancreatic tail and spleen was performed (Figure 3). An accessory spleen was confirmed by pathologic diagnosis (Figure 4).

DISCUSSION

Accessory spleen is a congenital focus of healthy splenic tissue that is separated from the main body of spleen[1]. It results from the fusion failure of splenic anlage, which is located in the dorsal mesogastrium to fuse[2,3]. Accessory spleen, a relatively common congenital defect, is seen in 10%-30% of patients at autopsy[1,2,4]. Splenic hilus is the most common site of an accessory spleen followed by pancreatic tail. In an autopsy study of 3000 patients, 61 of 364 (17%) accessory spleens identified were found

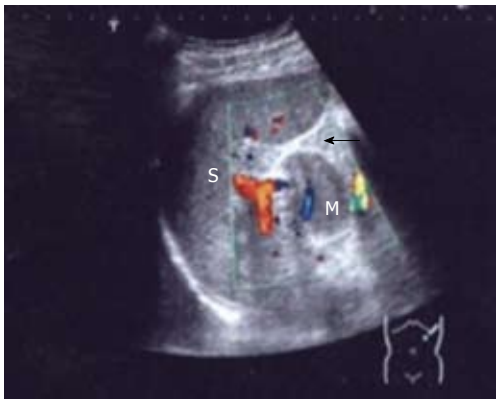

Figure 1 Transverse grayscale sonography image showing a homogeneous and isoechoic nodule (M) in pancreatic tail (arrow) near the spleen hilus with its echogenicity similar to that of the spleen (S).

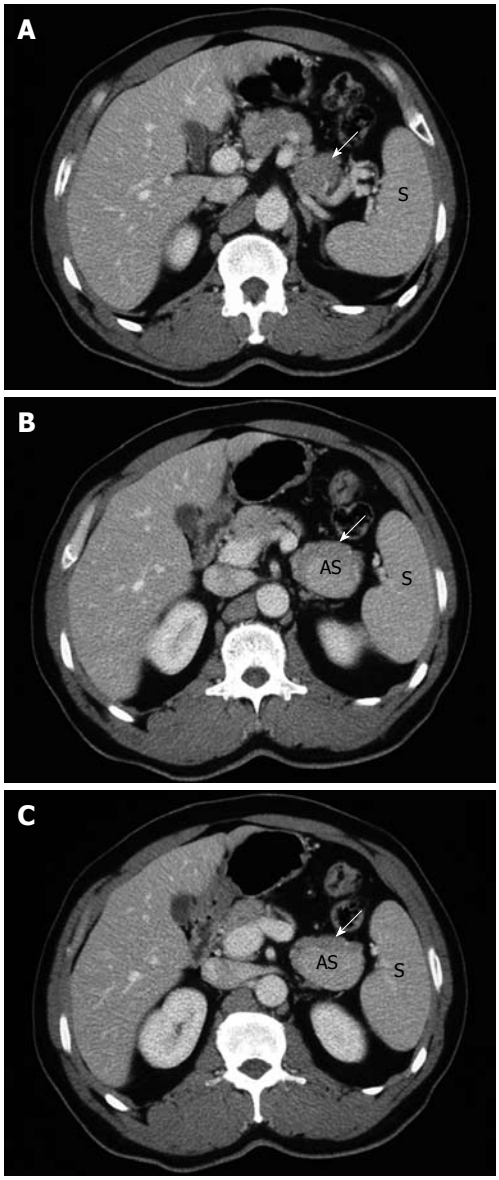

Figure 2 Axial CT image obtained in the portal venous phase showing an ovoid, well-enhanced nodule (AS) in pancreatic tail (arrow). The density of this lesion was higher than that of the pancreatic parenchyma and similar to that of the spleen (S). However, this finding is similar to that of pancreatic hypervascular tumors.

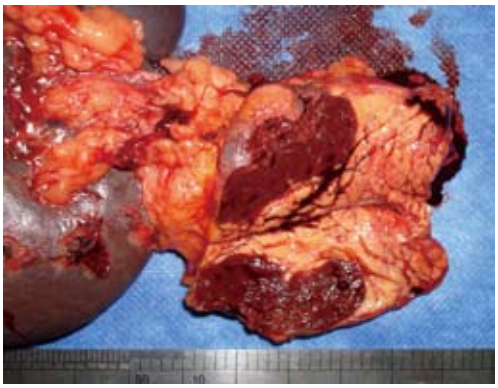

Figure 3 Excision of pancreatic tail and spleen with a well-defined lesion found in pancreatic tail.

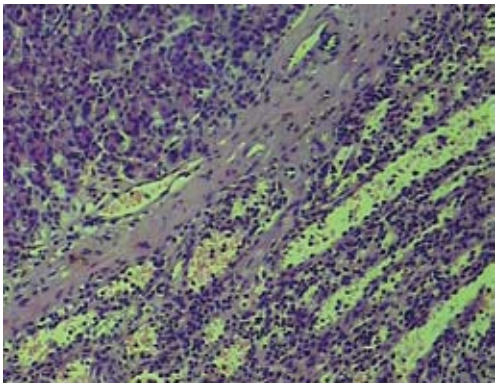

Figure 4 An intrapancreatic accessory spleen confirmed by histological imaging (HE, × 100).

in pancreatic tail[5]. Although an accessory spleen usually appears as an isolated asymptomatic abnormality, it may have clinical significance in some situations. When an accessory spleen is located in the pancreas, it may mimic an islet cell tumor or a pseudopapillary neoplasm[6-8]. Because an accessory spleen does not usually require treatment, accurate preoperative diagnosis is important.

At present, the main preoperative diagnostic technique for intrapancreatic accessory spleen is radiological. On baseline sonography, intrapancreatic accessory spleens are well defined and round, ovoid or lobulated. The echogenicity of most intrapancreatic accessory spleens is low compared with pancreatic parenchyma. In all intrapancreatic accessory spleens, the echogenicity is homogeneous and identical to that of the main spleen. On color or power Doppler sonography images, blood supply to intrapancreatic accessory spleens from splenic artery or vein can be seen in some patients.

In recent years, contrast-enhanced ultrasound has been more frequently used to diagnose spleen abnormalities. Levovist is an ultrasound contrast agent containing air microbubbles. In the vascular phase, it increases signal intensities in both grey-scale and Doppler modes. In the delayed phase, also known as "hepatosplenic phase", microbubbles are trapped almost exclusively by the hepatic and splenic parenchyma. By increasing the acoustic pressure, the

trapped microbubbles are disrupted and produce a non-correlated Doppler signal, which can be visualized as a strong transient enhancement. This method is also known as sonoscintigraphy, loss of correlation, stimulated acoustic emission and transient scattering[9]. Kim *et al*[10] used this technique to study 6 patients with accessory spleen. In the vascular phase, the vascular pedicle was clearly visualized in 3 patients, including a patient with a suspected vascular pedicle on color Doppler sonography images. In the arterial phase, there was an inhomogeneous enhancement in 3 patients and a homogeneous enhancement in the other three patients. In all the 6 patients, the intrapancreatic accessory spleen became homogeneous in the portal phase, showing a dense and persistent enhancement for 3-5 min. In comparison with pancreatic parenchyma, the intrapancreatic accessory spleen appeared to be hyperechoic during all dynamic sonography phases. The echo enhancement of all intrapancreatic accessory spleens, however, was identical to that of the spleen in all phases.

Mortelé *et al*[11] performed abdominal CT scans on 1000 consecutive patients. Of these patients, 156 (15.6%) had at least one accessory spleen, and 21 of these patients (13%) had more than one accessory spleen, with a maximum of three accessory spleens per patient, resulting in a total of 180 accessory spleens. Their anteroposterior diameter ranged 4-29 mm, with a mean of 11.9 mm. Their transverse diameter ranged 4-25 mm, with a mean of 11.6 mm. All accessory spleens were well defined, round in 141 patients (78.3%), ovoid in 27 (15%) and triangular in 12 (6.7%). The location of accessory spleens was variable. Most accessory spleens were located at the hilus of spleen. Intrapancreatic accessory spleens were seen in 2 patients. The findings suggest that most accessory spleens have a characteristic appearance on CT, and are well-defined, round masses that are smaller than 2 cm in diameter. Homogeneous enhancement on contrast-enhanced images is another important feature. However, in these case series, 32% of the accessory spleens were hypodense compared with the main spleen. Because all the accessory spleens were smaller than 1 cm in diameter, their attenuation may have been caused by partial volume effects. It is likely that, when thinner collimation (e.g. $\leqslant$ 5 mm) is used, accessory spleens appear similar to the spleen.

The most specific imaging method for diagnosing ectopic splenic tissue is nuclear scintigraphy using technetium-99m-labeled sulfur colloid or 99mTc-labeled heat-damaged RBCs. However, this technique offers far inferior anatomic resolution to CT or MRI, increasing likelihood of misdiagnosis[7].

RES-specific contrast media are particles, such as superparamagnetic iron oxides, that are phagocytosed by the reticuloendothelial system (liver, spleen, lymph nodes, and bone marrow). Resovist-enhanced imaging can show an uptake of the contrast agent in normal splenic parenchyma because of its endothelial and Kupffer's cells. Negative enhancement or loss of signal intensity of normal splenic parenchyma is delineated using T2-weighted images because of the more effective T2 shortening in these sequences[12].

Endoscopic ultrasound (EUS)-guided fine-needle aspiration (FNA) biopsy is a very sensitive test, particularly for the evaluation of pancreatic lesions. Schreiner[13] and his colleagues reported three cases of intrapancreatic accessory spleen diagnosed by EUS-guided FNA, which revealed predominantly small lymphocytes with a subset of histiocytes, conspicuous eosinophils, and plasma cells. There was also characteristic CD8 positive immunostaining of endothelial cells in cell block sections.

In conclusion, when an asymptomatic intra-pancreatic mass is detected, the possibility of an accessory spleen should be considered. Although some radiological techniques are not widely used in clinics, well-defined round masses in pancreas should be considered accessory spleens, especially when its contrast-enhanced images are similar to those of the splenic parenchyma during all dynamic phases.

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CASE REPORT

Malrotation causing duodenal chronic obstruction in an adult

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Abstract

Congenital duodenal obstruction is rare in adulthood. An unusual presentation of this condition has led to difficult preoperative diagnosis. We present a case of proximal jejunal obstruction by a congenital band in an adult and review the literature.

Key words: Malrotation; Duodenal obstruction; Surgical procedures; Congenital band; Adult

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INTRODUCTION

Midgut malrotation is an anomaly of fetal intestinal rotation that usually presents in the first month of life. It is rare in adulthood. Congenital duodenal obstruction (atresia or stenosis) is associated with various congenital anomalies[1,2]. Midgut malrotation is a congenital anomaly referring to either lack of or incomplete rotation of the fetal intestines around the axis of the superior mesenteric artery during fetal development[3]. Most patients present with bilious vomiting in the first month of life because of duodenal obstruction or a volvulus. The true incidence in adults is difficult to estimate because most patients remain asymptomatic and their conditions are, therefore, never diagnosed. A literature review by von Flüe *et al*[4] cites 40 cases from 1923 to 1992. Approximately 90% of patients with malrotation are diagnosed within the first year of life, of whom 80% are diagnosed within the first month of life[4]. Surgical therapy remains the mainstay of treatment regardless of age at presentation. The most commonly used approach is the Ladd procedure, which involves counterclockwise reduction of the volvulus if present, division of any coloduodenal bands, widening of the mesenteric base to prevent repeated volvulus, and prophylactic appendectomy[5]. We present a case of malrotation in an adult who presented with chronic abdominal pain.

CASE REPORT

A 21-year-old woman was admitted to our hospital with a 15-year history of postprandial epigastric pain. Pain relieved after vomiting. The presentations since her teenage years with similar symptoms had failed to identify the cause of her pain. She had no changes in bowel habits, and no significant past medical history. On physical examination, the patient's vital signs were pulse 72, and regular, blood pressure 108/76, respirations 18, and temperature 36.8℃. She was a normally-developed and in no acute distress. Her abdomen was minimally distended on inspection. A succession splash was readily elicited. Normal bowel sounds were auscultated. She exhibited no peritoneal signs. She denied any history of disease and no history of abdominal surgery; her family history was negative for GI disease. She was on no current medications and denied alcohol or tobacco use. On admission, her rectal examination was normal, and her stool occult test was negative. Hemoglobin, white blood cell count, and basic chemical panel were all within normal values. Abdominal X-ray showed dilatation of the stomach and the proximal part of the duodenum. A computed abdominal tomography taken 6 mo before was normal. Upper gastrointestinal contrast studies showed the duodenum not crossing the lumbar spine. The entire small bowel was noted to be sequestered on the right side of the abdomen (Figure 1) Abdominal decompression was accomplished after placement of a nasogastric tube. The patient underwent

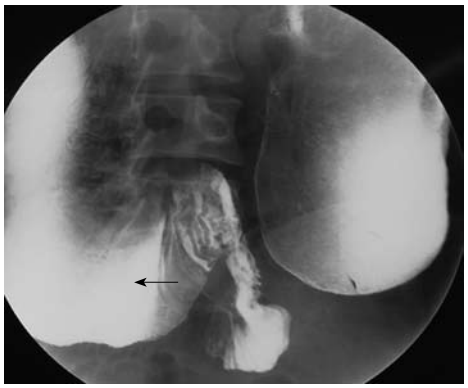

Figure 1 Barium image from upper gastrointestinal series reveals duodenal obstruction, demonstrating high-grade stenosis of the fourth portion of the duodenum and extreme dilation of proximal duodenum (arrow).

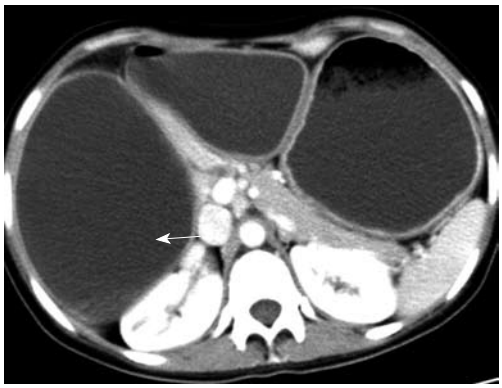

Figure 2 CT scan shows duodenum does not cross behind the superior mesenteric artery and the superior mesenteric vein, and extreme dilation of proximal duodenum (arrow).

aggressive fluid and electrolyte resuscitation, and parenteral nutrition was instituted for a suspected partial duodenal obstruction. On day 4 after admission to the hospital, the patient underwent abdominal exploration through a midline laparotomy, and was found to have a massively dilated stomach and proximal duodenum, and intestinal malrotation was confirmed. The small bowel and duodenum were on the right, with the transverse and descending colon positioned in the right upper quadrant. The duodenum was not posterior to the superior mesenteric artery, compressed between the peritoneal bands superiorly. Cecal bands attaching to the duodenum were immediately noted (Figure 2). The cecum was subsequently returned to the left abdomen. The appendix was not removed. The patient underwent the Ladd's procedure. Upon entering the abdomen, bands were lysed, and the duodenum and right colon were mobilized. Adhesions surrounding the superior mesenteric artery were also lysed (Figure 3). Postoperatively, the patient did well, tolerated a regular diet on postoperative day 4. The patient's postoperative course was uncomplicated. She was discharged from hospital on postoperative day 8. There were no complications with the surgery and the patient made a full and uneventful recovery and no recurrence was found after 4 mo of follow-up.

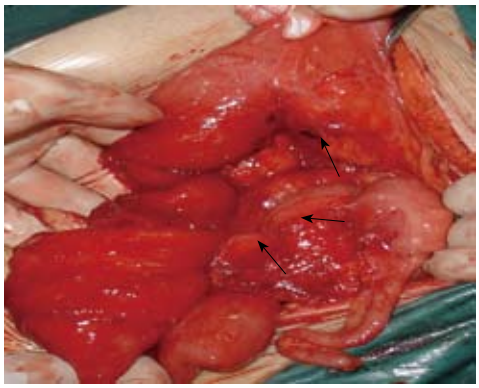

Figure 3 One band running from the anti-mesenteric wall of proximal jejunum to cecum has been lysed (arrows) and no Treitz's ligament is found.

DISCUSSION

The embryology of malrotation was described by Mall in 1898[6]. It was decribed in detail in 1923 by Dott. Intestinal malrotation was further classified by the specific embryologic abnormalities. Rotational abnormalities of the intestine occur when the normal embryologic rotation and fixation of the intestinal mesentery fail to take place[7]. Although the true incidence of intestinal rotation disorders is unknown, autopsy studies estimate that it may be as high as 1% of the total population[8]. Congenital anomalies of intestinal rotation are often seen in infants and children; however, they are uncommon in adults[9]. Yanez and Spitz[10] reported that only 50%-70% of patients actually present during the first 4 wk of life. In either event, there are a substantial number of discovered cases of malrotation that present after the neonatal period. In adults, it may cause chronic, but nebulous symptoms that are often difficult to diagnose. Adult presentation of malrotation is a difficult diagnosis because of the low incidence of the disorder. Patients with intestinal malrotation who were not diagnosed until adulthood may present with a variety of chronic symptoms, including nausea, vomiting, diarrhea, vague abdominal pain, early satiety and bloating, dyspepsia, and peptic or duodenal ulcer disease. Unfortunately, many patients never receive surgical referral and are instead labeled with functional or psychiatric disorders[11]. There may be a significant number of patients with malrotation who were undetected in the neonatal period either because they were asymptomatic, or because their symptoms were mild and misinterpreted. As these patients grow into adolescence and adulthood, they may continue to have misinterpreted symptoms, remain asymptomatic, or present with new onset of acute or chronic symptoms later in life, as did our patient. Adults, however, present with vague symptoms such as vomiting (bilious or non-bilious), weight loss, and recurrent or colicky abdominal pain (often postprandial)[10,12,13]. Intestinal obstruction, diarrhea, malabsorption, peritonitis, and septic shock also have been reported in the adult group[12]. Timing and frequency of the pain also can be variable[14]. It is these vague symptoms along with the relative rarity of adult

presentation of malrotation that often lead to a delay in diagnosis. In recent years, there has been increasing recognition of the various CT findings associated with malrotation in adults, leading to enhanced diagnostic accuracy[15]. In addition, the number of adults with malrotation misdiagnosed with non-abdominal (including psychiatric) pathology only reinforces the importance of obtaining routine imaging studies when the cause of chronic intermittent abdominal pain is unclear[16]. Generally, barium duodenography may still play an important role in the diagnosis of duodenal disorders. For the best management of duodenal diseases, barium studies in combination with cross-sectional imaging modalities may offer detailed evaluation of the duodenum and its surrounding organs. However, CT, US and MRI all provide excellent cross-sectional anatomic orientation, which allows accurate pre-operative evaluation[17]. Surgical therapy remains the mainstay of treatment regardless of age at presentation. For this reason, it is crucial that all surgeons operating on adult patients have firm knowledge of intestinal embryology and its anatomic variations. The most commonly used approach is the Ladd procedure, which involves counterclockwise reduction of the volvulus if present, division of any coloduodenal bands, widening of the mesenteric base to prevent repeated volvulus, and prophylactic appendectomy[5]. Although symptomatic malrotation after infancy requires prompt recognition and treatment, many patients with malrotation may remain asymptomatic into adulthood. Symptomatic or autopsy findings of malrotation were identified at a rate of 3 per 10 000 (0.03%) in a population-based birth defects study[18]. In 1996, a minimally invasive laparoscopic method was developed for performing Ladd's procedure in the case of malrotation without vo1vu1us[19].

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LETTERS TO THE EDITOR

Lubiprostone: Clinical applications beyond constipation

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Abstract

In comparison to polyethylene glycol, lubiprostone offers other advantages and is increasingly being used as an adjunctive agent in diagnostic as well as management strategies not only in gastroenterology, but in other fields. For instance, lubiprostone exerts beneficial effects in cystic fibrosis tissues. It augmernts the chloride secretion in these cells by activating non-cystic fibrosis transmembrane regulator (CFTR) secretion of chloride by afflicted respiratory epithelia. Lubiprostone also seems to improve visualization of the gastrointestinal tract during procedures such as colonoscopy. This is especially true if the lubiprostone is administered prior to bowel cleansing with agents such as polyethylene glycol electrolyte (PEG-E). Lubiprostone also enhances and stimulates contraction in colonic as well as gastric muscles and may thus further contribute as a prokinetic agent. Besides these effects, lubiprostone also causes hyperpolarization in other tissues such as uterine muscle cells. This may prove to be of significant clinical benefit in the management of uterine pathologies in the near future.

Key words: Lubiprostone; Cystic fibrosis; Colonoscopy; Uterine muscle; Prokinetic agent

Kapoor S. Lubiprostone: Clinical applications beyond constipation. *World J Gastroenterol* 2009; 15(9): 1147 Available from: URL: http://www.wjgnet.com/1007-9327/15/1147.asp DOI: http://dx.doi.org/10.3748/wjg.15.1147

TO THE EDITOR

I read with great interest the recent article by Moeser *et al*[1]. The authors have provided an interesting comparison of lubiprostone and polyethylene glycol. In comparison with polyethylene glycol, lubiprostone offers other advantages and is increasingly being used as an adjunctive agent in diagnostic as well as management strategies not only in gastroenterology, but in other fields.

For instance, lubiprostone exerts beneficial effects in cystic fibrosis tissues. It augments the chloride secretion in these cells by activating non-cystic fibrosis transmembrane regulator (CFTR) secretion of chloride by afflicted respiratory epithelia[2]. Lubiprostone also seems to improve visualization of the gastrointestinal tract during procedures such as colonoscopy. This is especially true if the lubiprostone is administered prior to bowel cleansing with the agents such as polyethylene glycol electrolyte (PEG-E)[3]. Lubiprostone also enhances and stimulates contraction in colonic as well as gastric muscles and may, thus, further contribute as a prokinetic agent[4]. Besides these effects, lubiprostone also causes hyperpolarization in other tissues such as uterine muscle cells[5]. This may prove to be of significant clinical benefit in the management of uterine pathologies in the near future.

It is clear from the above examples that lubiprostone has an array of clinical features that may enhance its clinical application in gastroenterology. Further studies are needed to evaluate lubiprostone as an effective agent for the management of other diseases besides constipation.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1179 experts in gastroenterology and hepatology from 60 countries.

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The major task of *WJG* is to rapidly report the most recent results in basic and clinical research on gastroenterology, hepatology, endoscopy and gastrointestinal surgery fields, specifically including autoimmune, cholestatic and biliary disease, esophageal, gastric and duodenal disorders, cirrhosis and its complications, celiac disease, dyspepsia, gastroesophageal reflux disease, esophageal and stomach cancers, carcinoma of the colon and rectum, gastrointestinal bleeding, gastrointestinal infection, intestinal inflammation, intestinal microflora and immunity, irritable bowel syndrome; liver biology/pathobiology, liver failure, growth and cancer; liver failure/cirrhosis/portal hypertension, liver fibrosis; *Helicobacter pylori*, hepatitis B and C virus, hepatology elsewhere; pancreatic disorders, pancreas and biliary tract disease, pancreatic cancer; transplantation, genetics, epidemiology, microbiology and inflammatory disorders, molecular and cell biology, nutrition; geriatric gastroenterology, pediatric gastroenterology, steatohepatitis and metabolic liver disease; diagnosis and screening, endoscopy, imaging and advanced technology.

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insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/14.doc.

Abbreviations

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

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