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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

^[3]Passed away on June 14, 2008



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INTRODUCTION

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

Kathie Béland, Pascal Lapierre, Fernando Alvarez

Kathie Béland, Pascal Lapierre, Fernando Alvarez, Division of Gastroenterology, Hepatology and Nutrition, CHU Sainte-Justine, 3175 Côte Ste-Catherine, Montreal (Quebec) Canada H3T 1C5, Canada

Author contributions: Béland K wrote the paper; Lapierre P and Alvarez F helped writing and revising the paper.

Correspondence to: Fernando Alvarez, MD, Division of Gastroenterology, Hepatology and Nutrition, CHU Sainte-Justine, 3175 Côte Ste-Catherine, Montreal (Quebec) Canada H3T 1C5, Canada. fernando.alvarez@umontreal.ca

Telephone: +1-514-3454626 Fax: +1-514-3454999

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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.

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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,

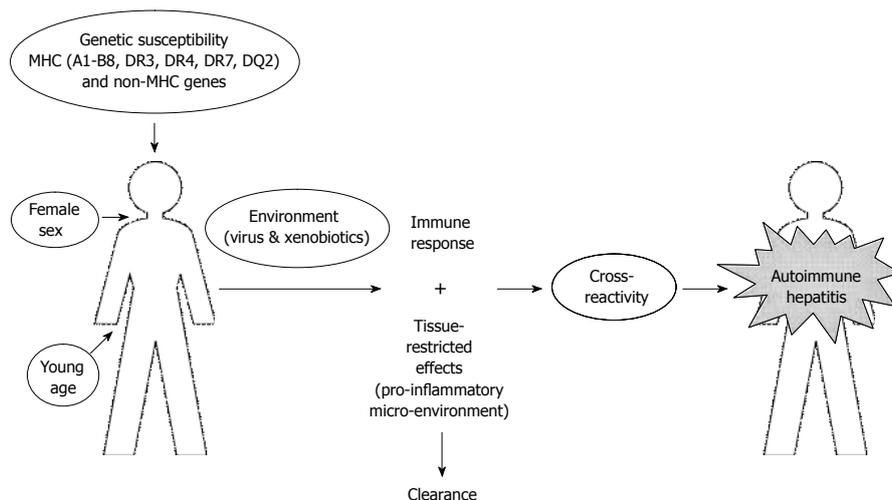


Figure 1 Pathogenesis of autoimmune hepatitis (molecular mimicry hypothesis). AIH can occur in an individual of a particular sex and age with a genetic background of susceptibility. To develop AIH, this individual must encounter an environmental trigger such as an infection or exposure to a xenobiotic. 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

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].

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

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]. Xenoinmunisation 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, xenoinmunisation 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. 17 β -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*, 17 β -estradiol (E2) protects C57BL/6 mice from experimental autoimmune encephalomyelitis (EAE)^[57]. However, 17 β -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 lectin 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 to the 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-bromohexanoate 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-bromohexanoate. 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 hypothetical 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 of 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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Update on autoimmune hepatitis

Andreas Teufel, Peter R Galle, Stephan Kanzler

Andreas Teufel, Peter R Galle, Department of Medicine I, Johannes Gutenberg University, Mainz 55099, Germany

Stephan Kanzler, Department of Medicine II, Leopoldina Hospital, Schweinfurt 97422, Germany

Author contributions: Teufel A, Galle PR, and Kanzler S all contributed equally to literature search and manuscript preparation.

Correspondence to: Stephan Kanzler, MD, Department of Medicine II, Leopoldina Hospital, Gustav Adolf Strasse 8, Schweinfurt 97422, Germany. skanzler@leopoldina.de

Telephone: +49-9721-7302482 Fax: +49-9721-7302484

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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.

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

Peer reviewers: Yun Ma, MD, PhD, Doctor, Institute of Liver Studies, King's College Hospital, Denmark Hill, London SE5 9RS, United Kingdom; Satoshi Yamagiwa, MD, PhD, Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori, Chuo-ku, Niigata, 951-8510, Japan

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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 anti-neutrophil 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 "plasmacell 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 maintenance 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 budenosis in combination with azathioprine 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 ASSOCIATIONS

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 screened 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, autoantibodies (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

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 criterion. 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.

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).

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 independent 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 efficacy 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 significant 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 budesonide may highly beneficial if its efficiency were comparable to the conventional prednisolone. Over the past decade, several smaller studies have suggested

the efficacy of budesonide for remission induction^[45]. However, one study failed to demonstrate such efficacy in AIH^[46].

Recently, Manns *et al*^[47] have compared combined budesonide 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, hirsutism, striae and glaucoma.

Budesonide 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 budesonide group reached the predefined primary endpoint of biochemical remission without typical adverse effects of steroids (47% *vs* 18.4%, $P < 0.00001$). Furthermore, for secondary endpoints, especially biochemical remission, budesonide 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, budesonide 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, budesonide 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 budesonide 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

Harry HX Xia, PhD, MD, Series Editor

Spontaneous bacterial peritonitis

Anastasios Koulaouzidis, Shivaram Bhat, Athar A Saeed

Anastasios Koulaouzidis, Endoscopy Unit, Centre of Liver & Digestive Disorders, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh, Scotland, EH16 4SA, United Kingdom

Shivaram Bhat, Gastroenterology, Belfast Trust Matter Hospital, 47-51 Crumlin Road, Belfast, Northern Ireland, BT14 6AB, United Kingdom

Athar A Saeed, Gastroenterology Department, Queen Elizabeth Hospital, Gateshead, Tyne & Wear, NE9 6SX, United Kingdom

Author contributions: Koulaouzidis A, Bhat S and Saeed AA contributed equally to this work; Koulaouzidis A designed the review, wrote the introduction, pathogenesis, laboratory diagnosis, and variants, and had overall supervision; Bhat S wrote management; Saeed AA wrote clinical manifestations.

Correspondence to: Anastasios Koulaouzidis, MD, MRCP, Endoscopy Unit, Centre of Liver & Digestive Disorders, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh, Scotland, EH16 4SA,

United Kingdom. akoulaouzidis@hotmail.com

Telephone: +44-131-2421603 Fax: +44-131-2421618

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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

Peer reviewer: Diego Garcia-Compean, MD, Professor, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, N.L. Mexico

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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°C 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%

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

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(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.

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

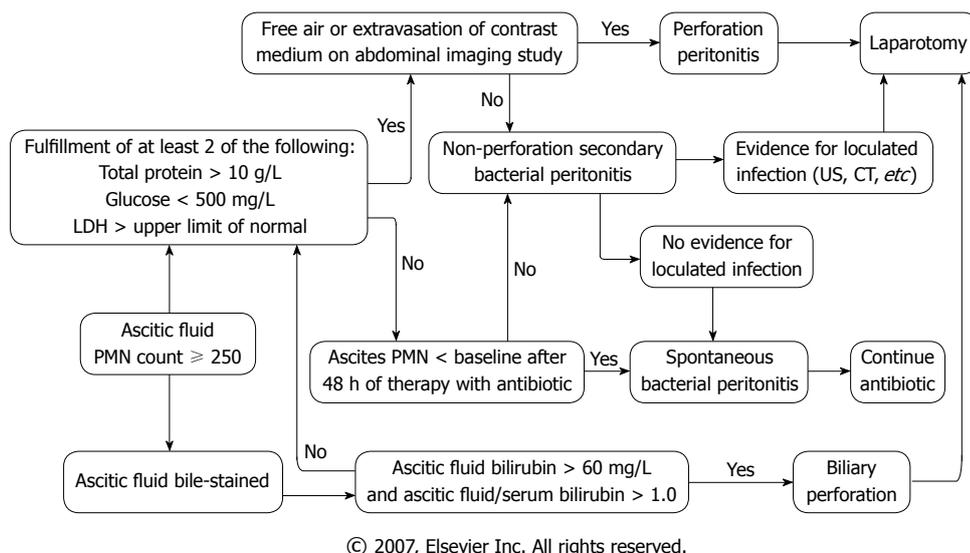


Figure 1 Algorithm for differentiating spontaneous from secondary bacterial peritonitis in patients with neutrocytic ascites (i.e. neutrophil count of 250 cells/mm³ or greater) in the absence of hemorrhage into ascitic fluid, tuberculosis, peritoneal carcinomatosis, or pancreatitis. CT: Computed tomography; LDH: Lactate dehydrogenase; PMN: Polymorphonuclear neutrophil; US: Ultrasound (Reproduced with permission from Akriadias 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 $\geq 250/\text{mL}$ ^[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 $\geq 10\,000/\text{mL}$), 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 ≤ 7.35 and blood-ascitic fluid odds ratio (OR) ≥ 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 $< 250/\text{mm}^3$ 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 $> 250/\text{mm}^3$, the current recommendation is to treat as for SBP. If the PMN count is $< 250/\text{mm}^3$, but the second set of cultures is positive, treat again as for SBP. If the PMN count is $< 250/\text{mm}^3$ 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 $> 250/\text{mm}^3$ but fluid cultures fail to grow any bacteria. It is considered to represent the expected 20% failure rate of culture to isolate microorganisms,

Table 2 Pathogens in ascitic fluid infection

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

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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; ²Amoxicillin-clavulanic acid. © 2007, BMJ publishing group, all rights reserved.

and it requires antibiotic treatment as if it were SBP. However, the term is now considered obsolete^[28,55].

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 amoxicillin/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

Harry HX Xia, PhD, MD, Series Editor

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

Ana Luiza Werneck-Silva, Ivete Bedin Prado

Ana Luiza Werneck-Silva, Casa da AIDS-Infectious Disease Division, School of Medicine, University of São Paulo, Rua Frei Caneca 255, CEP:05403-000, São Paulo, Brazil

Ivete Bedin Prado, University of São Paulo, Hospital das Clínicas da FMUSP Av. Ovídio Pires de Campos, 225, 6 andar CEP:05403-010, São Paulo, Brazil

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Correspondence to: Ana Luiza Werneck-Silva, Rua Ourânia 100 apto 91, CEP:05445-030, São Paulo,

Brazil. alwerneck@uol.com.br

Telephone: +55-11-38148919 Fax: +55-11-30919308

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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 be 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/mm³). 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.

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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/mm³ 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 as 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/ μ L) 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 immunosuppression.

***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 $CD4 \leq 200$ cells/mm³^[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/mm³; mean = 154.3 cells/mm³), 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 immunosuppressed 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 bienersi* 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. *Histoplasma 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 immunosuppressed (mean CD4 count 154.3 cells/mm³), 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/mm³ and upper GI complaints should parallel those of any other patient when immunodeficiency is not advanced. Patients with CD4 ≤ 200 cells/mm³, 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

Ashok K Singh, Nishit Pancholi, Jilpa Patel, Natalia O Litbarg, Krishnamurthy P Gudehithlu, Perianna Sethupathi, Mark Kraus, George Dunea, Jose AL Arruda

Ashok K Singh, Krishnamurthy P Gudehithlu, Mark Kraus, George Dunea, Jose AL Arruda, Department of Medicine, Stroger Hospital of Cook County, Chicago IL 60612, United States
Ashok K Singh, Nishit Pancholi, Jilpa Patel, Krishnamurthy P Gudehithlu, Perianna Sethupathi, Mark Kraus, George Dunea, Hektoen Institute of Medicine, Chicago IL 60612, United States

George Dunea, Jose AL Arruda, University of Illinois at Chicago and the Chicago VAMC, Chicago IL 60612, United States

Natalia O Litbarg, Perianna Sethupathi, Loyola-Hines Medical Center, Maywood IL 60612, United States

Author contributions: Singh AK, Arruda JAL, Gudehithlu KP and Kraus M designed the experiments; Pancholi N, Patel J, Gudehithlu KP, Litbarg NO and Sethupathi P performed the experiments; Litbarg NO contributed new reagents/analytic tools; Singh AK, Arruda JAL, Gudehithlu KP and Pancholi N analyzed the data; Singh AK, Dunea G and Arruda JAL wrote the paper.

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Correspondence to: Ashok K Singh, PhD, Stroger Hospital of Cook County, 637 South Wood St, Durand Bldg 2nd Floor, Chicago IL 60612, United States. singhashok@comcast.net

Telephone: +1-312-8644613 Fax: +1-312-8649569

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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.

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

Peer reviewers: James M Millis, Professor, University of Chicago, Section of Transplantation, MC 5027, 5841 S. Maryland Avenue, Chicago, IL 60637, United States; Isabel Fabregat, PhD, Associate Professor, Laboratori d'Oncologia Molecular, Institut d'Investigació Biomèdica de Bellvitge, Gran Via, Km 2,7, L'Hospitalet, 08907 Barcelona, Spain; Bruno Stieger, Professor, Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich 8091, Switzerland

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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.

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 $\mu\text{mol/L}$; 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,



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.

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-H₂O₂ (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	285	NM_031144	81822
	R (1210) 5'-CCTAGAAGCATTGCGGTGCACGATG-3'			
Wnt-4	F (127) 5'-GAAACGTGCGAGAAGCTCAAAG-3'	387	NM_053402	84426
	R (513) 5'-AAAGGACTGTGAGAAGGCTACG-3'			
WT-1	F (1059) 5'-TGAGAAACCATAACCAGTGTGAC-3'	400	NM_031534	24883
	R (1458) 5'-GTAGGTGAGAGGGAGGAATTTTC-3'			
Nanog	F (541) 5'-ATCCATGTCAGCTATTCTCAGG-3'	310	XM_575662	414065
	R (850) 5'-CTTCCAAATTCGCTCCAAATC-3'			
AFP	F (1421) 5'-CAGTGAGGAGAAAACGGTCCG-3'	252	NM_012493	24177
	R (1672) 5'-ATGGTCTGTAGGGCTCGCC-3'			
Oct-4	F (633) 5'-GGAGATATGCAAATCGGAGACC-3'	352	NM_001009178	294562
	R (984) 5'-CGAGTAGAGTGTGGTCAAATGG-3'			
HNF-6	F (1698) 5'-AAGACCAGGACCTCAAGATAGC-3'	304	NM_022671	25231
	R (2001) 5'-GCAGTGTGGTGAACAGATAAG-3'			

¹Wnt-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]; ²Accessed from <http://www.ncbi.nlm.nih.gov>; ³Numbers 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 μ g 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

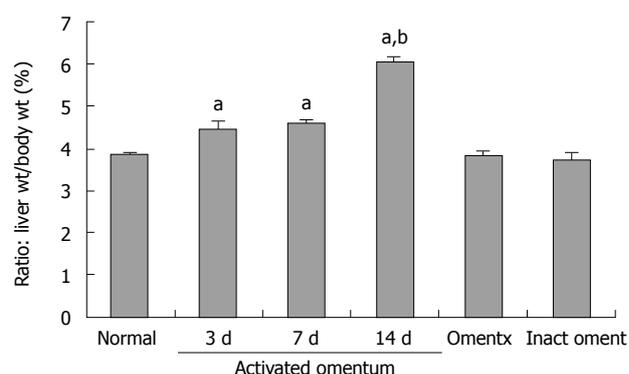


Figure 2 Liver mass (as a ratio of body weight) at different times after injury and fusing of the activated omentum to the wound. The ratio of liver wt/body wt in normal rats was established to be $3.85\% \pm 0.07\%$. 'Omentx' are rats in which the omentum was removed before liver injury ($n = 12$) and '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. ^aDenotes statistical difference from normal or 'omentx' or 'inact oment' groups at $P < 0.05$. ^bDenotes 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; $n = 3$).

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).

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

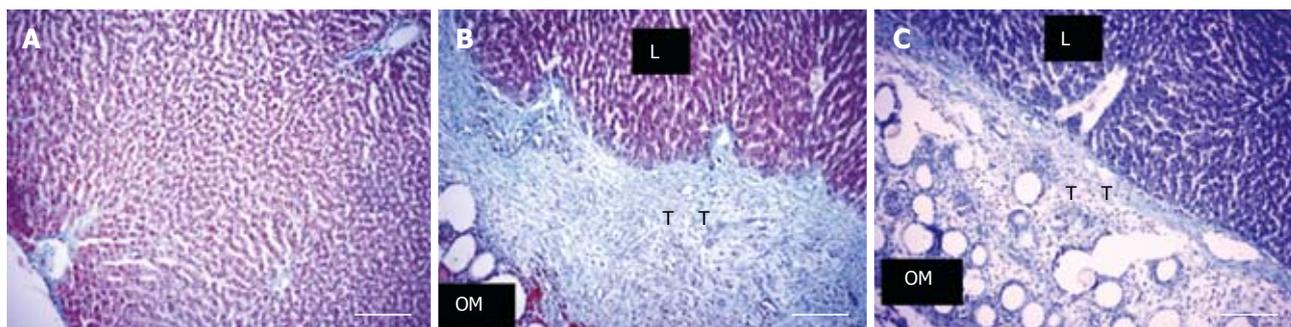


Figure 3 Histology of the boundary between the growing edge of the liver and the activated omentum. A: Normal rat liver; B: 7 d after liver injury the liver and the omentum were separated by a wide and compact interlying tissue (400-600 μm). On one side of the interlying tissue (T) lay the omental tissue (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 .

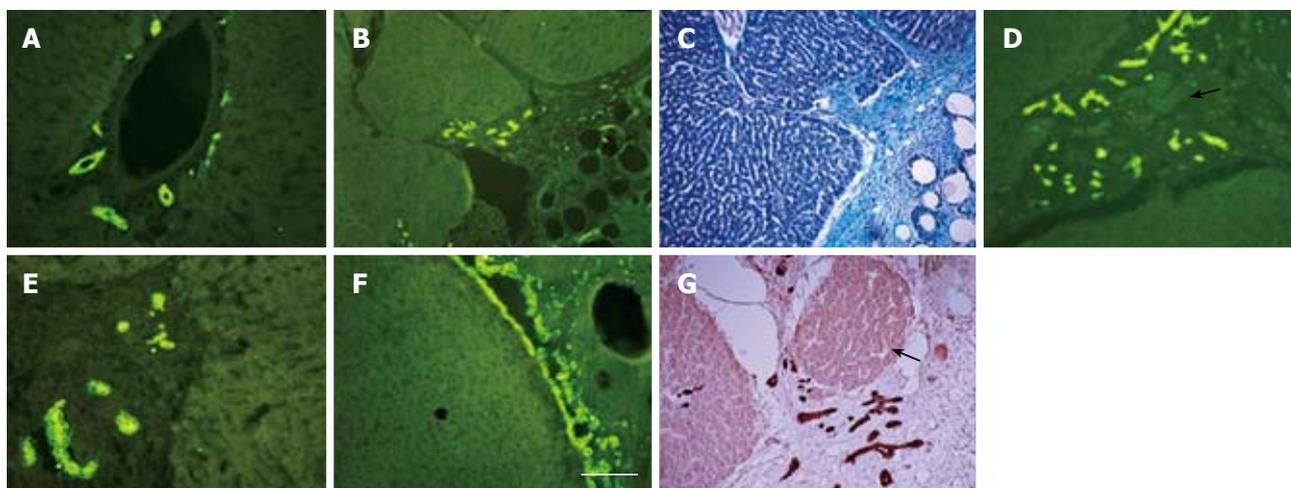


Figure 4 Immunostaining of normal and regenerated rat liver (from activated omentum) for cytokeratin-19, a marker of oval cells. A: Normal or uninjured liver lobe showing widespread presence of oval cells in the lining of bile ducts lying around a central vein; B, D, E, G: Different areas of injured 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.

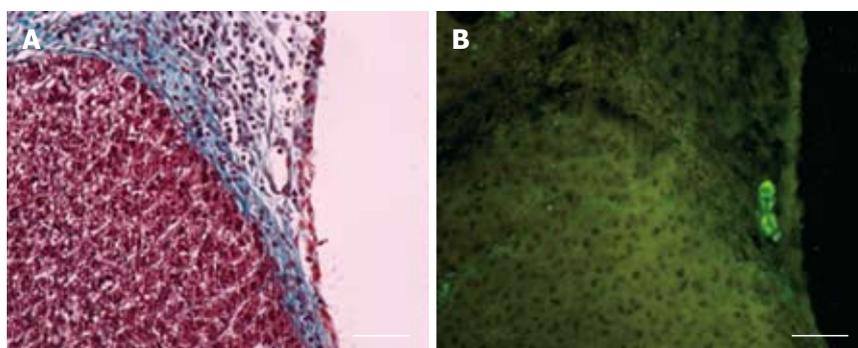


Figure 5 Histology and cytokeratin-19 immune staining of the liver at the boundary between the growing edge of the liver and the inactivated omentum at day 3 or 7 after injury. A: Trichrome stained section showing the adherent omental tissue with a thinner interlying tissue (blue stained) than that seen 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 [$n = 3$

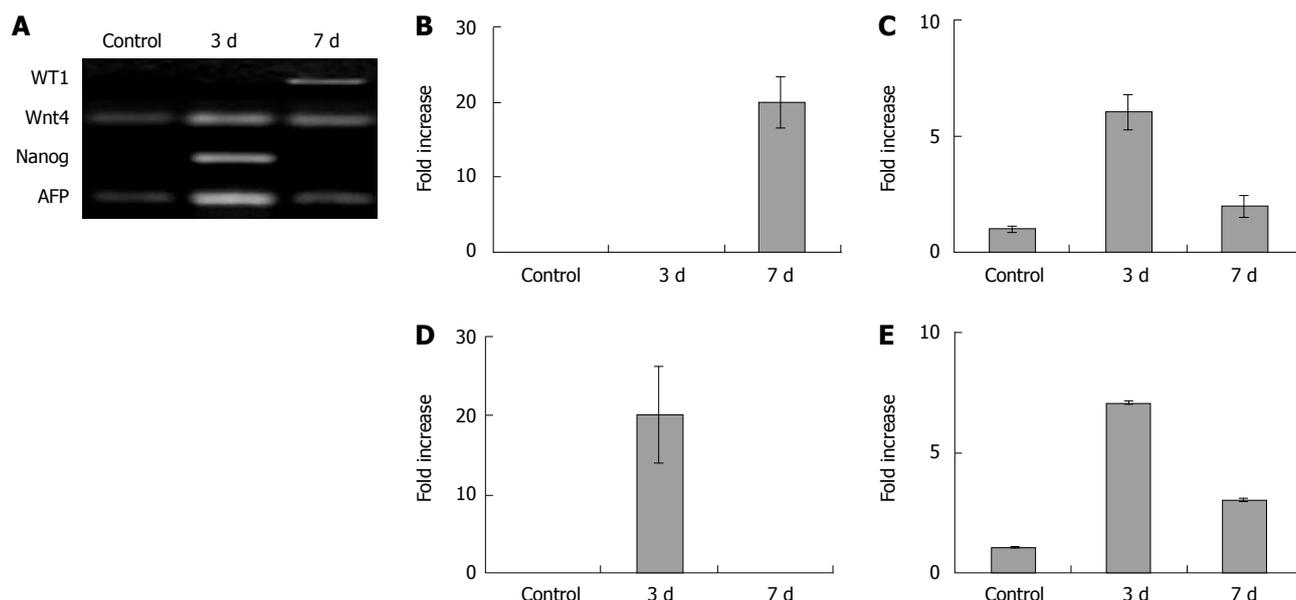


Figure 6 Activation of developmental genes in the regenerated liver at the wound site at days 3 and 7 after injury and fusion of the activated omentum. The regenerating part of the liver (part of liver attached to the omentum) showed high expression levels (7 to 20-fold) of WT-1, Wnt-4, Nanog, AFP by 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 $\mu\text{mol/L}$) (Figure 3B) and became thinner (100–150 $\mu\text{mol/L}$) 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 re-activated 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

Nora A Mohamad, Graciela P Cricco, Lorena A Sambuco, Máximo Croci, Vanina A Medina, Alicia S Gutiérrez, Rosa M Bergoc, Elena S Rivera, Gabriela A Martín

Nora A Mohamad, Graciela P Cricco, Lorena A Sambuco, Vanina A Medina, Alicia S Gutiérrez, Rosa M Bergoc, Elena S Rivera, Gabriela A Martín, Laboratory of Radioisotopes, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 954 (C1113AAB), Buenos Aires, Argentina

Máximo Croci, Institute of Immunooncology Dr. Ernesto Crescenti, Av. Córdoba 3200 (C1187AAS), Buenos Aires, Argentina

Rosa M Bergoc, Gabriela A Martín, National Research Council (CONICET), Av. Rivadavia 1917 (C1033AAJ), Buenos Aires, Argentina

Author contributions: Mohamad NA and Gutiérrez AS performed the *ex vivo* experiments; Cricco GP and Sambuco LA performed the *in vivo* experiments; Bergoc RM and Croci M performed the microscopical observation; Cricco GP and Martín GA designed the study; Rivera ES and Medina VA were involved in editing the manuscript and in critical review; Martín GA and Rivera ES participated in acquisition of funding; Mohamad NA, Cricco GP and Martín GA analyzed the data and wrote the manuscript.

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Correspondence to: Dr. Gabriela A Martín, Laboratory of Radioisotopes, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 954 (C1113AAB), Buenos Aires, Argentina. gamartin@ffyb.uba.ar

Telephone: +54-11-49648277-34 Fax: +54-11-49648277-31

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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.

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

Peer reviewers: Isabel Fabregat, PhD, Associate Professor, Laboratori d'Oncologia Molecular, Institut d'Investigació Biomèdica de Bellvitge, Gran Via, Km 2,7, L'Hospitalet, 08907 Barcelona, Spain; Francesco Feo, Professor, Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, Via P, Manzella 4, 07100 Sassari, Italy

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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 (O₂⁻) 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 μ L 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 mm³ they were excised, cut into 27 mm³ 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 mm³. The control group was left without treatment. Tumor size was measured with a caliper three times a week and volume was calculated as [(major diameter + minor diameter)/4]³ \times π /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 5.00TM. 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 PrismTM. 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- μ m 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 \pm SD)

Treatment	Doubling time (d)
Control	7.8 \pm 0.3
AG 1 mg/mL	7.8 \pm 0.3
AG 2 mg/mL	10.0 \pm 0.5 ^a
AG 4 mg/mL	11.1 \pm 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 \times and the remaining determinations at 630 \times . 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 cytoplasm 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 Prism™. Peritumoral vascularity was assessed by screening trichrome stained sections at 50 \times magnification to identify the largest vascular areas around the tumor. In these hot spots, individual vessel count was evaluated at 400 \times magnification using an ocular grid. Intratumoral vascularity was evaluated on trichrome and CD34 stained slices, counting vessels inside the tumor at 400 \times 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 mm³. 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.

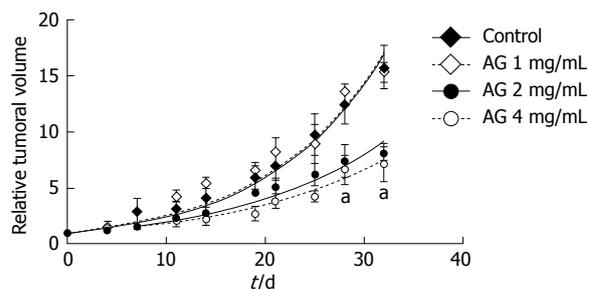


Figure 1 Antitumor effect of AG in PANC-1 xenografted mice. Tumor volumes were determined and referred to the initial volume for each treatment. ^a $P < 0.01$ for AG 2 and 4 mg/mL vs control, two way ANOVA and Bonferroni post test.

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 \pm 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

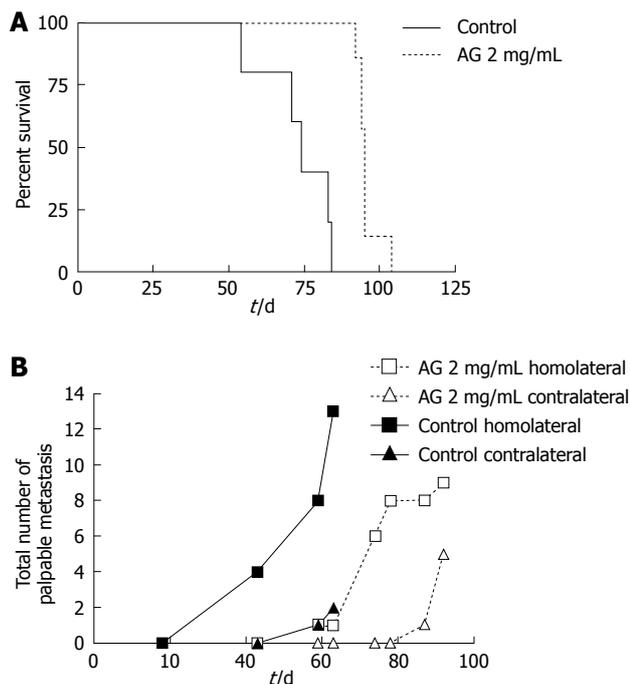


Figure 2 Action of AG on survival and metastases in PANC-1 xenografted mice. A: Time of spontaneous death of mice was determined, Kaplan-Meier survival curves were plotted and median survival was calculated. $P < 0.01$ for AG vs control, log-rank test; B: The development of palpable homolateral and contralateral 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 \pm 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 \pm SD). In the same way, the expression of Bax, evaluated as percent of positive cells (mean \pm 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 \pm 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 \pm 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

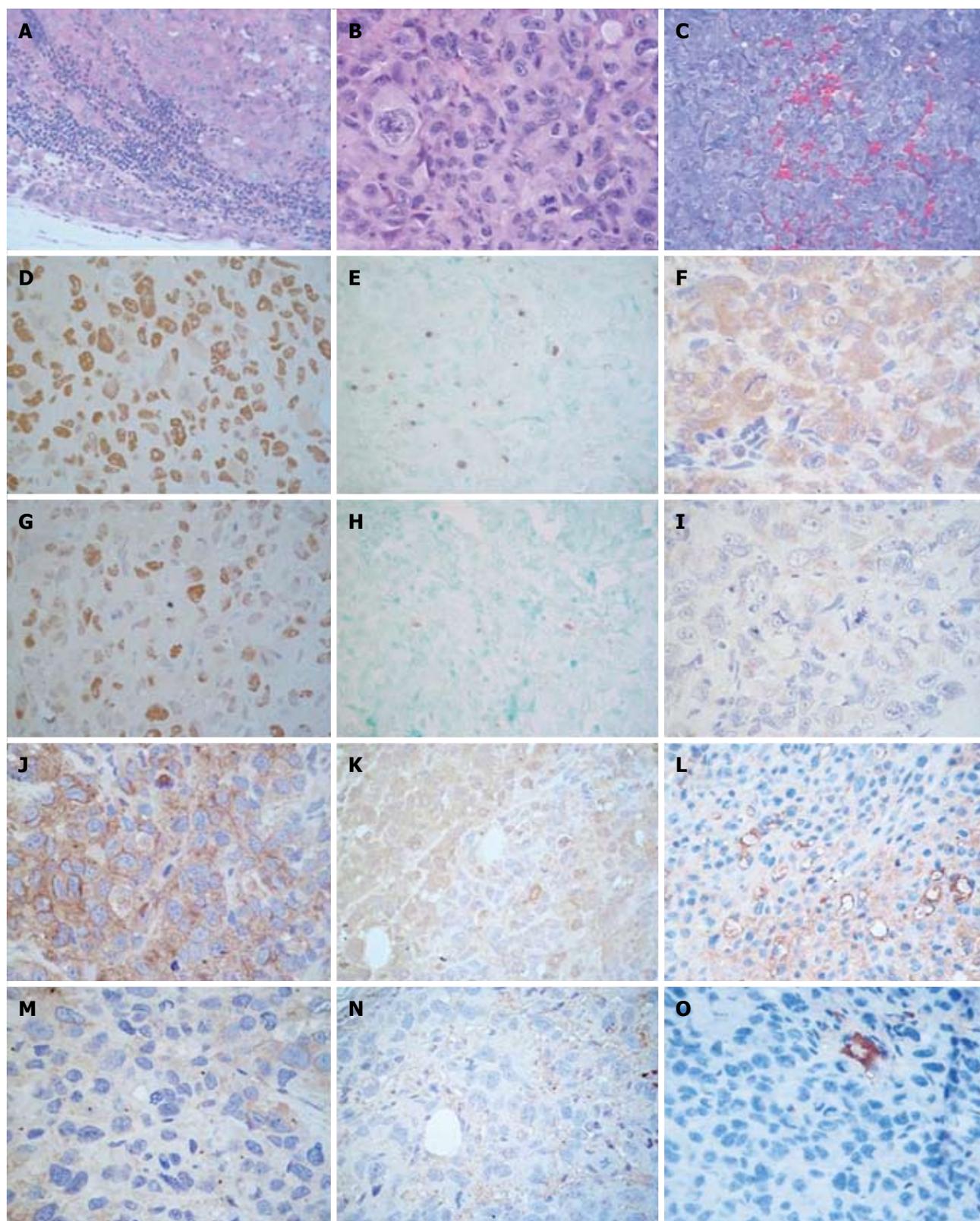


Figure 3 Histopathology and immunohistochemistry of PANC-1 xenografted mice. Formalin fixed paraffin embedded tissue sections of control mice were stained with HE. A: Metastatic lymph node ($\times 400$); B: Xenografts ($\times 630$). Immunohistochemistry in control and AG treated mice. Tissue sections were stained with DAB and counterstained with hematoxylin; C: Formalin fixed paraffin embedded tissue sections of control mice were stained with trichromic solution ($\times 400$); D, G: Ki-67 ($\times 630$); E, H: Tunel ($\times 400$); F, I: Bax ($\times 630$); J, M: VEGF ($\times 630$); K, N: eNOS ($\times 630$); L, O: CD34 in control ($\times 400$) and AG treated ($\times 630$) 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- κ B 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-tetramethylimidazole-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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Suppression of matrix metalloproteinase-2 *via* RNA interference inhibits pancreatic carcinoma cell invasiveness and adhesion

Ying-Hui Zhi, Mao-Min Song, Pi-Lin Wang, Tie Zhang, Zi-Yi Yin

Ying-Hui Zhi, Mao-Min Song, Pi-Lin Wang, Tie Zhang, Zi-yi Yin, Department of General Surgery, Affiliated Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China
Author contributions: Zhi YH performed the majority of experiments; Wang PL, Yin ZY and Zhang T provided vital reagents and analytical tools and were involved in editing the manuscript; Song MM provided financial support for this work; Zhi YH and Song MM designed the study and wrote the manuscript.

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Correspondence to: Dr. Mao-Min Song, Department of General Surgery, Affiliated Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China. smaomin@sohu.com

Telephone: +86-10-67096589 Fax: +86-10-67096593

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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 silencing 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.

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

Peer reviewer: Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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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°C, 5% CO₂) incubator, grown in 75-cm² 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 silencing 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'-GCTCCACCACCTACAACCTTTG-3' (sense) and 5'-CAAAGTTGTAGGTGGTGGAGC-3' (antisense); MMP-2 siRNA no. 3, 5'-GCAAACAGGA CATTGTATTTG-3' (sense) and 5'-CAAATACAAT GTCTGTTTGC-3' (antisense); MMP-2 siRNA no. 4, 5'-GGAGATACAATGAGGTGAAGA-3' (sense) and 5'-TCITCACCTCATTGTATCTCC-3' (antisense). The target sequence of negative control group which is named pGPU6 (-) is 5'-GTCTCCGAACGTGTCACG T-3' (sense) and 5'-ACGTGACACGTTCCGAGAAT-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 + *Hind*III. 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°C, 5% CO₂) 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°C in a CO₂ 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. Glutarylaldehyde-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 TTCTTGTGTCGCGGT-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°C for 10 min, then 37°C for 60 min. PCR amplification was performed under the following reaction conditions: 94°C for 30 s, 55°C (MMP-2) or 53°C (GAPDH) for 30 s, 72°C for 1 min, and a final extension at 72°C 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) \times 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°C. 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) \times 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 μ L of 5 mg/ μ L 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°C 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) \times 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 \pm 0.02
pGPU6 (-)	0.91 \pm 0.03
pGPU6-1	0.29 \pm 0.02 ^{b,d}
pGPU6-2	0.33 \pm 0.04 ^{b,d}
pGPU6-3	0.45 \pm 0.03 ^{b,d}
pGPU6-4	0.88 \pm 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 μ L serum-free medium and placed into the upper compartment of the Transwell chambers. The lower compartment of the chambers was filled with 200 μ L 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 \times 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 \pm 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).

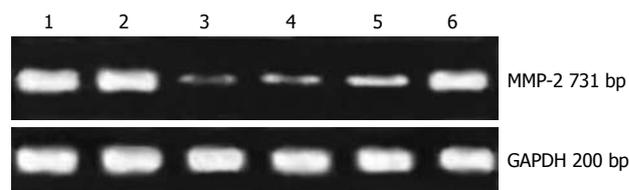


Figure 1 Down-regulation of MMP-2 mRNA expression detected by RT-PCR. Lane 1: Blank control; Lane 2: pGPU6 (-); Lane 3: pGPU6-1; Lane 4: pGPU6-2; Lane 5: pGPU6-3; Lane 6: pGPU6-4.

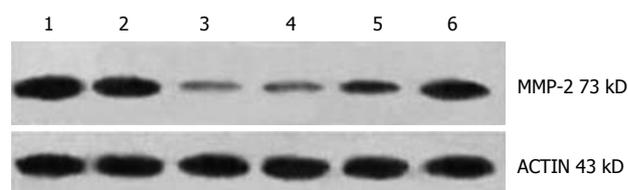


Figure 2 Down-regulation of MMP-2 protein expression detected by Western blot. Lane 1: Blank control; Lane 2: pGPU6 (-); Lane 3: pGPU6-1; Lane 4: pGPU6-2; Lane 5: pGPU6-3; Lane 6: pGPU6-4.

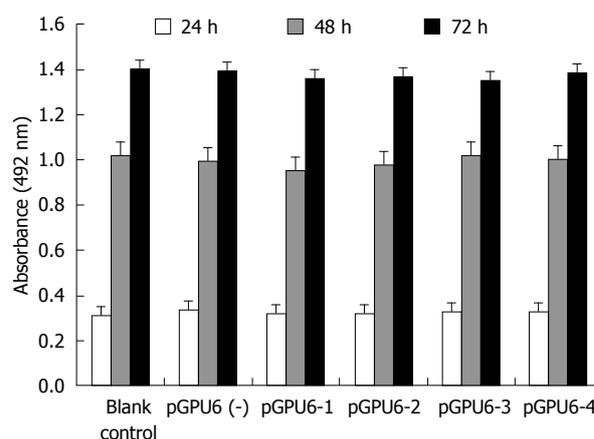


Figure 3 Effects of MMP-2 siRNA on tumor cell proliferation.

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

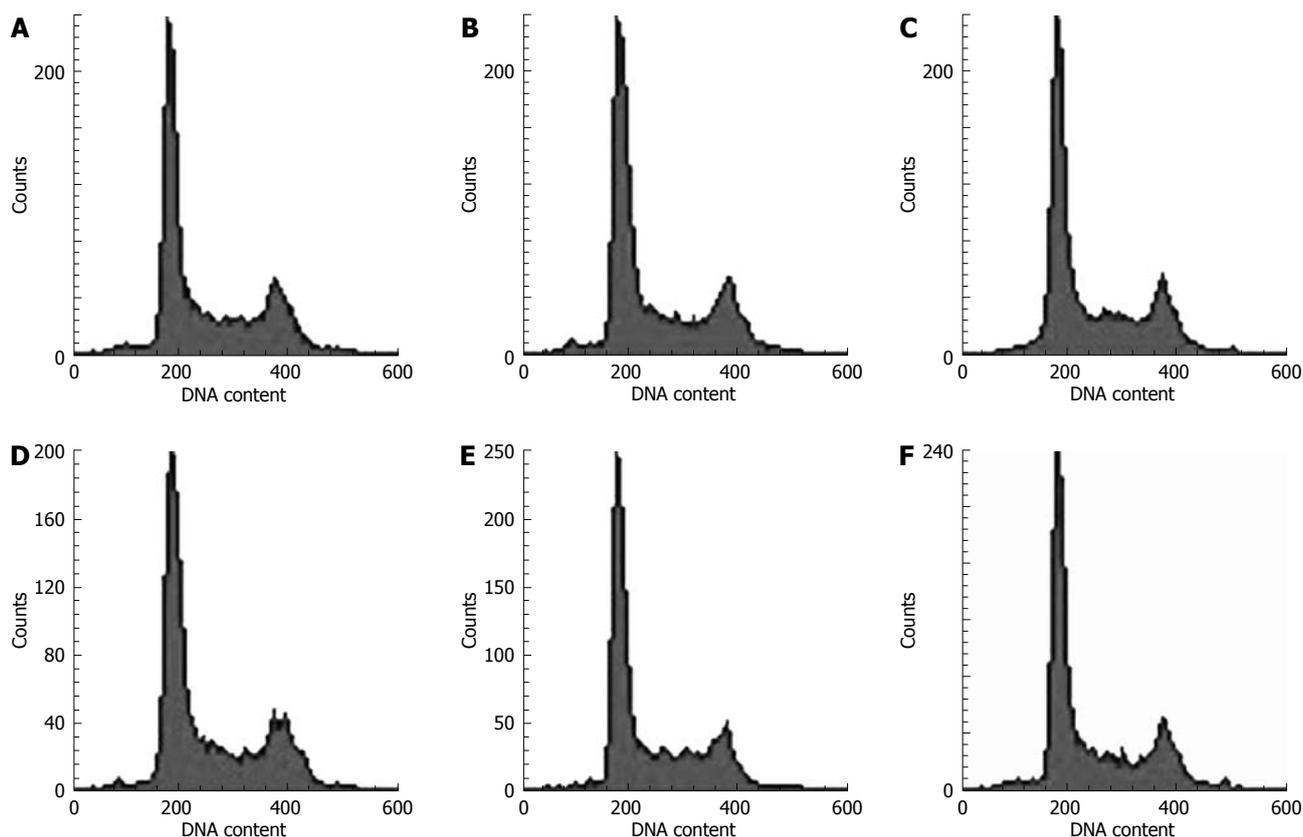


Figure 4 Effects of MMP-2 siRNA on tumor cell apoptosis. A: pGPU6-1; B: pGPU6-2; C: pGPU6-3; D: pGPU6-4; E: pGPU6 (-); F: Blank control.

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].

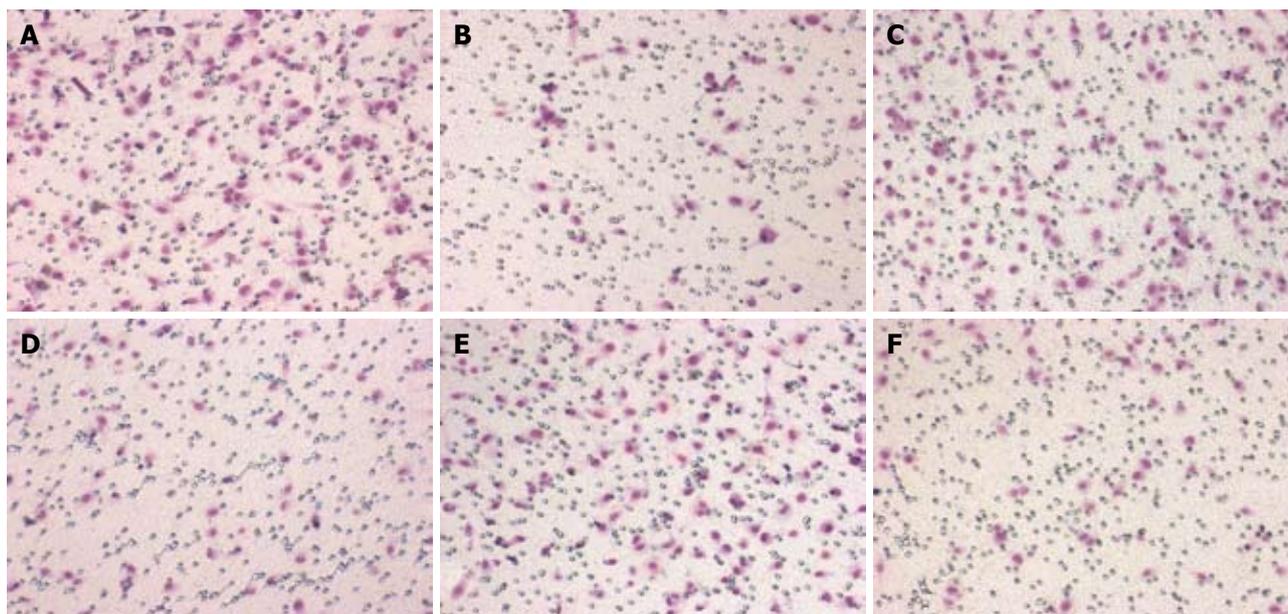


Figure 5 Cell invasion observed by CV stained ($\times 200$). A: Blank control; B: pGPU6-1; C: pGPU6-2; D: pGPU6-3; E: pGPU6-4; F: pGPU6-6.

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

Piet Habel, Karsten H Weylandt, Katja Lichopoj, Johannes Nowak, Martin Purschke, Jing-Dong Wang, Cheng-Wei He, Daniel C Baumgart, Jing X Kang

Piet Habel, Karsten H Weylandt, Katja Lichopoj, Johannes Nowak, Martin Purschke, Jing-Dong Wang, Cheng-Wei He, Jing X Kang, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, United States

Piet Habel, Karsten H Weylandt, Katja Lichopoj, Johannes Nowak, Daniel C Baumgart, Department of Medicine, Division of Gastroenterology and Hepatology, Charité Medical Center-Virchow-Hospital, Medical School of the Humboldt-University of Berlin, 13344 Berlin, Germany

Author contributions: Habel P and Weylandt KH contributed equally to this work; Habel P, Weylandt KH, Kang JX designed research; Habel P, Lichopoj K, Nowak J, Purschke M, Wang JD, He CW performed experiments; Habel P and Weylandt KH analyzed data; Habel P, Weylandt KH, Baumgart DC, Kang JX prepared the manuscript.

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Correspondence to: Karsten H Weylandt, MD, PhD, Dr. Kang's Lab, Massachusetts General Hospital, 149-13th Street, Room 4433, Charlestown, MA 02129,

United States. karsten.weylandt@gmx.de

Telephone: +1-617-7268509 Fax: +1-617-7266144

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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 E₂ (PGE₂) generation in the presence of AA and DHA was measured using a PGE₂-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 PGE₂ formation. DHA also down-regulated COX-2 expression. In addition to the effect on PGE₂ formation, DHA directly reduced PGE₂-induced cell proliferation in a dose-dependent manner.

CONCLUSION: These results suggest that DHA can inhibit the pro-proliferative effect of abundant AA or PGE₂.

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Key words: Colorectal carcinoma; Colon cancer; Omega-3; Omega-6; Polyunsaturated fatty acids; Arachidonic acid; Docosahexaenoic acid; Prostaglandin E₂; Cyclooxygenase-2; Apoptosis

Peer reviewer: Meenakshisundaram Ananthanarayanan, Associated Professor, Department of Pediatrics, Annenberg Bldg, Rm.14-24A, Box 1664, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY, 10029, United States

Habel P, Weylandt KH, Lichopoj K, Nowak J, Purschke M, Wang JD, He CW, Baumgart DC, Kang JX. Docosahexaenoic acid suppresses arachidonic acid-induced proliferation of LS-174T human colon carcinoma cells. *World J Gastroenterol* 2009; 15(9): 1079-1084 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1079.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1079>

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 E₂ (PGE₂) 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 PGE₂ 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 PGE₂, 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 PGE₂ 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 PGE₂-triggered activation of proliferation has been demonstrated previously^[6,27-29], to test the effect of DHA co-incubation with AA or PGE₂ 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 PGE₂-induced cell growth.

MATERIALS AND METHODS

Cell culture

Cells were cultured in a saturated atmosphere of 5% CO₂ and 95% air at 37°C. 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 PGE₂ (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.

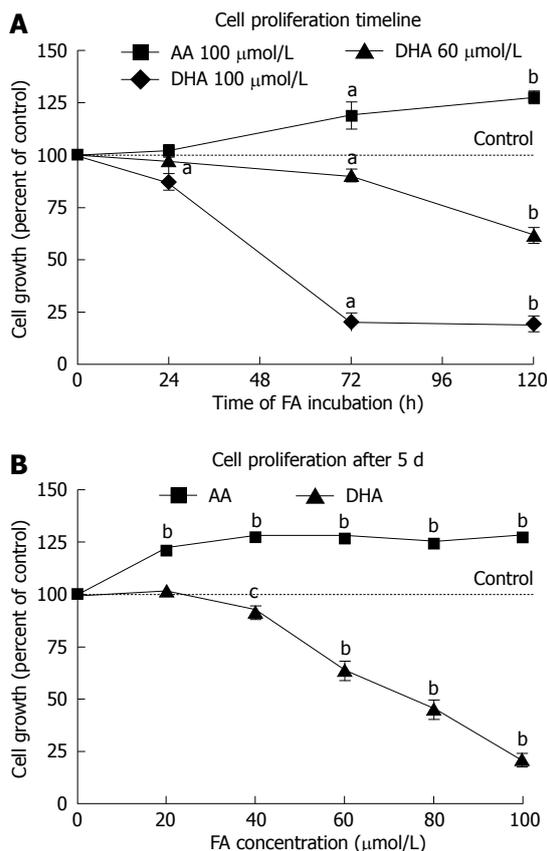


Figure 1 Effects of fatty acids on cellular viability. A: Growth of LS-174T cells during incubation with different concentrations of fatty acids in the medium with 1 mg/mL BSA. Data points represent at least five independent experiments. ^a*P* < 0.05 versus control, ^b*P* < 0.001 versus control. B: Concentration-dependent 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. ^c*P* < 0.01 versus control, ^b*P* < 0.001 versus control.

Cell proliferation assay

Cell viability was determined by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-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,

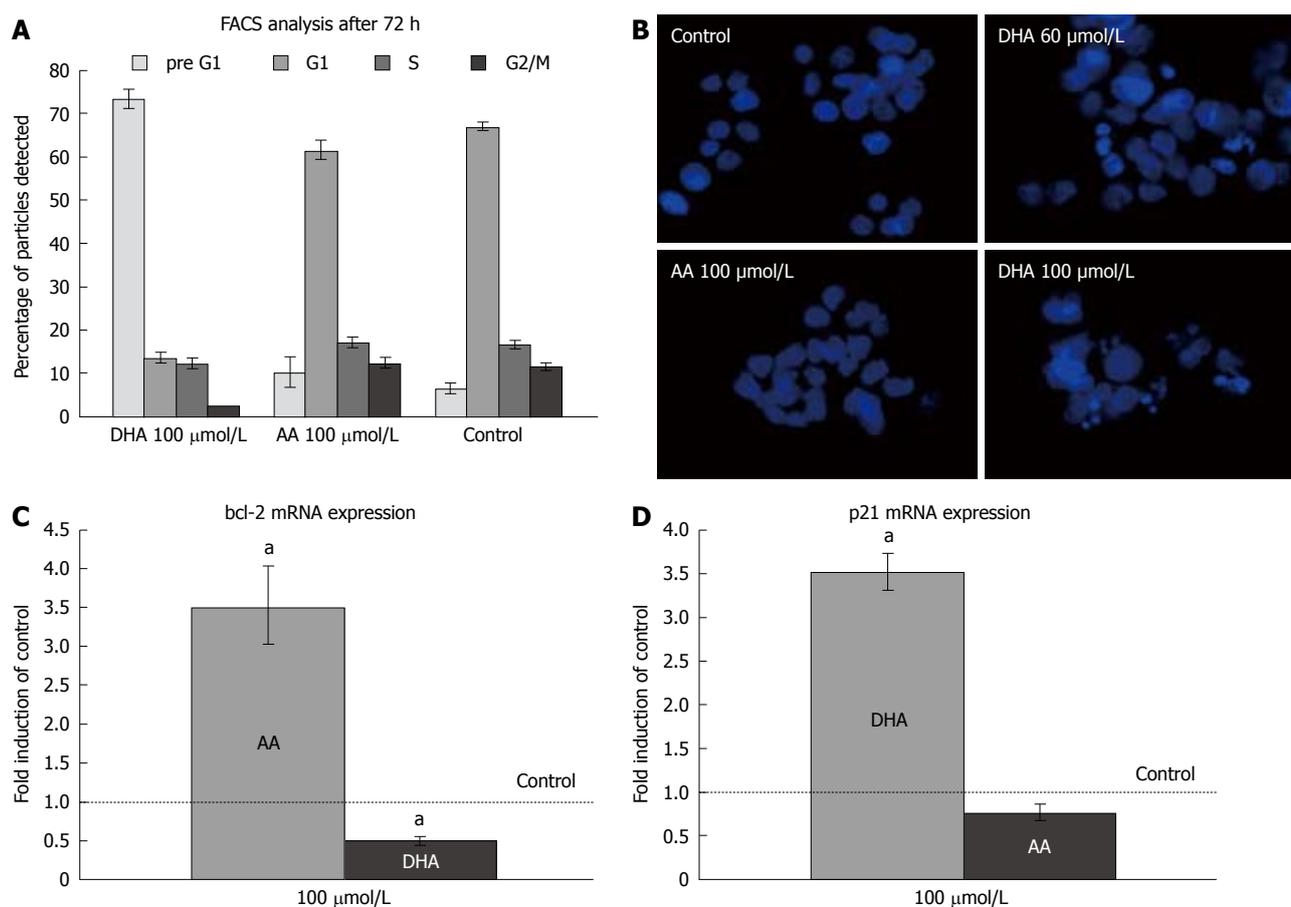


Figure 2 Effect of DHA and AA on cell cycle and apoptosis. A: Cell cycle analysis by flow cytometry. Induction of apoptosis is indicated by an increased pre-G1 fraction. Results represent five independent experiments. B: DAPI staining of LS-174T cells incubated with different concentrations of AA and 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 RNeasy 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 GTGGGGGCATCATCAAAAATCT, p21rev ACCCACCTTCCCCCTGCCTTCAC; bcl-2for

CATGCCAAGGGGAAACACCAGAA, bcl-2rev CACGGCCCCAGAGAAAGAAGAGG; GAPDHfor GGTGAAGGTCGGAGTCAAC, GAPDHrev CCATGGGTGGAATCATATTG.

PGE₂-ELISA

For PGE₂ analysis, cells were treated with PUFAs, as described above. The PGE₂-ELISA was then performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All results are presented as mean \pm 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 $2\Delta\Delta C_t$ 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

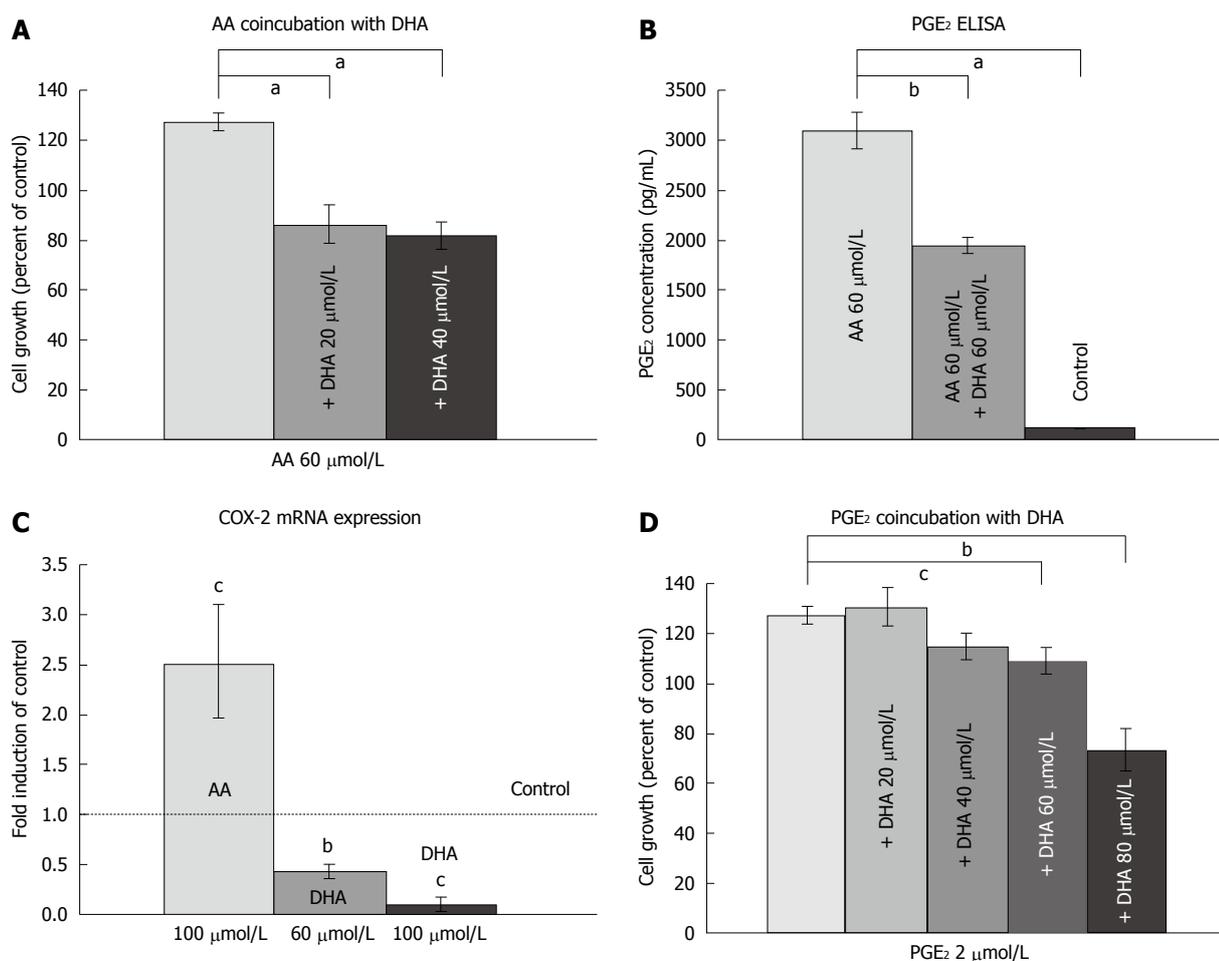


Figure 3 DHA suppresses AA- and PGE₂-induced proliferation. A: DHA inhibited AA-induced proliferation, ^a $P < 0.001$. Results represent six independent experiments. B: PGE₂ formation was induced by AA treatment and was suppressed by concomitant DHA incubation, ^a $P < 0.001$, ^b $P < 0.01$. Results represent the mean of PGE₂ 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 PGE₂-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 PGE₂-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 PGE₂ 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 PGE₂-formation, as co-incubation experiments with DHA and PGE₂ revealed that DHA also suppressed the PGE₂-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 PGE₂. In contrast, the n-3 PUFA DHA down-regulates anti-apoptotic factors, induces

apoptosis and decreases PGE₂ 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 PGE₂) can be suppressed by increasing the DHA content of the cells. Indeed, DHA can also directly inhibit PGE₂-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 PGE₂-levels.

COMMENTS

Background

Colon cancer is one of the leading causes of death in Western countries. It is known, that prostaglandin E₂ (PGE₂), 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 PGE₂ 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 PGE₂-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 PGE₂-levels.

Applications

The results suggest that supplementation of DHA may be a powerful tool to counteract AA- and PGE₂-promoted colon cancer cell growth that might be associated with the predominant Western diet.

Terminology

PGE₂ is generated from the n-6 PUFA AA *via* action of cyclooxygenases 1 and 2. PGE₂ 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

Jochim S Terhaar sive Droste, Mike E Craanen, Rene WM van der Hulst, Joep F Bartelsman, Dick P Bezemer, Kim R Cappendijk, Gerrit A Meijer, Linde M Morsink, Pleun Snel, Hans ARE Tuynman, Roy LJ van Wanrooy, Eric IC Wesdorp, Chris JJ Mulder

Jochim S Terhaar sive Droste, Mike E Craanen, Kim R Cappendijk, Linde M Morsink, Roy LJ van Wanrooy, Chris JJ Mulder, Department of Gastroenterology and Hepatology, VU University Medical Centre, PO Box 7057, 1007 MB, Amsterdam, The Netherlands

Rene WM van der Hulst, Gastroenterology and Hepatology, Kennemer Gasthuis, 2000 AK, Haarlem, The Netherlands

Joep F Bartelsman, Gastroenterology and Hepatology, Academic Medical Centre, 1100 DD, Amsterdam, The Netherlands

Dick P Bezemer, Epidemiology and Biostatistics, VU University Medical Centre, 1007 MB, Amsterdam, The Netherlands

Gerrit A Meijer, Pathology, VU University Medical Centre, 1007 MB, Amsterdam, The Netherlands

Pleun Snel, Gastroenterology and Hepatology, Slotervaart Hospital, 1006 BK, Amsterdam, The Netherlands

Hans ARE Tuynman, Gastroenterology and Hepatology, Medical Centre Alkmaar, 1800 AM, Alkmaar, The Netherlands

Eric IC Wesdorp, Gastroenterology and Hepatology, Sint Lucas Andreas Hospital, 1061 AE, Amsterdam, The Netherlands

Author contributions: Mulder CJJ, Bartelsman JF, Snel P, Tuynman HARE and Wesdorp EIC performed research, devised the study concept and designed research; Craanen ME, van der Hulst RWM and Meijer GA performed critical revision of the article for intellectual content; Bezemer DP performed statistical analysis; Cappendijk KR, Morsink LM and van Wanrooy RLJ were responsible for data acquisition and Terhaar sive Droste JS wrote the paper.

Correspondence to: Jochim S Terhaar sive Droste, MD, Department of Gastroenterology and Hepatology, VU University Medical Centre, PO Box 7057, 1007 MB, Amsterdam, The Netherlands. js.terhaar@vumc.nl

Telephone: +31-20-4440611 Fax: +31-20-4440554

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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.

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

Peer reviewer: Otto Schiueh-Tzang Lin, MD, C3-Gas, Gastroenterology Section, Virginia Mason Medical Center, 1100 Ninth Avenue, Seattle, WA 98101, United States

Terhaar sive Droste JS, Craanen ME, van der Hulst RWM, Bartelsman JF, Bezemer DP, Cappendijk KR, Meijer GA, Morsink LM, Snel P, Tuynman HARE, van Wanrooy RLJ, Wesdorp EIC, Mulder CJJ. Colonoscopic yield of colorectal neoplasia in daily clinical practice. *World J Gastroenterol* 2009; 15(9): 1085-1092 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1085.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1085>

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 ($\geq 25\%$ 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 ($\geq 25\%$ 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 $\leq 25\%$ 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

¹Known 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)

¹Known hereditary CRC syndromes like HNPCC, FAP or MYH-polyposis are excluded; ²Including 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)

¹Advanced 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

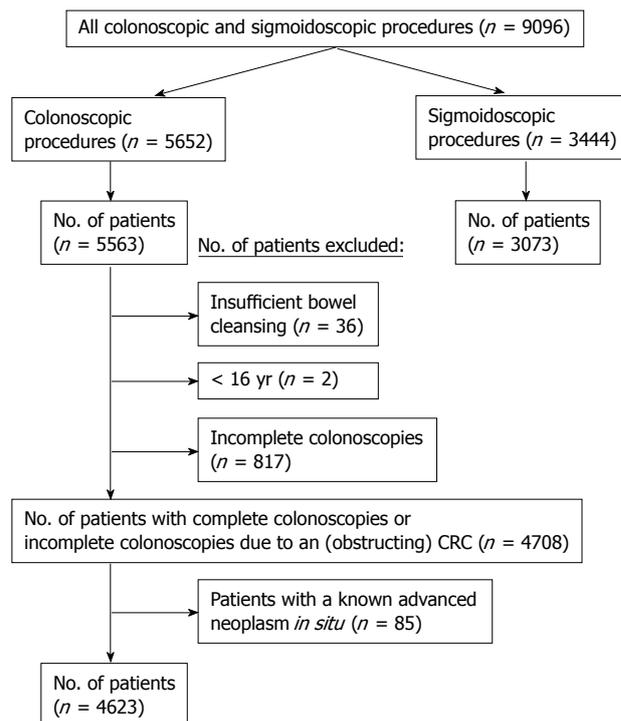


Figure 1 Number of patients included in the primary and secondary study objectives (*n* = 4623).

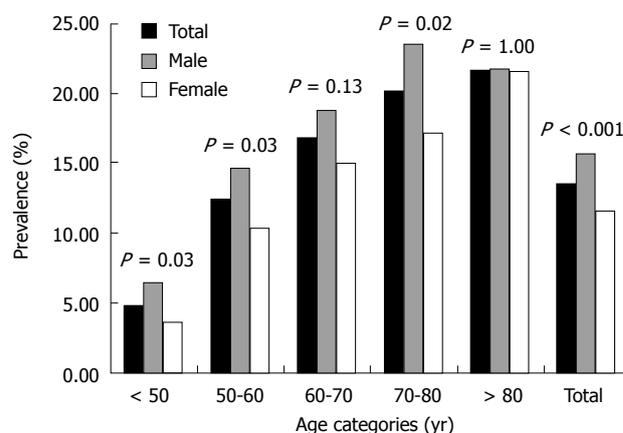


Figure 2 Yield of advanced neoplasia in different age categories (*n* = 4623). *P*-values for males compared to females.

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 ($P = \text{N.S.}$). 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 stand-

ardization, 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 United 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 ≥ 1.0 cm, an adenoma with villous or tubulovillous architecture ($\geq 25\%$ villous component) or an adenoma with high-grade dysplasia. Advanced colorectal neoplasm is defined as an adenoma ≥ 1.0 cm, an adenoma with tubulovillous or villous architecture ($\geq 25\%$ 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

Hüseyin Saadettin Uslusoy, Selim Giray Nak, Macit Gülten, Zeynep Bıyıklı

Hüseyin Saadettin Uslusoy, Selim Giray Nak, Macit Gülten, Uludag University Medical School, Department of Gastroenterology, Bursa 16059, Turkey

Zeynep Bıyıklı, Ankara University School of Medicine, Department of Biostatistics, Ankara 06100, Turkey

Author contributions: Uslusoy HS performed research; Nak SG wrote and translated the manuscript; Gülten M designed research and performed revisions; Bıyıklı Z performed the analysis and interpretation of data.

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Correspondence to: Dr. Hüseyin Saadettin Uslusoy, Uludag University Medical School, Department of Gastroenterology, Bursa 16059, Turkey. hulusoy.23@hotmail.com

Telephone: +90-354-2121070 Fax: +90-354-2120923

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Peer reviewers: Michael Torbenson, MD, Associate Professor of Pathology, Room B314 1503 E Jefferson (Bond Street Building), The Johns Hopkins University School of Medicine, Baltimore, MD 21231, United States; Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata 950-1104, Japan

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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 \geq 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 \geq 30 kg/m², waist/hip circumference ratio > 0.90 in men and > 0.85 in women, fasting blood glucose \geq 1100 mg/L, overt diabetes, presence of impaired glucose tolerance and/or IR,

triglycerides \geq 1500 mg/L, HDL-cholesterol < 400 mg/L in men and < 500 mg/L in women, arterial blood pressure \geq 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 necroinflammation 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 individual 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.

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

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	-
Hypertriglyceridemia					
(+)	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	-

¹69 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.

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. Interestingly, 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.

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 association 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%)

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.

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 prevalences 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 simple fatty liver cases). In our study, approximately 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, individuals 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

Supot Pongprasobchai, Sireethorn Nimitvilai, Jaron Chasawat, Sathaporn Manatsathit

Supot Pongprasobchai, Jaron Chasawat, Sathaporn Manatsathit, Division of Gastroenterology, Department of Internal Medicine, Siriraj Hospital, Bangkok 10700, Thailand
Sireethorn Nimitvilai, Department of Internal Medicine, Siriraj Hospital, Bangkok 10700, Thailand

Author contributions: Pongprasobchai S designed and conducted the study, analyzed the data and prepared the manuscript; Nimitvilai S and Chasawat J contributed equally in collecting the data; Manatsathit S critically edited and revised the manuscript.

Correspondence to: Dr. Supot Pongprasobchai, Division of Gastroenterology, Department of Internal Medicine, Siriraj Hospital, Bangkok 10700, Thailand. supotpong@hotmail.com
Telephone: +66-2-4197281 Fax: +66-2-4115013

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Abstract

AIM: To identify clinical parameters, and develop an Upper Gastrointestinal 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 $(3.1 \times \text{previous diagnosis of cirrhosis or signs of chronic liver disease}) + (1.5 \times \text{red vomitus}) + (1.2 \times \text{red NG aspirate})$, when 1 and 0 are used for the presence and absence of each factor, respectively. Using a cutoff

≥ 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 ≥ 3.1 for variceal bleeding were 79% and 97%, respectively.

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

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

Peer reviewer: Georgios Papachristou, MD, Assistant Professor of Medicine, Division of Gastroenterology, Hepatology and Nutrition, UPMC Presbyterian, Mezzanine Level, C-Wing, 200 Lothrop Street, Pittsburgh, PA 15213, United States

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INTRODUCTION

Upper Gastrointestinal 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 esophagogastroduodenoscopy (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 ≥ 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		<i>P</i>
	Variceal (<i>n</i> = 47)	Nonvariceal (<i>n</i> = 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)	

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

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

Laboratory findings	Causes of UGIB		<i>P</i>
	Variceal (<i>n</i> = 47)	Nonvariceal (<i>n</i> = 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.

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:

$$\text{UGIB Score} = (3.1 \times \text{previous diagnosis of cirrhosis or the presence of signs of chronic liver disease}) + (1.5 \times \text{presence of red vomitus}) + (1.2 \times \text{presence of red NG aspirate}).$$

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 \pm SD) n (%)

Clinical parameter	Cause of UGIB	
	Variceal (n = 46)	Non-variceal (n = 149)
Age (yr)	64.51 \pm 16	56.43 \pm 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 presence of varices, the need for hospitalization or interventions. Multiple scoring systems in UGIB,

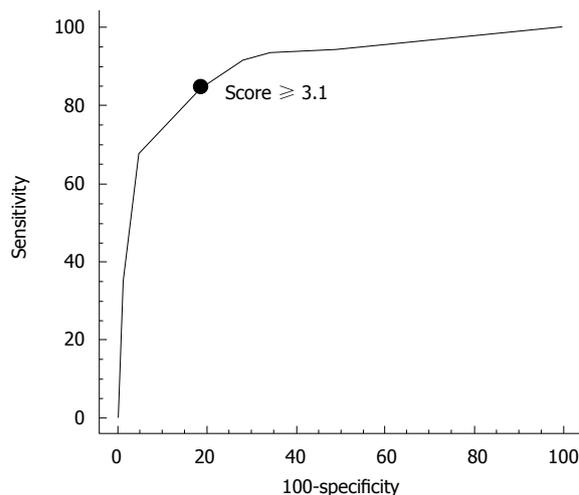


Figure 1 Receiver operating curve of the UGIB Etiology Score. The best cutoff point is the score ≥ 3.1 .

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 Gastrointestinal 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

Dhira Rani Saha, Simanti Datta, Santanu Chattopadhyay, Rajashree Patra, Ronita De, Krishnan Rajendran, Abhijit Chowdhury, Thandavaryan Ramamurthy, Asish Kumar Mukhopadhyay

Dhira Rani Saha, Division of Histology & Electron microscopy, National Institute of Cholera and Enteric Diseases, P 33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India

Simanti Datta, Abhijit Chowdhury, School of digestive diseases, Institute of Post Graduate Medical Education and Research, 244 AJC Bose Road, Kolkata 700020, India

Santanu Chattopadhyay, Rajashree Patra, Ronita De, Thandavaryan Ramamurthy, Asish Kumar Mukhopadhyay, Division of Bacteriology, National Institute of Cholera and Enteric Diseases, P 33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India

Krishnan Rajendran, Data management Division, National Institute of Cholera and Enteric Diseases, P 33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India

Author contributions: Saha DR and Mukhopadhyay AK designed the research; Chowdhury A performed the endoscopy and collected the biopsy samples; Chowdhury A, Dutta S and De R managed the patient data; Datta S, Chattopadhyay S, Patra R and De R processed and performed the genetic analysis of *H pylori* strains; Saha DR performed the histology; Chattopadhyay S, Patra R, Saha DR and Mukhopadhyay AK analysed the results; Rajendran K did the statistical analysis of data; Mukhopadhyay AK and Saha DR drafted the paper; Mukhopadhyay AK and Ramamurthy T revised the manuscript with intellectual inputs.

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Correspondence to: Asish Kumar Mukhopadhyay, PhD, Division of Bacteriology, National Institute of Cholera and Enteric Diseases, P 33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India. asish_mukhopadhyay@yahoo.com
Telephone: +91-33-23701176 Fax: +91-33-23705066

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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.

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

Peer reviewer: Özlem Yilmaz, PhD, Associate Professor of Microbiology, Dokuz Eylul University, School of Medicine, Department of Microbiology and Clinical Microbiology, Inciralti 35340, Izmir, Turkey

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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
<i>vacA</i> s1 or <i>vacA</i> s2	VA1-F	5'-ATGGAAATACAACAAACACAC	[7]
	VA1-R	5'-CTGCTTGAATGCGCCAAAC	
<i>vacA</i> m1 or <i>vacA</i> m2	VAG-F	5'-CAATCGTCCAATCAAGCGAG	[28]
	VAG-R	5'-GCGTCAAAATAATTCCAAGG	
<i>cagA</i> (5' end)	cag5c-F	5'-GTTGATAACGCTGTCGCTTC	[28]
	cag3c-R	5'-GGGTTGTATGATATTTTCCATAA	
<i>cag</i> -PAI empty site	Luni 1	5'-ACATTTTGGCTAAATAAACGCTG	[7]
	R5280	5'-GGTTGCACGCATTTTCCCTTAATC	
<i>iceA1</i>	IceA1F	5'-TATTTCTGGAACCTGCGCAACCTGAT	[7]
	M.Hpy1R	5'-GGCCTACAACCGCATGGATAT	
<i>iceA2</i>	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 μ L 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°C in double-gassed incubator, which maintains 10% CO₂, 5% O₂ and 85% N₂ 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°C for 1 h. After incubation, CTAB/NaCl (10% cetyl trimethyl ammonium bromide in 0.7 mol/L NaCl) was added and incubated at 65°C 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°C 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 MgCl₂ in standard PCR buffer for 30 cycles generally under the following conditions: 94°C for 40 s, 55°C for 40 s, and 72°C 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 μ m) 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 (<i>n</i> = 50)	Asymptomatic TR (<i>n</i> = 36)	<i>P</i> -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)

¹Indicates 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 less 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

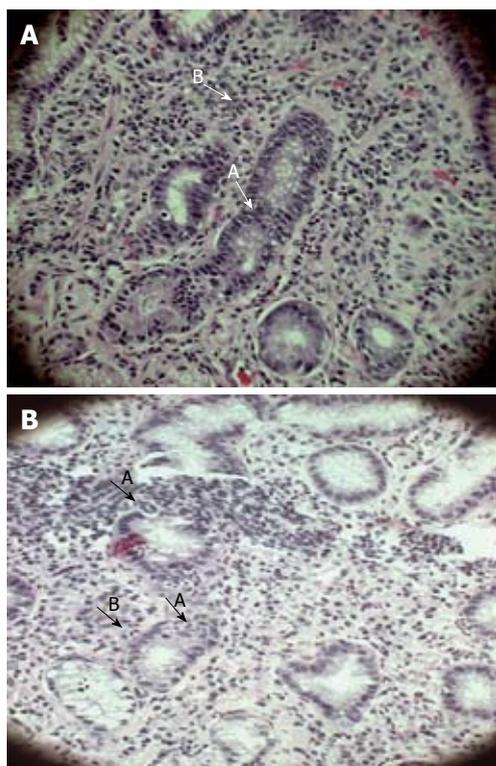


Figure 1 The histopathological features of active gastritis in DU and TR. A: Polymorphonuclear cells infiltration inside glands (HE, $\times 400$) (A) and in lamina propria (B)-DU (HE, $\times 400$); B: Neutrophils in glands (A) (HE, $\times 400$) and in lamina propria (B)-TR (HE, $\times 400$).

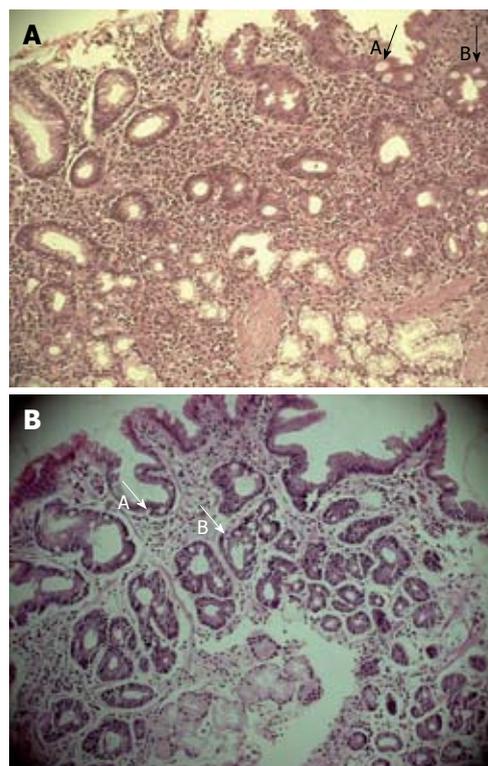


Figure 3 Intestinal metaplastic changes in DU and TR. A: Metaplastic changes with goblet cells in glands (B) and surface epithelium of gastric mucosa (A)-DU (HE, $\times 200$); B: Metaplastic cells replacing the normal gastric epithelium in glands (B) and surface mucosal epithelium (A)-TR (HE, $\times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

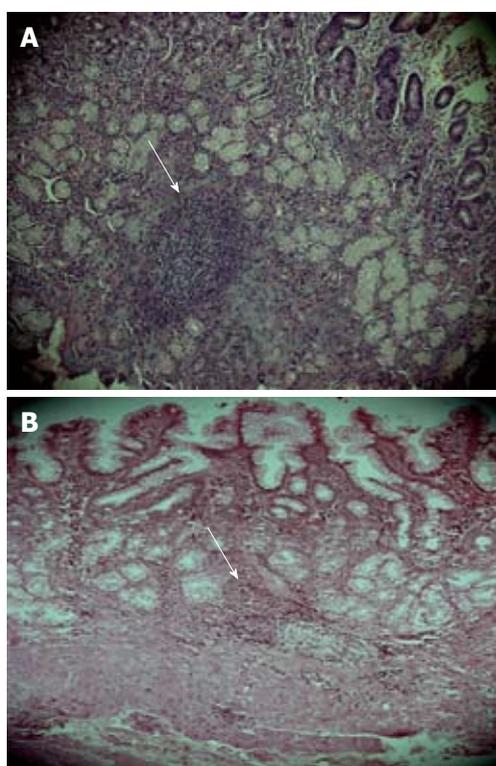


Figure 2 Mononuclear cells, lymphoid follicles and aggregates in DU and TR. A: Arrow indicates mononuclear cells in lamina propria with a lymphoid follicle in DU (HE, $\times 200$); B: Arrow indicates mononuclear cells in lamina propria with lymphoid aggregate in TR (HE, $\times 200$).

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

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 *vacAs1* versus nontoxigenic *vacAs2* 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)
<i>cagA</i> positive only	47 (94)	36 (100)
<i>cagA</i> negative only	1 (2)	0 (0)
Both <i>cagA</i> ⁺ and <i>cagA</i> ⁻	2 (4)	0 (0)
<i>vacA</i> s1 only	47 (94)	36 (100)
<i>vacA</i> s2 only	1 (2)	0 (0)
<i>vacA</i> s1 and s2 mixed	2 (4)	0 (0)
<i>vacA</i> m1 only	31 (62)	26 (72.2)
<i>vacA</i> m2 only	17 (34)	7 (19.4)
<i>vacA</i> m1 and m2 mixed	2 (4)	3 (8.3)
<i>iceA1</i> only	29 (58)	15 (41.7)
<i>iceA2</i> only	18 (36)	14 (38.9)
<i>iceA1</i> and <i>iceA2</i> 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 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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Effects of different periods of renal ischemia on liver as a remote organ

Mehri Kadkhodae, Fereshteh Golab, Maryam Zahmatkesh, Rana Ghaznavi, Mehdi Hedayati, Hossein Ali Arab, Seyed Naser Ostad, Manoocher Soleimani

Mehri Kadkhodae, Fereshteh Golab, Maryam Zahmatkesh, Rana Ghaznavi, Department of Physiology, School of Medicine, Tehran University of Medical Sciences, 14155-6447 Tehran, Iran

Mehdi Hedayati, Endocrine and Metabolism Research Center, Shahid Beheshti University of Medical Sciences, 193954763 Tehran, Iran

Hossein Ali Arab, Department of Pharmacology, School of Veterinary Medicine, Tehran University, 141556453 Tehran, Iran

Seyed Naser Ostad, Cell culture laboratory, Faculty of Pharmacy, Tehran University of Medical Sciences, 14155-6447 Tehran, Iran

Manoocher Soleimani, Department of Medicine, University of Cincinnati, MSB G259 OH, United States

Author contributions: Kadkhodae M conception and design, and drafting of the article; Zahmatkesh M conception and design, analysis and interpretation of data; Golab F acquisition, analysis and interpretation of data; Ghaznavi R, Hedayati M, Arab HA, Ostad SN acquisition of data; Soleimani M conception and design, final approval of the version to be published.

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Correspondence to: Dr. Mehri Kadkhodae, Department of Physiology, School of Medicine, Tehran University of Medical Sciences, 14155-6447 Tehran, Iran. kadkhodm@tums.ac.ir

Telephone: +98-21-88259862 Fax: +98-21-66419484

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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.

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

Peer reviewer: Valentin Fuhrmann, MD, Department of Internal Medicine 4, Intensive Care Unit, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

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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 $37 \pm 1^\circ\text{C}$. 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 laparotomy 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 $\times 400$ 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 \pm 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 μ mol/g, $P < 0.05$) and 60 min (25.86 ± 1.71 μ mol/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 μ mol/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 \pm 25.7	128 \pm 27.5
30 min ischemia + 1 h reperfusion	275 \pm 52.6	119 \pm 20.8
45 min ischemia + 1 h reperfusion	459.7 \pm 17.7 ^a	128 \pm 15.5
60 min ischemia + 1 h reperfusion	491.5 \pm 74.8 ^a	444.6 \pm 198.6 ^a

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

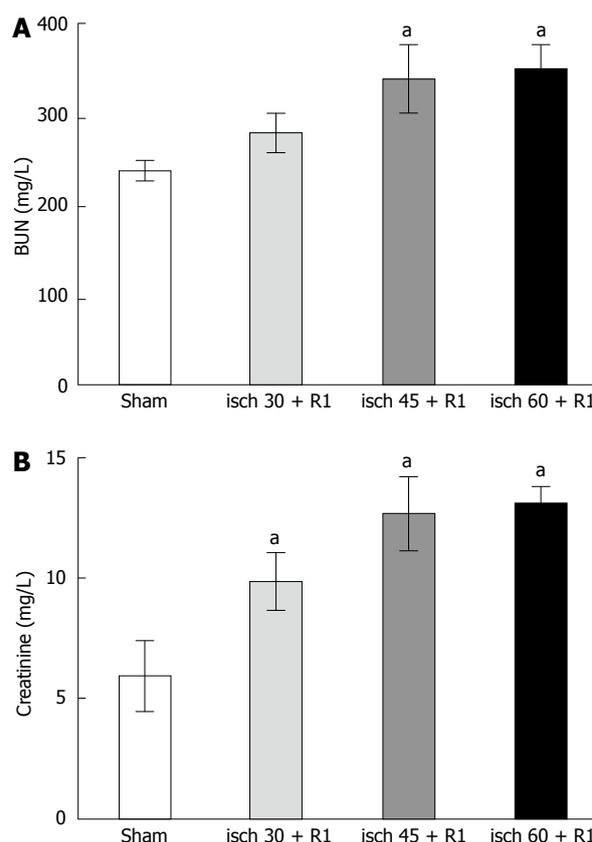


Figure 1 Alterations in renal function during different renal I/R periods. A: BUN; B: Plasma creatinine. The data are presented as mean \pm SE. ^a $P < 0.05$ vs sham-operated group. All ischemic periods were followed by 1 h reperfusion.

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,

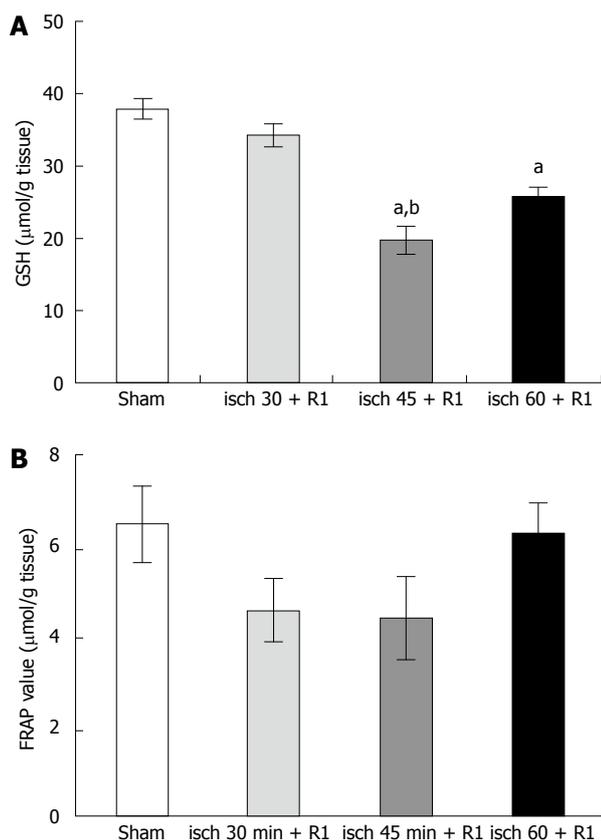


Figure 2 Alterations in liver GSH (A) and FRAP (B) during different renal I/R periods. The data are presented as mean \pm SE. ^a $P < 0.05$ vs sham-operated group; ^b $P < 0.05$ vs 30-min ischemia group. All ischemic periods were followed by 1 h reperfusion.

$P < 0.05$) 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).

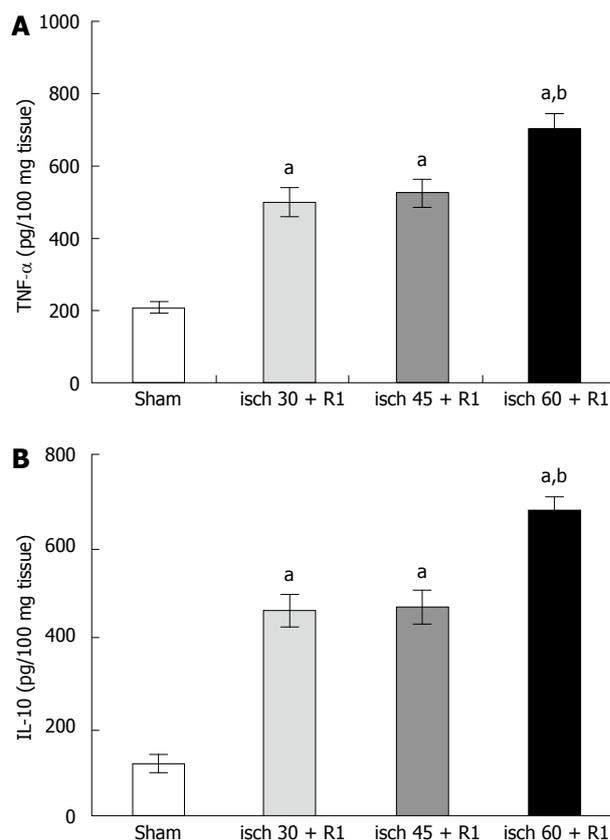


Figure 3 Alterations in liver TNF- α (A) and IL-10 (B) during different renal I/R periods. The data are presented as mean \pm SE. ^a $P < 0.05$ vs sham-operated group; ^b $P < 0.05$ vs other groups. All ischemic periods were followed by 1 h reperfusion.

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^[15]. 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

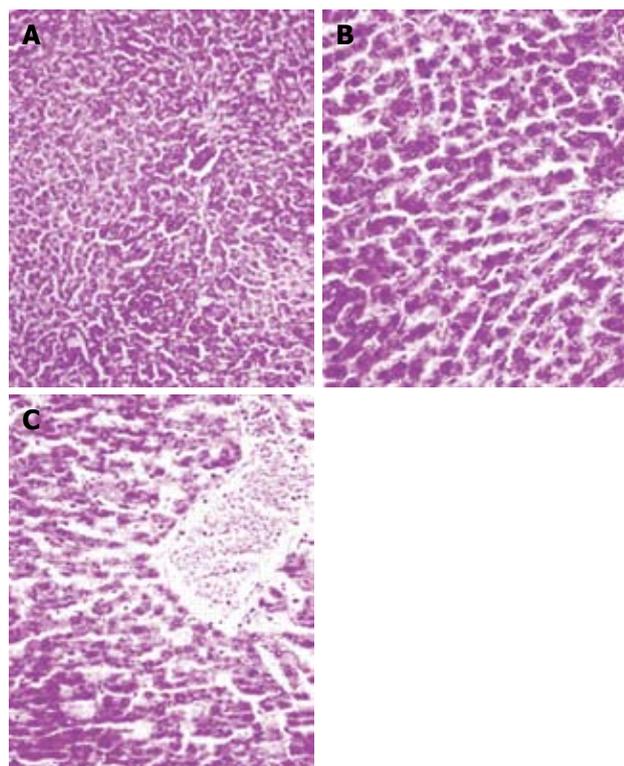


Figure 4 Hematoxylin and eosin-stained sections of rat liver. A: Sham-operated group. B: I/R group, vacuolization was frequent in the 45 min ischemia-reperfused tissues. Irregularity, pale (atypical) nuclei, disintegrated cytoplasm, and infiltration of leukocytes were seen. C: Similar changes were observed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

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

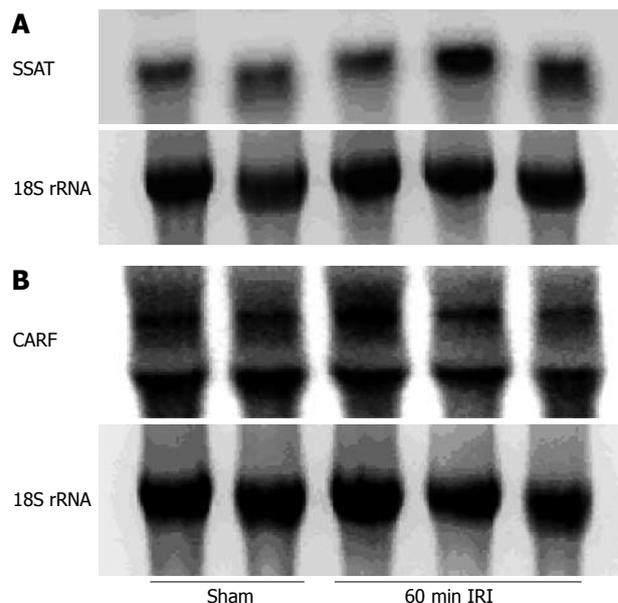


Figure 5 Alterations in liver SSAT and CARF expression during 60 min renal ischemia followed by 1 h reperfusion. A: SSAT mRNA levels in the liver of renal ischemic samples were $139 \pm 7\%$ vs the sham-operated group, after adjustment for RNA loading as determined by 18S rRNA. $P < 0.05$ vs sham-operated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

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

Mirosław Jarosz, Ewa Rychlik, Magdalena Siuba, Wioleta Respondek, Małgorzata Ryżko-Skiba, Iwona Sajór, Sylwia Gugąła, Tomasz Błażejczyk, Janusz Ciok

Mirosław Jarosz, Ewa Rychlik, Magdalena Siuba, Wioleta Respondek, Małgorzata Ryżko-Skiba, Iwona Sajór, Sylwia Gugąła, Tomasz Błażejczyk, Janusz Ciok, Department of Dietetics and Nutrition in Hospitals with Clinic of Metabolic Diseases and Gastroenterology, National Food and Nutrition Institute, 02-903 Warsaw, Poland

Author contributions: Jarosz M designed the research; Rychlik E, Respondek W, Ryżko-Skiba M, Sajór I, Gugąła S, Błażejczyk T, Jarosz M performed the research; Rychlik E, Siuba M analyzed the data; Jarosz M, Rychlik E, Siuba M, Ciok J wrote the paper.

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Correspondence to: Mirosław Jarosz, Professor, National Food and Nutrition Institute, Powsińska St. 61/63, 02-903 Warsaw, Poland. jarosz@izz.waw.pl

Telephone: +48-22-5509677 Fax: +48-22-8421103

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Jarosz M, Rychlik E, Siuba M, Respondek W, Ryżko-Skiba M, Sajór I, Gugąła S, Błażejczyk T, Ciok J. Dietary and socio-economic factors in relation to *Helicobacter pylori* re-infection. *World J Gastroenterol* 2009; 15(9): 1119-1125 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1119.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1119>

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.

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

Table 1 Characteristics of the examined groups

	Group I HP (+)			Group II HP (-)		
	<i>n</i>	Mean	Range	<i>n</i>	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

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 $P < 0.05$.

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

¹Number 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 (+) <i>n</i> = 111 ¹	Group II HP (-) <i>n</i> = 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)
Additional work outside the main job	No	4 (18)	12 (32)
	Yes	6 (8)	8 (7)
Feeling tired	No	65 (92)	110 (93)
	Very often	58 (53)	96 (55)
	Rather often	16 (14)	27 (16)
	Rather rarely	26 (24)	40 (23)
Self-assessed stress exposure	Hardly ever	10 (9)	11 (6)
	Very often	47 (43)	89 (51)
	Rather often	15 (13)	27 (16)
	Rather rarely	37 (34)	42 (24)
Smoking	Hardly ever	11 (10)	15 (9)
	Yes	36 (32)	54 (31)
	No	75 (68)	120 (69)

¹Number 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?

Winita Hardikar, Shivani Kansal, Ronald P J Oude Elferink, Peter Angus

Winita Hardikar, Peter Angus, Victorian Liver Transplant Unit, Austin Hospital, Melbourne 3084, Australia

Winita Hardikar, Shivani Kansal, Department of Gastroenterology & Clinical Nutrition, Royal Children's Hospital, Melbourne 3052, Australia

Winita Hardikar, Department of Paediatrics, University of Melbourne, Melbourne 3052, Australia

Winita Hardikar, Shivani Kansal, Murdoch Children's Research Institute, Melbourne 3052, Australia

Ronald P J Oude Elferink, AMC Liver Centre, Academic Medical Centre, Amsterdam 1105AZ, The Netherlands

Author contributions: Hardikar W, Angus P, Kansal S and Oude Elferink RPJ contributed equally to this work; Kansal S and Hardikar W reviewed the cases and wrote the manuscript; Angus P identified the cases, ordered the investigations and assisted in writing the manuscript; Oude Elferink RPJ analysed the DNA and proof read the manuscript.

Correspondence to: Winita Hardikar, Professor, Department of Gastroenterology, Royal Children's Hospital, Flemington Road, Parkville, Melbourne 3052, Australia. winita.hardikar@rch.org.au

Telephone: +61-3-93455060 Fax: +61-3-93456240

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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*

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.

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

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

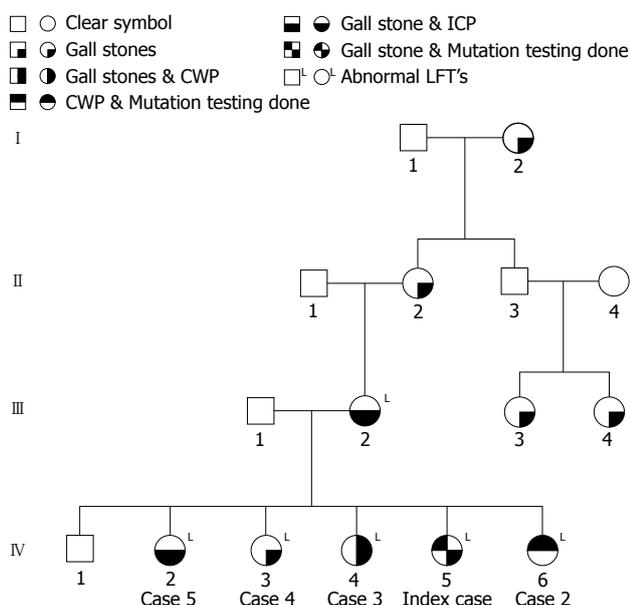


Figure 1 Pedigree showing affected family members with ICP, gallstones, and cholestasis with the oral contraceptive (CWP).

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).

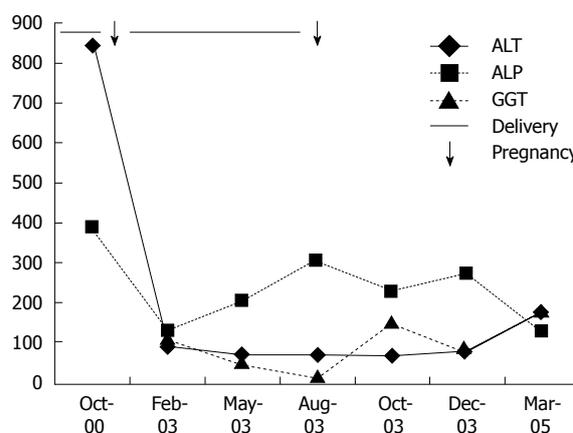


Figure 2 Liver function tests of the index case with an MDR3 mutation.

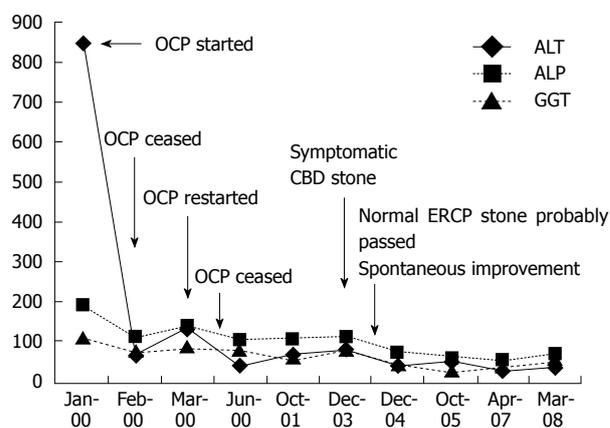


Figure 3 Liver function tests of case 2 with an MDR3 mutation.

These symptoms improved after discontinuation of contraceptives; however, they recurred when she recommenced 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 of 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

Panagiotis Katsinelos, Jannis Kountouras, Georgios Dimitriadis, Grigoris Chatzimavroudis, Christos Zavos, Ioannis Pilpilidis, George Paroutoglou, George Germanidis, Kostas Mimidis

Panagiotis Katsinelos, Grigoris Chatzimavroudis, Ioannis Pilpilidis, George Paroutoglou, George Germanidis, Kostas Mimidis, Department of Endoscopy and Motility Unit, Central Hospital, 54635 Thessaloniki, Greece

Jannis Kountouras, Christos Zavos, Department of Gastroenterology, Second Medical Clinic, Aristotle University of Thessaloniki, Ippokration Hospital, 54635 Thessaloniki, Greece

Georgios Dimitriadis, First Department of Urology, Aristotle University of Thessaloniki, Central Hospital, 54635 Thessaloniki, Greece

Author contributions: Katsinelos P was the main endoscopist; Zavos C and Pilpilidis I analyzed and interpreted the patient data; Paroutoglou G, Germanidis G and Mimidis K reviewed the relative literature; Katsinelos P and Chatzimavroudis G wrote the paper; Kountouras J and Dimitriadis G were contributors in revising the manuscript critically for intellectual content.

Correspondence to: Dr. Panagiotis Katsinelos, Department of Endoscopy and Motility Unit, Central Hospital, Ethnikis Aminis 41, 54635 Thessaloniki, Greece. gchatzim@med.auth.gr

Telephone: +30-2310-963341 Fax: +30-2310-210401

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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.

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

Peer reviewer: Nageshwar D Reddy, Professor, Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad 500082, India

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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

Dimitris P Korkolis, Chrysanthi Aggeli, George D Plataniotis, Emmanuel Gontikakis, Helen Zerbinis, Nikitas Papantoniou, Dimitris Xinopoulos, Nikiforos Apostolikas, Perikles P Vassilopoulos

Dimitris P Korkolis, Chrysanthi Aggeli, George D Plataniotis, Emmanuel Gontikakis, Helen Zerbinis, Perikles P Vassilopoulos, Department of Surgical Oncology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, Athens 14561, Greece

Nikitas Papantoniou, Dimitris Xinopoulos, Department of Gastroenterology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, Athens 14561, Greece

Nikiforos Apostolikas, Department of Surgical Pathology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, Athens 14561, Greece

Author contributions: Korkolis DP designed the research; Korkolis DP, Aggeli C, Plataniotis GD, Gontikakis E, Zerbinis H, Vassilopoulos PP performed the research; Apostolikas N performed the data analysis; Papantoniou N and Xinopoulos D contributed to the interpretation of data.

Correspondence to: Dimitris P Korkolis, MD, PhD, Department of Surgical Oncology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, 22 Socratous Street, 1st Floor, Kifissia, Athens 14561, Greece. dkorkolis_2000@yahoo.com

Telephone: +30-210-8083743 Fax: +30-210-8012689

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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.

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

Peer reviewers: Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan; Kazuhiro Hanazaki, MD, Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

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>

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 tomography (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-fetoprotein (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

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, extra-hepatically 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.

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 esophago-jejunal 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 treat-



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

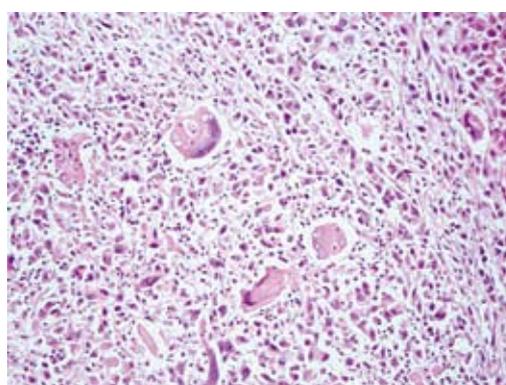


Figure 3 Microscopic appearance of a poorly differentiated HCC developed in liver cirrhosis. It was characterized by pleomorphic, multinucleated giant cells and extensive areas of tissue necrosis (HE, × 100).

ment 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.

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

Akira Kanamori, Takashi Kumada, Seiki Kiriya, Yasuhiro Sone, Makoto Tanikawa, Yasuhiro Hisanaga, Hidenori Toyoda, Hiroki Kawashima, Akihiro Itoh, Yoshiki Hirooka, Hidemi Goto

Akira Kanamori, Takashi Kumada, Seiki Kiriya, Yasuhiro Sone, Makoto Tanikawa, Yasuhiro Hisanaga, Hidenori Toyoda, Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki 503-8502, Japan

Hiroki Kawashima, Akihiro Itoh, Hidemi Goto, Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan

Yoshiki Hirooka, Hidemi Goto, Department of Endoscopy, Nagoya University Hospital, Nagoya 466-8560, Japan

Author contributions: All authors contributed equally to this work; Akira Kanamori wrote the paper.

Correspondence to: Akira Kanamori, Department of Gastroenterology, 4-86 minaminokawa-cho, Ogaki, Gifu, 503-8502, Japan. tkumada@he.mirai.ne.jp

Telephone: +81-584-813341 Fax: +81-584-755715

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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. Endoscopic 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.

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

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Kanamori A, Kumada T, Kiriya 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 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 2 Hypotonic duodenography demonstrating a mass situated 15 mm proximal to the major papilla, which was raised highly from the duodenum.



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



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

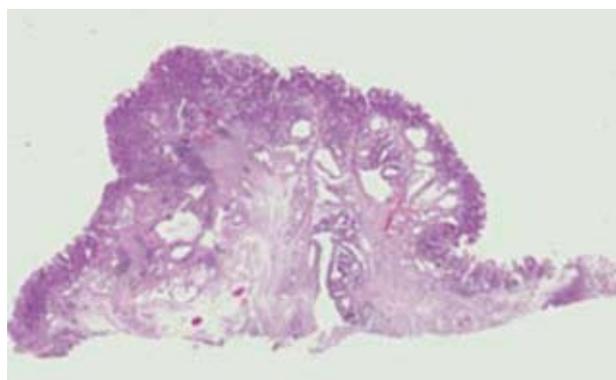


Figure 6 Histopathological findings of the specimen showed tubular adenoma and the margin of the tumor was negative; however, a slight infiltration of the pancreatic duct system was revealed (HE stain, × 4).

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

Wei Guo, Wei Han, Jun Liu, Lan Jin, Jian-She Li, Zhong-Tao Zhang, Yu Wang

Wei Guo, Wei Han, Jun Liu, Lan Jin, Jian-She Li, Zhong-Tao Zhang, Yu Wang, Department of General Surgery, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

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.

Correspondence to: Zhong-Tao Zhang, Department of General Surgery, Beijing Friendship Hospital, Capital Medical University, Beijing 100050,

China. zhangzht@medmail.com.cn

Telephone: +86-10-63138768 Fax: +86-10-63138457

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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.

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

Peer reviewers: Dr. Massimo Raimondo, Division of Gastroenterology and Hepatology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States; Kiichi Tamada, MD, Department of Gastroenterology, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Kawachigun, Tochigi 329-0498, Japan

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

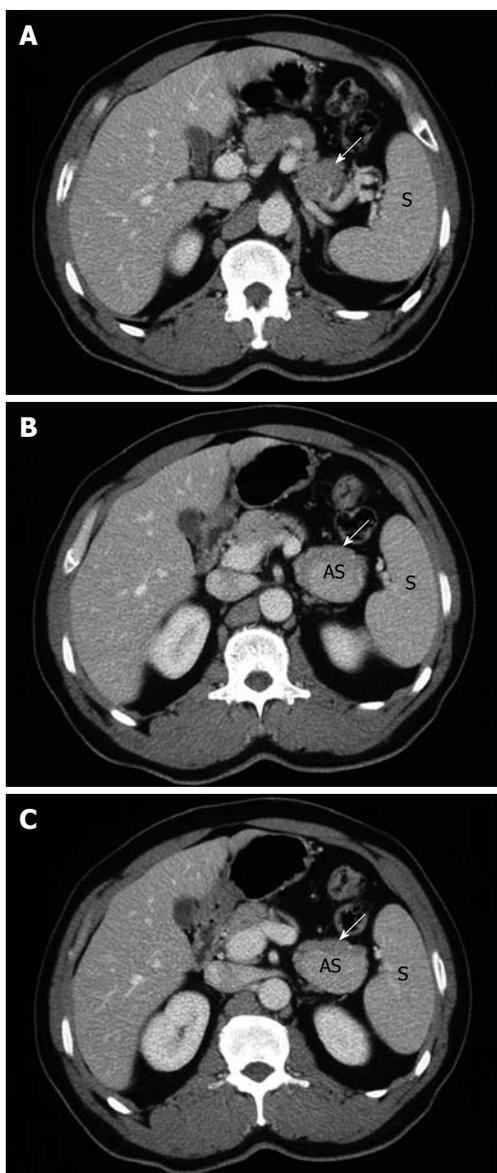


Figure 2 Axial CT image obtained in the portal venous phase showing an ovoid, well-enhanced nodule (AS) in pancreatic tail (arrow). The density of this lesion was higher than that of the pancreatic parenchyma and similar to that of the spleen (S). However, this finding is similar to that of pancreatic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

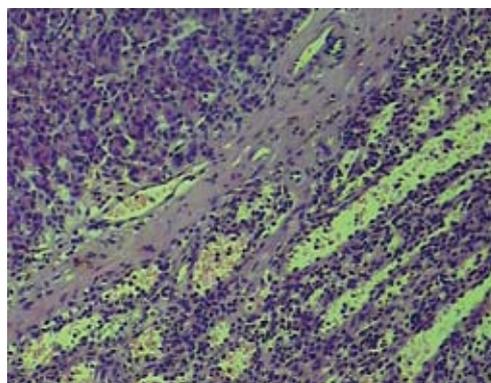


Figure 4 An intrapancreatic accessory spleen confirmed by histological imaging (HE, $\times 100$).

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 grayscale 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. ≤ 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 ^{99m}Tc-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

Jun Gong, Zhen-Jiang Zheng, Gang Mai, Xu-Bao Liu

Jun Gong, Zhen-Jiang Zheng, Gang Mai, Xu-Bao Liu, Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Gong J prepared the manuscript and literature review; Liu XB performed the surgery and revised the manuscript; Zheng ZJ collected the data and performed the surgery; Mai G revised English of the manuscript.

Correspondence to: Xu-Bao Liu, Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. xbliu@medmail.com.cn

Telephone: +86-28-85422474 Fax: +86-28-85422476

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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.

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

Peer reviewer: Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

Gong J, Zheng ZJ, Mai G, Liu XB. Malrotation causing duodenal chronic obstruction in an adult. *World J Gastroenterol* 2009; 15(9): 1144-1146 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1144.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1144>

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°C. 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).

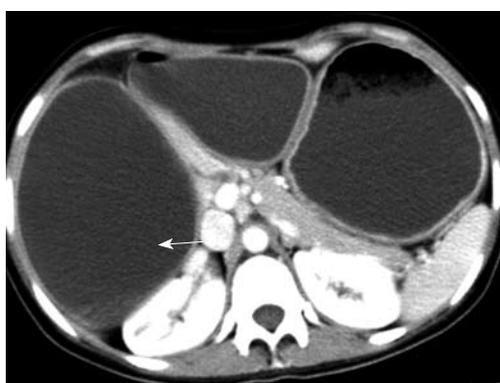


Figure 2 CT scan shows duodenum does not cross behind the superior mesenteric artery and the superior mesenteric vein, and extreme dilation of proximal duodenum (arrow).

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 described 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 10000 (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 volvulus^[19].

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S- Editor Li JL L- Editor Ma JY E- Editor Yin DH

Lubiprostone: Clinical applications beyond constipation

Shailendra Kapoor

Shailendra Kapoor, Kristin 24, Schaumburg, IL 60195, United States
Author contributions: Kapoor S contributed all to this work.
Correspondence to: Shailendra Kapoor, MD, Kristin 24, Schaumburg, IL 60195, United States. shailendrakapoor@yahoo.com
Telephone: +1-847-8866789 Fax: +1-847-8979878
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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 augments 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.

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

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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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Akira Andoh, MD

Department of Internal Medicine, Shiga University of Medical Science, Seta Tukiowa, Otsu 520-2192, Japan

Jamie S Barkin, MD, Professor of Medicine, Chief

Sinai Medical Center Division of Gastroenterology, Mt. Sinai Medical Center, University of Miami, School of Medicine, 4300 Alton Road, Miami Beach, FL 33140, United States

Dr. Francesco Costa

Dipartimento di Medicina Interna-U.O. di Gastroenterologia Università di Pisa-Via Roma, 67-56122-Pisa, Italy

Zong-Jie Cui, PhD, Professor

Institute of Cell Biology, Beijing Normal University, 19 XinJieKou-WaiDaJie, Beijing 100875, China

Benedicte Y De Winter, President, MD, PhD, Professor, Associate Professor, Chief

Laboratory of Gastroenterology, University of Antwerp, Campus Drie Eiken, Universiteitsplein 1, 2610 Antwerp, Belgium

Conor P Delaney, MD MCh PhD FRCSI FACS, Professor of Surgery

Case Western Reserve University, Chief, Division of Colorectal Surgery, Vice-Chairman, Department of Surgery, Director, Institute for Surgery and Innovation, University Hospitals, Case Medical Center, 11100 Euclid Avenue Cleveland, OH 44106-5047, United States

Sharon DeMorrow, Assistant Professor

Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

Abdel-Rahman El-Zayadi, Professor

Department of Hepatology and Gastroenterology, Ain Shams University and Cairo Liver Center, 5, El-Gergawy St. Dokki, Giza 12311, Egypt

Zvi Fireman, MD, Associate Professor of Medicine, Head

Gastroenterology Department, Hillel Yaffe Med Ctr, POB 169, 38100, Hadera, Israel

Peter Raymond Gibson, Professor

Department of Medicine, Box Hill Hospital, Box Hill, Victoria 3128, Australia

Ignacio Gil-Bazo, MD, PhD

Cancer Biology and Genetics Program, Memorial-Sloan Kettering Cancer Center, 1275 York Avenue, Box 241, New York 10021, United States

Salvatore Gruttadauria, MD, Assistant Professor

Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

Sanjeev Gupta, MD, Professor

Albert Einstein College of Medicine, Ullmann Building, Room 625, 1300 Morris Park Avenue, Bronx, NY 10461, United States

Kazuhiro Hanazaki, MD, Professor and Chairman

Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

Dr. Pietro Invernizzi

Division of Internal Medicine, Department of Medicine, Surgery, Dentistry, San Paolo School of Medicine, University of Milan, Via Di Rudinfi 8, 20142 Milan, Italy

Roger Jones, Professor

Department of General Practice and Primary Care, King's College London, 5 Lambeth Walk, London SE11 6SP, United Kingdom

Serhan Karvar, MD, Assistant Professor of Medicine

University of Southern California, Keck School of Medicine, Division of Gastrointestinal & Liver Diseases, 2011 Zonal Avenue, HMR 101, Los Angeles, CA 90089, United States

Jae J Kim, MD, PhD, Associate Professor

Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Anders E Lehmann, PhD, Associate Professor

Senior Principal Scientist, Bioscience, AstraZeneca R&D Mölndal, Mölndal, Sweden

Robin G Lorenz, Associate Professor

Department of Pathology, University of Alabama at Birmingham, 845 19th Street South BBRB 730, Birmingham, AL 35294-2170, United States

Shigeru Marubashi, MD, PhD

Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita 565-0871, Japan

Fanyin Meng, MD, PhD, Assistant Professor

Department of Internal Medicine, Ohio State University, Room 514A Medical Research Facility, 420 West 12th Avenue, Columbus, Ohio 43210, United States

Ibrahim A Al Mofleh, Professor

Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Yehuda Ringel, MD, Assistant Professor of Medicine

Gastroenterology & Hepatology, University of North Carolina at Chapel Hill, 130 Mason Farm Road, CB 7080, 4107 Bioinformatics Building, Chapel Hill, NC 27599-7080, United States

Ian C Roberts-Thomson, Professor

Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

Damian Casadesus Rodriguez, MD, PhD

Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Motoko Sasaki, MD

Department of Human Pathology, Kanazawa University Graduate School of Medicine, Takaramachi 13-1, Kanazawa 920-8640, Japan

Alain L Servin, PhD

Faculty of Pharmacy, French National Institute of Health and Medical Research, Unit 756, Rue J.-B. Clément, F-922296 Châtenay-Malabry, France

Tomohiko Shimatani, Assistant Professor

Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Ulrike S Stein, PhD, Assistant Professor

Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 13125 Berlin, Germany

Saúl Villa-Trevio, MD, PhD

Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN (Cinvestav), Ave. IPN No. 2508. Col. San Pedro, Zacatenco, CP 07360, México, DF, Mexico

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Mouse Models of Cancer

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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
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<http://www.apasl2009hongkong.org/en/home.aspx>

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AGAI/AASLD/ASGE/ACG Training Directors' Workshop

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www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
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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.apdwcgress.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

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John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Za-zhi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 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. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary 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. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

^[3]Passed away on June 14, 2008



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INTRODUCTION

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Islet transplantation and antioxidant management: A comprehensive review

Seyed Sajad Mohseni Salehi Monfared, Bagher Larijani, Mohammad Abdollahi

Seyed Sajad Mohseni Salehi Monfared, Mohammad Abdollahi, Faculty of Pharmacy, Pharmaceutical Sciences Research Centre, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Seyed Sajad Mohseni Salehi Monfared, Bagher Larijani, Endocrinology and Metabolism Research Center, Tehran University of Medical Sciences, Tehran 1411413137, Iran

Author contributions: Mohseni Salehi Monfared SS drafted the paper; Larijani B read the paper and commented; Abdollahi M supervised, reviewed, and edited the paper.

Correspondence to: Mohammad Abdollahi, Professor, Faculty of Pharmacy, Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran. mohammad.abdollahi@utoronto.ca

Telephone: +98-21-66959104 Fax: +98-21-66959104

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Abstract

Islet transplantation as a promising treatment for type 1 diabetes has received widespread attention. Oxidative stress plays an essential role in cell injury during islet isolation and transplantation procedures. Antioxidants have been used in various studies to improve islet transplantation procedures. The present study reviews the role of oxidative stress and the benefits of antioxidants in islet transplantation procedures. The bibliographical databases Pubmed and Scopus were searched up to November 2008. All relevant human and animal *in-vivo* and *in-vitro* studies, which investigated antioxidants on islets, were included. Almost all the tested antioxidants used in the *in-vitro* studies enhanced islet viability and insulin secretion. Better control of blood glucose after transplantation was the major outcome of antioxidant therapy in all *in-vivo* studies. The data also indicated that antioxidants improved islet transplantation procedures. Although there is still insufficient evidence to draw definitive conclusions about the efficacy of individual supplements, the benefits of antioxidants in islet isolation procedures cannot be ignored.

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Key words: Antioxidant; Diabetes; Free radical; Islet; Transplant

INTRODUCTION

Diabetes mellitus which is characterized by hyperglycemia has become an important disorder with major costs and complications worldwide. In a genetically susceptible person with an environmental trigger such as viruses and toxins, autoimmune destruction of β cells can occur causing type 1 diabetes usually in childhood and in young adults^[1]. Type 2 diabetes usually results from dysfunction of β cells and peripheral insulin resistance. It is accepted that oxidative stress is increased in both type 1 and type 2 diabetes, and it has been shown in many studies that biochemical markers of oxidative stress are higher in tissue samples and in the pancreas of diabetic patients^[2]. However, secondary complications comprising micro- and macro-vascular disorders which result in frequent amputation, end-stage renal failure, and blindness have motivated various investigators to identify new therapeutic approaches to cure diabetes. With this aim, whole pancreas transplantation was first carried out in 1966 by Kelly and Lillehei at the University of Minnesota and was then performed worldwide. Marked morbidity following pancreas transplantation prompted researchers to find other possible ways of curing this disease. As reviewed by Fontaine *et al*^[3], since 1974 when the first human islet transplantation was conducted by Sutherland and his colleagues, and then up to 1999, among approximately 1000 patients who received islet allotransplantations, most of the results were disappointing and only 10% remained insulin-independent for longer than one year.

In 1999, use of the Edmonton protocol with its steroid-free immunosuppressive regimen was an impressive leap in achieving insulin-independence after islet transplantation^[4]. Although the Edmonton protocol

succeeded in achieving insulin-independence, two or more donors are still needed to achieve normoglycemia following islet transplantation.

GREAT EFFORTS TO OPTIMIZE ISLET TRANSPLANTATION

Recent years, many studies have been carried out to optimize the Edmonton protocol to obtain the final goal of one-donor islet transplantation. Donor characteristics and pancreas procurement are the first steps. The quality of the donor pancreas depends largely on donor factors, such as age, body mass index, serum glucose levels, and hemodynamic stability^[5]. In the procurement phase, improved surgical techniques such as *in-vivo* atraumatic dissection and *in-situ* separation have made sufficient advancements in minimizing warm ischemia before isolation. Moreover, using perfluorocarbon (PFC) and oxygen in the University of Wisconsin (UW) solution during cold ischemic preservation, which is identified as the Two layer method (TLM), has enhanced the final quality of islets^[6].

One of the most important areas of research in islet transplantation is the isolation procedure, which remains a major topic of islet transplant investigations. At present, the maximum rate of purified islets at leading centers is about 50% to 70%^[7]. The largest reduction in islet yield occurs during the islet isolation phase. Below we will look in detail at the islet isolation phase, which has been the focus of a large number of studies.

Other efforts have focused on new approaches relating to the best site of transplantation, better revascularization of islet-grafts, visualization after engraftment, and further anti-rejection strategies which are not covered in this review. For the latest progress in these aspects of islet transplantation, readers are referred to two recently published review articles^[8,9].

ISLET ISOLATION AND ROLE OF OXIDATIVE STRESS

Successful islet transplantation would enable patients to live without tedious multiple insulin injections and reduce the risk of hypoglycemia. As previously mentioned, the main restriction in this procedure is the loss of healthy islets at the end of the operation due to the inevitable prolonged time required for islet isolation. In the clinical setting, the isolated islets are transplanted immediately or within a few days after harvesting from donors. Isolated islets are avascular and are therefore ischemic from the time of isolation through to the period required for revascularization. Prolonged ischemia has profound deleterious effects on the islets, resulting in a significant loss of islet cells. Two major factors which are expected to cause potential cell damage include hypoxia and enzymatic/mechanical trauma related to the experimental procedures.

Reactive oxygen species (ROS) in physiological concentrations provide normal conditions to protect

cells, for instance they are important within white cells to allow effective defense against infection. Nevertheless, whenever ROS are accumulated in excess and for long periods they can destroy cells. Free radicals cause damage to cellular proteins, membrane lipids and nuclear nucleic acids. The only protective mechanism present in the body to protect cells against excessive free radicals is the antioxidant enzyme system. Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are the main antioxidant enzymes. Hypoxia which occurs during the islet transplantation procedure initiates a cascade of biochemical reactions which results in the production of ROS causing necrosis and apoptosis *via* intracellular pathways.

Another important point which can lead to worsening of this condition is that β cells contain low levels of antioxidative enzymes such as CAT, SOD and GPx and thus they can only weakly defend against oxidative stress^[10-12]. These findings have demonstrated the major destructive role of oxidative stress in islet transplantation and have encouraged investigators to use antioxidants during the isolation phase and the entire transplantation process to overcome the final lack of healthy islets.

Various strategies such as modifications in enzymatic digestion^[13], purification with iodixanol instead of ficoll^[14], incubation of purified islets in culture medium, genetic manipulation for overexpression^[15,16] or silencing^[17] of specific genes, employment of different anti-inflammatory or other supplements such as small intestinal submucosa^[18] and serecin^[19] have been considered in an attempt to increase islet quality and yield. In a previous publication we hypothesized that using phototherapy could improve islet function before transplantation^[20].

This review focuses on antioxidant management in islet transplantation procedures and evaluated both *in-vitro* and *in-vivo* studies.

ANTIOXIDANT RECRUITMENT IN ISLET TRANSPLANTATION STUDIES

To perform a comprehensive survey and obtain all related studies we searched Pubmed and Scopus databases up to November 2008. Search terms were "antioxidant", "islet", "transplant" and "oxidative stress". Individual antioxidants such as vitamin E or C were also searched with the term "islet".

There were many studies in which various methods other than utilizing antioxidants were used to increase function and viability of islets which were not included in this review. Table 1 lists the various antioxidant agents which have been added to islets during the isolation procedure or to the culture medium. Table 2 shows the *in-vivo* studies.

Metabolite and vitamin antioxidants

Vitamin E or tocopherol was the supplement most used to enhance the viability of islets. This lipid-soluble

Table 1 *In-vitro* effects of antioxidants on pancreatic islets

Authors	Substance/Dose	Sample	Study design	Duration	Assessments
Campbell <i>et al</i> ^[21] , 2008	Sodium selenite (30 nmol/L)	Rat	Islets were incubated with/without Na ₂ SeO ₃	72 h	Insulin content ↑ Insulin secretion ↑ Glucose-stimulated insulin secretion ¹
Kanitkar <i>et al</i> ^[22] , 2008	Curcumin (10 μmol/L)	Rat	Cryopreservation of islets with/without curcumin Assessments were performed after 24 h culture post-thawing	Cryopreserved	Intact islets ↑ ROS ↓ Insulin Secretion ↑ Expression of Hsp70 and HO-1 ↑
Thomas <i>et al</i> ^[23] , 2007	Peptide SS-31 (1 nmol/L)	Mice	SS-31 was added to all reagents which were used for islet isolation	Isolation procedure	Islet apoptosis ↓ Islet yield ↑
		Human	Islets were incubated in culture medium with without SS-31	72 h	Islet apoptosis ↓
Hara <i>et al</i> ^[24] , 2007	Epigallocatechin-3-gallate (EGCG) (36, 72, 360 μmol/L)	Rat	Islets cultured under normal or H/R condition with/without EGCG	48 h	Islet apoptosis ↓ LDH ↓ Insulin secretion ↑
Xiong <i>et al</i> ^[25] , 2006	Puerarin (10, 50, 100 μmol/L)	Rat	Islets cultured under normal or H ₂ O ₂ stress conditions with/without Puerarin	24 h	Islet apoptosis ↓, Islet viability ↑ CAT & SOD activity ↑, ROS ↓ Insulin secretion ↑
Marzorati <i>et al</i> ^[26] , 2006	Glutathione (1, 5, 10 mmol/L) Vitamin E (2 × 10 ⁻⁵ mmol/L) Ascorbic acid (0.3 mmol/L)	Human	Antioxidant agents were added to culture medium individually	48 h	Glutathione : CCL2/MCP-1 release ↓ Insulin secretion ¹ Vitamin E: CCL2/MCP-1 release ¹ Ascorbic acid: CCL2/MCP-1 release ¹
Amoli <i>et al</i> ^[27] , 2006	Curcumin (10, 20 μmol/L)	Rat	Curcumin was added to culture medium	18 h + 24 h	MCP-1 release ↓
Rao <i>et al</i> ^[28] , 2005	MCI - 186	Rat	Islets were treated with H ₂ O ₂ in the presence or absence of MCI-186	18 h	Cell death ↓
Avila <i>et al</i> ^[29] , 2005	L-glutamine (5 mmol/L)	Human	Pancreas were manually perfused through the main pancreatic duct with either the standard HBSS or with HBSS + L-glutamine	0 → 24 h	Islet yield ↑ Lipid peroxidation (MDA) ↓ Glutathione (GSH) ↑ Viability ¹ Insulin secretion ¹
Brandhorst <i>et al</i> ^[30] , 2005	Free L-glutamine (2.5 & 5 mmol/L/L) Stable L-glutamine [NALG] (2.5 & 5 mmol/L)	Pig	Islets pretreated with free L-glutamine or NALG for 24 h and then stressed with H ₂ O ₂ , ETA or cytokine mix	24 h	Viability: L-glutamine ↑ NALG ¹
Giovagnoli <i>et al</i> ^[31] , 2005	Encapsulated enzymes (100 mg/35 mL)	NPCCs	NPCCs were co-cultured with/without entrapped enzymes	9 d	Viability ↑, Insulin secretion ↑ Insulin/DNA ratio ↑ mRNA expression of insulin and Glut-2 ↑
Bottino <i>et al</i> ^[32] , 2004	MnTDE (34 μmol/L)	Human	MnTDE was added as a supplement to culture or isolation medium	60 h	Islet yield ↑, Insulin secretion ↑ NF-κB DNA-binding ↓ IL6 & MCP-1 ↓, IL8 ¹ PARP activation ↑
Arata <i>et al</i> ^[33] , 2004	Ascorbic Acid-2 Glucoside (AA2G) (100 μg/mL)	Human	Cryopreservation of islets with UW solution or AA2G + UW solution	Cryopreserved	Viability ↑ Insulin secretion ↑ Insulin gene expression ↑
Luca <i>et al</i> ^[34] , 2003	Free vitamin D3 (2 μmol/L) Encapsulated vitamin D3 (20 μmol/L)	Rat	Islets were treated with/without vitamin D3	9 d	Insulin secretion ↑
Hardikar <i>et al</i> ^[35] , 2001	Taurine (0.3 & 3 mmol/L)	Rat	Cryopreservation of islets with/without taurine	Cryopreserved	Viability ↑ Lipid peroxidation ↓
Luca <i>et al</i> ^[36] , 2000	Vitamin D3 Vitamin E	NPCCs	NPCC cells were treated with/without VitD3 & VitE during their maturation and differentiation process	16 d	Large & intact islets ↑ Insulin secretion ↑
Tajiri <i>et al</i> ^[37] , 1999	Vitamin E (50 μg/mL)	Rat	Islets were co-cultured with/without vitamin E	1 d	Insulin secretion ↑
Shewade <i>et al</i> ^[38] , 1999	Riboflavin	Rat	Inclusion of riboflavin in the cryopreserved medium	Cryopreserved	Viability ↑ Lipid peroxidation ↓ GSH ↑, Insulin secretion ↑
Jindal <i>et al</i> ^[39] , 1996	Zinc (20 μmol/L)	Rat	Preservation of islets in UW and Hanks solution with/without Zinc	1, 3, 6 d	Viability ¹

↑: Significant increase compared with non-treated group; ↓: Significant decrease compared with non-treated group; ¹No significance difference between groups. Hsp70: Heat shock protein 70; HO-1: Heme oxygenase-1; H/R: Hypoxia/Reoxygenation; LDH: Lactate dehydrogenase; CCL2/MCP-1: Monocyte chemoattractant protein 1; HBSS: Hanks balanced salt solution; NALG: N-acetyl-L-alanyl-L-glutamine; MDA: Malondialdehyde; MnTDE: Manganese (III) 5,10,15,20-tetrakis (1,3-diethyl-2-imidazolyl) porphyrin; NF-κB: Nuclear factor-κB; PARP: Poly (ADP-ribose) Polymerase indicative of ongoing cell damage and death; UW: University of Wisconsin; NPCCs: Neonatal pancreatic porcine cell clusters.

Table 2 *In-vivo* effects of antioxidants on pancreatic islets

Author	Substance/Dose	Sample	Procedure	Output
Thomas <i>et al</i> ^[23] , 2007	Peptide SS-31 (3 mg/kg)	Mice → Mice	Injection of SS-31 to donor mouse + adding SS-31 during islet isolation	Better glucose control after transplant
Avila <i>et al</i> ^[29] , 2005	L-glutamine (5 mmol/L)	Human → Rat	Transplantation of human islets which were treated before with/without intraductal L-glutamine to nude rats	Normoglycemia percentage ↑ (83% vs 26%) Time to reach normoglycemia ↓ (1.83 d vs 7.6 d)
Brown <i>et al</i> ^[40] , 2005	Pyruvate Vitamin E Vitamin C	Rat → Rat	Oral administration of pyruvate, vitamin E and vitamin C during perioperative period	Pyruvate enhanced engraftment & functionality of suboptimal islet mass Vitamin E and vitamin C failed to enhance
Olcott <i>et al</i> ^[41] , 2004	Salen-manganese [EUK-8] (100 mg/kg)	Mice → Mice	Daily treatment with/without EUK-8 (IP injection) to recipient NOD mice	Better glucose control until 20 d after transplant
Winter <i>et al</i> ^[42] , 2002	Vitamin A (500 µg) Ascorbic Acid (40 mg) Vitamin E (10.6 mg) Selenium (2 µg)	Rat → Rat	Oral antioxidants, 24 h prior to transplantation	Slight better glucose control
Bottino <i>et al</i> ^[43] , 2002	SOD mimetic compound (34 µmol/L)	Human → Mice	Culture of isolated islet in the presence of SOD mimetic for at least 2 h before transplantation	Better glucose control
Hardikar <i>et al</i> ^[35] , 2001	Taurine (0.3 & 3 mmol/L)	Rat → Mice	Transplantation of islets which were previously cryopreserved with/without taurine	No significant difference was observed
Tajiri <i>et al</i> ^[37] , 1999	Vitamin E (40 mg/kg)	Rat → Rat	Every other day IP injection of vitamin E to transplanted rats	Treatment for 2 not for 6 wk enhanced basal insulin release and arginine induced insulin release
Vajkoczy <i>et al</i> ^[44] , 1997	Vitamin E (150, 8000 mg/kg)	Rat → Rat	Oral vitamin E after xenograft	Reduction of xenograft leukocyte-endothelium interaction at day 6 Adequate development of functional capillary density
Gembal <i>et al</i> ^[45] , 1993	Allopurinol Vitamin E Chlorpromazine	Rat → Rat	Transplantation of islets which were pretreated with/without combination of allopurinol, alpha-tocopherol and chlorpromazine.	Better glucose control after 3 d

↑: Significant increase compared with non-treated group; ↓: Significant decrease compared with non-treated group.

vitamin protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction^[46]. Luca *et al*^[36] and Tajiri *et al*^[37] have shown that vitamin E can increase secretion of insulin from islets when added *in-vitro* to the culture medium. Likewise, when vitamin E was administered before, after, or during the transplantation period^[37,42,44], the islet transplantation outcome was better and glucose control was slightly superior compared with control groups. Another study demonstrated that digestion buffer which contained vitamin E, resulted in healthy isolated islets, however, this study was not designed to evaluate the effect of vitamin E and therefore a control group was absent. An investigation into the effect of a mixture of allopurinol, vitamin E and chlorpromazine on islet transplantation concluded that a combination of free radical scavengers, antioxidants and membrane stabilizing drugs may be used to increase the effectiveness of islet transplantation^[45].

In contrast to the above-mentioned positive effects of vitamin E, Brown and colleagues^[40] showed that vitamin E and C could not enhance the function of rat engrafted islets; although the authors did not specify the dose which they applied. In a different study^[26], the use of vitamin E and ascorbic acid did not decrease the release of monocyte chemoattractant protein 1 (MCP-1)

from human islets. MCP-1 is a chemokine secreted from pancreatic islets to recruit the immune system and plays an important role in the clinical outcome of islet transplantation because of its proinflammatory property^[47,48]. Nevertheless, this review did not assess the secretion of insulin or the viability of islets following the use of vitamin E, thus we can not conclude that islets may or may not benefit from vitamin E.

Ascorbic acid or vitamin C is a monosaccharide antioxidant. This water-soluble vitamin is a reducing agent and can neutralize oxygen species. This metabolite antioxidant in the form of ascorbic acid-2 glucoside (AA2G) supplies a stable form of vitamin C in the culture medium and cryopreservation solution and thus improves viability, secretion and expression of insulin in cryopreserved human islets^[53]. In the study by Winter *et al*^[42], the combined administration of vitamin C and E, but not vitamin A and selenium, led to a significant improvement in functional islet graft survival, associated with augmented islet engraftment. In contrast, Brown *et al*^[40] and Marzorati *et al*^[26] showed that the combination of vitamin E and C was unable to improve graft survival or decrease *in-vitro* MCP-1 release, respectively.

Glutathione (GSH), as a cysteine-containing peptide, is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other

metabolites and enzyme systems as well as reacting directly with oxidants in the cells. When GSH was added to culture medium, in contrast to vitamin E and C, it reduced MCP-1^[26]. L-glutamine as a precursor of GSH was shown to increase islet yield when added intra-ductally during human pancreatic islet isolation. This study indicates that intra-cellular GSH levels can be increased by means of intra-ductal glutamine administration prior to the isolation procedure. Consequently, authors found lower cellular lipid peroxidation in islets isolated from glutamine-pre-treated pancreata, indicating less oxidative damage. Although they could not detect any differences in cell viability and islet function *in vitro*, the islets isolated from glutamine-pre-treated pancreata performed significantly better than controls after transplantation in diabetic nude mice^[29]. Supporting this study, Brandhorst *et al.*^[30] demonstrated that pig islet culture will significantly improve if L-glutamine is administered in an unbound (free) form compared with the stable compound N-acetyl-L-alanyl-L-glutamine (NALG).

Taurine (2-amino ethanesulfonic acid) an end-product of sulfur amino acid metabolism, is one of the most abundant free amino acids in the body. The membrane stabilizing, free radical scavenging, and osmoregulatory roles of taurine have been well documented^[49,50]. In one study, taurine enhanced viability and reduced lipid peroxidation in cryopreserved islets. However, no significant difference was observed in the islet insulin content between groups following cryopreservation, and taurine could not enhance glucose control following transplantation of treated islets^[51].

1, 25 dihydroxyvitamin D3, the active form of vitamin D3 is a membrane antioxidant and can prevent lipid peroxidation at the cell membrane^[51]. Luca and colleagues treated neonatal porcine cell clusters (NPCCs) with vitamin D3 at a certain time in their maturation and differentiation process. Insulin recovery showed that vitamin D3, unlike untreated controls, resulted in preservation of islet function for significantly long periods of time. Furthermore, this group exhibited sustained release vitamin D3 which entrapped vitamin D3 in microspheres. They showed that this form of vitamin D3 caused more insulin secretion compared with free vitamin D3 and both forms caused more insulin secretion than the untreated control group^[34,36].

Another study evaluated the benefit of 1, 25 dihydroxyvitamin D3 (1, 25 D3) on cytokine-induced human pancreatic islets. Addition of 1, 25 D3 significantly reduced nitrite release, IL-6 production and MHC class I expression which were not significantly different from controls. The authors suggested that vitamin D3 could affect this *via* reduction of cytotoxic challenges. Hence, it might play a role in the prevention of islet allograft rejection. However, more *in-vivo* studies are required to affirm this suggestion.

Riboflavin (vitamin B2) although it is not a pure antioxidant, has been used in one study^[38]. The authors showed that inclusion of this vitamin in the cryopreservation medium could protect islets comparable to healthy fresh isolated islets.

Trace elements

Selenium as an essential trace element plays an important role in the expression of some selenoproteins and selenoenzymes such as GPx. The antioxidative property of this element has been confirmed in many different cell types^[52]. The insulin-mimetic effect of selenium has been found both *in-vitro* and *in-vivo*^[53]. To ameliorate islet function, Campbell *et al.*^[21] showed that sodium selenite could increase islet content and secretion but not glucose-stimulated insulin secretion in culture media. In the study by Winter, when a combination of vitamin E, C, A and selenium were administered orally to transplanted mice, no significant improvement in functional islet graft survival was seen when compared with mice administered only vitamin C and E.

Furthermore, selenium in the insulin-transferrin-selenium (ITS) compound is a commercially available media supplement for the culture of mammalian cells under serum-free or near serum-free conditions. In islet culture, when Fraga and colleagues used ITS supplement instead of FBS, ITS was capable of maintaining viable islets for up to two months^[54].

Zinc, another trace element antioxidant has a role in the storage, synthesis, and secretion of insulin in islet cells. In one study^[39] when investigators added zinc to cold preservation UW and Hanks solution, no difference in islet viability was seen between the groups. The authors concluded that despite the integral role of zinc in islet metabolism, they were unable to find a beneficial role for zinc in cold storage solutions for the purposes of islet preservation.

Manganese as a trace element is a component of antioxidant enzymes and is used in new SOD mimetic compounds which will be discussed later.

Herbs

Curcumin is a polyphenolic compound commonly found in the dietary spice turmeric^[55,56]. Curcumin is an inhibitor of nuclear factor- κ B (NF- κ B) and has various biological activities such as anti inflammatory, antioxidant, antiseptic and anticancer effects^[57,58]. In two different studies, curcumin was shown to decrease MCP-1 release from islets^[27], decrease generation of ROS, increase secretion of insulin, and present more intact islets^[22].

Puerarin, the main isoflavone glycoside found in the Chinese herb, Radix of Pueraria lobata (Willd) Ohwi, has been used for various medicinal purposes in traditional Chinese medicine. It has been shown that puerarin has antioxidant activities such as radical scavenging and increasing SOD activity as well as antihyperglycemic effects^[59]. Xiong *et al.*^[25] found that if islets pretreated with puerarin for 48 h were exposed to H₂O₂, this did not result in loss of islet viability. They showed that this protective effect resulted from inhibition of free radical generation. Puerarin was also found to increase CAT and SOD activities.

Epigallocatechin-3-gallate (EGCG) is the main ingredient found in green tea. Anticarcinogenic,

antioxidant, and antiangiogenic activities have been attributed to EGCG as a constituent of green tea^[60]. In the study by Hara and colleagues, EGCG was shown to protect islets from hypoxia/reoxygenation injury. Insulin secretion was increased and apoptosis was inhibited by EGCG^[24].

Enzymes and new antioxidants

Two forms of enzymes, encapsulated slow release SOD and CAT and SOD-mimetic compounds were shown to increase viability, islet yield, and to decrease the release of MCP-1 and IL-6 from islets.

Encapsulated SOD and CAT in Poly D, L-lactide-co-glycolide (PLGA) microspheres were analyzed with NPCC cells. These powerful antioxidizing agents were shown to significantly improve morphology, viability and function, as assessed by microscopy, molecular, biochemical and functional studies on the incubated NPCCs^[51].

SOD mimetics: There are four main classes of SOD mimetics including desferrioxamine, macrocyclics, salen compounds, and mesoporphyrins^[61]. Two types of these compounds have been employed to possibly enhance islet survival by counteracting oxidative stress.

MnTDE is a manganese-porphyrin pentachloride, a synthetic porphyrin protein, which has been prepared as a SOD mimetic^[62]. Synthetic probes with MnTDE (AEOL10113 or AEOL10150) were used in the culture medium of human islets to ameliorate islet yield and insulin secretion. When treated islets were transplanted into diabetic mice, glucose control was better than in the non-treated group^[32,43].

MnTMPyP Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin, is a composition of porphyrin SOD mimetics. MnTMPyP preserved islet viability upon exposure to a nitric oxide donor in culture medium^[61].

EUK-8 a salen compound SOD mimetic is one of a new class of synthetic salen-manganese compounds with SOD, peroxidase, and CAT activities. EUK-8 treatment prolonged the survival of islet allografts in newly diabetic non-obese diabetic (NOD) mice^[41].

Peptide SS-31 (D-Arg-2-, 6-dimethyltyrosine-Lys-Phe-NH₂) is a novel peptide shown to target the inner mitochondrial membrane and prevent oxidative damage to cells. It has been shown to decrease islet apoptosis and increase islet yield^[23].

MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one; Edaravone) is a new free radical scavenger produced for use in some clinical conditions. It is a strong scavenger of hydroxyl radicals and was shown to have benefits in myocarditis and cerebral infarction. MCI-186 prevented islet cell death dose-dependently when cells were treated with H₂O₂^[28].

DISCUSSION

Most studies on antioxidants and diabetes in the literature have evaluated the possible effects of antioxidants in preventing β cell glucose toxicity and

cytokine-mediated cell damage in the field of type 1 diabetes pathophysiology. The role of antioxidants in type 2 diabetes is still undetermined due to scientific conflict. Vitamin E and C, selenium and the majority of antioxidant trace elements, as well as herbs and drugs have been used or are being used to try to manage diabetes and its complications^[2,63]. Moreover, there are, as seen in this review, few studies available which used antioxidants to improve islet transplantation outcome. However, a review of the existing studies indicates that many antioxidants are able to enhance cellular defense mechanisms against oxidative stress in islet cells. With deeper inspection of the presented studies, the effects of these drug compounds can be divided into two separate sub-categories.

The first sub-category concerns the direct antioxidant effects of drug compounds on islet viability (direct inactivation of free oxygen species). For instance, vitamin E and C were shown to increase viability of islets but they failed to decrease MCP-1 release from the cells. This shows that these vitamins do not act *via* inflammatory pathways. Enzyme antioxidants also promote islet yield and activity by direct inactivation of free radicals. The role of nitric oxide (NO) in early islet transplantation rejection should not be overlooked. NO was shown in experimental studies to harm islet cells in culture medium early after transplantation. N (G)-monomethyl-L-arginine (NMA) (a reversible inhibitor of NO synthase) prevented the dysfunction or destruction of cultured islets and markedly decreased the time needed to restore euglycemia after intraportal transplantation of islets in diabetic rats^[64]. Direct antioxidants such as vitamin E and salen-manganese compounds can prevent NO and nitrite radicals as well as ROS^[65,66] thus may play a protective role.

The second sub-category of effects concerns the action of these drug compounds on the modulation of beta cell apoptosis initiation and signaling. One potential ROS-dependent target molecule is the nuclear transcriptional factor NF- κ B. It is now known that NF- κ B is a key transcription factor involved in regulating proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes. Some antioxidants block the effects of NF- κ B including curcumin^[22,27], glutathione and MnTDE porphyrin^[32] and have been shown to ameliorate islet yields by reducing MCP-1 release as a consequence of NF- κ B blockade.

However, the two above-mentioned sub-categories may change following further investigations. Newer studies, especially those after 2004, have focused on the possible pathways by which antioxidants may improve the function of islets.

As previously stated, antioxidant enzymes are found in low levels in islets compared with other tissues. In addition, minimal amounts of GPx protein and mRNA expression as well as GPx activity in islets have been detected^[67], showing that GPx is low compared with SOD and CAT in islets. It is important to develop methods to increase islet survival and the number of islets during isolation and transplantation, thus

the suggestion of using antioxidants to improve islet transplantation is reasonable.

However, based on current data from *in-vitro* studies, we can conclude that the addition of an antioxidant during islet isolation procedures would result in better islet function. Furthermore, the overexpression of intracellular antioxidant enzymes and proteins^[68], as well as transgenic islets^[15] has been found to improve the function of islets, which is in agreement with our conclusions.

Interestingly, all the *in-vivo* studies with the exception of one on taurine^[35] showed better control of blood glucose using antioxidant supplementation mainly in the early stages of islet transplantation. However, similar to *in-vitro* studies, there was no significant advantage of any one antioxidant on glucose control following transplantation. Of course, the lack of human studies limits our conclusions regarding *in-vivo* research and necessitates further investigations to establish the benefits of antioxidants in human islet transplantation. Fortunately, there is sufficient evidence on the existence of oxidative stress in diabetes and the significant role of antioxidants in the reduction of diabetic complications^[69-76].

CONCLUSION

The collective data reviewed here show that different antioxidants improve islet transplantation procedures both *in-vitro* and *in-vivo*. We recommend antioxidant supplementation in islet isolation protocols, however, there is still insufficient evidence to draw definitive conclusions about the efficacy of individual supplements and to support profitable antioxidant management *in-vivo* particularly in humans.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

Alcohol and liver

Natalia A Osna

Natalia A Osna, Department of Internal Medicine, University of Nebraska Medical Center, Liver Study Unit R151, VA Medical Center, 4101 Woolworth Ave, Omaha, NE 68105, United States

Author contributions: This review was written by Osna NA.

Correspondence to: Natalia A Osna, MD, PhD, Department of Internal Medicine, University of Nebraska Medical Center, Liver Study Unit R151, VA Medical Center, 4101 Woolworth Ave, Omaha, NE 68105, United States. nosna@unmc.edu

Telephone: +1-402-9953735 Fax: +1-402-4490604

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FROM THE EDITOR

Liver is a primary site of ethanol metabolism, which makes this organ susceptible to alcohol-induced damage. Alcoholic liver disease (ALD) has many manifestations and complicated pathogenesis. In this Topic Highlight, we included the key reviews that characterize new findings about the mechanisms of ALD development and might be of strong interest for clinicians and researchers involved in liver alcohol studies.

Being the primary site of alcohol metabolism, liver is severely influenced by alcohol drinking. The combination of toxic effects of alcohol and numerous predisposing factors usually form the basis for ALD development. This disease has many manifestations, which are triggered by multiple pathogenic factors, causing progression in liver damage from steatosis to liver cirrhosis and hepatocarcinoma. The progression between various stages of ALD is driven by so-called "second hits", which trigger ALD development. In 2007, in *World Journal of Gastroenterology*, we published Topic Highlight: Alcohol liver injury: Pathological feature and models. There, we reviewed the role of alcohol in changes of iron metabolism, proteasome function, immune response, signaling mechanisms, transmethylation reactions, as well as apoptosis and mitochondrial damage. Current Topic Highlight is a logical continuation of the previous one, which further expands our understanding of the mechanisms of ALD progression and complexity of ALD pathogenesis, thereby providing important information for hepatologists about the modern directions in alcohol research.

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- 1 Bardag-Gorce F. Nuclear effects of ethanol-induced proteasome inhibition in liver cells. *World J Gastroenterol* 2009; 15: 1163-1167
- 2 Dolganiuc A, Szabo G. *In vitro* and *in vivo* models of acute alcohol exposure. *World J Gastroenterol* 2009; 15: 1168-1177
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Natalia A Osna, MD, PhD, Series Editor

Nuclear effects of ethanol-induced proteasome inhibition in liver cells

Fawzia Bardag-Gorce

Fawzia Bardag-Gorce, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1124 W. Carson St. Torrance, CA 90502, United States

Author contributions: Bardag-Gorce F wrote this review.

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Correspondence to: Fawzia Bardag-Gorce, PhD, The Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1124 W. Carson St. Torrance, CA 90502,

United States. fgorce@labiomed.org

Telephone: +1-310-2221846 Fax: +1-310-2223614

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Abstract

Alcohol ingestion causes alteration in several cellular mechanisms, and leads to inflammation, apoptosis, immunological response defects, and fibrosis. These phenomena are associated with significant changes in the epigenetic mechanisms, and subsequently, to liver cell memory. The ubiquitin-proteasome pathway is one of the vital pathways in the cell that becomes dysfunctional as a result of chronic ethanol consumption. Inhibition of the proteasome activity in the nucleus causes changes in the turnover of transcriptional factors, histone modifying enzymes, and therefore, affects epigenetic mechanisms. Alcohol consumption has been associated with an increase in histone acetylation and a decrease in histone methylation, which leads to gene expression changes. DNA and histone modifications that result from ethanol-induced proteasome inhibition are key players in regulating gene expression, especially genes involved in the cell cycle, immunological responses, and metabolism of ethanol. The present review highlights the consequences of ethanol-induced proteasome inhibition in the nucleus of liver cells that are chronically exposed to ethanol.

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Key words: Alcohol liver injury; Proteasome inhibition; Epigenetic mechanisms

Peer reviewer: Maria Concepción Gutiérrez-Ruiz, PhD, Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, DCBS, Av San Rafael Atlixco 186, Colonia Vicentina, México, DF 09340, México

INTRODUCTION

Proteasomes of mammalian cells exhibit a complex heterogeneity. Different proteasome variants exist and perform subtle activities. The 20S proteasome catalytic core binds to different activators (19S, 11S and PA200), and the resulting complexes perform specific functions, such as ATP-ubiquitin proteolysis, immunological response, cell cycle regulation, nuclear factor (NF) κ B activation, response to hypoxia, and transcription^[1]. Upon immune response, or interferon (IFN) γ treatment, the three catalytic subunits, β 1, β 2 and β 5, are replaced by three different subunits, LMP2, LMP7 and MECL-1, respectively, which form the immunoproteasome^[2]. Several chemical compounds, proteins and regulatory complexes, function as activators or inhibitors of the catalytic core 20S proteasome^[3]. Some of them are termed gate-openers because they provoke the opening of the gate at the alpha-type subunits of the 20S proteasome^[4], and facilitate the access of designated proteins to be degraded to the catalytic chamber formed by the beta-type subunits. The best known of these activators are the regulatory complex 19S, which is involved in the ubiquitin-proteasome pathway (UPP), the PA28 α / β and PA28 γ complexes, which, among other functions, are important for the generation of epitope peptides presented to the major histocompatibility complex^[5], and PA200, which is implicated in DNA repair^[6]. Recently, the hybrid form of the 26S proteasome, which contains both 19S and PA28 complexes associated at the two opposite sides of the 20S core particle, has also been characterized^[7], and other activators and proteins that modulate the proteasome activity are yet to be discovered.

As complex as is the UPP, it does not work alone. These regulatory complexes and proteasome interacting proteins (PIPs) complement the UPP to perform specific functions. Proteasome failure occurs in the liver cells because chronic ethanol exposure interferes with

the binding of the 20S catalytic core to its regulatory complex and to its PIPs. The 26S proteasome is responsible for the ATP-ubiquitin degradation of short-lived cellular proteins and the elimination of damaged and misfolded proteins, in addition to providing basic housekeeping functions^[8-10]. Therefore, damaged and ubiquitinated proteins accumulate, causing protein aggregation, such as Mallory-Denk body (MDB) formation^[11-13], when, because of chronic ethanol ingestion, the 20S is unable to bind to the 19S to form the 26S proteasome, which leads to cell death and tissue damage^[14].

PROTEASOMES AND TRANSCRIPTION

The UPP plays an important role in a variety of cellular functions, primarily *via* its proteolytic activity. However, recent studies have implicated this pathway in the direct regulation of specific transcriptional factors^[15,16]. It is now believed that the proteolytic activity of the proteasome is critical in promoting the exchange of transcriptional factors on chromatin, and possibly facilitating multiple rounds of transcription initiation, hence controlling gene expression^[17]. Proteasomes are also now widely accepted as being essential for promoting the exchange of transcriptional factors on chromatin^[18,19]. A growing body of evidence demonstrates that the components of the UPP are directly, and mechanistically, involved in transcription and in regulating the gene expression of the DNA repair system^[20,21], cell cycle^[22,23], and chromatin modifying enzymes^[17]. Although the role of the UPP in regulating many transcription factors, such as p53, NFκB and hypoxia-inducible factor 1 α , is well established, the role of the UPP in regulating epigenetic mechanisms has only recently been given attention^[24].

Ethanol-induced oxidative stress in nuclei is believed to damage DNA and proteins, thereby disrupting genomic integrity^[25]. Oxidative stress has also been shown to cause adduct formation with lysine located in the N-terminal histone tails^[26]. These lysine residues are the site of a number of post-translational modifications that regulate chromatin function. Therefore, chronic accumulation of oxidative stress adduct modifications may cause epigenetic changes, which may have important implications in terms of histone function and genomic integrity. Oxidatively damaged histones are degraded by a nuclear 20S proteasome in an ATP- and ubiquitin-independent manner^[27], which indicates that damaged histones accumulate when the activity of the proteasome is inhibited and causes alteration of the epigenetic mechanisms.

However, the effect of ethanol-induced proteasome inhibition in the nucleus, and thus in regulating gene expression, is still not well known. Inhibition of proteasome function has been widely reported in models of alcoholic liver disease (ALD)^[28,29], but how proteasome dysfunction may enhance hepatotoxicity is not well defined. It has been reported that chronic ethanol exposure leads to post-translational

modifications of the alpha type subunits of the 20S proteasome that constitute the opening of the gate to the catalytic chamber of the 20S proteasome^[29], thus blocking the opening of the gate and causing proteasome activity to decrease. Recently, proteasome activity has been measured in the isolated nuclei from liver cells of rats fed ethanol chronically, and the proteasome was found to be significantly inhibited (personal observation, manuscript submitted to *World Journal of Gastroenterology*, 2008). The inhibition of proteasome activity in the nucleus is therefore etiologically involved in the accumulation of damaged proteins in the nucleus, and in the deregulation of transcription.

Thus, it seems reasonable to postulate that the nucleus should have a more important proteasomal activity than other cellular compartments. It is possible that proteasome inhibition, caused by chronic ethanol feeding in the nucleus, may be greater than the inhibition caused in the cytosolic compartment^[29].

EPIGENETIC MECHANISMS AND PROTEASOME INHIBITION

Chronic ethanol exposure causes an alteration in liver cell memory by changing the epigenetic mechanisms, thus causing significant liver pathology that persists after ethanol withdrawal^[30]. Dr. French's group has shown that cells forming MDBs have a memory of the exposure to the drug even four months after drug withdrawal^[31]. This memory is primarily associated with histone modifications that are recovered when s-adenosylmethionine (SAME) is added to the drug-primed mouse diet^[32].

Epigenetic mechanisms are involved in development, phenotype determination and maintenance, aging, and cancer. These epigenetic mechanisms are based on histone modifications controlled by histone acetyltransferases (HATs) and histone methyltransferases, and by DNA methylation, controlled by a regulated balance between DNA methyltransferase and demethylase. These epigenetic events can change rapidly in response to changes in cell signaling, which are initiated by environmental influences, such as inflammation, metabolism, or toxic injury. These changes cause the genome to respond globally or in a restricted manner, depending on the nature of the signals generated. During these responses, the histone modifications themselves are rapidly changed as well^[33]. The dynamics of this process are pervasive and extremely complex, which makes it difficult to connect the epigenetic responses to the final manifestations. The identification of key epigenetic changes, which are linked to alcohol liver injury, is of high interest and is at its very beginning. Histone modifying enzymes are the key target to regulate the histone modifications. Histone deacetylase (HDAC) inhibitors and demethylating agents are currently used in clinical trials to treat cancer. HDAC inhibitors, like trichostatin A, repress

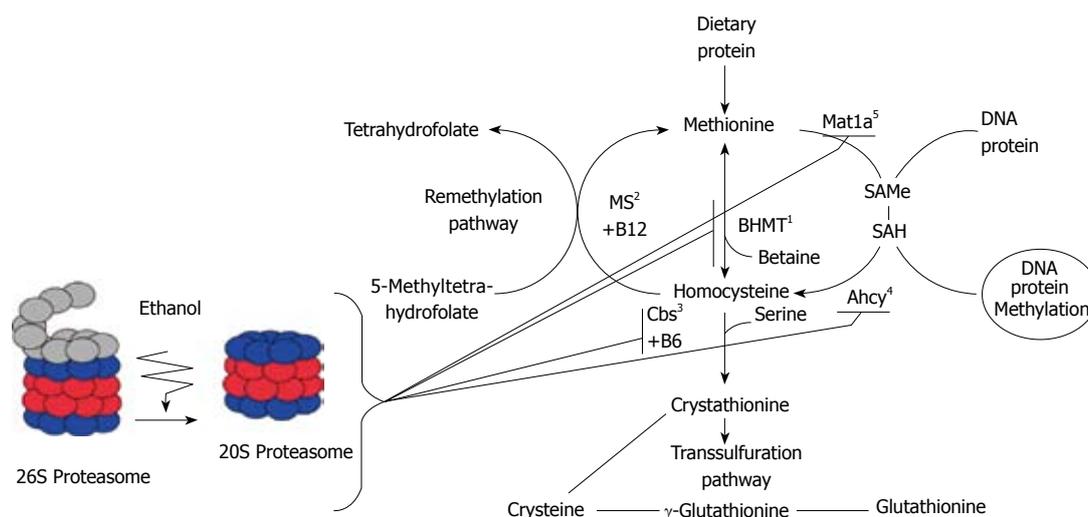


Figure 1 Illustration of methionine metabolism enzymes system and the effects of proteasome inhibition in the remethylation pathway. 1-BHMT: Betaine homocysteine Methyltransferase; 2-MS: Methionine synthase; 3-Cbs: Cystathionine beta-Synthase; 4-Ahcy: S'-adenosylhomocysteine transferase; 5-Mat1a: Methionine adenosyltransferase.

the expression of genes that induce growth arrest, cellular differentiation and apoptosis^[34]. Likewise, DNA methyltransferase inhibitors, like 5-azacytidine (Aza), are being used in clinical trials to treat cancer. Aza, a potent inhibitor of Dnmt 1 and Dnmt3a (not Dnmt3b), induces demethylation and reactivation of silenced tumor suppressor genes^[35]. Moreover, proteasome and HDAC inhibitors may be a prominent combination in future cancer therapy^[36], and specifically, in gastrointestinal cancer^[37].

HISTONE MODIFICATIONS AND HISTONE MODIFYING ENZYMES

The nucleosome surface interacts with several histone modifying enzymes that are responsible for histone tail lysine or arginine modifications^[38], operating as part of a predictive and initiated epigenetic code that defines patterns of gene expression^[39]. Histone modifications control the accessibility of DNA elements for transcription factors, which influences gene expression. These modifications control the recruitment of proteins involved in DNA methylation and affect chromatin structure and function^[40].

Histone modification alterations can change long-term effects on gene expression patterns by influencing chromatin structure throughout the cell cycle, and from one cell generation to the next^[41]. For instance, trimethylation of H3K9me3 and depletion of acetylation leads to gene silencing, because H3K9me3 acts as a binding site for heterochromatin-forming protein HP1. In the nuclear extract from liver cells of rats fed ethanol chronically, H3K9me3 is decreased and H3K9 acetylation is increased^[42,43]. These histone modifications are linked to DNA modifications and affect gene expression because DNA methylation is dependent on prior histone methylation^[44-47].

More epigenetic mechanisms still need to be elucidated with respect to alcoholism, particularly histone

modifications, such as methylation, phosphorylation and ubiquitinylation of lysine and arginine residues. The task will be to identify the modifications and the specific modifying enzymes that regulate gene expression and account for the cellular memory observed when ethanol is fed chronically^[30]. For example, it has been reported that a global increase in histone H3 lysine 9 acetylation, histone H3 lysine 4 dimethylation and H3 lysine 27 trimethylation is found in the liver of rats fed ethanol chronically^[48], which correlates with an increase and a decrease, respectively, in transcriptionally active promoters. However, the genes linked to these changes are still to be identified. Microarray analysis has shown that hundreds of genes are up-regulated and hundreds are down-regulated when rats are fed ethanol for 1 mo, which indicates that ethanol causes several epigenetic changes that affect gene expression^[25]. More specifically, gene expression of ALDH1a4^[48] and ADH^[49] are up-regulated, which suggests a change in the epigenetic events that regulate the expression of these genes in the liver of rats fed ethanol chronically. However, the linkage of ethanol-induced proteasome inhibition to the changes in the expression of these genes still needs to be determined. Data mining of microarray analysis of proteasome inhibition has shown that, similar to ethanol-treated rat liver, several genes are changed, which indicates an epigenetic phenomenon c proteasome inhibition^[17,50].

PROTEASOME INHIBITION AND REMETHYLATION PATHWAY

The changes in gene expression in the remethylation pathway, particularly for the enzymes that regenerate SAMe, cause deregulation of cellular methylation, thus resulting in abnormal cellular methylation.

Hyperhomocysteinemia (HHcy) is a leading cause of liver injury in ALD. A lack of the activity of the enzymes responsible for homocysteine metabolism,

particularly cystathionine b-synthase (Cbs) or 5,10-methylenetetrahydrofolate reductase, results in severe forms of HHcy. Additionally, nutritional deficiencies in B vitamin cofactors required for homocysteine metabolism, including folic acid, vitamin B6 (pyridoxal phosphate), and/or B12 (methylcobalamin), can induce HHcy (Figure 1).

Proteasome inhibition is believed to be involved etiologically in the changes in gene expression of these enzymes responsible for the hepatic transmethylation reactions. Indeed, analyses performed on the livers of rats given PS-341 (bortezomib, Velcade®), a dipeptide boronic acid used to inhibit proteasome activity, and used today as an anticancer agent in human myeloma^[51], have shown down-regulation of gene expression for methionine metabolizing enzymes. Proteasome inhibition causes a decrease in methionine adenosyltransferase (MAT1a), in S-adenosylhomocysteine hydrolase gene expression, and a marked decrease in gene expression of betaine-homocysteine methyltransferase^[50,52] and Cbs^[53], which affects cellular methylation. Rats fed ethanol chronically also exhibit a relatively low level of MAT1a, which is associated with low levels of SAME and faster growth^[54]. These observations indicate that SAME is an attractive agent for both chemoprevention and treatment of alcohol associated liver cancer^[54].

Proteasome inhibition, induced by ethanol feeding, is associated with histone modifications, and is involved in the regulation of histone modifying enzymes, such as the HAT p300^[42]. It is believed that proteasome inhibition affects gene expression, particularly genes of the transmethylation pathway, *via* an epigenetic mechanism that involves modified histone and transcriptional factors.

CONCLUSION

Regulation of gene expression of the transmethylation pathway is one of the promising approaches to counteract proteasome inhibition effects. Investigating the changes in the remethylation pathway, particularly the enzymes that regenerate SAME, the major methyl group donor, is essential to identify the key enzyme that is changed by chronic ethanol exposure and proteasome inhibition.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

In vitro and *in vivo* models of acute alcohol exposure

Angela Dolganiuc, Gyongyi Szabo

Angela Dolganiuc, Gyongyi Szabo, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324, United States

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Correspondence to: Angela Dolganiuc, MD, PhD, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605,

United States. angela.dolganiuc@umassmed.edu

Telephone: +1-508-8565955 Fax: +1-508-8564770

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Abstract

Alcohol abuse is a global problem due to the financial burden on society and the healthcare system. While the harmful health effects of chronic alcohol abuse are well established, more recent data suggest that acute alcohol consumption also affects human wellbeing. Thus, there is a need for research models in order to fully understand the effect of acute alcohol abuse on different body systems and organs. The present manuscript summarizes the interdisciplinary advantages and disadvantages of currently available human and non-human models of acute alcohol abuse, and identifies their suitability for biomedical research.

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INTRODUCTION

Alcohol abuse is widely spread around the globe^[1-9].

Alcohol is the third leading cause of preventable death in the United States and the third leading cause of healthy years lost to death and disability in developed nations^[9]. Humans use and abuse alcohol acutely or chronically, when alcohol consumption is frequent and dependence has developed^[10]. Although significant progress was made in the area of alcohol research during the last decades, the pathogenesis of alcohol use and abuse is not fully understood. Further, most research was focused on alcoholism, which is an advanced stage of alcohol abuse, involving chronic alcohol consumption, alcohol dependence and severe health and social consequences^[1-13]. Thus, research models are emergent in order to detail what drives human desire to consume alcohol, how the body responds to alcohol, and most important, what are the beneficial and harmful effects of acute alcohol consumption on the human body.

ACUTE ALCOHOL ABUSE (AAA): HOW BIG THE PROBLEM REALLY IS?

In the USA, a “drink” is defined as an equivalent of 14 g alcohol, which equals roughly 1 shot [1.25 oz of 40% (80-proof) liquor], 1 (12 oz) beer (4.2 mL/L, Ethanol), or 1 (4 oz) glass of wine (12 mL/L, Ethanol)^[14]. In other countries, the alcohol content of a serving is measured in “units”. One unit (about 25 mL of a 40% 80-proof liquor) contains 7.9 g of pure ethanol^[8,15]. However, in many countries the “standard drink” is used to quantify alcohol intake. More importantly, the standard drink varies significantly from country to country, from 10 mL (7.9 g) of alcohol in the UK to as high as 25 mL (19.75 g) in Japan^[16]. Current use includes at least one drink in the past 30 d; binge drinking is defined as five or more drinks on the same occasion within 2 h at least once in the past 30 d; and heavy use is defined as five or more drinks on the same occasion on at least 5 different days in the past 30 d^[11-13]. The 0.08% blood alcohol level (BAL) is the legal limit for most states in the US and it is achieved with consumption of five or more drinks for an adult male and four or more drinks for an adult female^[11-13].

Traditionally medical research focuses on the mechanisms of chronic alcohol abuse; this is due to the significant financial burden that society encountered primarily from chronic alcohol abusers^[1-13]. However, more recently acute alcohol abuse has emerged as a

Table 1 The characteristics of *in vitro* and *in vivo* models of AAA

AAA model	Advantages	Disadvantages	Area of research
<i>In vitro</i>	Low cost Technically easy to perform Large number of experimental groups Pure cell populations Single cell type or multi-cell type co-culture Strictly controlled settings yielding reproducible results	Limited alcohol metabolism Limited complexity at cellular and tissue levels Limited areas of research, not suitable for behavioral and social studies.	Behavioral and biomedical
<i>In vivo</i>	Availability of physiological routes of alcohol administration Complex interactions of all bodily organs and systems, including complex metabolism Controlled settings, caloric and composition controls Indications to individual and population variability	Ethical concerns High cost Limited information about the effect on one separate cell population.	All areas of research including biomedical, behavioral and social.

social problem^[17]. The National Survey on Drug Use and Health (NSDUH) estimated that in the USA about 4.4 million persons had used alcohol for the first time in 2004, which lead to about 12000 “new recruits” per day; this was significantly greater than in 2002 (3.9 million) and 2003 (4.1 million). Most (86.9%) of the 4.4 million recent alcohol initiates were younger than 21 years of age at the time of encounter. More than one fifth (22.%) of people age 12 or older participated in binge drinking at least once in the 30 d prior to the survey in 2004^[12]. Acute alcohol intake in the form of binge drinking in 2004 was highest for the 18- to 25-year-old age group compared with other age groups, with the peak rate occurring at age 21^[1,5-7,11-13]. The statistics also show that illness and death among young adults primarily result from lifestyle choices and behaviors, including excessive alcohol use^[18,19].

AAA: BIOMEDICAL IMPACT

The known biological effects of AAA include those of the central nervous system (CNS) and non-CNS origin. Alcohol use is characterized by symptoms of CNS intoxication, impaired brain activity, poor motor coordination, and behavioral changes^[20,21]. AAA leads to impaired CNS activity due to alcohol's effect on synthesis^[22], release^[23] and signaling^[23,24] of neurotransmitters, including serotonin^[25,26], glutamate^[27], GABA^[28], endocannabinoids^[29,30] and their receptors. AAA causes damage and functional impairment of the gastrointestinal (GI) tract, including luminal GI^[31-38], liver^[39-53], and pancreas^[56-62]; it also affects the protein, carbohydrate, and fat metabolism^[58,63-66]. AAA leads to insufficient immune system responses to infections; such deficiency was observed both in organ-specific^[67-69] and systemic infections^[70-72]. Acute alcohol intoxication impairs the ability of the host to counteract hemorrhagic shock^[73], augments corticosteroid release^[74] and delays wound healing^[75-78], thus contributing to higher morbidity and mortality^[79] and prolonged recovery from trauma^[80]. The pathogenesis of AAA effects on human health is not fully understood.

MODELS OF AAA

Research of acute alcohol consumption/abuse is entirely

based on models, due to their advantage of controlled settings. Currently there are *in vitro* and *in vivo* models of AAA; their characteristics are defined in Table 1. In contrast to chronic alcohol abuse, the research of AAA has not benefited from population studies due to recall bias^[81-84].

One important feature of AAA models is the definition of biologically meaningful levels of alcohol, either *in vitro* or *in vivo*, and their relationship to blood alcohol levels (BAL) in humans. This is an important requirement of the research models of AAA, because BAL can be detected as soon as minimal amounts of alcohol are ingested^[85], however measurable affects of alcohol on physiology and/or behavior is established at 0.08% or above this level, with individual variations depending on the species, metabolic particularities, age, gender and genetic background^[86-97]. It is also important to identify that AAA models differ by their route of alcohol delivery to achieve alcohol intoxication, some of them being physiological, such as oral administration, while others being non-physiological, when ethanol is administered by parenteral routes. Nevertheless, current research shows that the BAL levels, rather than the route of alcohol administration play a major role in the establishment of the biological effects of alcohol^[97].

Thus, optimal AAA models should fulfill several criteria: (1) Define the length of alcohol exposure. *In vitro* the length of acute alcohol treatment is variable in diverse published experimental settings and range from seconds to hours; it is currently accepted that treatment with alcohol for up to 24 h is considered as an acute setting^[98-106]. *In vivo* the consumption of alcohol in one setting implies that the entire dose of alcohol is consumed at once, while a ‘binge’ is defined by NIAAA as an excessive pattern of alcohol drinking that produces BAL greater than 0.08% within a 2-h period and may, or may not, be associated with dependence^[11,12,17,18]. Thus any model using consumption of biologically active amounts of alcohol within 2 h is considered an acceptable model of AAA^[81,107-121]. (2) Establish an exposure to an accurate concentration of ethanol. For *in vitro* studies the 10-100 mmol/L ethanol range is considered physiological, with 25 mmol/L ethanol being close to 0.08% BAL achieved *in vivo* after 4-5 drink equivalents^[7,11,12,98-106]. For the *in vivo* studies an 0.08% BAL or above this level yields

signs of intoxication and it is employed in the majority of biomedical studies^[107-121]. (3) Recruit individuals who are currently not and never have been alcohol abusers for *in vivo* studies and employ alcohol-naïve primary cells or cell lines for *in vitro* studies. Alcohol use habits of the study participants are usually determined by questionnaires^[122]. Among most frequently used questionnaires are those that incorporate the AUDIT and CAGE tests^[123-125]; the study parameters are usually permissive for males who had alcohol use of fewer than nine drinks/week, females < 6 drinks/week.

IN VITRO AAA MODEL

The *in vitro* alcohol treatment model is based on supplementation of culture media with pure alcohol, usually 200-proof ethanol. Currently supplementation of cell culture with a wide variety of alcohol concentrations, ranging from 1 to 500 mmol/L, is reported in the bio-medical literature. One of the major concerns with the *in vitro* alcohol treatment using concentrations above 100 mmol/L is the direct cytotoxic effect of alcohol on cells^[40,100]. At lower concentrations (< 100 mmol/L), alcohol changes the redox status of the cells and alters intercellular junctions^[33,126], increases the membrane fluidity of cells^[127-129] and affects the composition of lipid rafts^[106,130,131], all of which may contribute to alcohol-mediated increase in transcellular and paracellular permeability^[132,133] and thus affect cell function^[106,130-134]. Alcohol also affects the expression of adhesion molecules^[135], which may be a concern when using adherent cell types due to possible cell detachment. Additional concerns arise from the possibility of modified *ex vivo* function of some primary cells, including hepatocytes, stellate cells and their precursors, due to limited *ex vivo* environment compared to *in vivo* conditions^[136-138].

From a technical point, the acute alcohol exposure of cells *in vitro* may be hampered by alcohol evaporation. To avoid the fluctuation of alcohol concentration due to evaporation, investigators used settings where ethanol was added into the culture media and the cell culture plates were maintained for the entire duration of stimulation in a microclimate chamber at 37°C with gas mixture and an alcohol atmosphere^[139]. For example, if the desired alcohol concentration in the cell culture is 25 mmol/L, a Petri dish with 2 × the alcohol amount (50 mmol/L) was placed on the bottom of the chamber to ensure the saturation of the gas in the chamber; such conditions maintain the initial alcohol concentration ± 15% over a 24 h period^[139]. However, depending on the scientific question of the study, the declining alcohol levels *in vitro* may be desired to mimic the alcohol elimination *in vivo*; in these situations the *in vitro* experiments are disadvantaged by the absence/limitation of alcohol metabolism^[76,134].

The *in vitro* AAA model offers the possibility of primary *in vitro* exposure of alcohol-naïve cells to alcohol alone or its combinations with diverse pharmacological or naturally-derived substances^[24,28,31,35,36,42,55,68,71,72,96,103],

but also the investigation of the effects of *in vivo* exposure to alcohol followed by *ex vivo* exposure to other stimulants^[110,113,115] or *vice versa*. One other main characteristic of the *in vitro* AAA model is its simplicity, often considered as an advantage or disadvantage depending on the research goal. Most of the *in vitro* research involves culture of a single cell type^[134,139-142] or co-culture of several cell types^[143]; while such an approach brings forward the differential effect of alcohol on pure cell populations, and/or their intercellular interaction; it lacks the systemic alcohol metabolism and intercellular interactions. More recently significant efforts were invested in establishment of more complex *in vitro* systems, such as culture of cells in three dimensional systems^[100], organ slices^[144] or organ explants^[145]; while such systems are informative in the setting of chronic alcohol exposure to date there is no report of their use as an AAA model.

IN VIVO AAA MODELS

The *in vivo* models of AAA are more informative compared to the *in vitro* model due to complex physiological impact of alcohol on all bodily organs and systems, but also due to the availability of systemic alcohol metabolism. Currently there are human and non-human models of AAA, and the later include use of invertebrates^[146-147] and vertebrates^[21,25,37,44,46,47,53,65,72,86,93,94,98,104,110,111]. The invertebrate models (*Drosophila melanogaster*^[146,147], *Caenorhabditis elegans*^[105]) and those using lower vertebrates (*Zebra fish Danio rerio*)^[98] are invaluable for research of the effect of alcohol on behavior, development and maintenance of memory, and on basic signaling mechanisms. These models offer the advantage of a well-defined genetic background, high-turnover rate of experiments due to short life cycle and relatively low-cost; in light of these advantages they constitute an excellent resource for research of signaling pathways and are highly desirable for their drug-screening capacity. On the downside, significant differences in the structure and function of organs and systems compared to humans limit the informative value of invertebrate and lower vertebrate models of AAA.

The vertebrate models are preferred to those using invertebrates due to closer resemblance of their bodily structure, function, and metabolism to that of humans. However, because of intrinsic differences between humans and other vertebrates, no single non-human model is perfect since none of the models can represent all features of the complex human trait, such as motivation for social occasional or binge alcohol consumption, development of alcohol dependence and establishment of the impact on health. Further, the controlled setting of research models may not be completely satisfactory for psychology and social research, since they may not fully reproduce the social component, the motivation and the spontaneity of alcohol abuse. However, research models are invaluable for the understanding of the effects of alcohol and its

mechanisms of action on hardwired bodily systems, including the brain and all other organs and systems.

HUMAN MODELS OF AAA

Human alcohol intake in the experimental setting is the best available model of AAA, because it offers the advantage of the physiological route of alcohol consumption, the possibility to investigate human pathobiology and the availability of relatively large amounts of physiological bodily fluids for research. The disadvantages of human models of AAA include ethical concerns related to potential harmful health effects due to excessive or repeated intoxication, and the theoretical possibility of development of dependence or tolerance even after a one-time drinking session. Published models of human AAA are based on consumption of alcoholic beverages containing either distilled ethanol or wine; these models are physiological, as they involve alcohol drinking, and achieve a biologically meaningful BAL^[87,92,107,110-113,115]. The majority of the reported *in vivo* models of human AAA strictly control for the amount of alcohol based on the constant volume of alcohol per kg body weight, includes placebo-treated age and gender matched controls. However, most of these studies design the consumption of the alcohol beverage during a 2 h period of time^[92,107,110-113], which based on recent NIAAA and NSDUH classification qualifies as binge drinking^[11-13,17]. Thus the major disadvantage of the human models of AAA is that they (1) do not clearly distinguish between one-time and the binge alcohol consumption pattern, and, (2) for ethical reasons, do not allow longer binge sessions which are often observed in real-life and account for the majority of the heavy alcohol intake in young adults^[5,7,11-13,17-19].

To fulfill the requirement for an AAA model, the human studies usually include nonalcoholic individuals, who did not drink any alcohol at least 24 h prior to the study. Depending on the study design, some AAA human models require that the study participants did not take any medication, while others accept individuals taking moderate doses of anti-hypertensive medication and oral contraceptives^[107,110]. The study participants are usually required to abstain from food for at least 6 h before alcohol consumption and are allowed free access to water and a light meal before or shortly after the study^[107]. The human model of AAA is currently used for research in physiology^[86,92,111,122], hematology^[107,128] and immunology^[110,113,115].

CONSUMPTION OF DISTILLED ETHANOL MODEL

In this model the study individuals drink distilled alcohol (usually 80-proof vodka) in amounts of about 0.5-0.6 g/kg body weight, which is an equivalent of about 2 mL vodka/kg body weight in a standardized total volume of liquid (300-450 mL of water or orange juice)^[92,107,110-113].

CONSUMPTION OF NON-DISTILLED ETHANOL MODEL

In this model the study individuals drink wine to an equivalent of a pre-determined amount of ethanol/kg BW (for example, Fehr *et al.*^[107] reported use of 4.36 mL of red wine/kg of body weight as an equivalent of 0.5 g ethanol/kg BW to lead to a peak BAL of about 15 mmol/L in the first 2 h), while the control individuals are exposed to the same volume of fluid by mouth (usually water) per individual in a randomized way. The major disadvantage of this model is the use of controlled volumes of liquids that are not matched by calorie intake or by composition, which is technically challenging to achieve due to restricted availability of equivalent alcohol-free compounds. To bypass the bias concern some studies employ a cross-over approach, where each subject serves as its own control and repeats the study at least 2 wk after the first experiment with either alcohol or placebo consumption according to the cross-over design^[107].

NON-HUMAN AAA MODELS

Among non-human vertebrates commonly involved in alcohol research are primates^[90,91,148], pigs^[104,120], dogs^[114,121], mice^[70,72,74,86,89,96,109,118,119,141], rats^[88,94,108,149,150] and rabbits^[132]. The rodent AAA models (mice and rats) are used most frequently due to their relatively well-defined genetic background and the availability of diverse genetic traits, including those coding for high or low alcohol consumption^[88,89,96,109]. Most non-human AAA models currently in use^[93,95] examine relative oral self-administration from a bottle containing alcohol versus one^[86,94,108] or multiple bottles^[119] containing water (preference drinking) or administration of alcohol against the will, either by physiological (by mouth using gavage)^[54,71,72] or by non-physiological (parenteral)^[67,68] routes. Voluntary consumption of alcohol may be an optimal animal model of AAA, due to physiological route and pattern of alcohol consumption. However, in the self-administration models it is not clear when or if the animals drink to pharmacologically significant levels because the drinking is episodic and often occurs over a 24-h period. Nevertheless, these models are invaluable for research of neurobiology of acute intoxication with alcohol and for establishment of mechanisms of addiction. The AAA models using administration of alcohol against-the-will bypass all the above-mentioned inconveniences of AAA models using voluntary consumption. Alcohol administered either by physiological (by mouth using gavage) or by non-physiological (parenteral) routes yields comparable physiological effects on the central nervous system and on organs/systems that are not affected directly by the route of alcohol administration, such as muscle and brain^[97]. However, administration of alcohol per os is more physiological compared to administration via parenteral routes, yields meaningful levels of BAL and shows signs of acute alcohol intoxication^[54,71,72,132,149].

Table 2 The effect of acute alcohol abuse on GI system

GI segment	Effect of acute alcohol exposure
Oral cavity	Unknown
Esophagus	Low concentrations of alcohol (up to 5%) cause alterations in ion transports and affect the barrier function Concentrations of alcohol of 10% and above cause injury of mucosa Co-carcinogenic potency
Stomach	Motor dysfunction: decrease in lower esophageal sphincter pressure and amplitude Motor dysfunction: Inhibition of gastric emptying Mucosal damage, impaired barrier function, increased epithelial permeability Pro-inflammatory reaction: decreased gastric blood flow, vascular damage, polymorphonuclear neutrophils (PMN) dependent- and independent-mucosal damage Aggravation of <i>H pylori</i> infection
Intestine	Disruption of barrier function Epithelial apoptosis Enhanced bioavailability of some alcohol-soluble drugs and impaired absorption of key nutrients Increased paracellular intestinal permeability to toxins
Liver	Hepatocytes: Amplification of Fas-mediated hepatocyte death Generation of oxidative stress Hepatic mitochondrial dysfunction Increased free iron levels Imbalanced fatty acid metabolism Inhibition of IFN- α -induced antiviral response towards hepatotropic viruses including hepatitis C virus favors hepatitis C virus replication expression Induced histone H3 acetylation leading to increased gene expression in the liver Limited hepatic protein synthesis Arrest of liver regeneration early after partial hepatectomy and suppression of hepatic stimulator substance (HSS) activity by induction of liver cell cycle arrest Kupffer cells: Suppressed LPS-mediated priming for enhanced CC-chemokine release in vitro; up-regulated expression of CC-chemokine mRNA; primed the KC for enhanced RANTES release Desensitized HIV-1 gp120-induced CC-chemokine production Downregulates HIV-1 glycoprotein 120-induced KC and RANTES production Regulates production of reactive oxygen species Modulate the tolerance to LPS Stellate cells: Imbalanced redox potential owed to increased generation of reactive oxygen species upon GSH depletion
Pancreas	Stimulates islet blood flow, amplifies insulin secretion, induces hypoglycemia Lower baseline amylase output of acinar pancreatic cells, with the difference being significantly exacerbated by cerulein stimulation Interference with release of oxidized proteins in acinar cells Predisposes the pancreas to postprandial cholinergic stimulation that triggers cellular events leading to pancreatic inflammation Impaired apical exocytosis and redirected exocytosis to less efficient basolateral plasma membrane sites Augments elevated-[Ca ²⁺]-induced trypsin activation in pancreatic acinar zymogen granules, leading to premature activation of trypsin and tissue damage.

Among disadvantages of administration of alcohol per os are technical challenges, time consumption and high cost of the procedures. In contrast, alcohol administration by parenteral routes is relatively easy to perform technically and offers controlled settings (time, amount); on the downside, they may be less suitable for research of the effects of alcohol on organs/systems that are affected directly by the routes of alcohol administration. In this context, administration of alcohol by intraperitoneal route may be less suitable for research using peritoneal macrophages, or even liver and intestines, compared to other administration routes, such as intravenous or enteral. Further, some parenteral methods of alcohol administration are preferred over others, owing to differences in the level of technical difficulty of the procedure and the effect of alcohol on different cell types. For example, alcohol administration by intravenous route is known to affect the erythrocytes when present in high concentrations^[128]. Thus alcohol administration by intravenous route is currently

limited to creating an acute alcohol exposure during treatment of alcohol withdrawal symptoms^[151], while administration by intraperitoneal injections is widely preferred in research settings.

Similar to human AAA models, the non-human *in vivo* models employ either distilled alcohol^[53,67-72,74,89,90,96,109,116,119,121,132,135,141,152] or alcohol-containing beverages, such as wine^[152,153] and beer^[46]; the control groups are usually treated with alcohol-free caloric and composition equivalents. The vertebrate AAA models are widely used in research of biomedical effects of AAA, including brain^[23-30,116], gastrointestinal^[38-44,46-48,64-66,154], vascular^[73,153], muscle^[97] and immune^[68-72,74] systems.

THE PARTICULARITIES OF AAA MODELS FOR RESEARCH IN GASTROENTEROLOGY

In contrast to the abundance of the literature about the

effects of chronic alcohol abuse on the gastrointestinal system, research of the effects of acute alcohol abuse on the gastrointestinal (GI) tract is limited to certain cell types, as outlined in Table 2.

Currently the state of scientific knowledge suggests a tight interplay between organs and systems. The GI system is dependent on blood circulation and systemic availability of metabolites, is closely governed by both the central and the autonomous nervous system^[155,156] and contains a hallmark of resident and recruited immune cells^[157,158]. Thus, it is conceivable that the direct effects of alcohol on either of these systems will indirectly affect the function of the gastrointestinal system; this area is currently largely unexplored.

From a technical point, the GI research may take advantage of both *in vitro* and *in vivo* AAA models; however some *in vivo* models, such as those using parenteral administration of alcohol by the intraperitoneal route, may be less suitable due to the non-physiological direct contact between high concentrations of alcohol and GI tissues.

Alcohol use/abuse is associated with acute life-threatening conditions, including acute alcoholic hepatitis^[45] or acute pancreatitis^[159]. The majority of these patients report acute alcohol abuse, which is often overlapping with withdrawal from or even discontinued chronic alcohol abuse, or it follows an episode of binge drinking^[45,159]. As such, it is difficult to associate these diseases with the single-occasion AAA, yet they do not fit into the classic chronic alcohol abuse picture. This category of alcohol abuse, defined as “acute-on-chronic”, is in need of modeling for GI research.

In prospective, we currently lack in-depth knowledge in regards to the effects of acute alcohol abuse on different segments of the luminal GI tract, on liver functions, and on pancreas, including its endocrine and exocrine functions. Further, we do not know if acute alcohol consumption affects the GI stem cells and/or is involved in development of GI-derived tumors.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

Autophagy and ethanol-induced liver injury

Terrence M Donohue Jr

Terrence M Donohue Jr, Liver Study Unit, The Omaha Veterans Affairs Medical Center, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska 68105, United States

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Correspondence to: Terrence M Donohue Jr, PhD, Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Ave, Omaha, NE 68105, United States. tdonohue@unmc.edu

Telephone: +1-402-9953556 **Fax:** +1-402-4490604

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Abstract

The majority of ethanol metabolism occurs in the liver. Consequently, this organ sustains the greatest damage from ethanol abuse. Ethanol consumption disturbs the delicate balance of protein homeostasis in the liver, causing intracellular protein accumulation due to a disruption of hepatic protein catabolism. Evidence indicates that ethanol or its metabolism impairs trafficking events in the liver, including the process of macroautophagy, which is the engulfment and degradation of cytoplasmic constituents by the lysosomal system. Autophagy is an essential, ongoing cellular process that is highly regulated by nutrients, endocrine factors and signaling pathways. A great number of the genes and gene products that govern the autophagic response have been characterized and the major metabolic and signaling pathways that activate or suppress autophagy have been identified. This review describes the process of autophagy, its regulation and the possible mechanisms by which ethanol disrupts the process of autophagic degradation. The implications of autophagic suppression are discussed in relation to the pathogenesis of alcohol-induced liver injury.

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Peer reviewer: Minoti V Apte, Associate Professor, Pancreatic Research Group, South Western Sydney Clinical School, The University of New South Wales, Liverpool, NSW 2170, Australia

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INTRODUCTION

Lysosomes are the primary hydrolytic organelles of higher eukaryotic cells and have been appropriately named the cell's "digestive system". A large number of acid hydrolases inhabit these organelles. Together, these enzymes catalyze the breakdown of all forms of biopolymers, ranging from intracellular and extracellular proteins to storage carbohydrates (e.g. glycogen) to nucleic acids. Macromolecular catabolism takes place inside the lysosomal lumen at hydrogen ion concentrations that are 500-fold higher (pH about 4.7) than that in the cytoplasm (pH about 7.4)^[1]. Maintenance of such an acidic lysosomal interior occurs through the continuous action of ATP-dependent proton transporters that reside on its membrane. Lysosomes exhibit morphological and functional plasticity; they undergo continuous change as they mature from larger primary vesicles that segregate and carry hydrolase precursors, to mature hydrolytic organelles that execute intracellular digestion. As the catabolic organelles for protein degradation, lysosomes differ from proteasomes in their size, their complexity, their intracellular locations and in the types of substrates they degrade. While proteasomes have essentially supplanted lysosomes as the primary system that degrades intracellular proteins in higher eukaryotes, the crucial task of breaking down protein substrates that are more difficult to digest, as well as hydrolyzing all other forms of polymeric substrates, is left to lysosomes. Certainly, compelling evidence of their importance is the existence of a number of human abnormalities known as lysosomal storage diseases, in which afflicted individuals possess genetically defective lysosomal hydrolases. Clinically, this group of diseases exhibits a spectrum of impaired function, from neurodegeneracy to premature death^[2]. Furthermore, deficiencies in the pathways that participate in lysosome

biogenesis or that have a crucial function in supplying lysosomes with their substrates, can be lethal to the organism^[3]. Studies in the mammalian liver have provided a great deal of our current understanding of the lysosomal pathway, as this organ is highly responsive to changes in nutrient supply^[4,5]. The liver is also the predominant organ that metabolizes ethanol and liver injury is the principal clinical complication of alcohol abuse^[6]. This review will focus on the process of autophagy, its role in normal hepatic function and its alteration due to ethanol consumption. A portion of this review will consider how ethanol disrupts protein catabolism, which is believed to have a significant role in liver injury. The reader will note the citation of other excellent reviews that have been published on the process of autophagy and/or lysosomal proteolysis^[3,7-9]. The physiological and biomedical importance of autophagy is further underscored by the establishment of the international journal, *Autophagy*, the first issue of which was published in 2005.

FEEDING THE LYSOSOMAL APPARATUS; HETEROPHAGY AND AUTOPHAGY

In order for lysosomes to be “fed”, substrates of high molecular weight must gain entry to the interior of the organelle through a membrane that is normally impermeable to large molecules. Such access is accomplished by a number of mechanisms, each involving the active participation of membranes. During heterophagy extracellular materials are imported into the cell by either receptor-mediated or fluid phase endocytosis or by bulk phagocytosis of larger particles, including whole cells such as bacteria and apoptotic cells. All these are accomplished by invagination of the plasma membrane, thereby transporting the membrane-enclosed material, via endosomes to their final destinations, which, in many (but not all) cases is degradation in lysosomes. Heterophagy has a critical function in the normal turnover of senescent plasma proteins and in adaptive immunity, the latter involving antigen presentation through the action of endosomal proteases by the MHC class II pathway^[10].

Autophagy is exclusively an intracellular lysosomal process, which literally means “self eating”. It was originally described in the 1950s and later systematically analyzed by the classical work of de Duve and Wattiaux in rat liver. Autophagy of intracellular proteins occurs by three distinct mechanisms: Macroautophagy is the vacuolar engulfment by membranes of large portions of cytoplasm and collateral organelles, forming a double membrane vesicle called an autophagosome or autophagic vacuole. The outer membrane of the autophagosome later fuses with existing lysosomes to form an autolysosome in which the contents are degraded. Microautophagy describes membrane uptake by existing lysosomes of smaller portions of cellular constituents by lysosomal membrane invagination followed by rapid hydrolysis of those molecules.

Chaperone-mediated autophagy (CMA) is the singular uptake and degradation by lysosomes of specific protein substrates bearing a recognizable peptide motif, KFERQ. The latter process is mediated by molecular chaperones, the most prominent of which is a cytoplasmic form of heat shock protein 70 (HSC 70), which recognizes and binds the peptide motif of the substrate protein^[11,12]. The CMA substrate/chaperone complex then moves to the lysosome, where a specific receptor, the lysosome-associated membrane protein type-2A (LAMP-2A) recognizes and binds the complex; the substrate protein is then unfolded and translocated across the lysosome membrane and degraded in the lysosomal lumen. CMA is enhanced by oxidative stress, and, in addition to the proteasome, thereby serves a quality control function by degrading modified proteins. CMA also declines with age, largely due to the depletion of LAMP-2A. Recent studies have demonstrated that CMA can be restored in livers of aged mice that are transgenic for LAMP-2A. These animals, compared with their wild type littermates, showed improved cellular quality control and hepatic function^[13]. While microautophagy and CMA are largely ongoing constitutive processes, macroautophagy is more highly regulated. In general, when the term autophagy is used here, it will be used synonymously with macroautophagy.

Autophagy is clearly important for survival of the organism, particularly in times of nutrient deprivation when degradation of macromolecular constituents becomes necessary to recycle essential carbon sources to maintain viability. Autophagy was initially characterized morphometrically and biochemically in rat liver, one of the most sensitive organs to changes in nutrient supply. An illustration of this is when rats are fasted for 48 h, they exhibit a 40% loss of liver protein and a significant loss of liver mass, both of which are rapidly restored to normal levels 12 h after food is replenished. These respective changes correlate with an induction of autophagy by fasting followed by its nutrient-induced suppression after refeeding^[4,14,15]. A similar suppression of both autophagy as well as lysosome biogenesis was reported 24 h after partial hepatectomy in regenerating rat liver^[16]. Thus, autophagy rapidly responds to these changes and is an important component of cell adaptation. In fact, the half-life of the autophagosome is less than 10 min^[5], which indicates that the molecular machinery required to form autophagic vacuoles is constitutive for rapid degradation of cellular constituents. Furthermore, like the proteasome, autophagy has a significant role in removing misfolded proteins from the hepatocyte. Such is the case in alpha-1-antitrypsin (A-1AT) deficiency, in which the cellular accumulation of the mutated unsecretable form of A-1AT, known as ATZ (the isoform most prone to aggregation), is directed to autophagic vacuoles for degradation^[17]. Similarly, other aggregation-prone proteins are reported to have the same fate^[18,19]. It was recently demonstrated that inhibition of proteasome activity by treatment of mice with bortezomib caused Mallory-Denk-(MD)-like body formation. Activation of autophagy with

rapamycin treatment prevented the formation of these inclusions, indicating that proteasome inhibition causes the formation of M-D like bodies in livers of susceptible mice, while autophagosomes prevent M-D body accumulation by resorbing and degrading these insoluble inclusions^[20]. Thus, autophagy, not only functions in cell survival but also provides an additional line of cellular defense, the latter by removing aggregated, potentially toxic proteins, presumably resistant to degradation by the proteasome.

REGULATION OF AUTOPHAGY

Hormonal and nutrient regulators of autophagy include food deprivation or glucagon treatment and both are reliable autophagy inducers. Conversely, treatment with growth factors such as insulin or amino acids are well-known methods of autophagic suppression. The molecular mechanisms by which these agents actually induce or suppress autophagy have been investigated more recently and this has led to investigations of the protein components and signaling mechanisms that regulate macroautophagy to form the autophagosome. To date, over 30 autophagy-related gene products (Atgs) are now known. There are extensive molecular interactions that occur between these gene products in the early stages of autophagosome formation. The most well known mechanism is that of the class I phosphoinositol-3-kinase (PI-3K) pathway, which initiates an upstream signaling cascade to regulate the activity of the mammalian target of rapamycin (mTOR), a kinase, which is a major regulatory anabolic protein, and a suppressor of autophagy. Activation of mTOR leads to inhibition of Atg1, a key signal in autophagy induction. If Atg1 is not inhibited by mTOR, it is free to recruit the autophagy proteins, Atg11, Atg13 and Atg17 to form a complex which signals the induction of autophagy. The cellular machinery that initiates the formation of the autophagosome occurs through the activation of Beclin-1 (Atg6), which interacts with the class III PI 3K pathway and complexes with Atg 14. A second complex involves the interaction of Atg12, Atg16, Atg5, and Atg 7 complex, which is critical for the recruitment of Atg8, (also known as LC31) to the phagophore, the isolation membrane at which the macroautophagic response is initiated and the precursor of the autophagosome. Autophagic induction leads to cleavage of Atg8 (LC3- I), which is covalently bound to phosphatidylethanolamine (PE) to form a distinct form of the protein, LC3- II. The latter is associated with and is a commonly used marker for autophagosome membranes. Atg8 is believed to be involved in autophagosome closure as well as its relative size to surround portions of the cytoplasm for degradation^[21]. Eventual fusion of the autophagosome and the lysosome requires the participation of the LAMP-2 protein. Figure 1 depicts the major regulatory proteins and signaling factors in the autophagic response.

ETHANOL CONSUMPTION AND THE HEPATIC AUTOPHAGIC/LYSOSOMAL SYSTEM

The majority of ethanol is metabolized in the liver and individuals who abuse alcohol by routinely drinking 50-60 g (about 4 to 5 drinks) of ethanol per day are at risk for developing alcoholic liver disease^[6]. The pathogenesis of liver disease from alcohol abuse comes from the interaction of several factors, including the generation of oxidants and reactive metabolites from ethanol oxidation, which, in turn, causes other metabolic derangements. In addition, both acute and chronic ethanol administration cause enhanced formation of cytokines, especially TNF-alpha by hepatic Kupffer cells, which have a significant role in liver injury. This latter aspect is reviewed and described elsewhere^[22-24]. Besides the development of fatty liver (steatosis), another early sign of excessive ethanol consumption is liver enlargement and protein accumulation, both of which are common findings in alcoholics and heavy drinkers. Baraona *et al*^[25,26] originally described this in alcohol-fed rats. Later investigations sought to determine the origin of ethanol-elicited protein accumulation. They showed that chronic ethanol consumption slowed down the catabolism of long-lived proteins in rat liver^[27] and also depressed hepatic protein synthesis^[28]. Other studies confirmed that rats subjected to chronic ethanol feeding exhibited lower hepatic proteolysis than control rats. This was associated with reduced volume densities of autophagosomes and autolysosomes, as determined morphometrically^[29]. Our later studies, using isolated lysosomes from chronically ethanol-fed rats also revealed a reduced capacity of these organelles for proteolysis of endogenous as well as exogenous (i.e. akin to CMA) substrates *in vitro*. Furthermore, there was an enhanced tendency of lysosomes from ethanol-fed rats to leak their contents, but the intact organelles still exhibited lower levels of cathepsins, B and L compared with those from pair-fed control rats. A later report showed that lysosomes from ethanol-fed rats exhibited altered sedimentation properties on density gradients. Pulse-chase analyses, using isolated hepatocytes demonstrated that the reduced lysosomal content of cathepsin L was the result of delayed trafficking of its nascent precursor and impaired processing to its mature catalytic form in cells from ethanol-fed rats^[16,30,31]. Both flow cytometric and microscopy studies showed that vesicular movement and acidification of lysosomes in hepatocytes are both impeded after ethanol exposure^[32,33].

MECHANISMS OF ETHANOL-ELICITED SUPPRESSION OF AUTOPHAGY

The exact mechanisms responsible for the aforementioned ethanol-induced changes in hepatic autophagy and lysosomal proteolysis are not clear but some cogent explanations have been advanced. One is that oxidants (e.g. peroxynitrite) and reactive species

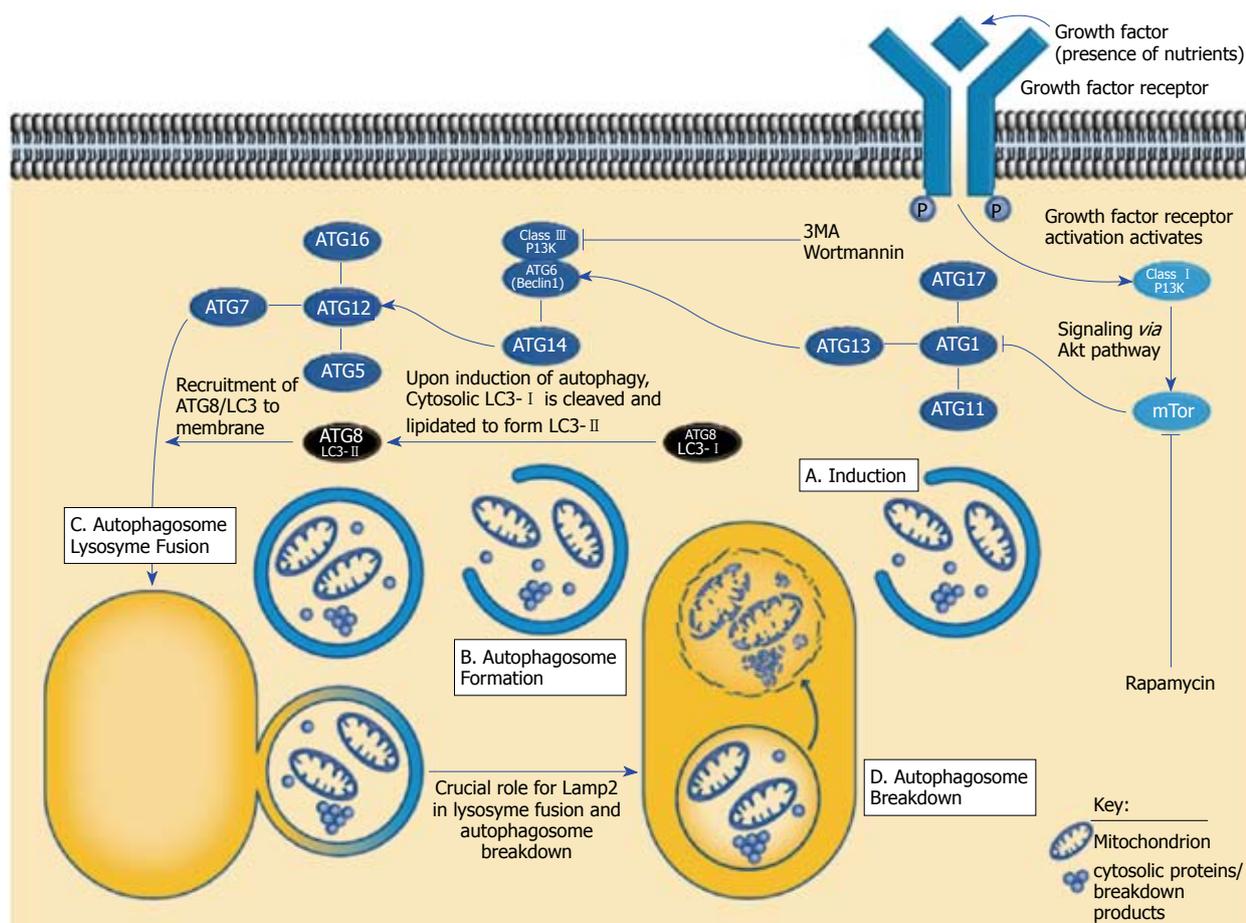


Figure 1 Interaction of gene products and pathways in the regulation of autophagy. Growth factors and nutrients activate the Class I P13K proteins, which, in turn, signal, via the AKT pathway to activate mTOR. This leads to inhibition of ATG1 - the primary signal for autophagy. Nutrient deprivation or inhibition of mTOR by rapamycin allows ATG1 to recruit ATG11, ATG13 and ATG 17 to form a complex to initiate formation of the autophagosome. This is dependent on the formation of 2 complexes: ATG 6 (Beclin1) which interacts with the Class III P13K protein complexes with ATG14. Another complex involves ATG12, ATG16, ATG5 and ATG7. The latter complex recruits ATG8 (LC3). Upon induction of autophagy, cytosolic LC3-I (ATG8) is cleaved and lipidated to form LC3-II. LC3-II is a marker for the autophagosome membrane. Fusion between the autophagosome and the lysosome and subsequent breakdown of the contents of the autophagic vacuole requires LAMP2 protein. Reproduced in modified form with permission (Abcam, Inc.) <http://www.abcam.com/ps/pdf/cardiovascular/autophagic.pdf>.

(e.g. acetaldehyde and malondialdehyde-acetaldehyde)^[34] derived from ethanol metabolism may impair autophagy, similar to that which occurs with the proteasome^[35-37]. Some of the current evidence for this is circumstantial, but nevertheless compelling as there is evidence of lysosomal damage, as judged by enhanced lysosomal fragility, which could result from either altered lipid metabolism, oxidative stress or both^[15]. By analogy, other instances of lysosome fragility by iron-induced oxidative stress are documented^[38,39]. However, ethanol administration has little to no effect on the activities of lysosomal hydrolases^[31] and, because a significant number of cathepsins rely on a reduced sulfhydryl group for their catalytic activity in their active centers, the alteration of lysosomal glutathione or cysteine content by ethanol seems unlikely. It is worth noting that lysosomes and proteasomes seem to exhibit differential sensitivity to ethanol levels in the serum. Animal experiments have revealed that proteasome activity declines in animals that have higher (i.e. > 40 mmol/L) serum ethanol concentrations^[40]. The lysosomal system, on the other hand, appears to be impaired by lower serum ethanol levels, as recently reported in livers of

female ethanol-fed rats^[41].

Ethanol-induced suppression of autophagy may result from alterations in hepatic amino acid pool sizes, especially those of leucine, phenylalanine, methionine, histidine, tryptophan, glutamine, proline, and tyrosine, which have been deemed regulatory amino acids and suppressors of macroautophagy^[5]. L-leucine appears to be one of the strongest autophagic suppressors in this group. It is noteworthy that reports indicate that chronic ethanol administration in rats increases the intrahepatic levels of leucine by 1.4 to 1.8-fold over pair-fed controls^[27,42]. Thus, the association of an ethanol-induced reduction in autophagy with higher levels of intrahepatic leucine may partially explain autophagic suppression in the ethanol-fed state. However, it remains to be conclusively demonstrated whether this association represents a causal relationship. It is also paradoxical that ethanol feeding results in higher levels of leucine during a slowdown in protein catabolism when one would expect the opposite situation. Still, leucine accumulation could reflect a reduced ability of the liver to synthesize proteins, which indeed occurs in ethanol-fed animals^[42,43].

A third likely mechanism of autophagic suppression

by ethanol is its well-documented ability to disrupt protein trafficking in the liver. Autophagy requires the action of cytoskeletal elements, including the microtubules and microfilaments. Both are necessary for autophagosome formation and their fusion with other vesicular bodies, as demonstrated by blockage of these processes with specific inhibitors, including nocadazole and vinblastine (microtubules) and the cytochalasins (microfilaments)^[44,45]. Disruption of vesicular movement within the hepatocyte by ethanol treatment occurs by mechanisms that are independent of the molecular motors, dyenin and kinesin, although there is evidence for alterations in the protein, dynamin^[32]. Trafficking of exogenous proteins into the hepatocyte by endocytosis^[46,47] and the intracellular delivery of proteases to lysosomes^[31] are both inhibited by ethanol consumption. Furthermore, the anti-secretory properties of ethanol in the liver are well documented. Studies with liver slices and cultured cells indicate that ethanol metabolism is required for disruption of these protein trafficking events^[48-51]. *In vitro* investigations also revealed that acetaldehyde, the initial product of ethanol oxidation, inhibits the polymerization of tubulin to form microtubules, indicating that the reactive metabolite may impair protein trafficking by forming adducts with tubulin subunits, thereby blocking their polymerization into microtubules^[52]. Further, the finding that acetaldehyde can undergo secondary reactions with malondialdehyde (MDA) to form more bulky substituents on proteins, known as malondialdehyde acetaldehyde adducts (MAA) with proteins, makes this mechanism of autophagic suppression an attractive hypothesis.

ETHANOL-INDUCED ALTERATIONS IN CELLULAR SIGNALING: REGULATION OF AMP KINASE

While the foregoing mechanisms of autophagic suppression by ethanol are plausible, it is likely that ethanol and/or its metabolites impact signaling events that govern the autophagic response. There have been significant advances in our understanding of these processes at the molecular level, as recently reviewed^[3]. Because autophagy is an energy-dependent process and requires ATP^[53], it follows that ATP depletion naturally suppresses autophagy. Ethanol consumption has been reported to inhibit ATP production by mitochondria^[54], in part by enhancing oxidative modification and inactivation of proteins within these organelles^[55]. However, others have reported an increased efficiency of mitochondrial oxidative phosphorylation in livers of ethanol-fed animals^[56]. Thus, reduced ATP availability from mitochondria may not fully explain such downstream effects on autophagy. Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric protein that is, itself, activated by elevated ratios of AMP/ATP and is a significant regulator of a variety of metabolic and signal transduction pathways,

including autophagy. Elevated AMP/ATP ratios are indicators of low intracellular energy charge. Therefore, when AMP activates the AMPK it generally down-regulates energy requiring pathways and stimulates catabolic pathways. Interestingly, however, inhibition of AMPK also suppresses autophagy^[57]. These findings are consistent with the suppression of autophagy by ethanol consumption, which significantly reduces AMPK activity in liver^[58]. Such a decline in AMPK activity would, in turn cause the release of suppression of mTOR activity by AMPK^[59], thereby permitting mTOR to suppress proteolysis. Conversely, AMPK activation suppresses mTOR, thereby allowing autophagy to proceed. Recent work with cultured hepatoma cells determined that ethanol metabolism is required for suppression of AMPK phosphorylation. This occurs by an inactivation of two upstream kinases, LKB-1 and protein kinase C-zeta. Simultaneously, ethanol exposure activates protein phosphatase 2A (PP2A), which causes dephosphorylation (and inactivation) of AMPK^[60]. The latter findings seem paradoxical, as AMPK is generally activated by reactive oxygen and nitrogen species^[22,61]. Thus, while ethanol metabolism similarly generates oxidants and reactive species, including acetaldehyde, these molecules down-regulate upstream kinases and up-regulate the downstream phosphatase, PP2A, resulting in AMPK inactivation, which, in turn, can cause autophagic suppression. AMPK also has an important role in regulating lipid metabolism and AMPK suppression by ethanol allows activation of rate-limiting enzymes involved in lipid biosynthesis, which contribute to ethanol-induced fatty liver^[62,63]. Figure 2 depicts the putative mechanisms of autophagic suppression.

PATHOLOGICAL CONSEQUENCES OF AUTOPHAGIC SUPPRESSION

Protein accumulation due to ethanol-elicited decline in protein catabolism probably contributes to the formation of Mallory Denk (M-D) bodies in liver cells. These inclusions are prominent histological hallmarks of liver disease in alcoholics^[64]. M-D bodies contain the cytokeratins 8 and 18 as well as ubiquitin, and p62, an adaptor protein. As pointed out earlier, M-D bodies comprise an aggresome, consisting of filamentous ubiquitylated misfolded proteins that are believed to represent a failed attempt by the proteasome to degrade them. Recent evidence indicates that autophagy can degrade these insoluble complexes^[20]. However, clearance of such cellular debris is hampered by an ethanol-elicited suppression of autophagy, a situation that may have a perilous outcome if drinking continues.

In addition to the accumulation of potentially toxic proteins, ethanol also causes mitochondrial damage. Changes ranging from morphologically detectable mitochondrial swelling, to DNA fragmentation, to depolarization of the inner membrane, (commonly known as the mitochondrial permeability transition or MPT) to disruption of mitochondrial gene products

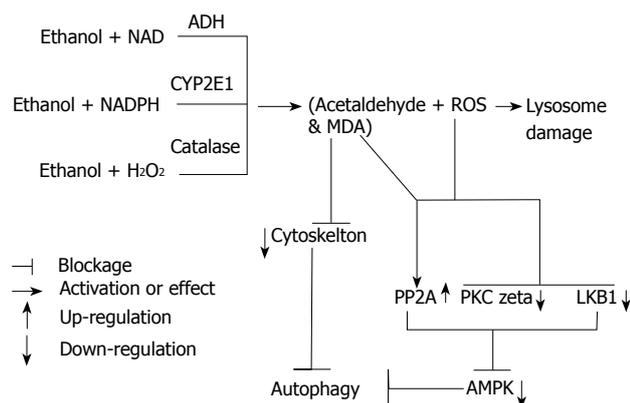


Figure 2 Putative mechanisms of autophagy suppression by ethanol. Ethanol metabolism by the three major pathways, alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase generates acetaldehyde, which can undergo secondary reactions with malondialdehyde (MDA) to form MAA. In addition, reactive oxygen species (ROS) are generated by CYP2E1 catalysis. Reactive oxidants (ROS) may also contribute to lysosomal damage. The reactive aldehydes are known to form adducts with tubulin and other cytoskeletal elements to block trafficking and movement of autophagic vacuoles and their formation. The combined formation of ROS and acetaldehyde putatively cause upregulation of protein phosphatase 2A (PP2A) and a downregulation of LKB1 and PKC zeta. The latter changes cause inactivation of AMPK, which in turn suppresses autophagy due to up regulation of mTOR (not shown) as described in the text.

as well as decreases in glutathione occur in livers of ethanol-fed animals^[55,65-72]. It is crucial that such damaged organelles be removed from the cell and there is evidence that selective engulfment of mitochondria (called mitophagy) into autophagic vacuoles occurs in cells as a quality control device^[73-75]. Similarly, there appears to be rather selective autophagy of ER components (ER-phagy) as well as peroxisomes (pexophagy) in cells under conditions where there is an accumulation of these organelles^[76,77]. It remains to be demonstrated whether such selective autophagy represents actual recognition of damaged organelles or simply occurs because of mass action due to their accumulation, however, reports show that the permeability transition may act as a signal for autophagic destruction of the mitochondrion^[74]. While there is no firm evidence of ethanol-elicited suppression of mitophagy, the detection of increased numbers of damaged mitochondria in livers of ethanol-fed animals provides circumstantial evidence of mitophagy inhibition. In this regard it is also worth noting that mitochondrial damage also occurs by non-oxidative products of ethanol metabolism, namely fatty acid ethyl esters (FAEE). These condensation products between ethanol and fatty acids are generated enzymatically and have been shown to cause mitochondrial damage^[78]. Thus a mechanism for ethanol-induced injury in nonhepatic tissue such as pancreas, heart and brain could occur by the generation of FAEE.

CONCLUSION

This review has summarized the evidence for the ethanol-elicited suppression of autophagy as a mechanism by which liver cells can accumulate damaged

proteins and organelles. Suppression of proteolysis in general can be lethal to cells, as normal turnover is disrupted and the removal of potentially toxic proteins is prevented. As an organ that is sensitive to nutrients as well as toxins, the liver is highly active in autophagy. The inability to respond to depletion of nutrient supply in the alcoholic state is a potentially perilous condition. Still, while there have been numerous investigations of hepatic autophagy, the actual mechanism(s) by which ethanol may influence this process remain(s) to be conclusively determined. From this summary of results from our laboratory and from the literature, we hypothesize that ethanol consumption probably influences pathways upstream of the autophagic process, including inhibition of AMPK, but this awaits experimental confirmation. Finally, while this review has specifically focused on autophagy in hepatic tissue, a recent report indicates that ethanol exposure also slows autophagy in neural cells^[79], indicating that this effect of ethanol is not exclusively liver-specific. In conclusion, while autophagy has been recognized for decades as an indispensable means of macromolecular disposal, its roles in other cellular processes from differentiation to cell death are now being documented, thus rekindling new interest in this fascinating field of investigation.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

Is the iron regulatory hormone hepcidin a risk factor for alcoholic liver disease?

Duygu Dee Harrison-Findik

Duygu Dee Harrison-Findik, Division of Gastroenterology and Hepatology, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, 68198-5820, United States

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Correspondence to: Duygu Dee Harrison-Findik, Division of Gastroenterology and Hepatology, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, 68198-5820, United States. dharrisonfindik@unmc.edu

Telephone: +1-402-5596355 Fax: +1-402-5596494

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Abstract

Despite heavy consumption over a long period of time, only a small number of alcoholics develop alcoholic liver disease. This alludes to the possibility that other factors, besides alcohol, may be involved in the progression of the disease. Over the years, many such factors have indeed been identified, including iron. Despite being crucial for various important biological processes, iron can also be harmful due to its ability to catalyze Fenton chemistry. Alcohol and iron have been shown to interact synergistically to cause liver injury. Iron-mediated cell signaling has been reported to be involved in the pathogenesis of experimental alcoholic liver disease. Hepcidin is an iron-regulatory hormone synthesized by the liver, which plays a pivotal role in iron homeostasis. Both acute and chronic alcohol exposure suppress hepcidin expression in the liver. The sera of patients with alcoholic liver disease, particularly those exhibiting higher serum iron indices, have also been reported to display reduced prohepcidin levels. Alcohol-mediated oxidative stress is involved in the inhibition of hepcidin promoter activity and transcription in the liver. This in turn leads to an increase in intestinal iron transport and liver iron storage. Hepcidin is expressed primarily in hepatocytes. It is noteworthy that both hepatocytes and Kupffer cells are involved in the progression of alcoholic liver disease. However, the activation of Kupffer cells and TNF- α signaling has been reported not to be involved in the down-regulation of hepcidin expression by alcohol

in the liver. Alcohol acts within the parenchymal cells of the liver to suppress the synthesis of hepcidin. Due to its crucial role in the regulation of body iron stores, hepcidin may act as a secondary risk factor in the progression of alcoholic liver disease. The clarification of the mechanisms by which alcohol disrupts iron homeostasis will allow for further understanding of the pathogenesis of alcoholic liver disease.

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Peer reviewer: Shivendra Shukla, Professor, Department of Medical Pharmacology and Physiology, University of Missouri School of Medicine, 1 Hospital Drive, M530 Medical Sciences Bldg., Columbia MO 65212, United States

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INTRODUCTION

Many eukaryotes require iron for critical biological processes^[1,2]. However, excess iron is toxic, causing lipid peroxidation and oxidative stress^[3]. Due to it being a “double edged sword”, iron uptake and transport must therefore be tightly regulated^[4-7]. Hepcidin, a circulatory peptide synthesized by the liver, performs this important task by regulating iron transport in different parts of the body including duodenum, spleen and bone marrow^[8,9]. Alcohol has been shown to suppress hepcidin expression in the liver leading to an increase in intestinal iron transport^[10-15]. Both iron and alcohol act synergistically to cause liver injury. Disruption of hepcidin synthesis by alcohol may therefore be one of the “second hit” mechanisms leading to the progression of alcoholic liver disease.

ALCOHOL-INDUCED DISTURBANCES IN IRON METABOLISM

Alcohol consumption has long been associated with

changes in iron homeostasis. The reported changes range from anemia to iron overload^[16-22]. Blood loss is common in patients with alcoholic cirrhosis^[23]. Anemia and iron deficiency can be due to gastrointestinal blood loss arising from the complications of alcohol abuse. Nutritional deficiencies, such as folate deficiency, can also be a common cause of anemia^[24-27]. Megaloblastic and sideroblastic anemias, macrocytosis of alcoholism, elevated mean corpuscular volume, and iron deficiency have been reported to be common among hospitalized chronic alcoholics^[23,27-30]. However, it has been demonstrated that anemia and megaloblastic and sideroblastic changes do not occur if an adequate diet is provided during chronic alcohol administration to human volunteers^[25,31]. This has also been shown to be the case for relatively well-nourished alcoholics^[25,26,32,33]. These findings therefore suggest that alcohol by itself does not induce iron deficiency or anemia.

In fact, wine has a high iron content and increases iron absorption^[34]. Decreasing the alcohol content of red wine has been reported to significantly reduce the absorption of non-heme iron in human subjects^[35]. Beer consumption has been suggested to have a more significant effect on serum ferritin levels than wine and spirits, both in males and females^[18]. Alcohol consumption (up to 2 alcoholic drinks per day) has also been shown to exert a protective effect by reducing the risk for iron deficiency anemia in adult participants of the Third National Health and Nutrition Examination Survey^[36]. On the other hand, heavy alcohol consumption (more than two alcoholic drinks per day) elevates the risk of iron overload^[36]. In both male and female adolescents (aged 16-19 years), who participated in the First National Health and Nutrition Examination Survey, serum iron concentration was significantly related to drinking frequency^[17]. Alcohol consumption was associated with elevated serum iron concentration in both male and female adolescents. Interestingly, male adolescents also exhibited increased transferrin saturation and hemoglobin concentration^[17]. Drinking frequency was unrelated to dietary iron intake, poverty index or race^[17]. A Danish population survey also reported a correlation between alcohol intake and elevated serum ferritin in healthy adult males and females (aged 30-60 years)^[37]. African Americans who consume alcohol (4 drinks per day) exhibit a higher prevalence of ferroportin Q248H allele, which is implicated in iron accumulation, and alcohol has been suggested to contribute to higher body iron stores in this population^[38]. Alcohol elevates iron absorption and patients with alcoholic cirrhosis often exhibit elevated liver iron content^[39,40]. *In vivo* whole-body retention studies have demonstrated a two-fold increase in intestinal iron absorption in chronic alcoholics^[41]. Alcoholic cirrhosis patients with higher liver iron content display increased mortality rates^[42]. Experimental animal models of prolonged alcohol exposure also display increased liver iron deposition and hepatocellular injury^[19,43]. In mild cases of alcoholic liver disease (ALD), iron has been reported to accumulate in hepatocytes^[44]. However, in patients with advanced ALD, both parenchymal cells and Kupffer

cells exhibit iron staining^[20].

In some alcoholics, the elevated serum iron indices have been reported to significantly decrease within 2-6 wk of abstinence, suggesting a direct role for alcohol consumption^[45]. However, the mechanisms by which alcohol disrupts iron metabolism are unclear. Recent studies indicate a role for hepcidin in this process.

HEPCIDIN

The discovery of the circulating peptide hormone hepcidin has revolutionized our understanding of iron metabolism. Hepcidin sequence is highly conserved between species, from fish to mammals. Hepcidin was first isolated from human urine and serum as short cysteine-rich peptide forms^[46,47]. Hepcidin is homologous to the members of the defensin family of antimicrobial peptides and has been reported to exhibit antimicrobial activity *in vitro*^[46]. It is the studies with transgenic mice that have highlighted the importance of this protein in the regulation of iron metabolism. Namely, hepcidin knockout mice, deficient in the expression of hepcidin, develop severe iron overload, whereas mice overexpressing hepcidin display iron deficiency and anemia^[48,49]. Moreover, the synthesis of hepcidin has also been shown to be responsive to the body iron levels. Iron overload state induces hepcidin synthesis whereas iron deficiency and anemia have the opposite effect^[50,51].

Liver is the main site of hepcidin synthesis in the body^[50]. It is synthesized as an 84 amino acid precursor protein and is subsequently cleaved into the biologically active 25 amino acid peptide form^[8,9,50,52]. In the liver, hepcidin is primarily expressed in the hepatocytes (Figure 1). Although to a much lesser extent, other cells of the liver and other organs also display hepcidin expression. The exciting discovery of the interaction between hepcidin and the iron exporter protein ferroportin was pivotal to our understanding of how hepcidin regulates iron metabolism^[53,54]. The binding of hepcidin to ferroportin induces the internalization and degradation of the ferroportin protein, which in turn inhibits iron from being transported into the circulation. This is how hepcidin accomplishes the regulation of iron mobilization between distant locations (i.e. duodenum, bone marrow, reticuloendothelial macrophages) in the body (Figure 1)^[8].

The synthesis of hepcidin in the liver is modulated by numerous upstream regulators^[1,2]. Transferrin receptor 2, the genetic hemochromatosis gene product, Hfe, and the juvenile hemochromatosis gene product, HJV, are positive regulators of hepcidin expression^[55-62]. Mutations in these genes result in an increase in body iron stores (i.e. hemochromatosis) due to the down-regulation of hepcidin expression. Recently, a novel candidate TMPRSS6, a transmembrane serine protease also known as matriptase 2, has been identified. Interestingly, TMPRSS6 is the first identified negative regulator of liver hepcidin expression. In other words, TMPRSS6 keeps hepcidin expression under control in the liver^[63,64]. Accordingly, patients expressing TMPRSS6 mutations exhibit iron-refractory iron deficiency anemia due to elevated hepcidin produc-

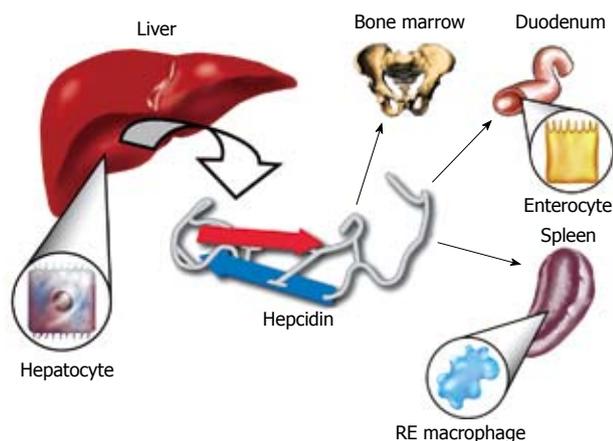


Figure 1 The Iron regulatory hormone hepcidin. Hepcidin is synthesized in the hepatocytes of the liver as an 84 amino acid precursor protein. It is subsequently cleaved into the 25 amino acid biologically active peptide form and is released into the circulation. Hepcidin plays a central role in the regulation of iron metabolism by inhibiting the release of iron from the enterocytes of the duodenum and from reticuloendothelial macrophages. Hepcidin blocks the export of iron from these cells by binding to the iron exporter protein, ferroportin, which induces the internalization and degradation of ferroportin protein. As a soluble mediator, hepcidin establishes the cross-talk between distant organs in the body in order to maintain iron homeostasis.

tion^[63]. Moreover, the splicing defects in the *TM6SF2* gene lead to the mask phenotype, a recessive, chemically induced mutant mouse phenotype^[64]. This phenotype results from the reduced absorption of dietary iron due to high levels of hepcidin expression^[64]. However, the signaling mechanisms involved in these regulatory processes (negative or positive) are as yet unknown.

KUPFFER CELLS, ALCOHOL AND HEPCIDIN

Liver macrophages (Kupffer cells) also play a pivotal role in the progression of ALD. Alcoholics have elevated levels of lipopolysaccharide (LPS) in their circulation, which is believed to prime the macrophages leading to the release of proinflammatory cytokines, particularly $\text{TNF-}\alpha$, and reactive oxygen species^[65-67]. The importance of Kupffer cells in ALD has been demonstrated in studies where their depletion or inactivation blocked alcohol-induced symptoms, including inflammation, fatty liver and necrosis, in experimental animal models of chronic alcohol exposure^[68,69].

In advanced forms of ALD and in experimental animal models of ALD, besides parenchymal cells, Kupffer cells also exhibit iron accumulation^[20,70,71]. Reticuloendothelial (RE) macrophages play a major role in iron trafficking and recycling to meet changes in the body's iron demand^[72]. Like the parenchymal cells of the liver, the RE system also stores large amounts of iron bound to the storage protein, ferritin. RE macrophages acquire iron mainly by phagocytosing senescent red blood cells. However, macrophages also express the divalent metal transporter1 (DMT1), natural resistance associated macrophage protein1 (Nramp1), hemoglobin scavenger receptor (CD163) and transferrin receptor1 (TrfR1), all

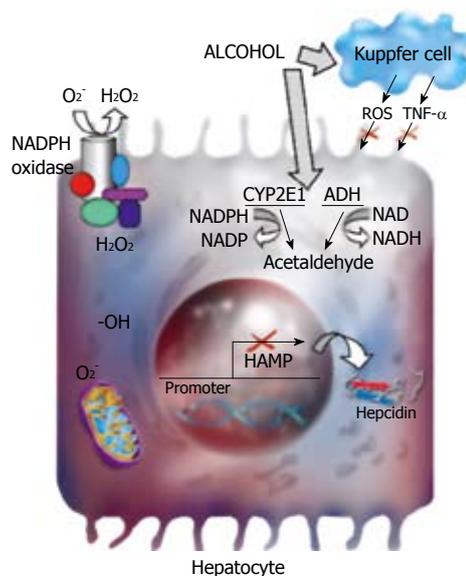


Figure 2 Hepcidin and alcohol. Alcohol is metabolized by alcohol dehydrogenase (ADH) and cytochrome P4502E1 (CYP2E1) in the liver. Alcohol-induced oxidative stress leads to the suppression of hepcidin promoter activity and hepcidin transcription in the liver. The parenchymal, but not the non-parenchymal cells of the liver are involved in the regulation of hepcidin transcription by alcohol-induced oxidative stress. The activation of CYP2E1 or NADPH oxidase and changes in mitochondrial functions are involved in alcohol-induced oxidative stress in hepatocytes. The role of these pathways in the regulation of hepcidin transcription by alcohol requires further investigation.

of which are involved in iron uptake and transport^[72-75]. The livers from experimental animal models of ALD and Japanese patients with ALD have been reported to exhibit significantly more TrfR1 mRNA or protein expression, respectively, compared to control animals or healthy human subjects^[76,77]. Macrophages can take up transferrin-bound iron *in vitro*. However, a significant uptake of transferrin-bound iron by macrophages has not been observed *in vivo* in humans^[72,78]. Although an increase in TrfR1 mRNA expression was observed in Kupffer cells of experimental animals, the increase in TrfR1 protein expression in Japanese patients with ALD was observed mainly in the hepatocytes^[20,76,77]. It should also be noted that mice with disrupted TrfR1 gene, deficient in the global expression of TrfR1 receptor, display abnormalities only in erythropoiesis and neurologic development^[79]. The Kupffer cells of rats exposed to chronic alcohol have also been shown to display an increase in mRNA levels of Hfe, the gene for genetic hemochromatosis^[77]. Hfe gene product does not bind iron and is not directly involved in iron uptake. However, it is involved in the regulation of iron metabolism as shown by the fact that mice deficient in Hfe expression develop iron overload^[80,81]. Hfe is believed to achieve this by modulating the expression and iron responsiveness of hepcidin in the liver^[57,58,82]. On the other hand, the iron release from macrophages is regulated by the iron exporter protein ferroportin^[83,84]. The multicopper ferroxidase, ceruloplasmin may also play a role in iron efflux in macrophages^[85-87]. The Kupffer cells of animals subjected to alcohol exposure have been shown to display an increase in both ferroportin mRNA and protein,

indicative of increased iron export^[77]. Of note, hepcidin binds to ferroportin and induces its internalization and degradation^[53,54]. The increase in ferroportin expression in the Kupffer cells of these animals may be due to suppressed hepcidin expression. Alcohol has been shown to down-regulate hepcidin expression both in animal models of ALD and in patients with ALD^[11-13]. However, Xiong *et al*^[77] have reported no change in plasma pro-hepcidin levels and a decrease in hepcidin mRNA levels in Kupffer cells in their experimental model.

Iron has been reported to prime Kupffer cells for alcoholic liver injury in rats exposed to chronic intragastric alcohol infusion^[70]. The proinflammatory cytokine TNF- α is a key player in alcohol-induced liver injury^[88-90]. Iron has been shown to activate the transcription factor, NF- κ B in hepatic macrophages and induce the synthesis and release of TNF- α ^[76,91]. Following erythrophagocytosis, Kupffer cells have been shown to display elevated levels of LPS-induced NF- κ B activation^[70]. Treatment of cultured Kupffer cells *in vitro* and *ex vivo* with iron chelators blocked both NF- κ B activation and TNF- α synthesis^[70,71]. Proinflammatory cytokines (IL-1, IL-6, TNF- α) have been suggested to increase iron uptake into the monocytes of patients with rheumatoid arthritis^[92]. It is therefore possible that alcohol and/or iron-induced release of TNF- α may serve as a feedback loop for further iron uptake into the Kupffer cells. On the other hand, Olynyk *et al*^[93] have reported that deposition of iron in Kupffer cells impairs LPS-induced proinflammatory cytokine production in these cells. Xiong *et al*^[77] have reported that the intracellular labile non-heme iron pool is involved in peroxynitrite or LPS-mediated activation of NF- κ B and TNF- α release in Kupffer cells isolated from experimental animal models of chronic alcohol exposure. The intracellular labile iron pool is a transitory, free (non-ferritin-bound) pool of iron, which is chelatable by commonly used iron chelators. This pool of iron contributes to the generation of reactive oxygen species but its biological relevance is unclear^[94]. Transient changes in the dynamics of the labile iron pool is not only affected by increased iron uptake but also by prooxidant chemicals, which induce the reductive release of iron from intracellular stores^[95,96]. Xiong *et al*^[77] have demonstrated that iron dextran treatment of cultured Kupffer cells *in vitro* does not affect the basal TNF- α release. Interestingly, Kupffer cells isolated from mice two weeks after iron dextran injection, exhibited significant increases in basal TNF- α release (independent of peroxynitrite or LPS stimulation). These findings suggest that either other cells in the liver (besides Kupffer cells) or yet unknown signals contribute to this process *in vivo*. It is also possible that the discrepancy between *in vitro* and *in vivo* iron loading regarding basal TNF- α release from Kupffer cells may not be due to iron (iron loading) *per se* but rather due to the differences in the level of oxidative stress.

The synthesis of hepcidin in the liver is mediated by several stimuli including iron and inflammation. Kupffer cells have been shown not to play a role in the regulation of hepcidin expression by iron *in vivo*^[97,98]. The data in

the literature regarding the involvement of Kupffer cells in the regulation of liver hepcidin expression by inflammation is contradictory^[97-99]. Montosi *et al*^[97] have reported a role for Kupffer cells in the regulation of hepcidin expression by inflammation in mice. On the other hand, Lou *et al*^[98] and Theurl *et al*^[99] have demonstrated that the depletion of Kupffer cells in mice does not abrogate the up-regulation of liver hepcidin expression by LPS.

The role of Kupffer cell activation and TNF- α signaling in the regulation of hepcidin expression by alcohol *in vivo* has also been reported^[100]. The inactivation of Kupffer cells by gadolinium chloride in rats paired with alcohol Lieber DeCarli diets for 6 wk or mice fed with ethanol in the drinking water for 1 wk did not reverse the alcohol-induced suppression of hepcidin expression in the liver^[100]. Moreover, similar results were obtained when Kupffer cells were depleted by liposomes containing clodronate^[100]. When phagocytosed by the Kupffer cells, clodronate released from the liposomes induces apoptosis and thereby depletes the Kupffer cell^[101]. When co-cultured, Kupffer cells have been suggested to exert a negative effect on hepcidin synthesis in hepatocytes^[99]. However, the depletion or inactivation of the Kupffer cells has been reported to not induce any significant changes in basal hepcidin expression levels in the livers of both control and alcohol-treated animals *in vivo*^[100]. Interestingly, one week of ethanol treatment (in the drinking water) was sufficient to induce NF- κ B activation and TNF- α and IL-6 release in mice, compared to control mice fed with plain water^[100]. The neutralization of TNF- α inhibited the activation of NF- κ B^[100]. However, neither the neutralization of TNF- α nor the absence of TNF- α receptor I and II expression altered the effect of alcohol on hepcidin expression (i.e. down-regulation)^[100]. These findings therefore strongly suggest that the activation of Kupffer cells and TNF- α signaling are not involved in the regulation of hepcidin expression by alcohol *in vivo*. These findings are also in agreement with Lou *et al*^[98], who demonstrated that Kupffer cells are not required for the regulation of liver hepcidin expression by LPS-induced inflammation.

The suppression of hepcidin synthesis in the liver by alcohol occurs very early (within days) following alcohol exposure and involves hepatocytes, but not Kupffer cells (Figure 2)^[12,100]. It is noteworthy that hepatocytes are the main site of hepcidin synthesis in the liver^[50]. Alcohol-induced oxidative stress in hepatocytes is one of the main mechanisms by which hepcidin expression in the liver is down-regulated by alcohol^[12]. The decrease in liver hepcidin synthesis leads to an increase in intestinal iron transport and liver iron content^[10-12]. Hence, alcohol dysregulates iron homeostasis by suppressing hepcidin expression in the liver. Although Kupffer cells are not involved in the initial stages of this process induced by alcohol, the increase in iron stores (due to low levels of hepcidin) will further activate Kupffer cells, and thereby lead to the release of proinflammatory cytokines. It is therefore possible that unlike LPS, the priming of Kupffer cells by iron^[70] occurs in later stages of ALD and exacerbates the inflammatory processes by facilitating further

release of TNF- α , and thereby contributing to liver injury.

ALCOHOL-INDUCED OXIDATIVE STRESS AND HEPCIDIN

Alcohol metabolism generates reactive oxygen species and lipid peroxidation products (malondialdehyde, 4-hydroxynonenal) during the oxidation of ethanol by alcohol dehydrogenase and cytochrome P4502E1 to form acetaldehyde. Serum thioredoxin levels are also an indicator of oxidative stress and they have been reported to be significantly higher in patients with ALD compared to healthy subjects^[102]. Due to its capacity to take part in the Fenton reaction as a transition metal, iron itself induces the generation of reactive oxygen species (superoxide, hydroxyl iron), which subsequently damage cellular membranes *via* lipid peroxidation^[3,103]. Iron and alcohol act as a deadly cocktail to exacerbate liver injury. Orally effective iron chelators have been reported to attenuate alcohol-induced hepatic lipid peroxidation in rats^[104].

Alcohol-induced oxidative stress is involved in the suppression of hepcidin promoter activity and hepcidin transcription in the liver *in vivo* by inhibiting the DNA-binding activity of the transcription factor, C/EBP α ^[12]. Both Kupffer cells and hepatocytes are involved in alcohol-induced oxidative stress. However, a role for Kupffer cells in the regulation of hepcidin expression by alcohol in the liver has been excluded (Figure 2)^[100]. This strongly suggests the involvement of hepatocytes in the regulation of hepcidin expression, and thereby iron metabolism by alcohol. Multiple pathways are involved in alcohol-induced oxidative stress in hepatocytes; redox state changes, the activation of CYP2E1 or NADPH oxidase and changes in mitochondrial functions^[105-109]. Alcohol metabolism, which is accompanied by oxidation and reduction reactions, causes an imbalance in the redox state of the cell by generating excess NADH (reduced nicotinamide adenine dinucleotide). CYP2E1 enzyme, present in liver microsomes, has a high redox potential and is also metabolically active in the absence of alcohol^[107]. It produces reactive oxygen species such as superoxide and hydrogen peroxide, which is increased by alcohol exposure leading to lipid peroxidation, oxidative stress and tissue injury. NADPH oxidase catalyzes the reduction of molecular oxygen to generate superoxide^[110,111]. The liver expresses both phagocytic and non-phagocytic isoforms of NADPH oxidase^[112,113]. Alcohol-induced liver pathology was not observed in mice with deficient NADPH oxidase activity suggesting a role for this enzyme in the progression of alcoholic liver disease^[109]. Mitochondria play a key role both in iron biogenesis and alcohol metabolism^[3,108,114]. Alcohol induces lesions in the proteins of the mitochondrial electron transport chain and decreases the rate of hepatic ATP synthesis^[114-116]. This induces the transfer of unpaired electrons to molecular oxygen and elevates the production of reactive oxygen species. The involvement of

these changes in the regulation of hepcidin expression in hepatocytes by alcohol needs further investigation (Figure 2).

Several factors including iron, viral infection, endotoxin and reactive oxygen species have been implicated to act as second hit factors in the progression of alcoholic liver disease. Of note, hepcidin expression in the liver is regulated by iron, alcohol, hepatitis C viral proteins, inflammation, hypoxia and oxidative stress^[10-12,48,100,117-120]. Nishina *et al*^[119] have recently shown that hepatitis C viral protein-mediated oxidative stress suppresses hepcidin transcription by altering the activity of the transcription factor, C/EBP α . These findings are in agreement with our previously published results demonstrating a role for alcohol-mediated oxidative stress in the inhibition of C/EBP α activity and hepcidin transcription^[11,12]. Hepcidin may therefore act as a potential second hit factor in the progression of alcoholic liver disease.

CONCLUSION

Patients with alcoholic liver disease frequently display elevated iron stores. Recent reports by several groups strongly suggest that this may be a regulated mechanism rather than iron simply leaking out of injured intestinal cells. Hepcidin, an iron regulatory hormone synthesized in hepatocytes, plays a key role in the regulation of iron metabolism. Alcohol-mediated oxidative stress suppresses hepcidin expression in the liver leading to increased intestinal iron uptake and liver iron storage. Reduced hepcidin expression may be one of the mechanisms leading to elevated iron stores following alcohol exposure. Hepcidin may therefore act as a second hit in the progression of ALD. Both hepatocytes and Kupffer cells play a role in ALD. However, Kupffer cells have been reported to not be involved in the regulation of liver hepcidin expression by both acute and chronic alcohol exposure. Hepatocytes are the main site of alcohol metabolism in the liver leading to the generation of reactive oxygen species and the depletion of antioxidants. Alcohol-induced oxidative stress in hepatocytes is therefore one of the mechanisms leading to the suppression of hepcidin synthesis and thereby to iron overload, which in turn acts as a secondary risk factor in ALD. Accordingly, iron has been shown to prime Kupffer cells leading to the release of TNF- α in experimental animal models of ALD. The disruption of hepcidin synthesis and thereby iron metabolism by alcohol may have clinical relevance. Hepcidin therefore holds promise as an early diagnostic marker and as a candidate for therapeutic strategies for ALD.

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TOPIC HIGHLIGHT

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Impact of asialoglycoprotein receptor deficiency on the development of liver injury

Serene ML Lee, Carol A Casey, Benita L McVicker

Serene ML Lee, Departments of Internal Medicine and Biochemistry & Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68105, United States

Carol A Casey, Benita L McVicker, Liver Study Unit, Department of Veterans Affairs Medical Center, and Departments of Internal Medicine and Biochemistry & Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68105, United States

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Correspondence to: Carol A Casey, PhD, Veterans Affairs Medical Center, Research Service (151), 4101 Woolworth Avenue, Omaha, NE 68105, United States. ccasey@unmc.edu

Telephone: +1-402-9953737 Fax: +1-402-4490604

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Abstract

The asialoglycoprotein (ASGP) receptor is a well-characterized hepatic receptor that is recycled *via* the common cellular process of receptor-mediated endocytosis (RME). The RME process plays an integral part in the proper trafficking and routing of receptors and ligands in the healthy cell. Thus, the mis-sorting or altered transport of proteins during RME is thought to play a role in several diseases associated with hepatocyte and liver dysfunction. Previously, we examined in detail alterations that occur in hepatocellular RME and associated receptor functions as a result of one particular liver injury, alcoholic liver disease (ALD). The studies revealed profound ethanol-mediated impairments to the ASGP receptor and the RME process, indicating the importance of this receptor and the maintenance of proper endocytic events in normal tissue. To further clarify these observations, studies were performed utilizing knockout mice (lacking a functional ASGP receptor) to which were administered several liver toxicants. In addition to alcohol, we examined the effects following administration of anti-Fas (CD95) antibody, carbon tetrachloride (CCl₄) and lipopolysaccharide (LPS)/galactosamine. The results of these studies demonstrated that the knockout mice sustained enhanced liver injury in response to all of the treatments, as shown by increased indices of liver

damage, such as enhancement of serum enzyme levels, histopathological scores, as well as hepatocellular death. Overall, the work completed to date suggests a possible link between hepatic receptors and liver injury. In particular, adequate function and content of the ASGP receptor may provide protection against various toxin-mediated liver diseases.

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Key words: Asialoglycoprotein receptor; Asialoglycoprotein receptor deficient mice; Receptor-mediated endocytosis; Alcohol; Carbon tetrachloride; Anti-Fas; Lipopolysaccharide/galactosamine; Toxicant-induced liver injury

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THE ASIALOGLYCOPROTEIN RECEPTOR AND ITS POTENTIAL ROLE IN LIVER INJURY

The asialoglycoprotein (ASGP) receptor, also termed the hepatic binding protein or the Ashwell receptor, was discovered nearly four decades ago by Ashwell and Morell, and was described as a hepatocellular surface carbohydrate that binds glycoproteins lacking terminal sialic acid residues (asialoglycoproteins)^[1,2]. Subsequently, many studies have contributed to the detailed characterization of the ASGP receptor, describing its functional role in the binding, internalization and transport of a wide range of glycoproteins, which have exposed galactose or N-acetylgalactosamine residues, *via* the process of receptor-mediated endocytosis (RME)^[3-6]. However, translating altered ASGP receptor function and its altered clearance of serum glycoproteins to disease states remains a topic of current

research efforts. This ongoing interest is fueled by the knowledge that the ASGP receptor can bind a variety of important plasma proteins that include transport proteins (i.e. transferrin)^[7], enzymes such as alkaline phosphatase^[8], immunoglobulins including IgA^[9], apoptotic hepatocytes^[10,11], fibronectin^[12] and platelets^[13]. Additionally, the expression of the ASGP receptor has been clinically correlated to the level of hepatic function that is lost during liver diseases related to cancer, viral hepatitis, and cirrhosis^[14,15]. Overall, the quest to identify and understand the physiological role(s) of the ASGP receptor, and the consequences that may result from alterations in the function and/or expression of this abundant hepatocellular binding protein, continues.

In search of the physiological roles of the ASGP receptor, our lab initially concentrated on characterizing the role of the ASGP receptor and RME events during a serious and common form of liver injury, alcoholic liver disease (ALD). Alcoholism, and resultant ALD, are indeed significant biomedical problems. Specifically, recent data has noted that chronic liver disease and cirrhosis was the 12th leading cause of death in the United States in the year 2005, and that out of those deaths, approximately 47% of them were due to ALD^[16]. Therefore, defining potential contributing mechanisms (such as altered protein trafficking and impaired hepatic receptor functions) may aid in the elucidation of potential therapeutic treatments for ALD. In that effort, our laboratory has extensively studied the RME process and parameters of the ASGP receptor following the administration of ethanol to rodents.

The ASGP receptor consists of major and minor subunits, which in the rat were identified as rat hepatic lectin (RHL) 1 and RHL 2/3, that have respective molecular weights of 42, 49 and 54 kDa^[17]. The selective binding and uptake of terminal galactosyl bearing proteins requires the formation of hetero-oligomers between these major and minor forms, and that binding activity was calcium and pH dependent^[2,5,18]. Also, the subcellular distribution of the receptor revealed that approximately one-third of the total ASGP receptor pool was associated with the plasma membrane located on the basolateral surface of the hepatocyte^[19]. Additionally, it was shown that the total ASGP receptor population consisted of two functionally distinct receptor populations, designated State 1 and State 2, which were involved in the endocytosis and intracellular processing of ligands by different pathways^[20,22].

Utilizing these known properties, we studied the effects of ethanol on the ASGP receptor itself, as well as endocytic processes, using isolated hepatocytes, whole liver sections, and perfused livers obtained from rats voluntarily fed an ethanol containing diet over a time course of administration. In summary, differential effects were observed over the time course of treatment in the ability of ethanol and resultant metabolites to affect the ASGP receptor and RME events. Specifically, after early periods of ethanol feeding (1-2 wk), we found that the observed decrease in ligand binding capacity of the ASGP receptor could be attributed to inactivation and redistribution of the receptor^[23]. However, after more

chronic ethanol administration (5-8 wk), the functional alterations of the receptor were found to be reflective of reductions in the content, synthesis, and mRNA expression of the receptor^[23]. Also, it was determined that ethanol treatment caused equal inactivation of both State 1 and State 2 receptors, suggesting that ethanol may be unique compared to other agents (e.g. monensin, vanadate, and chloroquine) that are known to inflict post-translational modifications, such as acylation, selectively to just the State 2 population^[24]. In other studies, it was revealed that the ASGP receptor was hyperphosphorylated over the time course of treatment, which could contribute to the aberrant activity of the receptor by disrupting the phosphorylation/dephosphorylation state associated with normal recycling of the receptor^[25]. We were also able to demonstrate that the ASGP receptor is involved in the recognition and uptake of apoptotic cells and that this process was significantly altered in hepatocytes obtained from ethanol fed rats^[11]. Overall, the results from these studies revealed that ethanol administration impairs multiple aspects of RME by the hepatic ASGP receptor, such that binding, internalization and degradation of ligands internalized by the receptor were found to be significantly altered. Additionally, it was shown that these defects are associated with alterations in the ASGP receptor's physiologically relevant role of clearing apoptotic cells. Taken together, our findings have important implications for the pathogenesis of alcoholic liver injury and potentially for other forms of liver diseases in which RME is profoundly affected. In more recent studies, a mouse model lacking the ASGP receptor was used to gain a better understanding of the associations that may exist between alterations in receptor function and the generation of pathological liver injury.

THE ASGP RECEPTOR-DEFICIENT MOUSE MODEL

The ASGP receptor in mice is a hetero-oligomeric receptor composed of 2 subunits that are both required for its function. These subunits have been named murine hepatic lectin (MHL), with the major subunit called MHL-1 and the minor subunit called MHL-2^[26]. ASGP receptor-deficient (RD) mice have a complete lack of the MHL-2 protein and were generated by homologous recombination with a gene replacement vector in embryonic stem cells^[27]. MHL-2 appears to be required for the post-translational stability of MHL-1, as these mice have substantially reduced protein content of MHL-1, even though MHL-1 mRNA expression remains the same^[27]. Although the MHL-1 protein is still detected in low levels in the RD mice, these levels are unable to induce a measurable clearance of 125I-labeled asialo-orosomucoid^[27]. Despite lacking functional ASGP receptors, these knockout mice remain viable and fertile, and appear to have a normal lifespan. In addition, these mice do not display any obvious phenotypic abnormalities^[27,28].

As previously mentioned, we have found that chronic alcohol administration markedly decreased mRNA expression and content of the ASGP receptor

in rats prior to the appearance of pathology such as fibrosis^[23,29]. Thus, it was felt that the RD mice might provide a powerful tool to examine the role of the ASGP receptor and help delineate pathways by which liver injury occurs in general, as well as during alcoholic liver injury. Currently, we are utilizing the knockout mouse model to examine the link between ASGP receptor function and liver injury, in the context of various models of toxic liver injury such as alcohol, anti-Fas, carbon tetrachloride (CCl₄) and lipopolysaccharide (LPS)/galactosamine. In this report, we present a brief overview of our findings to date.

MODELS OF LIVER INJURY AND THEIR EFFECTS ON ASGP RECEPTOR-DEFICIENT MICE

Alcohol

Alcohol-induced liver injury has previously been found to be related to several events, including ethanol metabolism (*via* alcohol dehydrogenase^[30-33]), generation of reactive oxygen species (*via* cytochrome isoforms such as CYP2E1^[34-36]), interaction of other liver products (such as cytokines^[37,38]) and the induction of apoptosis through the Fas death receptor system^[39]. In the search for cellular signaling and mechanisms resulting as a consequence of these events, studies were performed that examined the effects of ethanol on hepatocellular protein trafficking, particularly the process of RME utilizing the hepatic ASGP receptor.

As mentioned previously, we examined the effects of ethanol administration using a rat model exclusively; the rats showed decreased ligand binding, internalization and degradation of several ligands including asialo-orosomucoid, which are processed by RME^[4,23,40-43]. In order to assess the effect of ethanol administration on RME by the ASGP receptor using a mouse model, we obtained wild-type (WT) mice possessing abundant ASGP receptor activity and ASGP receptor-deficient (RD) mice lacking MHL-2 from the Jackson Laboratories (Bar Harbor, ME). The mice were fed a Lieber de-Carli liquid diet (with or without 5% by volume ethanol) for ten days^[44]. When hepatocytes from these mice were incubated with 125I-ASOR (a representative ligand for the ASGP receptor), WT mice showed ethanol-induced alterations that were consistent with our observations for rats, with an approximately 50% decrease in ligand binding, internalization and degradation in isolated hepatocytes^[4,23,44]. However, binding, internalization and degradation of the ligand by RD hepatocytes was negligible, regardless of diet^[44]. In addition, the presence of apoptotic bodies was found to be approximately three-fold higher in the livers of RD mice compared to WT mice, irrespective of diet^[44]. As a result of this work, it is hypothesized that a potential consequence of altered ASGP receptor function is impaired clearance of ethanol-generated apoptotic cells, resulting in the observed accumulation of apoptotic bodies. Furthermore, other work has shown that these bodies have the potential to promote a variety of responses

within the liver, such as the activation of Kupffer cells and the subsequent release of proinflammatory and profibrogenic substances, leading to the enhanced susceptibility to hepatocellular damage that is observed following ethanol administration^[11,45].

Anti-Fas

Anti-Fas is an antibody that specifically recognizes and works as an agonist of the Fas antigen^[46]. Fas is a member of the TNF receptor superfamily and is a key mediator of apoptosis^[47]. This receptor is found in hepatocytes, cholangiocytes, sinusoidal endothelial cells, stellate cells and Kupffer cells^[48]. Ligation of Fas results in the recruitment of adaptor proteins, such as Fas-associated death domain (FADD) and procaspase 8, to form the death-inducing signaling complex (DISC)^[47]. Caspase 8 can then either directly or indirectly cleave procaspase 3 to mediate apoptosis^[47]. A variety of studies have shown that the injection of anti-Fas into mice causes widespread apoptosis and ultimately results in focal hemorrhage and hepatocyte necrosis, making Fas injection a model for fulminant hepatic failure^[46,49,50].

From studies related to Fas-mediated cell death in our laboratory, we have shown that the metabolism of ethanol in WIF-B cells (hepatoma hybrid cells) was involved in enhanced Fas protein localization to the membrane, leading to increased activity of the upstream initiator caspases (caspase 2 and caspase 8) and the subsequent downstream activation of caspase 3^[39]. As an extension to these studies, aimed to characterize the role of Fas-mediated death in injured hepatocytes, anti-Fas (0.1 or 0.2 µg/g body weight) was injected intraperitoneally into WT and RD mice, which were monitored for up to 48 h^[51]. Receptor-deficient mice showed an enhancement of liver injury with higher aspartate transaminase (AST) and alanine transaminase (ALT) activities in the serum compared to the enzyme levels detected in WT mice^[51]. Similarly, pathology showed that the RD mice had increased steatosis, inflammation and necrosis compared to the WT mice^[51]. As expected, caspase 3 activities were found to be increased 5- to 6-fold in WT mice at 2 h and 16 h after anti-Fas injection, with caspase activities returning to baseline levels by 24 h^[51]. However, the activity of caspase 3 remained elevated in the RD livers at all times following treatment and was significantly enhanced over the WT livers at 24 h and 48 h post anti-Fas injection^[51]. Overall, the livers of the RD mice were found to be more susceptible than the livers of the WT mice to anti-Fas injection; showing greater apoptosis and increased ECM deposition of collagen and fibronectin^[51].

Carbon tetrachloride

Another agent used to study liver injury is carbon tetrachloride (CCl₄), which can cause liver damage through a number of mechanisms. Carbon tetrachloride is metabolized through the action of the mixed function cytochrome P450 system of the endoplasmic reticulum to form the trichloromethyl free radical (CCl₃•), which can subsequently be converted to the trichloromethyl peroxy radical (CCl₃OO•) in the presence of oxygen^[52,53].

These free radicals are highly reactive and can bind covalently to cellular macromolecules forming nucleic acid, protein and lipid adducts. When these radicals attack the polyunsaturated fatty acids of the cellular membranes, the fatty acid free radicals generated initiate autocatalytic lipid peroxidation, ultimately resulting in the loss of membrane integrity^[52,53]. Carbon tetrachloride can also induce cellular hypomethylation, leading to inhibition of protein synthesis (possibly through ribosomal RNA hypomethylation) and defects in lipid and lipoprotein metabolism^[53]. Finally, CCl₄ also affects hepatocellular calcium homeostasis, either by disrupting membrane integrity or by opening certain membrane calcium channels. High levels of Ca²⁺ in the cell can then activate Ca²⁺-responsive enzymes such as proteases, endonucleases and phospholipases and lead to cell death *via* apoptosis and necrosis^[52,53]. The consequences of CCl₄ toxicity include centrilobular steatosis, inflammation, apoptosis and necrosis^[52-54].

In our studies, WT and RD mice were injected with CCl₄ (1 mL/kg body weight) and monitored up to a week after injection^[55]. Carbon tetrachloride injection caused greater liver injury in the RD mice, as evidenced by the RD mice having increased AST and ALT activities in the serum, compared to the WT mice 48 h post CCl₄ injection^[55]. Histologically, centrilobular liver damage was observed in WT mice by 48 h after injection^[55]. At this time point, RD mice had more severe damage, showing a greater number of neutrophilic inflammatory infiltrates^[55]. In order to elucidate the mechanisms by which this damage is caused, malondialdehyde (MDA), deposition of α -smooth muscle actin (α -SMA) and the percentage of TUNEL-positive hepatocytes was measured^[55]. Levels of MDA in the WT mice were not significantly increased throughout the course of the experiment^[55]. In contrast, RD mice showed increased contents of MDA as early as 24 h post CCl₄ injection and these levels were maintained up to 48 h^[55]. α -SMA was increased significantly in both the WT and RD mice, with the RD mice having a more prolonged increase (between 48 h to 72 h) than the WT mice (only at 72 h)^[55]. In addition, α -SMA content was approximately 2-fold of that in the WT liver at 48 h, 96 h and 7 d following injection^[55]. Finally, RD mice had significantly more TUNEL-positive hepatocytes than the WT mice at 48 h post injection (2.4-fold more)^[55]. This suggests that the absence of functional ASGP receptor resulted in increased lipid peroxidation, perturbations in ECM turnover and increased apoptosis^[55].

LPS/galactosamine

Lipopolysaccharide (endotoxin) and galactosamine can be used either alone or in combination with each other to cause liver injury in mice. The metabolism of galactosamine leads to hepatotoxicity by depleting uridine nucleotides and UDP-hexoses and concurrently increasing UDP-hexosamines, primarily in hepatocytes^[56,57]. The depletion of uridine nucleotides (as mentioned above) results in an inhibition of RNA and protein synthesis^[58]. It has also been suggested that metabolism of galactosamine results in an impaired biosynthesis of macromolecular cell

constituents^[56]. This impairment leads to plasma membrane injury, which results in an influx of calcium ions and a commitment to cell death^[58]. In rats, galactosamine leads to an inflammatory infiltrate of polymorphonuclear leukocytes and lymphocytes and foci of hepatocellular necrosis, resembling the effects of human viral hepatitis^[57,59].

Mice and rats are relatively resistant to the lethal effects of LPS^[60]. Thus, LPS is injected in concert with galactosamine for a model of fulminant hepatitis^[61]. It is thought that galactosamine-induced suppression of RNA synthesis leads to an increased tumor necrosis factor (TNF) production by macrophages, resulting in an increased susceptibility to LPS^[60-62]. Tumor necrosis factor was proposed as the agent responsible for the lethality, because lethality was retained by substituting LPS with TNF and was inhibited by anti-TNF antibody^[63,64]. Thus, in LPS/galactosamine injection, apoptosis induced by TNF occurs initially and is followed subsequently by necrosis^[65].

In our studies, a sub-lethal dose of LPS (50 μ g/kg body weight) combined with galactosamine (350 mg/kg body weight) was injected into WT and RD mice *via* the intraperitoneal route and the mice were monitored up to 4.5 h^[66,67]. After LPS/galactosamine injection, WT mice maintained normal liver lobular architecture^[66]. However, RD mice showed considerable liver injury with areas of portal inflammation, hepatocellular necrosis, increased inflammatory cell infiltration and hemorrhage^[66]. These histological observations were further corroborated by the RD mice having increased serum AST and ALT activities at 4.5 h after LPS/galactosamine injection, which were not observed in the WT mice^[66]. Also, RD mice showed increased apoptosis, having significantly enhanced caspase 3 activities and TUNEL-positive cells at 4.5 h post injection, whilst there were no changes measured in WT mice^[66]. Additionally, the serum content of the pro-inflammatory cytokine interleukin 6 (IL-6) was increased in RD mice compared to WT mice at 3 and 4.5 h after LPS/galactosamine injection^[67]. Overall, the results demonstrate that the RD mice are more susceptible to the development of fulminant liver injury as a result of sub-lethal treatment with LPS/galactosamine^[66,67]. Given that the induction of apoptosis is a consequence of LPS/galactosamine treatment, the enhanced susceptibility to liver damage observed in the RD mice may be related to the inability of hepatocytes to phagocytose and clear the dying apoptotic cells *via* the ASGP receptor.

THE LINK BETWEEN FUNCTIONAL ASGP RECEPTOR AND LIVER INJURY

After the administration of the four toxicants mentioned above (alcohol, anti-Fas, CCl₄ and LPS/galactosamine), RD mice consistently sustained greater liver injury than WT mice, as evidenced by increased indices of liver damage (serum AST and ALT activities) and worse pathology (light microscopy). These four agents of liver injury cause damage through different biochemical pathways or signaling cascades. Therefore, it appears that proper functioning of the ASGP receptor may provide

universal protection against liver injury from these toxicants and possibly others. Although it is not known exactly how an adequately functioning receptor can protect the hepatocyte, the use of the knockout mice treated with these four toxicants highlight some of the possible mechanisms.

It appears that with all four toxicants, caspase 3 or TUNEL-positive cells are increased. Apoptosis is a highly regulated mode of cell death that helps to maintain tissue homeostasis in a healthy organ^[68]. However, it appears that when apoptotic death factors are inappropriately expressed due to the introduction of pathological stimuli, such as the four toxicants, apoptosis becomes one of the common pathways by which liver injury is caused. Increased accumulation of apoptotic cells has been shown to occur during alcohol-induced liver injury in a variety of species, including humans, and is thought to play an important role in the progression of liver injury^[69-75]. During the process of forming apoptotic cells, glycoconjugates on the cell surface losing their sialic acid masks the increase^[10,76]. Since the ASGP receptor binds to desialylated proteins, the receptor recognizes and binds the altered glycans on apoptotic bodies, resulting in phagocytosis and efficient removal of the dying cells^[11]. Thus, we speculate that the ASGP receptor exerts protection by removing apoptotic bodies in a timely fashion.

Another way that the ASGP receptor might be protective is through its role in regulating turnover of ECM. Anti-Fas and CCl₄ treatments lead to increased deposition of collagen, fibronectin or α -SMA in the RD mice in comparison to the WT mice. The ASGP receptor has a direct link to cellular fibronectin clearance because cellular fibronectin displays terminal galactose residues, making it a ligand of the ASGP receptor^[12]. Cellular fibronectin is one of the first ECM proteins that accumulates during fibrosis^[77-79] and thus impairments of ASGP receptor function could lead to increased ECM deposition and hence lead to fibrosis and cirrhosis.

Two additional ways that liver injury could be mediated is through increased lipid peroxidation (MDA content) or by increased contents of pro-inflammatory cytokines, such as IL-6. At present, it is not known how ASGP receptor function is related to perturbations in levels of these two substances. In addition, there may be dysregulation of other asialoglycoproteins not examined at this point. The total absence of ASGP receptor function does not result in a measurable increase in the steady state concentrations of galactose-terminating glycoproteins in the plasma of knockout mice^[28]. Thus, the ASGP receptor is unlikely to be involved in the normal turnover of serum glycoproteins. However, in toxicant-induced injuries, acute surges of asialoglycoproteins may overwhelm the alternative galactose recognition systems^[28]. Thus, the ASGP receptor might function to prevent an acute increase in potentially harmful asialoglycoproteins. Altogether, the ASGP receptor knockout mouse model provides an excellent tool to elucidate the relationship between ASGP receptor function and liver injury.

Table 1 Changes in ALT activity, TUNEL positive cells, AST activity, caspase 3 activity, collagen content, IL-6 content, MDA content and α -SMA content in RD mice compared to WT mice

Challenge	RD mice compared to WT mice
Alcohol	Liver TUNEL-positive hepatocytes
Anti-Fas	Liver TUNEL-positive hepatocytes Serum ALT Serum AST Liver caspase 3 activity
CCl ₄	Collagen deposition in extracellular matrix Fibronectin deposition in the extracellular matrix Liver TUNEL-positive hepatocytes Serum ALT Serum AST Liver MDA content Liver α -SMA content
LPS/galactosamine	Liver TUNEL-positive hepatocytes Serum ALT Serum AST Liver caspase 3 activity Serum IL-6 content

ALT: Alanine transaminase; AST: Aspartate transaminase; IL-6: Interleukin 6; MDA: Malondialdehyde; α -SMA: α -smooth muscle actin; RD: Receptor-deficient; WT: Wild-type. The various conditions the mice were challenged with were alcohol (Lieber de-Carli ethanol diet for 7 d), anti-Fas (0.2 μ g/kg body weight), CCl₄ (1 mL/kg body weight) and LPS/galactosamine (50 μ g LPS/kg body weight and 350 mg galactosamine/kg body weight).

CONCLUSION

The ASGP receptor is an abundant hepatic receptor that recognizes desialylated ligands. After binding to its ligand, the receptor internalizes and facilitates transport of specific ligands by the process of receptor-mediated endocytosis. Previously, studies have shown that ASGP receptor function is impaired in disease states such as alcoholic liver disease. This gave us the impetus to examine if proper ASGP receptor function offers protection against liver injury or if defects in function occurred as a result of liver damage. To examine this, we utilized a knockout mouse model, which lacked functional ASGP receptor in comparison to wild-type animals in the context of various toxic challenges (alcohol, anti-Fas, CCl₄ and LPS/galactosamine). After all four challenges, the receptor-deficient mice consistently showed more liver injury than the wild-type animals (Table 1), proving that ASGP receptor function is protective. Thus, these studies highlight receptor-mediated endocytosis as a novel mechanism that may be involved in the induction of toxin-induced liver injury. At present, the precise nature of the specific ligands involved or the pathways that lead to further injury have not been determined. However, in the studies reviewed here, it is likely that impaired clearance of apoptotic bodies, perturbations in extracellular matrix deposition, oxidative stress, and cytokine dysregulation may play roles in the progression of disease. In the future, further clarification of the pathways by which liver injury occurs (including altered ASGP receptor-mediated endocytosis) will provide new therapeutic leads.

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Natalia A Osna, MD, PhD, Series Editor

Hepatitis C virus and ethanol alter antigen presentation in liver cells

Natalia A Osna

Natalia A Osna, Department of Internal Medicine, University of Nebraska Medical Center, Liver Study Unit R151, VA Medical Center, 4101 Woolworth Ave, Omaha, NE 68105, United States
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Correspondence to: Natalia A Osna, MD, PhD, Department of Internal Medicine, University of Nebraska Medical Center, Liver Study Unit R151, VA Medical Center, 4101 Woolworth Ave, Omaha, NE 68105, United States. nosna@unmc.edu
Telephone: +1-402-9953735 Fax: +1-402-4490604
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Abstract

Alcoholic patients have a high incidence of hepatitis C virus (HCV) infection. Alcohol consumption enhances the severity of the HCV disease course and worsens the outcome of chronic hepatitis C. The accumulation of virally infected cells in the liver is related to the HCV-induced inability of the immune system to recognize infected cells and to develop the immune responses. This review covers the effects of HCV proteins and ethanol on major histocompatibility complex (MHC) class I - and class II-restricted antigen presentation. Here, we discuss the liver which functions as an immune privilege organ; factors, which affect cleavage and loading of antigenic peptides onto MHC class I and class II in hepatocytes and dendritic cells, and the modulating effects of ethanol and HCV on antigen presentation by liver cells. Altered antigen presentation in the liver limits the ability of the immune system to clear HCV and infected cells and contributes to disease progression. HCV by itself affects dendritic cell function, switching their cytokine profile to the suppressive phenotype of interleukin-10 (IL-10) and transforming growth factor beta (TGF β) predominance, preventing cell maturation and allostimulation capacity. The synergistic action of ethanol with HCV results in the suppression of MHC class II-restricted antigen presentation. In addition, ethanol metabolism and HCV proteins reduce proteasome function and interferon signaling, thereby suppressing the generation of peptides for MHC class I-restricted antigen presentation. Collectively, ethanol exposure further impairs antigen

presentation in HCV-infected liver cells, which may provide a partial explanation for exacerbations and the poor outcome of HCV infection in alcoholics.

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Key words: Alcohol; Antigen presentation; Hepatitis C Virus; Interferon alpha and gamma; Liver; Major histocompatibility complex (MHC) class I ; MHC class II

Peer reviewer: Eva Herrmann, Professor, Department of Internal Medicine, Biomathematics Saarland University, Faculty of Medicine, Kirrberger Str., 66421 Homburg/Saar, Germany

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INTRODUCTION

In non-cytolytic viral infections, the immune system (and mainly T-lymphocytes) is necessary to clear the infected cells. The most specialized and effective homeostatic control is provided by cytotoxic T lymphocytes (CTLs) (CD8+ T-lymphocytes). For clonal expansion, CTLs need activating “help” from CD4+ T-lymphocytes, which, in turn, require recognition of viral antigens on professional antigen-presenting cells (APC), including dendritic cells (DC), macrophages and B-lymphocytes.

Recognition of antigens by T-lymphocytes depends upon the expression of the major histocompatibility complex (MHC) on the surface of APC. MHC molecules involved in antigen presentation include MHC class I, MHC class II and MHC-like CD1 molecules^[1]. MHC class I molecules are expressed on the nuclear cells and load protease-generated peptides to CD8+ T-lymphocytes. MHC class II molecules are expressed on the surface of specialized APC and load peptides generated in the endocytic compartment to CD4+ T cells. CD1 molecules bind acyl chains, therefore allowing T cells to recognize fatty acids, glycolipids and lipopeptide antigens (self or foreign)^[2].

Hepatitis C virus (HCV) is an example of an intra-

cellularly persistent virus, which targets liver cells and is eliminated by immune cells^[3,4]. In acute HCV infection, antigen presentation plays a pivotal role in the activation of immune response, while HCV protein-induced defects in antigen presentation lead to insufficient activation of the immune system, which controls the killing of infected hepatocytes^[5]. Accumulation and persistence of virally infected cells provide a basis for the chronic course of HCV infection. The reduced clearance of virus and increased viral load usually observed in immunodeficient and in alcohol-consuming patients are associated with a high risk of hepatocarcinoma development^[6,7].

LIVER AS AN IMMUNE PRIVILEGE ORGAN

Liver is considered an immunological organ^[8]. Liver cells are involved in the clearance of foreign antigens, which come from the gastrointestinal tract. The physiological role of the liver is to face antigenic flow from the gastrointestinal tract and to respond to this intervention by activating the immune response. To escape immune activation, liver cells use a mechanism of immune tolerance, which prevents unnecessary immune-mediated damage of hepatocytes.

Some liver cells, such as liver dendritic, Kupffer, endothelial and stellate cells serve as antigen presenting cells^[9]. Kupffer cells (KC) are the resident macrophage population, which act as incompetent antigen-presenters^[10]. They produce IL-10, which down-regulates CD4+ T-cell activation^[11,12]. Reactive oxygen species (ROS) production is an essential trigger of antigen presentation in KC, accompanied by induction of MHC class II and co-stimulatory molecule expression^[13]. In contrast to KC, liver endothelial cells (LSEC) are known as potent APC that present antigens in a MHC class II-restricted manner to CD4+ T-cells^[9]. The efficacy of their antigen presentation is comparable to DC^[8]; however, IL-10 and TGF β secreted by KC and LSEC as well as lipopolysaccharide (LPS), which comes with blood from the gastrointestinal tract, can down-regulate their antigen-presenting properties^[14,15]. Both KC and LSEC express MHC antigens, costimulatory and adhesion molecules and produce IL-1 and IFN γ , suggesting that these are relatively mature cells^[16]. In contrast, liver DC are immature cells, which express MHC antigens on their surface, but express a relatively small amount of co-stimulatory molecules, which does not allow them to efficiently stimulate naïve T-cells^[17]. Hepatic DC play an important role in the induction and regulation of immune responses, by interacting with CD4+, CD8+ lymphocytes and natural killers (NK) cells. These cells typically reside only around portal triads and like other DC, they capture, process and transport antigens to regional lymphoid tissues^[16]. Two subpopulations of DC, myeloid and plasmocitoid cells are found in liver at a low density. Exposure of progenitor DC to IL-10 and TGF β generates

suppressive and tolerogenic effects^[18]. In the liver, they are resistant to DC maturation stimuli, such as IFN γ and TNF α , while matrix proteins, such as collagen type 1, induces maturation^[19]. However, even being tolerogenic in the liver, hepatic DC are the main professional liver APC, which can further migrate to lymphoid tissue to undergo maturation^[20]. In addition to other liver cells, stellate cells have also recently been characterized as having antigen presentation properties^[21].

Almost 60% of liver cells are hepatocytes. Due to specific liver architecture, hepatocytes are exposed to a mixture of portal venous and hepatic arterial blood. Liver parenchyma is actively involved in immune response by expressing a variety of receptors. Because liver is a site of apoptotic CD8+ T-lymphocyte accumulation^[22], it raises the question whether liver cells, including hepatocytes, induce apoptosis in activated CD8+ T-lymphocytes, thereby promoting a tolerogenic response or they specifically attract apoptotic T-lymphocytes without causing T-lymphocyte death. Resting hepatocytes act as APC for MHC class I-restricted T-cells, while MHC class II and CD1 are not constitutively expressed on hepatocytes^[23]. However, the role of hepatocytes as potent APC activating intrahepatic lymphocytes is contradictory. While some studies indicate that activation of naïve CD8+ T-lymphocytes does not happen in case the primary activation occurs within the liver, other studies suggest that the presentation of antigens in the liver showed no immunosuppressive effect on the activation of CD8+ T-cells and that hepatocytes are an excellent priming site for naïve CD8+ T-cells^[24-26].

ANTIGEN PROCESSING AND PRESENTATION IN THE CONTEXT OF MHC CLASS I

Proteolysis is the first step in the antigen processing and presentation pathway. Peptide products are transported by transporters associated with antigen processing (TAP) to endoplasmic reticulum (ER), where they assemble in a trimolecular complex with β 2-microglobulin and the heavy chain of MHC. Assembly is facilitated by TAP and a number of chaperones and allows class I molecule to bind the peptide to achieve optimal MHC class I loading. These complexes are transported to the plasma membrane, where they interact with the T-cell receptor (TCR) of a T-lymphocyte. TAP has sequence preference and peptide-size limitation, which is matched to MHC class I. To be presented in a context of MHC class I, antigenic proteins undergo the processing to peptides and then the peptides are further cleaved, to fit into the MHC class I groove. Certain proteases participate in the cleavage of peptides for antigen presentation, and the major protease is proteasome.

For activation, CD8+ cells require presentation of MHC class I-peptide complexes on professional APC cells. Peptides can be generated from viral proteins sensitized by infected APC or from proteins originally synthesized by other infected "donor" cells (cross-priming, or cross presentation). Cross-priming can be carried by mo-

lecular chaperones and come from apoptotic cells^[27]. In addition, it is suggested that these peptides are captured by the lysosomal compartment, with further trafficking to cytosol followed by proteasomal processing for MHC class I loading^[28]. However, other authors question the role of proteasomal processing in the cross-presentation mechanism^[29].

PROTEASOME, AMINOPEPTIDASES AND INTERFERON GAMMA-INDUCED GENERATION OF PEPTIDES FOR ANTIGEN PRESENTATION

Degradation of intracellular proteins into oligopeptides for antigen presentation is catalyzed by the proteasome^[30]. This multicatalytic enzyme exists as a 26S particle, which is ATP-dependent and recognizes ubiquitinated polypeptides. Another form, the 20S proteasome, can degrade substrate proteins in an ubiquitin-independent manner. Both the 26S and 20S proteasome particles degrade antigenic proteins that may later be presented as peptides, depending on the properties of the protein. The chymotrypsin-like and the trypsin-like activities of the proteasome are related to antigen presentation due to their ability to cleave peptide bonds after hydrophobic and basic amino acids, respectively^[30]. In cells treated with IFN γ , proteasome particles acquire two novel MHC-encoded low molecular weight subunits, known as LMP-2 and LMP-7, and a third subunit, MECL-1, encoded by genes outside the MHC locus^[31]. The acquisition of these three subunits converts the constitutive proteasome to the immunoproteasome and alters its peptidase activities for the generation of uniform sized 8-10-mer peptides^[32]. The importance of immunoproteasome induction for antigen presentation has been confirmed using LMP-2 and LMP-7-knockout mice, which demonstrated impaired antigen presentation, reduced expression of MHC class I and poor CTL response to viral epitopes^[33,34]. The immunoproteasome is responsible for cleavage of peptides at their C-termini^[35]. However, some antigenic peptides have N-terminal extensions that are unable to bind to the MHC class I groove. Aminopeptidases, namely, cytosolic leucine aminopeptidase (LAP), finally trims the epitopes to 8-9 amino acid residues necessary to form the complexes with MHC class I^[36]. This enzyme is also IFN γ -inducible^[37]. Trimmed peptides are transported to the endoplasmic reticulum (ER) by TAP. In the ER lumen, the peptide can be further trimmed by downstream endoplasmic reticulum aminopeptidase I (ERAP1)^[38] to generate a stable complex with MHC class I heavy and light chains (β 2-microglobulin). These complexes, with the help of molecular chaperones, traffic to the cell surface to be recognized by CD8+ T-cells^[39,40]. The immune system relies heavily on intracellular protein degradation to recognize a complex of surface MHC class I molecules with short peptide fragments of eight to nine amino acids^[41]. Induction of CTLs is programmed by the spec-

trum of presented MHC class I-peptide complexes on the cell surface^[42].

HCV, MHC CLASS I-RESTRICTED ANTIGEN PRESENTATION AND EFFECTS OF ETHANOL

The effects of HCV proteins on MHC class I-restricted antigen presentation are not widely studied and most of the findings are related to MHC class II-restricted antigen presentation. In HCV infected patients, associations between the human MHC and sustained virological response have been found, indicating that the various immunogenetic backgrounds of chronic hepatitis C patients are related to the differences in the course of the disease^[43].

Processing of CTL epitopes in HCV infected cells may be altered due to mutations generated by HCV, which interfere with the ability of the peptide to be cleaved by the proteasome^[44]. Nevertheless, interferon alpha-mediated induction of immunoproteasome or aminopeptidase expression has been shown as a necessary step for CD8+ T-cell activation in acute HCV infected chimpanzees^[45,46]. In addition to HCV-restricted mutations of viral proteins, proteasome activity is also modulated by HCV. Thus, proteasome activity in the nuclear compartment is up-regulated by PA28 γ activator which forms a complex with HCV core protein^[47], while non-structural HCV protein, NS3, interacts with LMP-7, an immunoproteasome subunit, and reduces its activity, potentially providing a negative effect on the generation of peptides for antigen presentation^[48]. Our previous studies on HCV core protein expressing Huh7 cells, which also express CYP2E1, revealed that core protein slightly enhances 20S proteasome activity, by 20S proteasome-core protein direct interactions and by induction of low CYP2E1-dependent oxidative stress^[49]. However, this proteasome activation is reversed after ethanol exposure, where ethanol treatment considerably reduces proteasome function due to induction of high oxidative stress^[49]. Indeed, the dependence of proteasome on the level of oxidative stress has been previously demonstrated^[50,51], and ethanol is known to suppress proteasome function in liver cells^[52-55]. Ethanol-elicited suppression of proteasome activity in the liver ultimately results in reduced generation of antigenic peptides^[56] and reduced MHC class I-restricted antigen presentation on hepatocytes (Osna *et al*, Hepatology, in press). Presentation of peptide-MHC class I complexes on virally infected hepatocytes (HCV, HBV infections, *etc*) as target cells is crucial for CTLs because when clonal expansion of CTLs is established, the next important restriction for elimination of infected cell is the availability of peptide-MHC class I complexes on the surface of target cells (hepatocytes), which are recognized by CTLs. Thus, even if CD8+ T-cells are activated by the presentation of HCV peptides on professional APC, the recognition of the peptide-MHC class I complexes on hepatocytes

(which are target cells for CTLs) may be limited under ethanol-induced oxidative stress.

MHC CLASS II-RESTRICTED ANTIGEN PRESENTATION

Loading of MHC class II molecules with antigenic peptides requires the involvement of specialized APC, such as DC, B-lymphocytes and macrophages. Phagocytosis is the main way to capture antigenic proteins by DC or macrophages, while B-lymphocytes use the antigen specific B-cell receptors^[57]. Antigens are digested into peptides (which are longer than those required for MHC class I-restricted antigen presentation) by proteases at endosome compartments. Cathepsins, intracellular acidic proteases, play a pivotal role in the processing of internalized antigens into class II-presentable T cell epitopes^[58]. These peptidases play a dual role by generating the peptides or by destroying them. The coupling of peptides with MHC class II is controlled by chaperones and takes place in late endocytic vesicles^[44].

When pathogen-associated molecular patterns (PAMPs) on macrophages are recognized by innate immunity receptors (such as Toll-like (TLR)-, mannose-, Fc- and complement- receptors), they induce production of pro-inflammatory cytokines, namely, IFN γ and colony-stimulating factor (GM-CSF). These cytokines increase the expression of MHC class II and co-stimulatory molecules in the cells. Chronic TLR signaling induced by LPS may impair MHC class II-restricted antigen presentation^[59]. A large number of class II-bound proteins are derived from cytosolic proteins. Because the autophagy inhibitor has been shown to block the presentation of cytosolic peptides by MHC class II molecules, some authors discuss the role of autophagy in delivery of these cytosolic peptides to the endocytic route^[60].

After immature DC engulf pathogens, they undergo biochemical changes, such as secretion of TNF α , IL-6, IL-12, IL-10 and IFN α and expression of co-stimulatory molecules, including CD80, CD86 and CD40. These cells traffic to the nearest lymph node for presentation to T-lymphocytes. However, only mature DC can efficiently prime naïve T-lymphocytes, because only these cells generate MHC class II molecules, by redistribution of class II molecules and cathepsins to peptide-loading compartments and by enhancement of lysosomal acidification^[61]. It is still unclear how the peptide-MHC class II complex is recruited to the cell surface from the endocytic compartment in DC. Human DC are subdivided into two big categories: myeloid DC and plasmacytoid DC. In addition to phenotypic differences, they express different TLRs and secrete a different spectrum of cytokines. Thus, myeloid DC express TLR3 and upon stimulation, produce IL-12p70, thereby promoting Th1 response, while plasmacytoid DC express TLR7 and TLR9 and secrete IFN α ^[62]. Therefore, myeloid DC cells are considered classic antigen presenters, while plasmacytoid DC have a limited ability to capture, process and load antigen onto MHC molecules^[63]. Activation of DC

is positively regulated by IFN γ and additional CD40 ligation; anti-inflammatory cytokines, such as IL-10, inhibit both IL-12 and IFN α expression, which results in pathogen survival^[64].

MHC CLASS II-RESTRICTED ANTIGEN PRESENTATION, HCV AND ETHANOL

HCV infection affects DC function in many ways. Firstly, there is a decrease in the frequency of peripheral myeloid or plasmacytoid DC in chronically infected patients^[65]. This may be, in part, related to an accumulation of intrahepatic DC in HCV infection, due to altered DC trafficking^[66]. However, another study suggested that HCV directly targets mature cells because some HCV proteins (core, NS3 and NS5) induce apoptosis in DC^[67]. Secondly, myeloid DC from chronic HCV patients have a decreased capacity to stimulate allogenic T-lymphocytes^[68]. Furthermore, HCV core and E1 genes introduced in DC obtained from uninfected donors, lower their capacity to stimulate allogenic T-cell response^[69]. In addition, core, NS3 and NS4 viral proteins were reported to influence the differentiation of DC from monocytes, due to IL-10 secretion^[70]. The function of myeloid DC obtained from HCV patients is also decreased due to a reduced level of co-stimulatory molecules and down-regulated HLA-DR expression^[5,68]. In addition, in chronic HCV patients, the production of IL-12 by DC is suppressed by HCV proteins, but can be restored by successful antiviral therapy^[71]. Thirdly, the impairment of plasmacytoid DC function has been reported in HCV patients and reduced IFN α production upon DC stimulation with TLR ligands has also been observed^[72]. This decrease in IFN α production can be attributed to monocyte-derived TNF α and IL-10^[73]. Interestingly, the other studies did not demonstrate defective abilities in plasmacytoid DC to initiate immune response^[74], thereby questioning the altered presentation of HCV proteins by these cells to CD4+ cells.

The proposed mechanisms of ethanol-HCV interactions were summarized elsewhere^[75]. Ethanol drastically enhances the negative effects of HCV on DC function. A possible reason for this is that alcohol stimulates HCV expression at HCV RNA and proteins levels, which is, in part, related to up-regulation of the Cox2 pathway^[76]. Chronic ethanol feeding suppresses CD4+ T cell proliferation in response to antigens as well as CTL activity after DNA-based vaccine immunization with NS5 and HCV-expressing plasmids^[77-79]. This CD4+T-cell response was restored by co-administration of IL-2-expressing plasmid, while the restoration of CTL activity required administration of GM-CSF^[77,78]. Alcohol doubles the effects of HCV by decreasing expression of co-stimulatory molecule, B7, and IL-12 production, increasing IL-10 production and lowering allostimulatory capacity^[80-82]. Furthermore, human studies demonstrated that ethanol affects the allostimulatory activity of resting and activated DC generated from peripheral blood mononuclear cells in the presence of cytokines^[81],

indicating that HCV infection impairs DC function, which is further exacerbated by ethanol. Recent studies performed on alcohol-fed mice, immunized with HCV DNA-based vaccine that induces immune response to NS5 HCV protein, demonstrated that ethanol exposure reduced the number of splenic DCs without altering endocytosis capacity. It also reduced the lymphoid DC population, as well as expression of co-stimulatory molecules, CD40 and CD86, altered cytokine profile (enhanced production of IL-1 and IL-10 and decreased secretion of IL-12, IFN γ and IL-6)^[83]. Finally, CTL response to NS5 was shown to be impaired, but was corrected by syngeneic transfer of control DC^[83].

HCV, IFN SIGNALING AND ETHANOL

Antigen presentation is so tightly regulated by IFNs that it should be analyzed in the context of IFN signaling. The reported effects of HCV proteins on IFN signaling are quite controversial. Most studies revealed down-regulation of STAT1 phosphorylation in response to IFN α ^[84-86] attributed to SOCS3 activation by HCV core or NS5A proteins. In addition to that, NS3 protein plays a role in dissociation of the adaptor molecule, MAVS that disrupts the early activation of IFN β signaling by the virus^[87,88]. Subsequently, the disruption of STAT1 phosphorylation by the NS3/NS4 complex was documented^[89]. In some studies, suppressed STAT1 phosphorylation was linked to either the ability of the core protein to specifically interact with the SH2 domain, preventing STAT1 hetero- or homodimerization^[90] or to direct targeting of STAT1 with core protein and subjecting activated STAT1 to degradation by the proteasome^[91]. The latter hypothesis is consistent with our recent findings that core protein activates proteasome function^[49]. Core protein also inhibits the IFN-induced nuclear import of STAT1 and STAT2 and transcription of antiviral genes^[92,93]. HCV proteins interfere with IFN α signaling related to the attachment of activated STAT1 to DNA. Methylation on arginine residues, which prevents the formation in STAT1-PIAS1 complexes, is altered in chronic hepatitis C patients^[94,95]. Protective effects of the methylation regulators, S-adenosyl methionine and betaine, were shown to correct HCV-induced inhibition of IFN α signaling *in vitro*^[96]. Interestingly, in other studies, core protein has been shown to activate interferon stimulated response element (ISRE) and gamma activated site (GAS) sequence promoters in the presence or absence of IFN α and γ and to stimulate INOS promoter, INOS protein induction as well as activation of IFN-inducible 2'5'-oligoadenylate synthetase (2'5'-OAS)^[97,98]. Less is known about the interference of HCV with IFN γ -induced signaling, which is up-regulated by core protein, due to enhanced IFN γ R2 expression^[99]. In a cell culture system, prolonged treatment with IFN γ attenuated IFN α -signaling^[100]. Acute ethanol treatment of hepatoma cells inhibited anti-viral actions of IFN against HCV replicon, induced serine STAT1 phosphorylation, but blocked tyrosine phosphorylation^[101]. Recently, it has

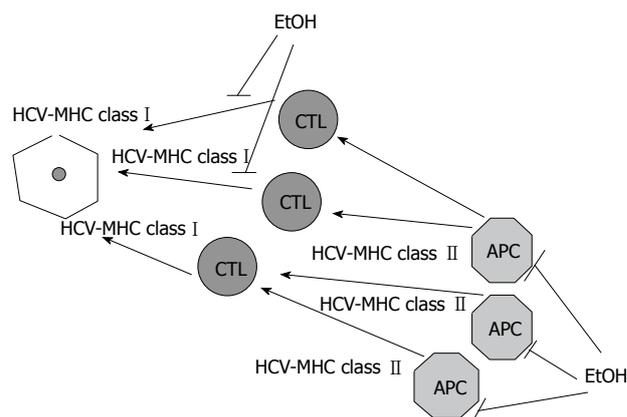


Figure 1 Alcohol (ethanol) reduces antigen presentation on HCV-infected liver cells. HCV interferes with the capacity of professional antigen presenting cells (APC) to prime the immune response. Ethanol (EtOH) further dysregulates antigen presentation, suppressing both MHC class I-restricted antigen presentation on infected hepatocytes and MHC class II-restricted antigen presentation on APC.

been shown that alcohol metabolism induces increased replication of HCV and attenuates the antiviral effects of IFN^[102]. However, the number of studies regarding the combined effects of ethanol and HCV proteins on IFN signaling is very limited.

CONCLUSION

HCV alters antigen-presentation capacities of professional APC, thereby contributing to the persistence of virally infected cells. The most prominent effects were observed on MHC class II-restricted antigen presentation and DC functions. Ethanol potentiates the effects of HCV, providing further suppression of both MHC class I- and class II-restricted antigen presentation on DC, hepatocytes and others hepatic APC, which diminish CTL response. IFNs which support antigen presentation by activating APC and peptidases lose this property in the presence of HCV and alcohol, because HCV and alcohol, separately or in combination, reduce IFN signaling. This, in part, explains why alcohol consumption exacerbates the course, worsens the outcome and reduces the responsiveness to interferon alpha treatment in chronic HCV infection. The effects of alcohol (ethanol) on HCV-restricted antigen presentation are summarized in Figure 1.

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Alcohol metabolites and lipopolysaccharide: Roles in the development and/or progression of alcoholic liver disease

Courtney S Schaffert, Michael J Duryee, Carlos D Hunter, Bartlett C Hamilton 3rd, Amy L DeVeney, Mary M Huerter, Lynell W Klassen, Geoffrey M Thiele

Courtney S Schaffert, Michael J Duryee, Carlos D Hunter, Bartlett C Hamilton 3rd, Amy L DeVeney, Mary M Huerter, Lynell W Klassen, Geoffrey M Thiele, Department of Internal Medicine, University of Nebraska Medical Center, 986350 Nebraska Medical Center, Omaha, NE 68198-6350, United States; Veterans Administration Alcohol Research Center, Omaha Veterans Administration Medical Center, 4101 Woolworth Avenue, Omaha, NE 68105, United States

Geoffrey M Thiele, Department of Pathology and Microbiology, University of Nebraska Medical Center, 986495 Nebraska Medical Center, Omaha, NE 68198-6495, United States

Author contributions: Schaffert CS, Duryee MJ, Hunter CD, Hamilton BC 3rd, DeVeney AL, Huerter MM, Klassen LW, Thiele GM contributed equally to the writing and preparation of this manuscript.

Correspondence to: Michael J Duryee, Omaha Veterans Administration Medical Center, Research Service 151, Rm 321, 4101 Woolworth Avenue, Omaha, NE 68105, United States. mduryee@unmc.edu

Telephone: +1-402-9953429 Fax: +1-402-449060

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tion and fibrosis, and play a role in the development and/or progression of ALD.

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Peer reviewers: Dr. Sheikh Mohammad Fazle Akbar, Assistant Professor, Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791-0295, Japan; Mark D Gorrell, PhD, Professor, Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

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Abstract

The onset of alcoholic liver disease (ALD) is initiated by different cell types in the liver and a number of different factors including: products derived from ethanol-induced inflammation, ethanol metabolites, and the indirect reactions from those metabolites. Ethanol oxidation results in the production of metabolites that have been shown to bind and form protein adducts, and to increase inflammatory, fibrotic and cirrhotic responses. Lipopolysaccharide (LPS) has many deleterious effects and plays a significant role in a number of disease processes by increasing inflammatory cytokine release. In ALD, LPS is thought to be derived from a breakdown in the intestinal wall enabling LPS from resident gut bacterial cell walls to leak into the blood stream. The ability of adducts and LPS to independently stimulate the various cells of the liver provides for a two-hit mechanism by which various biological responses are induced and result in liver injury. Therefore, the purpose of this article is to evaluate the effects of a two-hit combination of ethanol metabolites and LPS on the cells of the liver to increase inflamma-

INTRODUCTION

It has become increasingly clear that alcohol alone is not solely responsible for the initiation and/or progression of alcoholic liver disease (ALD). While alcohol consumption does increase fatty liver, lipid peroxidation, and reactive oxygen species (ROS), these insults are typically not enough to induce the onset of more severe forms of liver disease^[1]. The "two-hit" proposal of ALD is of interest because inflammation caused by the metabolites of ethanol [ROS, aldehyde modified proteins or lipopolysaccharide (LPS)] increase the levels of cytokines/chemokines resulting in a deleterious positive feedback loop that propagates liver inflammation, infiltration of inflammatory cells^[2,3], and fibrosis. To support this, aldehyde modified proteins^[4,5] and endotoxin (LPS)^[6,7] have been detected in the serum and/or livers of patients with ALD. These substances have been shown to increase the release of TNF- α , interleukin-1 β , and prostaglandin by Kupffer cells, sinusoidal endothelial cells and stellate cells. This release in turn promotes an influx of

inflammatory cells leading to an increase in cellular damage promoting the development of necrosis and eventually liver failure^[8-11]. The purpose of this review was to more closely examine how a “two-hit” model of ethanol metabolism and LPS interaction affects resident liver cells in the progression and/or development of ALD. Many excellent reviews^[12-14] exist concerning inflammatory cell activation and recruitment to the damaged liver during the development of ALD and therefore will not be discussed here^[15-17].

ANIMAL MODELS OF ETHANOL AND LPS-INDUCED LIVER INJURY

Current animal models of ALD have provided many valuable findings^[18]. However, in general, these models do not produce the type of end-stage liver failure observed in patients with ALD. To counter this, models have been developed that combine ethanol treatment strategies with an additional injury cofactor. One way of developing more overt injury (e.g. steatohepatitis and fibrosis) in order to mimic the human disease is to combine ethanol and LPS treatment. LPS is a component from the cell walls of bacteria found in the gut as normal flora. Typically, when gram-negative bacteria break down, LPS is released and removed by endothelial cells lining the blood vessels or Kupffer cells (KCs) in the liver. If the normal activity of the gut epithelium is disrupted, as has been shown to happen with acute or chronic ethanol ingestion, the LPS released from degrading bacteria can cross into the bloodstream^[6,7,19]. Even though the exact mechanism of this is unknown, it is suggested that with chronic ethanol consumption, ethanol can damage the cells lining the interior of the intestine and increase the amount of LPS entering the blood stream. In addition, ethanol impairs KCs and prevents them from clearing LPS from the bloodstream^[11,20]. When LPS enters into the bloodstream and moves to the liver, it activates KCs by interacting with the CD14 and Toll-like receptor 4 (TLR-4) molecules on the surface of the cell. This interaction causes a cascade which results in production of ROS and release of inflammatory cytokines (i.e. TNF- α , IL-1 β , IL-6, IL-10), which in turn activates signaling cascades and causes injury to the primary liver cells, the hepatocytes^[8,21].

There have been a number of different approaches utilized to investigate the effects of ethanol consumption and LPS on ALD. The first hit from ethanol metabolism results in the production of ethanol metabolites (i.e. acetaldehyde and acetate). These metabolites increase redox state, steatosis, production of ROS, and lipid peroxidation. This in turn, increases other reactive aldehydes like malondialdehyde and 4-hydroxynonenal. These aldehydes can react with or adduct proteins to alter normal liver functions, induce cell death, and/or liver inflammation. This makes the liver more susceptible to a second hit, probably by LPS. The second hit perpetuates liver injury and fibrosis as a result of LPS-induced oxidative stress, cytokine release, and subsequent infiltration of immune cells^[2].

Two-hit animal models

In a two-hit model of ALD described by Koteish *et al*^[22], C57BL-6 mice were maintained on diets containing 4 mL/L ethanol for five weeks. Pair-fed control mice were fed an identical volume of ethanol-free diet. At the end of the five weeks, the mice received one 10 μ g injection of LPS and sacrificed at 0.5, 1.5 and 6 h post-injection. Histological results showed that the livers from control pair-fed mice had features of apoptosis, but no inflammatory cell infiltrates or hemorrhage. In contrast, hepatocytes in the ethanol-fed mice showed fat accumulation, inflammatory cell infiltration, and hemorrhage.

Procaspase-3, procaspase-8, Jun N-terminal kinase (JNK), TNF- α and TNFR-1 are mediators of LPS-induced hepatotoxicity. Apoptosis in this model was evaluated by determining the levels of procaspase-3 and procaspase-8. Immunoblot analysis to examine procaspase-3 levels showed that the ethanol-fed mice exhibited a decreased content of procaspase-3 relative to the pair-fed mice before and after LPS injections. Evaluation of procaspase-8 showed that it was elevated in the ethanol-fed mice compared to the pair-fed mice.

Examination of JNK activity demonstrated an eight-fold increase 1.5 h after LPS exposure alone. In contrast, LPS had no effect on JNK activity in ethanol-fed mice. TNF- α can interact with TNFR-1 and initiate a signaling cascade that activates procaspase-3 and inducing apoptosis. The expressions of TNF- α and TNFR-1 were found to be steady between the ethanol-fed and control pair-fed mice. Cytokines (IL-10, IL-15 and IL-6) that inhibit TNF- α activity have been shown to prevent liver damage from LPS. These cytokines were up-regulated in both groups but to a greater extent in the ethanol-fed mice.

Thus, the Koteish mouse animal model provides an excellent way to set up a long-term study showing the interaction of ethanol and LPS. This model is beneficial as there is no surgery to perform and only one injection of LPS is administered at the end of the study.

In the von Montfort *et al*^[23] model, four groups of C57BL-6J mice were given different treatments with epinephrine and ethanol. In the first group, 2 mg/kg per day of epinephrine was administered *via* an osmotic pump implanted in the dorsal area for five days. In previous studies this epinephrine dose had been shown to effectively mimic the effects of ethanol. The second group (controls) underwent sham surgery to mimic the implantation of the osmotic pump. The third group was injected with ethanol (6 g/kg per day) for three days. The last group was given a maltose/dextrin injection that was comparable calorifically to the ethanol injection. An injection of 10 mg/kg LPS was administered after the 5-d period and 24 h after the last ethanol dose, the mice were sacrificed at one, eight and 24 h later.

Histological results showed normal hepatocytes in the control animals and in the animals with the epinephrine pump/no LPS injection. Mice injected with LPS alone showed mild inflammation, whereas the mice receiving the epinephrine pump/LPS showed enhanced

liver damage. Importantly, the mice that were given ethanol/LPS showed dramatic liver damage. Plasma AST levels showed that the mice infused with epinephrine alone had no significant increase in plasma AST levels as compared with control mice. Animals treated with LPS only showed a progressive increase in AST with values approximately two-fold higher than the controls. Exposure of animals to epinephrine and ethanol with LPS exposure showed a significant increase in AST levels after LPS exposure by a factor of approximately four and six, respectively. The expression of pro-inflammatory genes, TNF- α , IL-6, and PAI-1 (plasminogen activator inhibitor 1) were analyzed by RT-PCR. In this part of the study, LPS alone stimulated all three genes as early as one hour after injection. Epinephrine pretreatment alone did not affect the expression of these genes, but paired with LPS showed an increased expression of all three genes. The induction of PAI-1 caused by LPS was significantly greater (two-fold) in the presence of epinephrine. The peak expression of TNF- α and IL-6, one hour after LPS exposure was not enhanced by epinephrine pre-exposure. However, these levels were significantly elevated when compared with the LPS alone group at the 8 h time point. Twenty-four hours after LPS injection, mRNA levels of all three returned to basal levels with no difference between the LPS and epinephrine/LPS groups.

Inflammation by LPS is not only enhanced by increased production of pro-inflammatory cytokines, but by impairing the expression of anti-inflammatory genes. Key anti-inflammatory genes such as IL-10, SOCS-1 and SOCS-3 were not significantly changed following epinephrine infusion alone, or in the sham-treated mice.

Curcumin (CMN) has been shown to exhibit anti-inflammatory, anti-bacterial, anti-oxidant properties, and reducing oxidative stress caused by ethanol. This animal model effectively shows how CMN pretreatment protects from LPS-induced liver damage. Kaur *et al.*^[24] used CMN in a model where six groups of male Wistar rats were administered different treatments in conjunction with CMN. In these studies CMN was administered at different doses (5, 30, 60 mg/kg) in the presence or absence of LPS. Rats were sacrificed six hours after an LPS injection on the last day.

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and alkaline phosphatase (AP) were analyzed in all rats which showed a marked rise in serum levels, following LPS injection causing increased liver damage. LPS also caused a rise in serum bilirubin and a decrease in serum total protein compared to control rats. Treatment with CMN significantly decreased the elevated levels of AST, ALT, AP, and bilirubin in response to LPS challenge. Serum nitrite levels and lipid peroxidation were decreased, whereas hepatic GSH, reduced glutathione, and superoxide dismutase (SOD), were increased. Additionally, TNF- α and IL-6 cytokines increased in LPS-administered rats compared to normal rats. CMN administration for seven days ef-

fectively blocked the rise of TNF- α and IL-6 cytokines in the LPS-challenged rats. Histology results did not reveal any morphological alterations in the control group but did show marked morphological disruption in LPS-administered rats. Treatment with CMN attenuated these morphological changes.

In the Gustot *et al.*^[25] animal model, C57BL-6J mice were fed a liquid ethanol diet adapted from the Lieber-DeCarli diet *ad libitum* for 10 d followed by an injection intraperitoneally with one of the following: 60 or 120 μ g lipoteichoic acid (LTA), 60 or 120 μ g peptidoglycan (PGN), 60 or 120 μ g polyinosine-polycytidylic acid (polyIC), 30 or 60 μ g LPS, 60 μ g flagellin or 60 or 120 μ g 7-allyl-8-oxoguanosine (loxoribine). Control mice were pair-fed with a control diet and injected with the same volume of saline solution after 10 d. Mice were sacrificed eight hours after injection.

The ethanol diet led to an increased liver weight compared to the control mice. Histology showed nearly 75% of hepatocytes in ethanol-fed mice exhibited steatosis. Compared to the control-fed mice, the ethanol-fed mice showed significant increase in serum (ALT) levels, and TNF- α mRNA expression. After 10 d, the expression of Toll-like receptors (TLR) 1, 2, 4, 6, 7, 8 and 9 was measured and found to have significant increases over control-fed mice. However, TLR3 and TLR5 were not statistically different in the two groups. To look at the role that endotoxin plays in liver damage in ethanol-fed mice, antibiotics were administered before the experiment began. Cultures from the ethanol-fed mice treated with antibiotics showed a clear decrease in gram-negative bacteria from the mice not treated with antibiotics prior to the start of the experiment. Addition of antibiotics reduced the severity of fatty liver, as shown by decreases in liver weight, serum ALT levels, and steatosis. An increase of lipid peroxidation products (e.g. malondialdehyde, 4-hydroxyaldenals) and a decrease of antioxidant levels (e.g. glutathione) demonstrated hepatic oxidative stress in the livers of ethanol-fed mice.

This animal model is effective because it provides a way to study ethanol consumption and LPS in a way that shows the importance of LPS in the liver for damage to occur. It is important to note that clinically, antibiotics have demonstrated some success in the treatment of ALD patients^[26-28].

The studies above used different approaches, but have shown very similar results when comparing the short-term and long-term effects of ethanol. Overall the short-term affects show that ethanol provides a protective affect from LPS by altering the expression of endotoxin receptors and intracellular signaling molecules^[20]. In contrast, the long-term effect shows an increased sensitivity to LPS and ethanol resulting in liver damage. Clearly, while these models have helped in gaining new insights into the mechanisms of ALD, the development of new and innovative animal models are needed to better elucidate the development and/or progression of ALD.

EFFECTS OF ETHANOL, METABOLITES, AND LPS *IN VITRO*

Liver sinusoidal endothelial cells (SECs)

SECs are flattened, highly fenestrated, and lack a basement membrane. This leads to their common characterization as “sieve-like”. Scanning electron microscopy has shown healthy fenestrae are approximately 150 nm in diameter and make up 6%-8% of the sinusoidal surface. These cells are active filters, selectively moving liquids and solutes from the portal blood into the Space of Disse where they are exposed to parenchymal hepatocytes and lipid storage cells. SECs are also highly endocytotic using scavenger receptors (SRs) to clear the blood of many molecular waste products. They have much higher permeability than other capillaries, and there is constant bidirectional exchange between hepatic parenchyma and blood *via* SECs. The size of the molecules that can pass through SECs is only constrained by fenestral diameter^[29]. These cells are the first line of defense against harmful molecules that could potentially damage the liver. There are many immunological functions of SECs. They have been shown to efficiently remove small (< 200 nm) molecules from the blood using innate immune mechanisms such as scavenger and mannose receptors^[29,30]. They have also been shown to express MHC class II, and co-stimulatory molecules (CD40, CD80, and CD86). They also express intercellular adhesion molecules including CD54 and CD106, which attract immune cells during inflammation. This suggests that they are involved in antigen processing and presentation as well as leukocyte recruitment^[30].

Capillarization of the SECs occurs when they form basement membranes that have been shown to precede fibrosis^[31]. Also, hepatic stellate cells (HSCs), when activated to their collagen producing form, induce fibrosis^[32]. It has been shown *in vitro* that quiescent rat HSCs maintain their inactivity and active HSCs revert to quiescence when grown in co-culture with healthy, nitric oxide (NO) producing SECs in the presence of vascular endothelial growth factor (VEGF). However, this effect is not observed when HSCs are co-cultured with capillarized SECs or those not producing NO^[33].

There is also evidence that malondialdehyde and acetaldehyde-two ethanol metabolites-form an adduct (MAA) which modifies proteins such that they increase the expression of fibrotic molecules by SECs. One such molecule is fibronectin. Fibronectin is expressed following insult to the liver and is known to activate HSCs and induce fibrosis. After stimulation with MAA-modified bovine serum albumin (MAA-Alb), isolated rat SECs show significant increases over negative controls in expression of soluble fibronectin, cellular fibronectin, and of the EIIIA fibronectin variant (the form most closely associated with activation of HSCs)^[34]. Incubation of MAA-Alb with isolated rat SECs for four hours has been shown to elicit increases in pro-inflammatory cytokines/chemokines: an eight-fold increase in TNF- α ; a two-fold increase in MCP-1; and a four-fold increase in MIP-2^[8]. This increase in fibrotic molecules and pro-

inflammatory cytokines provides a potential mechanism by which damage to the liver is mediated by metabolites of ethanol adducted proteins.

Due to their participation in the clearance of LPS from the blood, SECs have mechanisms for the control of inflammatory, leukocyte-mediated response to LPS, while maintaining the population of SRs necessary for toxin and waste clearance. Upon initial contact with LPS, cultures of mouse hepatic SECs release and activate IL-6. This occurs without regard to the presence of TNF- α and requires functional TLR4. SEC responsiveness is decreased following repeated stimulation by LPS. This occurs *via* reduced nuclear translocation of transcriptionally active nuclear factor $\kappa\beta$ (NF $\kappa\beta$). Importantly, repeated exposure to LPS diminishes SEC scavenger function but does not eliminate it. Initial exposure to LPS results in an increased expression of adhesion molecules on leukocytes including CD54, CD106, MCP-1, and IFN-inducible protein 10. None of the above are up-regulated in cultured SECs with previous LPS exposure, and in the case of CD54, this was shown to occur *via* reduced gene expression^[35].

Exposure to LPS *in vivo* has been shown to increase both the quantity of small (20 nm) latex beads and the maximum size (from 100 nm to 500 nm) of latex beads that can be ingested by mouse SECs. Increases were not seen in the rate of ingestion of 100 nm beads, which were the most aggressively ingested regardless of LPS stimulation. LPS was also shown to increase the uptake of both BSA (soluble protein) and dextran (soluble carbohydrate). These increases are attributed to LPS-induced actin remodeling by protein kinase C (PKC) and phosphoinositide 3-kinase (PI3-K) as indicated by increased expression of *src*-suppressed C kinase substrate (SseCKS) on the endothelial cells^[36].

Two-hit model effects on SECs

Lining the portal veins of the liver, the SECs are the first line of defense against LPS derived from the gut^[30]. Most of the attention in LPS clearance has been attributed to the KCs. However, it has been shown that SECs contribute to the regulation of LPS in the liver^[30,35,36]. This regulation of LPS under normal physiological conditions is kept in check by both KC and SECs, which keep the amount of inflammation to a minimum. If LPS levels are increased due to other factors (i.e. alcohol or aldehyde modified proteins), then the potential for disruption of normal homeostasis exists. The fact that alcohol increases both gut permeability (increasing the LPS)^[7] and aldehyde modified liver proteins^[4], provides a possible two-hit mechanism by which the liver could become damaged.

Chronic exposure to ethanol metabolites in the form of MAA-adducted albumin has been shown to alter the SEC response to LPS. In hepatic SECs isolated from rats, LPS-induced secretions of TNF- α , MCP-1, and MIP-2 all show at least two-fold increases in the presence of MAA modified albumin, but no increase in the presence of unmodified albumin. The TNF- α

response is decreased by chronic ethanol consumption, but MCP-1 and MIP-2 responses are not^[8]. The MAA-adduct has also been shown to bind to, and be degraded by, SECs *via* SRs on their surface^[37]. It has also been shown that SECs have CD14 and TLR receptors, which are involved in the uptake of LPS^[38,39]. The potential exists for both aldehyde modified proteins and LPS to bind their receptors simultaneously, increasing the normal release of pro-inflammatory factors that promote inflammation of immune cells to the liver.

While chronic ethanol consumption and LPS stimulation independently increase apoptosis of SECs (as measured by caspase-3 activity) in pre-ALD rat livers, the combination elicits no additional increases. However, data generated from this study did demonstrate that LPS treatment of animals increased the amount of malondialdehyde (MDA) in the hepatocytes. The increase in MDA provides additional substrate for the potential formation of MAA adducts. Therefore an increase in AA and MDA from alcohol metabolism and an increase in MDA from inflammation-induced cell damage could lead to increased MAA-adducted self cellular material and the subsequent initiation of an autoimmune disease^[40].

KCs

KCs are the resident macrophages found in the liver. It is believed that KCs play an important role in the development of ALD^[41]. When stimulated with LPS they become activated and release pro-inflammatory and fibrotic cytokines, along with ROS, which can contribute to liver injury^[11]. Interestingly, it has been shown that circulating LPS concentrations are increased in the blood of alcoholics, and in rats fed alcohol intragastrically, due to the effects ethanol on increasing the permeability on the intestinal mucosa^[42,43]. The circulating LPS derived from intestinal bacteria in turn activate KCs, which initiate their pro-fibrotic and pro-inflammatory effects. KCs can also be activated by interactions with proteins modified by reactive aldehydes associated with ethanol metabolism increasing oxidative stress due to [acetaldehyde (AA) and malondialdehyde (MDA)]. These modified proteins have been associated with ALD^[44-47]. LPS may further sensitize KC interactions with aldehyde-modified proteins^[8] and may actually be involved in their formation^[48].

Chronic ethanol does more than provide the KCs with LPS; it also directly affects their sensitivity to LPS. This section will examine the role that LPS activation of KCs plays in the development of ALD and whether ethanol directly affects KC responses to LPS. The role(s) of aldehyde modified proteins in KC activation and how LPS may affect KC sensitivity to stimulation by these modified proteins will also be examined.

Alcohol causes both tolerance and sensitization to KC activation by LPS^[49-51]. Studies have shown that acute ethanol administration inactivates KCs probably due to ethanol's effects on calcium channels and their requirement for TNF- α release^[52]. KCs isolated from rats two hours after ethanol treatment indeed lacked increased intracellular calcium normally observed when KCs are

treated with LPS. However, when KCs were isolated 24 h after the rats were treated with ethanol, the cells displayed their normal TNF- α production and histological changes upon LPS stimulation. These cells also displayed higher levels of the LPS binding receptor, CD14, which may explain their increased sensitivity to LPS. Treating the rats with antibiotics, which sterilizes the gut removing portal LPS, lowered CD14 expression on KCs isolated 24 h after the administration of ethanol, suggesting that gut derived LPS was the cause of the increased CD14 expression and resultant sensitivity to LPS^[53]. From these studies it can be concluded that a single dose of ethanol can either sensitize KCs or induce tolerance to LPS based on timing.

Two-hit model effects on KCs

As discussed above, chronic ethanol exposure seems to increase the sensitivity of KCs to LPS stimulation. One of the major cytokines released by KCs exposed to LPS is TNF- α , which plays a role in the development of ALD^[41]. Use of anti TNF- α antibody has been shown to protect against ALD in certain animal models^[54]. The role of TNF- α in ALD was also confirmed in studies using TNF- α receptor 1 knockout mouse. In these mice, the pathological changes associated with ethanol treatment were greatly diminished^[55]. KCs may become extra sensitive to LPS and as a result increase their production of TNF- α when chronically exposed to ethanol. This increases CD14 expression and changes the signaling cascade molecules involved in LPS stimulation induced by ROS. These events change transcription factor binding to DNA and increase stability of mRNA involved in TNF- α production.

KCs stimulated by LPS also have effects on HSCs. Hepatic fibrosis is characterized by an over deposition of extracellular matrix components. HSCs are involved with the production of extracellular matrix (ECM) in the liver and during fibrogenesis they undergo a process of activation and proliferation leading to excess collagen synthesis^[56]. LPS activated KCs have been shown to be capable of activating HSC *in vitro*, and levels of ECM production have been directly correlated to increased HSC proliferation^[57].

The *in vitro* effects that LPS-stimulated KCs have on HSCs may be similar to what is seen during *in vivo* fibrogenesis. Higher levels of LPS in the blood of chronic alcoholics may serve as the catalyst for KC activation and therefore may promote HSC activation and fibrogenesis. The production of TGF- β 1 by LPS-stimulated KCs is one of the most significant steps in the activation of HSCs^[57].

Studies have indicated that the detection of acetaldehyde (AA), malondialdehyde (MDA), and AA MDA hybrid (MAA) modified proteins adducts, correlate with increased liver enzymes and liver damage^[45,47,58]. Experiments have also demonstrated that administering gadolinium chloride with ethanol results in the decreased accumulation of MDA and especially AA protein adducts in the livers of rats chronically fed alcohol indicating a role for KCs in AA protein adduct formation^[48].

LPS stimulation of KCs and their ability to release

pro-inflammatory cytokines might also promote immune system recruitment and surveillance, which might help to promote an immune response to these modified proteins. Circulating antibodies to aldehyde-derived epitopes have been identified^[59]. KCs might be involved in the actual presentation of these molecules to the immune cells and be stimulated by these adducted proteins to release pro-inflammatory cytokines. SRs found on their surfaces can bind MAA modified proteins and subsequent binding of these proteins leads to increased levels of TNF- α . When KCs are stimulated with low levels of LPS in addition to MAA modified albumin (MAA-Alb), TNF- α secretion increases six to eight fold. The levels of LPS used for co-stimulation were so low that LPS alone did not result in any TNF- α secretion^[8].

In summary, KCs are a key component in the development and/or progression of ALD. LPS is a potent activator of KCs, causing them to release cytokines such as TNF- α and TGF- β 1, which have been indicated in the development of ALD. Acute administration of ethanol can lead to tolerance or sensitivity to LPS in isolated KCs, while chronic ethanol exposure usually induces a state of ethanol sensitivity marked by increased cytokine production. Cytokines produced by KCs stimulated by LPS can lead to proliferation and activation of HSCs resulting in ECM production. KCs might be involved in the formation of aldehyde protein adducts and these adducts might promote a pro-inflammatory response in KCs, especially in the presence of LPS.

Stellate cells (HSCs)

HSCs undergo activation and proliferation when under the influence of acute and chronic liver injury events. Liver fibrosis and cirrhosis occur in the chronic stages of injury and represent activation of HSCs and secretion of matrix from these cells. Activation of HSCs also occurs in liver injury in acute stages where this damage is known to be able to resolve on its own^[60]. Chronic liver injury events influenced by alcohol and LPS are of interest as they are associated with a constant hepatic insult that leads to life threatening complications with liver transplantation as the only viable option^[60]. As HSCs are involved in the pathway of the wound healing of the liver, their association with alcohol and LPS is an important relationship to understand.

Metabolism of ethanol by the liver is an extremely oxidative event resulting in the development of acetaldehyde (AA). AA is further metabolized into acetate *via* the mitochondrial enzyme acetaldehyde dehydrogenase (ALDH)^[61]. This is a slow reaction that allows for AA buildup over time when alcohol is consumed, and while ADH activity is not greatly influenced, CYP2E1 and other microsomal enzymes are greatly stimulated^[61]. This buildup of AA has also been shown to form stable aldehyde adducts on proteins, which stimulate collagen synthesis, activate protein kinase C, and promote the release of chemokines MCP-1 and MIP-2^[62-64]. Activation of HSCs increases the release of collagen and matrix proteins, which begin the fibrogenic response of wound healing^[32,61].

TGF β 1 is the major profibrotic cytokine in the nor-

mal wound healing process. TGF β 1 messenger expression in HSCs is elevated by ethanol and acetaldehyde, whereby collagen type I gene expression is up-regulated^[32]. Indeed, mouse α 2 (I) collagen promoter was shown to have greater activation in transient transfection experiments when TGF β 1 and acetaldehyde were introduced in tandem, rather than alone, suggesting that TGF β 1 could play a direct part in collagen I gene activation^[65].

Two-hit model effects on stellate cells

In rodent models, using ethanol to induce substantial fibrotic liver injury is problematic regardless of the concentration or length of treatment. Therefore two-Hit models have been used in rodents with the hope to better emulate the fibrotic injury found in human disease states, using some secondary factor with ethanol. Degradation of ethanol increases oxidative stress within the hepatic system, generating free radicals, leading to further hepatic events such as endotoxemia. These events increase gut permeability allowing for more LPS release making LPS clearance more difficult^[10,11,19].

Karaa *et al*^[66] looked for therapeutic agents that slow or possibly prevent ALD progression. S-adenosyl-L-methionine (SAME) has previously been shown to be a precursor in glutathione (GSH) synthesis in the transsulfuration pathway, which is an important hepatic antioxidant. The fact that chronic ethanol consumption greatly limits hepatic SAME storage, GSH synthesis, and increases gut permeability, allows LPS to act as a secondary agent in the two-hit model.

Karaa *et al*^[66] examined how SAME acted as an anti-fibrotic agent by looking at liver fibrosis, HSC activation, and collagen deposition. This model used Lieber DiCarli liquid diet containing ethanol (or a calorie matched diet) concurrently with twice weekly LPS injections during an eight week period. Ethanol alone led to steatosis, some immune cell infiltration (mainly neutrophils), HSC activation, and increased hepatic collagen production. When LPS was introduced with ethanol, hepatic infiltration of neutrophils was increased as well as increased activation of HSCs and preferential pericellular collagen deposition. When SAME was introduced with LPS and chronic ethanol administration, the antioxidative properties of SAME were apparent. GSH stores depleted by ethanol were replenished and hepatic oxidative stress was reduced.

Quiroz *et al*^[67] also looked at the effect of LPS on rat HSC (CFSC-2G) in relation to ethanol and AA. GSH, oxidized GSH (GSSG), IL-6, and collagen secretion were measured. The authors found that lipid peroxidation levels were increased in all experimental conditions versus controls (controls being HSCs with LPS, ethanol, or AA alone). MDA response to ethanol and acetaldehyde exposure did not show a significant change with LPS pretreatment. Experimental cells showed a 2.5 fold increase in GSSG content with LPS and ethanol, and a 5.5 fold increase with LPS and AA, with control values of GSH being much lower. Collagen content was also greatly enhanced with pretreatment of LPS, 120%

greater with ethanol and 209% greater with AA. TGF- β secretion was similar to that of the controls, but IL-6 was greatly up-regulated^[3].

Pretreatment of LPS resulted in an increase of intracellular GSSG, leading to the formation of mixed disulfides with protein thiols, thus lowering the ability to fight oxidative stress induced hepatic injury^[67]. The authors theorized that with the aforementioned change in GSH and GSSG levels, HSCs pretreated with LPS might also generate additional ROS. They also speculated that IL-6 not only promotes hepatocyte proliferation, but enhances collagen production by these activated HSCs. Therefore, "LPS pretreatment of HSC adds to the damage produced by ethanol and acetaldehyde by diminishing GSH content and increasing GSSG content, collagen, and IL-6 secretion"^[67].

Precision cut liver slices (PCLS)

Precision cut liver slices (PCLS) may provide an alternative to other *in vitro* model systems using isolated liver cells to study the combined effects of ethanol and LPS. PCLS are representative of the whole liver and have recently been developed as an *in vitro* model of ethanol induced liver injury^[68]. PCLS exhibit significant ethanol-induced damage in as little as 24 h. This model uses PCLS originating from Wistar rats, cultured in the presence or absence of 25 mmol/L ethanol in a roller system under 95% O₂^[68]. Over a 96 h time period this model efficiently metabolizes ethanol, produces AA, develops a reduced redox state and fatty liver, and exhibits impaired albumin secretion. All of these phenomena are characteristics of early liver injury. Interestingly, in the presence of 4-methylpyrazole (4-MP), an inhibitor of ethanol metabolism, all of the ethanol-mediated effects are ameliorated or significantly reduced, indicating that the metabolites of ethanol are responsible. In addition, recent studies have shown that 25 mmol/L ethanol induces sustained production of IL-6, depletion of GSH, increased lipid peroxidation, and induction of fibrogenesis (increased expression of smooth muscle actin and deposition of collagen in sinusoidal areas). All these phenomena are inhibited by 4-MP, implicating ethanol metabolites. The production of IL-6, GSH depletion, and lipid peroxidation all precede the induction of fibrogenesis, suggesting that inflammation and production of reactive oxygen species are responsible.

PCLS have also been used to examine the effects of LPS on the liver^[69,70]. LPS induced expression of TNF- α , IL-1 β , IL-6 and IL-10 within 24 h of incubation. Consistent with other studies indicating TNF- α induces expression of other cytokines, TNF- α expression was maximal by five hours, whereas expression of the other cytokines was maximal by 16-24 h^[69]. The authors attributed this to activation of KCs, but activation of SECs cannot be ruled out, as they also respond to LPS. Additionally, production of nitric oxide (NO) gradually increased after LPS treatment, starting at five hours after treatment, and continuing throughout the 24 h treatment period^[70]. This increase was paralleled by an increase in inducible nitric oxide synthase (iNOS) expression in the hepatocytes. Inhibition of

TNF- α and IL-1 β attenuated iNOS expression, indicating a paracrine effect by the cytokines induced by LPS treatment. Production of NO *in vivo* has been shown to have protective effects in inflammation and endotoxemia-induced hepatic injury^[71]. Thus, these results might indicate a compensatory response by the PCLS to the inflammatory response induced by LPS treatment.

One previous study examined the combined effects of LPS and ethanol on PCLS cytotoxicity^[72]. This study incubated PCLS in the absence or presence of 0.5%-8% ethanol and/or 0.1-100 μ g/mL LPS for up to 12 h. Ethanol at 1% or more exhibited a time- and dose-dependent hepatotoxicity by itself, whereas LPS had no appreciable effect. However, when combined, 2% ethanol and increasing concentrations of LPS exhibited additive effects on hepatotoxicity with 100 μ g/mL LPS inducing the most injury (70% viable) compared to 2% ethanol alone (90% viable) at 12 h. While these concentrations of both ethanol and LPS are extremely high, they do provide an upper limit for future studies to examine the combined effects of ethanol and LPS. In fact, preliminary data by this laboratory suggests that more physiological levels of ethanol (25 mmol/L) and LPS (10 ng) exhibit additive effects on inflammatory processes and induction of oxidative stress in PCLS.

CONCLUSION

Studies into the pathogenesis of ALD have demonstrated that ethanol, LPS, and the metabolites of ethanol all may have a significant role in the development and/or progression of this disease. However, the data also strongly suggests that while each of these components are necessary, alone they are not sufficient to induce the pathogenesis of ALD. Indeed, it is becoming more apparent that combinations of one or more of these components must occur to induce ALD, which would suggest that at the very least a "two-hit" model is involved in the pathogenesis of ALD. Figure 1 shows a proposed hypothesis of the "two-hit" model of ALD.

Support for this concept can be found in some very simple observations. Acute (down-regulation) versus chronic (no effect) ethanol exposure decreases the response of KCs and SECs to LPS, which is thought to be a major co-factor in ALD. It has also been shown that on some cell types, multiple receptors (Scavenger Receptor A; SRA-1) are present and may bind LPS and degrade this ligand before it binds to TLR4. When the expression of these SRs is altered (ethanol inhibits degradation), LPS is now free to bind to CD14/TRL4 and initiate pro-inflammatory responses. It has also been shown that multiple ligands, ROS, aldehyde-modified proteins, and hyaluronan are involved in the development and/or progression of ALD. Therefore, depending upon which ligands were to bind to their appropriate receptors, then the response would be totally different than if only one of these ligands were to bind. Couple this with the fact that each cell type in the liver expresses different receptors, and it is easy to imagine that the interactions

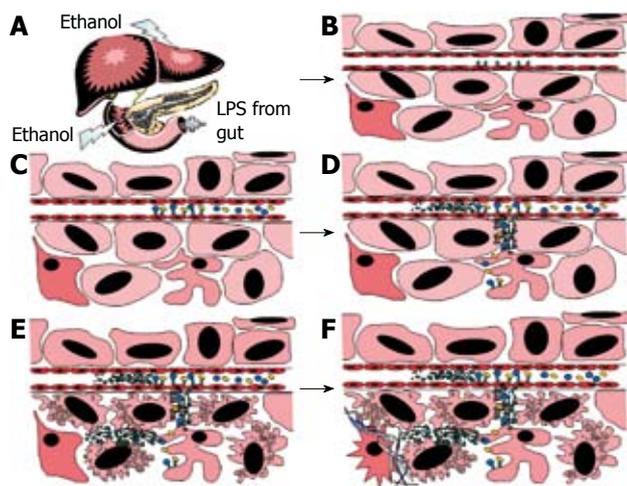


Figure 1 A proposed hypothesis of the two-hit model for the onset and/or progression of alcohol liver disease. A: The prolonged consumption of ethanol has been associated with an increase in gut permeability. Lipopolysaccharide (LPS) may leak out of the gut and into the blood stream, finding its way back to the liver. B: Meanwhile, the breakdown of alcohol and fats in the liver could modify cellular proteins with malondialdehyde and/or acetaldehyde and result in increased levels of these modified proteins. Receptors on endothelial cells (SECs) specific for LPS and aldehyde modified proteins might also be up-regulated. C: The various scavenger receptors bind LPS and/or aldehyde modified proteins circulating in the blood stream. D: Binding of these molecules causes an increased release of pro-inflammatory cytokines, which are dumped into the blood stream and into the liver through the Space of Disse. The release into the liver causes activation of kupffer cells, binding of LPS and modified proteins, and their release of more pro-inflammatory cytokines. E: The cytokine release from SECs and/or kupffer cells signals immune cells infiltrate into the liver and result in damage to the hepatocytes. F: Damage to the hepatocytes increases the amount of TGF- β and other cytokines, causing the stellate cells and SECs to secrete pro-fibrogenic factors. These factors help to rebuild and remodel the liver parenchyma initiating the wound healing responses. Following repair of the liver, there is scarring and some irreparable damage, which can get better if the insults (LPS and modified proteins) are taken away. However, if alcohol consumption remains there becomes a point whenentence, preferably as two shorter sentences, to clarify this?

between the different cell types might change depending upon the receptors expressed. As discussed in this review, it is apparent that these interactions do occur and the development and/or progression of ALD is a complex interaction that may be investigated utilizing a “two hit” or “multiple hit” model.

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Alcohol-induced protein hyperacetylation: Mechanisms and consequences

Blythe D Shepard, Pamela L Tuma

Blythe D Shepard, Pamela L Tuma, Department of Biology, The Catholic University of America, 620 Michigan Avenue, NE Washington, DC 20064, United States

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Correspondence to: Pamela L Tuma, PhD, Department of Biology, The Catholic University of America, 620 Michigan Avenue, NE Washington, DC 20064, United States. tuma@cua.edu

Telephone: +1-202-3196681 Fax: +1-202-3195721

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Abstract

Although the clinical manifestations of alcoholic liver disease are well-described, little is known about the molecular basis of liver injury. Recent studies have indicated that ethanol exposure induces global protein hyperacetylation. This reversible, post-translational modification on the ϵ -amino groups of lysine residues has been shown to modulate multiple, diverse cellular processes ranging from transcriptional activation to microtubule stability. Thus, alcohol-induced protein hyperacetylation likely leads to major physiological consequences that contribute to alcohol-induced hepatotoxicity. Lysine acetylation is controlled by the activities of two opposing enzymes, histone acetyltransferases and histone deacetylases. Currently, efforts are aimed at determining which enzymes are responsible for the increased acetylation of specific substrates. However, the greater challenge will be to determine the physiological ramifications of protein hyperacetylation and how they might contribute to the progression of liver disease. In this review, we will first list and discuss the proteins known to be hyperacetylated in the presence of ethanol. We will then describe what is known about the mechanisms leading to increased protein acetylation and how hyperacetylation may perturb hepatic function.

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INTRODUCTION

The liver is the major site of ethanol metabolism and thus sustains the most injury from chronic alcohol consumption. In the hepatocyte cytosol, alcohol dehydrogenase (ADH) converts ethanol to acetaldehyde, a highly reactive intermediate. The acetaldehyde is further metabolized in mitochondria to acetate by acetaldehyde dehydrogenase (ALDH). Alcohol is also metabolized by the resident ER enzyme, cytochrome P450 2E1 (CYP 2E1). CYP 2E1-mediated ethanol metabolism not only leads to the formation of acetaldehyde, but also to the formation of oxygen and hydroxyethyl radicals that in turn promote the formation of other highly reactive intermediates^[1]. All of these reactive metabolites can readily and covalently modify proteins, DNA and lipids^[2-7]. More recently, it has become apparent that alcohol exposure induces protein covalent-modifications that are part of the natural repertoire. To date, these post-translational modifications include increased methylation, phosphorylation and acetylation^[8-14]. In particular, numerous proteins have been identified that are hyperacetylated upon ethanol exposure, and this list is expanding rapidly. As for adduct formation, it is not clear how increased acetylation is related to the progression of alcohol-induced hepatotoxicity. In this review, we will first list and discuss hepatic proteins known to be hyperacetylated by ethanol exposure. We

will then describe our current understanding of the mechanisms and physiological consequences of protein hyperacetylation.

ETHANOL-INDUCED LYSINE HYPERACETYLATION

For over 40 years it has been recognized that proteins can be acetylated and that the modification comes in two forms^[15]. One is the irreversible, co-translational N-terminal acetylation of α -amino groups of mainly serine and alanine, but also of threonine, methionine and glycine. The other form is the reversible, post-translational modification of ϵ -amino groups on lysine residues located within a polypeptide^[15,16]. The reversibility of lysine acetylation and its presence on an ever expanding list of nuclear and nonnuclear proteins have led some to postulate that it might rival phosphorylation in its ability to regulate cellular processes^[16]. Thus, alcohol-induced protein hyperacetylation likely results in major physiological consequences that contribute to the progression of hepatotoxicity. In this section, we will first discuss individual hepatic proteins that are known to be hyperacetylated upon ethanol exposure (Table 1). We will then comment on recent work that implicates many more candidates for alcohol-induced lysine acetylation (Table 2).

Histone H3

Histones were the first proteins known to be acetylated^[15,16], and histone H3 was the first protein known to be hyperacetylated after ethanol treatment. This effect has been observed in isolated hepatocytes and in rat liver after both acute and chronic ethanol exposure^[8,17-21]. Although histone H3 encodes at least four acetylated lysines (lys 9, 14, 18 and 23), acetylation of lysine 9 is selectively increased after exposure to physiological ethanol concentrations^[8]. Because histone H3 hyperacetylation was prevented by both 4-methylpyrazole (4-MP) and cyanamide, it was concluded that the modification requires alcohol metabolism and is likely mediated by the ethanol metabolite, acetate^[8]. Interestingly, lysine 9 is also reversibly methylated, and in ethanol-treated hepatocytes, its methylation is decreased^[9]. Furthermore, alcohol also induces hypermethylation of lysine 4 and phosphorylation of neighboring serine residues (ser 10 and ser 28)^[10]. It will be important to consider all of these modifications as the functional consequences of histone H3 hyperacetylation are defined (see below).

p53

p53 is a tumor suppressor that is mutated in over half of all human cancers. It is activated by DNA damage and functions to stop cell cycle progression. For over a decade it has been known that p53 is reversibly lysine acetylated, and that this modification promotes enhanced

Table 1 Ethanol-induced hyperacetylated proteins

Protein	EtOH exposure	System	References
Histone H3	Acute and chronic	Hepatocytes, rat liver, stellate cells	[8,17-21]
p53	Chronic	Rat liver, VLA17 cells	[61]
PGC-1 α	Chronic	Rat liver, mouse liver	[12,14]
SREBP-1c	Chronic	H4IIEC3 cells	[12]
α -tubulin	Chronic	WIF-B cells, rat liver	[11,39,75]
AceCS2	Chronic	Rat Liver	[13]

Table 2 Newly identified ethanol-induced hyperacetylated proteins

Liver subcellular location ¹	EtOH exposure	Function	No. proteins identified
Cytosol	Chronic	AA metabolism	7
		Carbohydrate metabolism	4
		Other metabolic pathways	3
		Oxidative stress	3
		Other	2
		Mitochondria	Chronic
		AA metabolism	5
		Oxidative phosphorylation	2

¹Our unpublished results.

DNA binding^[15,16]. Very recently, p53 has been shown to be hyperacetylated upon chronic ethanol exposure in rat liver lysates^[14]. Although multiple lysines are known to be acetylated, it is not yet known which residue(s) is hyperacetylated after ethanol consumption. Furthermore, it is not yet known whether hyperacetylation requires ethanol metabolism or whether it alters p53 DNA binding properties.

Sterol response element binding protein-1c (SREBP-1c)

SREBPs are a family of transcription factors that regulate lipid and cholesterol synthesis. SREBP-1c is the major form expressed in liver and is known to activate numerous lipogenic enzymes. In rat hepatoma H4IIEC3 cells, ethanol treatment for 24 h led to a dose-dependent increase in SREBP-1c acetylation^[12]. This result was confirmed in livers from ethanol-fed mice indicating it has physiologic importance. It is not yet known whether hyperacetylation requires ethanol metabolism nor is it known which lysine is hyperacetylated. However, more is known about the mechanism by which SREBP-1c is acetylated/deacetylated and how that relates to SREBP-1c function in gene regulation and lipogenesis (see below).

Peroxisome proliferator-activated receptor γ coactivator α (PGC-1 α)

The nuclear transcriptional coactivator, PGC-1 α , is a key regulator of hepatic glucose homeostasis and lipid metabolism^[22]. It has also been implicated in regulating mitochondrial biogenesis and respiration^[22]. PGC-1 α is known to be acetylated on many lysines,

and in general, acetylation is correlated with decreased transcriptional activity resulting in decreased expression of genes involved in mitochondrial fatty acid oxidation and gluconeogenesis. Thus, it is notable that PGC-1 α hyperacetylation is induced by ethanol consumption^[12]. Currently, it is not known which lysine(s) is modified or whether PGC-1 α hyperacetylation requires ethanol metabolism. However, as for SREBP-1c, there is more known about some likely functional consequences of PGC-1 α hyperacetylation (see below).

Acetyl CoA synthetase 2 (AceCS2)

A recent proteomic survey of mammalian cell proteins identified nearly 200 lysine-acetylated nuclear and nonnuclear proteins^[23]. Remarkably, this survey further revealed that more than 20% of mitochondrial proteins were lysine-acetylated^[23]. Thus, it is not surprising that the acetylation of AceCS2, an enzyme involved in lipid metabolism, is increased upon alcohol exposure, probably on lysine 635^[13,24]. Acetylation of this residue correlates with decreased AceCS2 catalytic activity^[25], but whether its activity is decreased in ethanol-treated cells is not yet known. Interestingly, alcohol-induced acetylation of mitochondrial proteins, including AceCS2, was not observably altered in livers from CYP 2E1 knockout mice suggesting that the modification does not require CYP 2E1-mediated ethanol metabolism^[13]. However, whether ethanol metabolism by ADH and ALDH is required for mitochondrial protein hyperacetylation is not yet known.

Tubulin

Microtubules are one of the three major cytoskeletal systems of the cell. The polymer is made of repeating units of α - and β -tubulin heterodimers that form protofilaments, which in turn assemble into hollow tubes consisting of 13 protofilaments arranged in parallel. Microtubules exist as both dynamic and stable polymers. The latter population is characterized by a longer half-life, resistance to microtubule poisons (e.g. cold and nocodazole) and by specific post-translational modifications on the α -tubulin subunit^[26]. These modifications include the removal of a carboxy-terminal tyrosine, polyglutamylation, polyglycylation and acetylation of lysine 40^[26]. The functions of these modifications or whether they contribute to microtubule stability are still the subject of debate^[27]. Recently, it was determined that chronic ethanol exposure enhanced α -tubulin acetylation at lysine 40 in polarized WIF-B cells and livers from ethanol-fed rats^[11]. Increased acetylation correlated to increased stability suggesting that tubulin acetylation might in fact enhance microtubule stability. In WIF-B cells, increased tubulin acetylation and stability displayed both ethanol time- and dose-dependence^[11]. Furthermore, tubulin hyperacetylation and stability was prevented by 4-MP and potentiated by cyanamide indicating that ethanol metabolism was required for the effects^[11]. Thus, unlike acetate-mediated histone H3 hyperacetylation, tubulin hyperacetylation and increased stability are likely mediated by acetaldehyde. This

disparity is likely to be due to the different mechanisms leading to enhanced acetylation of either substrate (see below).

The expanding list

With the growing number of known acetylated proteins and the large number of modifying enzymes, it is likely that numerous hepatic proteins are hyperacetylated in ethanol-treated cells. We initiated a proteomics approach to identify other hyperacetylated proteins from cytosolic and total membrane fractions prepared from livers from control and ethanol-fed rats (manuscript in preparation). So far, about 40 nonnuclear proteins have been identified (but not yet confirmed), half of which were from the cytosolic fraction and half from the total membranes. Remarkably, all the hyperacetylated proteins in the latter fraction were from mitochondria and most were metabolic enzymes (Table 2). Seven of these mitochondrial proteins were also identified in the proteomic survey for acetylated lysines described above^[23] partially confirming our result. Also consistent with this finding is a recent study where purified mitochondria from alcohol-fed rats were immunoblotted with anti-acetylated lysine antibodies^[13]. Numerous immunoreactive species were observed (but not yet identified) suggesting massive mitochondrial protein hyperacetylation after ethanol exposure. Similarly, cytosolic fractions were highly hyperacetylated after ethanol exposure and the proteins identified varied widely in function, ranging from metabolic enzymes to proteins regulating oxidative stress to molecular chaperones. Efforts are currently underway to confirm the acetylation state of these proteins and to determine the functional consequences of their ethanol-induced hyperacetylation.

MECHANISMS OF ETHANOL-INDUCED LYSINE HYPERACETYLATION

Protein acetylation results from the coordinated activities of acetyltransferases and deacetylases^[15,16]. Histones were the first proteins known to be acetylated, and accordingly the modifying enzymes were initially named histone acetyltransferases (HATs) and histone deacetylases (HDACs). Although the list of acetylated proteins has since grown to include numerous nonhistone substrates, their names have remained. In this section, we will briefly describe the two classes of enzymes and whether they are expressed in the liver. We will also discuss what is known about how these HATs and HDACs may be responsible for the alcohol-induced increase in lysine acetylation.

HDACs

To date, there are at least 18 known deacetylases that are categorized into four general classes based on sequence homology and cofactor/coenzyme dependence^[28] (Table 3). Classes I, II and IV are closely related zinc-dependent enzymes whereas the class III HDACs are

Table 3 Deacetylases expressed in the liver

Class	Enzyme	In liver? (methods)	Location	Substrates	References
I	HDAC1	Yes (RT-PCR, IB, IHC, activity)	Nucleus	Histones, p53, retinoblastoma, STAT3, androgen receptor, estrogen receptor, Smad7, other transcription factors	[29,32,38,42,89-92]
	HDAC2	Yes (RT-PCR)	Nucleus	Histones, STAT3, other transcription factors	[29,32,38,42]
	HDAC3	Yes (RT-PCR, IB, activity)	Nucleus	Histones, STAT3, Smad7, other transcription factors	[29,32,38,42,91,93]
	HDAC8	Yes (RT-PCR)	Nucleus	Histones, transcription factors, smooth muscle actin	[29,32,38,94]
II	HDAC4	Yes/No (RT-PCR)	Cytoplasm Nucleus	Transcription factors	[34,38,42]
	HDAC5	Yes (RT-PCR)	Cytoplasm Nucleus	Transcription factors	[34, 38]
	HDAC6	Yes (RT-PCR, IB, IF)	Cytoplasm	Tubulin, cortactin, HSP90	[34,38-42,56,59]
	HDAC7	No	Cytoplasm Nucleus	HIF α , other transcription factors	[34]
	HDAC9	No	Cytoplasm Nucleus	Transcription factors	[34]
	HDAC10	Yes (RT-PCR)	Cytoplasm Nucleus	Phosphatase pp1	[34,95]
III	Sirt1	Yes (RT-PCR, IB)	Nucleus	Histones, PGC-1 α , LXR, p53, other transcription factors	[12,14,18,42-44,96-99]
	Sirt2	Yes/No (RT-PCR, IB)	Cytoplasm	Histone H4, tubulin	[39,42-46]
	Sirt3	Yes (RT-PCR, IB)	Mitochondria	Acetyl CoA synthetase 2, glutamine dehydrogenase, ICDH2	[13,43-45,47,48]
	Sirt4	Yes (RT-PCR)	Mitochondria	Glutamine dehydrogenase	[43-45,47,48]
	Sirt5	Yes (RT-PCR)	Mitochondria	Cytochrome c	[43-45,47,48,61]
	Sirt6	Yes (RT-PCR)	Nucleus	DNA polymerase β	[44,45]
	Sirt7	Yes (RT-PCR)	Nucleus	RNA polymerase I	[44,45]
IV	HDAC11	Unknown	Nucleus	Unknown	[29]

IB: Immunoblotting; IHC: Immunohistochemistry; IF: Immunofluorescence; HIF α : Hypoxia-inducible factor α ; LXR: Liver x receptor; ICDH2: Isocitrate dehydrogenase.

more distantly related, NAD⁺-dependent sirtuins^[28,29]. Phylogenetic analysis of bacterial HDAC relatives indicates that the evolution of the class I, II and IV family members preceded the evolution of histones^[29] suggesting that these HDACs have nonhistone substrates. This conclusion is consistent with the wide-range of mammalian HDAC substrates that have been identified (Table 3).

Mechanistically, classes I, II and IV all share a conserved catalytic core consisting of open α/β folds and a tubular active site pocket in which a zinc ion sits. The poised zinc mediates the nucleophilic attack of water on the acetylated lysine substrate resulting in the formation of a tetrahedral oxyanion intermediate. The nitrogen on the intermediate is then primed to accept a proton resulting in a charge relay system between histidine, aspartate, and tyrosine that results in the formation of acetate and the deacetylated lysine^[29-31]. This catalytic mechanism was deduced with the help of HDAC inhibitors that displace the zinc ion thereby disrupting the charge relay system. Many of these inhibitors are proving to be promising anti-cancer therapies. The most potent inhibitor, trichostatin A (TSA), is a near perfect fit for the HDAC active site and is often used in studies to confirm the affect of protein acetylation on function (see below)^[32].

The class I family members include HDACs 1, 2, 3 and 8 that are closely related to the yeast deacetylase, reduced potassium dependency 3 (RPD3)^[32] (Table 3). They are all ubiquitously expressed (including in liver, Table 3), are found almost exclusively in the nucleus, are roughly the same length (370-480 aa) and encode one

deacetylase domain^[29,30,32,33]. These four HDACs have all been shown to deacetylate histones as well as a variety of transcription factors and other nuclear proteins including STATs and Smads^[29,30,32,33] (Table 3). Recent reports suggest that class I enzymes may function together in multiprotein complexes that bind and deacetylate transcription factors^[29,30]. Future work is needed to understand how the deacetylase activities are specifically coordinated leading to changes in gene expression.

Class II enzymes are much more variable in size (700-1200 aa) and exhibit more tissue-specific expression patterns with only HDACs 4, 5, 6 and 10 identified in the liver (Table 3). Like for class I enzymes, known class II substrates are mainly nuclear transcription factors^[28-30,32-34]. However, the class II deacetylases encode both nuclear import and export signals allowing them to shuttle between the nucleus and cytoplasm where they bind to location-specific substrates^[30] (Figure 1, bottom panel). Although interactions between these enzymes and their substrates are thought to regulate their activity and location^[29,30], little is known how this process is regulated. The class II HDACs catalyze lysine deacetylation in much the same way as the class I enzymes but their active sites encode a glutamate instead of a glycine^[29]. This larger side chain decreases active site size restricting substrate access thereby increasing enzyme specificity. Interestingly, HDAC6 and HDAC10 encode two deacetylase domains in tandem^[28,34]. Whether the two sites deacetylate different substrates or whether both are needed for function is not yet clear^[35-37]. Of particular interest to this review is HDAC6, the only exclusively cytosolic HDAC found in liver^[38,39]. One

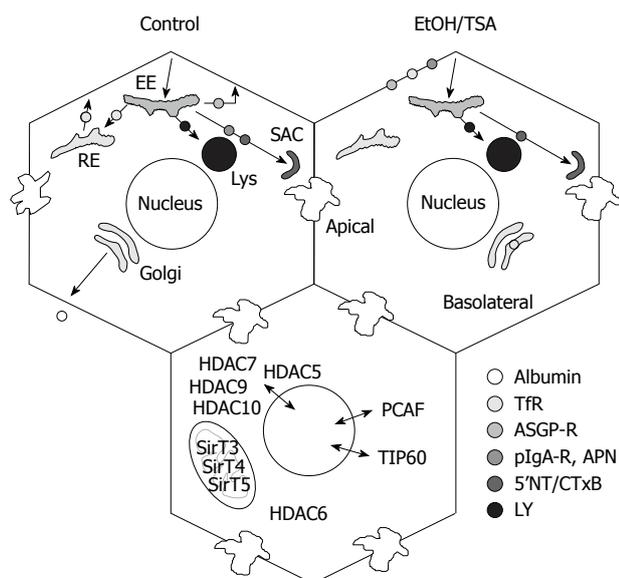


Figure 1 Alcohol-induced defects in protein trafficking can be explained by increased microtubule acetylation and stability. Cargo internalized from the basolateral plasma membrane is delivered to the early endosome (EE) and sorted into at least four different pathways. They are recycled directly back to the plasma membrane (e.g. ASGP-R), delivered to the recycling endosome (RE) before recycling back to the plasma membrane (e.g. Tf-R), delivered to lysosomes (lys) (e.g. LY) or to the apical plasma membrane via the sub-apical compartment (SAC) (e.g. APN, pIgA-R and 5'NT). Albumin secretion is also indicated. In ethanol (EtOH) or TSA treated cells, albumin secretion and the internalization of APN, pIgA-R, Tf-R and ASGP-R is impaired whereas the fluid-phase delivery of LY to lysosomes or the raft/caveolae-mediated internalization of 5'NT and CTxB are not changed. In the lower hepatocyte, the various deacetylases and acetyltransferases that may play a role in the acetylation of nonnuclear proteins are indicated.

of its known substrates is α -tubulin, one of the best characterized, nonnuclear, hyperacetylated proteins in ethanol exposed cells^[11,39-42].

Currently there is only one known class IV deacetylase, the recently identified HDAC11. As a result of its high sequence similarity to class I enzymes, HDAC11 is often considered a class I HDAC^[50]. At present, little is known about HDAC11's substrates, expression patterns or subcellular distributions. HDAC11 has been shown to act in a complex with other HDACs, most notably HDAC6, suggesting that it may not be an independent lysine deacetylase^[29]. Clearly, more work is needed on this class IV enzyme before we can understand its role in protein acetylation.

Unlike the three classes of zinc-dependent HDACs, class III deacetylases are referred to collectively as sirtuins. Sirtuin activity requires NAD^+ , and thus each family member encodes a highly conserved, elongated catalytic core containing a Rossmann fold characteristic of NAD^+ /NADH-binding proteins. Both the NAD^+ and the acetylated lysine substrate bind to the "C pocket" which is located in the cleft between the Rossmann fold and a smaller, more variable domain containing a zinc ion. This binding results in a nucleophilic substitution reaction where the NAD^+ and acetylated lysine substrate are converted to nicotinamide, a deacetylated lysine and a novel metabolite, 2'-O-acetyl-adenosine diphosphate ribose^[51]. In total, there are seven known sirtuins that all

exhibit interesting expression patterns. SirT1 is a widely expressed, well characterized nuclear sirtuin whose long list of substrates includes transcription factors, histones, p53, and PGC-1 α ^[42-44] (Table 3). SirT6 and 7 are two other nuclear sirtuins, and all three have been found in the liver by RT-PCR^[44,45]. SirT2 is the lone cytoplasmic sirtuin, and like its counterpart, HDAC6, its main substrate is α -tubulin^[46]. Although SirT2 was detected in liver homogenates by RT-PCR, the protein has not been detected at any level in liver lysates or WIF-B cells^[39]. SirT3, 4 and 5 are all mitochondrial-specific sirtuins that are ubiquitously expressed (Figure 1, bottom panel). So far, only SirT3 and 5 have confirmed deacetylase activity^[47,48]. Interestingly, recent reports suggest that SirT4 (and nuclear SirT6) are instead ADP ribosyltransferases that preferentially ADP-ribosylate acetylated substrates^[49,50]. Further studies are needed to examine the effects of ethanol on this activity.

HATs

To date, there are 17 families of HATs that have been grouped according to sequence homology. In general, the N and C terminal domains of the acetyltransferases are structurally and functionally diverse while the catalytic domain is highly conserved. All HAT active sites contain a structurally conserved loop- β strand core domain that binds acetyl Co-A^[51]. Also within this core domain is a glutamate that serves as a lysine docking site. The glutamate removes a proton from the docked lysine allowing acetyl transfer from acetyl CoA^[51]. From the 17 families, three major groups have emerged: Gcn5/PCAF, p300/CBP and MYST (Table 4). Little is known about the mammalian members of the Gcn5/PCAF family, but in general these HATs have been detected in liver, reside in the nucleus and have mainly nuclear substrates. Only PCAF has been found to distribute to both the nucleus and cytoplasm^[52-53] allowing for more varied substrates including histones, p53, and the actin binding protein, cortactin^[56-58]. The p300/CBP family consists of p300 and CBP that are nearly structurally identical and are referred to interchangeably^[57,58]. They also reside in the nucleus and are expressed in the liver. The least characterized MYST family consists of the mammalian enzymes, TIP60, HBO, MOZ, and MORF^[57,58]. Although each of these HATs have been shown to acetylate histones H3 and H4, much still needs to be learned about other possible substrates and whether they function alone or in large multi-HAT complexes.

General roles for HATs and HDACs in ethanol-induced protein acetylation

Changes in both HAT and HDAC expression have been correlated with global changes in protein acetylation. Thus, a simple explanation for ethanol-induced protein acetylation may be altered enzyme levels. The prediction is that either a decrease in HDAC expression or an increase in HAT levels will lead to increased protein acetylation. In addition, changes in enzyme activity or subcellular distribution may lead to hyperacetylation. For example, either a decrease in HDAC activity or a

Table 4 Histone acetyltransferases expressed in the liver

Family	HAT	In liver? (methods)	Location	Substrates	References
Gcn5/PCAF	PCAF	Yes (IB)	Nucleus Cytoplasm	Histones, p53, PGC-1 α , other transcription factors, cortactin	[52-58]
p300/CBP	CBP	Yes (IB)	Nucleus	Histones, E1A, p53, SRC-1, TIF2, ACTR, SREBP, other transcription factors	[54, 57, 58]
	p300	Yes (IB)	Nucleus	Histones, E1A, p53, SRC-1, TIF2, TAT, ACTR, SREBP, other transcription factors	[18, 54, 55, 57, 58]
MYST	TIP60	Yes (IB)	Nucleus Cytoplasm	Histones H3 and H4, androgen receptor	[54, 57]
	HBO	Unknown	Nucleus	Histones H3 and H4	[57, 58]
	MOZ	No	Nucleus	Histones H3 and H4	[100]
	MORF	Yes (RT-PCR)	Nucleus	Histones H3 and H4	[101, 102]

E1A: Adenovirus early region 1A; SRC-1: Steroid receptor coactivator; TIF2: Transcriptional intermediary factor 2.

loss of nuclear import could enhance nuclear protein acetylation. Additionally, the sirtuin deacetylases are NAD⁺-dependent enzymes. Because ethanol metabolism is NAD⁺-depleting, sirtuin activity may be impaired thereby leading to increased protein acetylation. Below, we summarize what is known about how alterations in HAT or HDAC activity/function contribute to ethanol-induced protein hyperacetylation.

Histone H3 hyperacetylation and p300

At present, the mechanisms responsible for ethanol-induced hyperacetylation of histone H3 are not known, but recent studies have provided some clues. In livers of ethanol-fed rats, p300 protein levels were increased about 3 fold, which correlated with increases in overall nuclear HAT activity^[17,18,21]. Acetate treatment also led to enhanced nuclear HAT activity and histone H3 hyperacetylation indicating ethanol metabolism is required for this modification^[17]. Interestingly, the protein expression of the closely related p300 homologue, CBP, was not altered^[18] indicating that ethanol-induced histone H3 acetylation may be specific to p300. These studies further suggest that these two HATs may not be completely interchangeable. To date, it is not known which (if any) HDAC is involved in histone H3 hyperacetylation in ethanol-treated cells. However, total nuclear HDAC activity was significantly decreased in ethanol-treated WIF-B cells, and that decreased activity correlated with increased histone H3 acetylation^[39]. In contrast, total nuclear HDAC activity was not altered in ethanol-treated isolated hepatocytes where enhanced histone H3 acetylation was observed^[17,21]. The reasons for these disparate results are not known but may be explained by differences in the cell systems used, acute vs. chronic alcohol-treatment, or differences in the deacetylase assay used. Further research is needed to elucidate this further.

SREBP-1c hyperacetylation and SirT1 and p300

As for histone H3, the mechanisms responsible for ethanol-induced hyperacetylation of SREBP-1c are not known, but work has provided some hints. From studies performed in H4IIEC3 cells, overexpressing SirT1 and p300 were found to deacetylate or acetylate SREBP-1c,

respectively, confirming that SREBP-1c is a substrate for both enzymes^[12]. Increasing ethanol concentrations enhanced p300-mediated SREBP acetylation in these cells while a concomitant decrease in SirT1 protein levels was observed^[12,14]. Thus, SREBP-1c hyperacetylation was the result of changes in both types of modifying enzymes. As for SREBP-1c, PGC-1 α hyperacetylation was also correlated with decreased SirT1 protein expression^[12,14]. At present, it is not known if changes in SirT1 or p300 activity levels correlate with changes in protein levels or whether SREBP-1c and PGC-1 α hyperacetylation requires ethanol metabolism. Future work is clearly needed to understand this fully.

Tubulin hyperacetylation and HDAC6

Since the specific microtubule acetyltransferase has not yet been identified, studies thus far have focused on the known hepatic tubulin deacetylase, HDAC6 (Table 3). Although ethanol treatment did not alter HDAC6 subcellular distributions in polarized WIF-B cells, it led to a 25% decrease in HDAC6 protein levels^[39]. HDAC6 binding to endogenous microtubules was also found to be significantly impaired by about 70% in ethanol-treated cells and this impairment partially required ethanol metabolism. Measuring HDAC6 tubulin deacetylase activity by two methods further revealed that ethanol did not impair HDAC6's ability to bind or deacetylate exogenous tubulin. This suggests that tubulin from ethanol-treated cells was modified, thereby preventing HDAC6 binding^[39].

Although decreased HDAC6 protein levels are a simple explanation for increased tubulin acetylation, it is likely that the impaired microtubule binding has more impact. HDAC6 is abundant in the liver^[56,59] such that a 25% decrease in levels may not likely have profound effects on tubulin acetylation. Rather, the 70% impairment in HDAC6 binding to microtubules may have a more dramatic effect; much less of the available enzyme can bind its substrate leading to decreased deacetylation. At present, the nature of the alcohol-induced tubulin modification is not known, but there are some interesting possibilities. Both impaired HDAC6 binding to microtubules and alcohol-induced tubulin hyperacetylation require ethanol metabolism,

and from studies using 4-MP, both events are likely mediated by acetaldehyde^[11,39]. Because this highly reactive ethanol metabolite can readily and covalently modify a highly reactive lysine in α -tubulin *in vitro*^[60], one provocative possibility is that tubulin acetaldehyde adducts impede HDAC6 binding. Because decreased HDAC6 binding to microtubules was only partially prevented by 4-MP, it is also possible that other reactive ethanol metabolites form detrimental tubulin adducts. Future studies are needed to elucidate this and other details.

Mitochondrial protein hyperacetylation and sirtuins

The ever-expanding list of ethanol-induced hyperacetylated mitochondrial proteins is likely to generate much interest in the mitochondrial-specific sirtuins, but so far, only protein levels of SirT3 and 5 have been examined. Although SirT3 is considered the predominant mitochondrial deacetylase, its expression levels were not changed in livers from ethanol-fed rats^[13]. In contrast, SirT5 protein levels were significantly decreased^[61]. To date, nothing is known about the subcellular distributions, activities or substrate specificities of these deacetylases in ethanol-treated cells. Furthermore, virtually nothing is known about the HATs required for mitochondrial protein acetylation. Clearly, this is a fertile area of investigation for many researchers.

Cytosolic protein hyperacetylation and nonnuclear HATs and HDACs

Recent proteomics studies have revealed that many cytosolic proteins are hyperacetylated upon ethanol exposure (Table 2). At present, very little is known about the HATs or HDACs responsible for these modifications. However, attention is being turned to those modifying enzymes that are exclusively cytosolic (HDAC6) or those that shuttle between the nucleus and cytoplasm (PCAF, TIP60 and HDACs 5, 7, 9 and 10) (Figure 1, bottom panel). Not only will future studies likely provide a better understanding of alcohol-induced protein acetylation and the progression of hepatotoxicity, they will also undoubtedly identify new substrates for these enzymes. As for studies on the mitochondrial modifying enzymes, this area of research promises to be fruitful.

CONSEQUENCES OF ETHANOL-INDUCED LYSINE HYPERACETYLATION

Although there is an ever-expanding list of proteins that are known to be hyperacetylated upon ethanol exposure, little is known about the functional consequences of this modification. In general, the added acetyl group likely neutralizes the positive charge on lysine while increasing the overall size and hydrophobicity of the side chain. Such changes may result in protein conformational changes that alter function, albeit to a much lesser extent than the addition of a large, highly

charged phosphoryl group. Also, lysine acetylation sites have been identified that overlap with nuclear localization signals^[23] such that the modification may induce altered protein subcellular distributions. Not only can lysine residues be acetylated, they can also be methylated, sumoylated and ubiquitinated such that ethanol-induced hyperacetylation may displace other modifications further altering protein function. In fact, p300 acetylation has been shown to prevent its sumoylation thereby repressing its activity^[62]. Clearly, mechanistic studies are required to not only understand the functional consequences of acetylation in the normal liver, but also how alcohol-induced hyperacetylation alters hepatic function in the alcoholic liver. In this section, we will describe some recent advances in our understanding of how ethanol-induced acetylation impairs hepatic function. Although it is too early for specific mechanistic details, the results provide an exciting framework for continued investigation.

Histone H3 hyperacetylation alters transcriptional regulation

Alcohol consumption has long been known to lead to changes in gene expression. Many genes are up-regulated including those encoding for enzymes involved in alcohol metabolism, lipogenesis and the regulation of oxidative stress^[63,64]. Many genes are also down-regulated, but fewer seem to functionally group together^[63,64]. The specific mechanisms responsible for changes in alcohol-induced gene expression are not well defined. Recent studies have been aimed at understanding the role of histone modifications in transcriptional regulation. The amino-termini of histones are characterized by at least six different modifications (acetylation, ubiquitinylation, methylation, phosphorylation, sumoylation and ADP-ribosylation) occurring on lysines, arginines, serines, threonines and histidines^[65]. In general, these reversible modifications are thought to change the net negative charge of the amino-terminal domain leading to altered DNA binding and changes in gene expression^[66]. Thus, the simple hypothesis is that alcohol-induced lysine 9 hyperacetylation will decrease the amino-terminal net negative charge thereby loosening histone H3 associations with DNA. The relaxed DNA becomes more accessible to the transcriptional machinery, leading to enhanced transcription.

This prediction was tested in ethanol-treated hepatocytes using a series of chromatin immunoprecipitations. In general, histone H3 with acetylated lysine 9 residues was found to be more highly associated with the promoters of genes known to be up-regulated by ethanol exposure (ADH and glutathione S-transferase)^[9], consistent with this hypothesis. Also consistent with this hypothesis is that histone H3 with methylated lysine 9 residues (this modification is associated with gene silencing) was more highly associated with promoters of genes known to be down-regulated by ethanol (L-serine dehydratase and CYP 2C11)^[9]. Somewhat surprisingly, histone H3 containing methylated lysine 4 residues was found to be highly associated with ADH and glutathione

S-transferase promoters, not with promoters of down-regulated genes^[9]. These associations suggest that lysine 4 hyperacetylation and lysine 4 methylation enhance gene expression while lysine 9 methylation represses transcription.

At present, the site-specific differences in promoter associations cannot be fully explained. One implication is that promoter regions are characterized by specific microenvironments that are differentially accessible to histone modifying enzymes and by extension, are differentially affected by ethanol exposure. In order to fully understand these site-specific differences, it will be necessary to fully account for all six possible histone H3 modifications and the modifying enzymes in control and ethanol-treated cells. Also, to determine how each modification or combination of modifications regulates gene expression, assays that directly monitor transcription activation (rather than promoter associations) will need to be developed. Furthermore, recent reports demonstrate that histone hyperacetylation may also lead to nucleosome instability allowing transcription at cryptic promoters resulting in aberrant gene product expression^[67,68]. This emerging hypothesis must be considered as the transcriptional consequences of ethanol-induced histone acetylation as elucidated.

Ethanol-induced acetylation of SREBP-1 and PGC-1 α leads to altered lipid metabolism

One of the first clinical manifestations of alcohol consumption is the appearance of a fatty liver (steatosis). This is correlated with the up-regulation of many lipogenic enzymes that leads to the alcohol-induced synthesis of hepatic triglycerides and phospholipids^[69]. An active area of steatosis research is aimed at identifying members of the transcriptional machinery that regulate gene expression of the enzymes involved in lipid metabolism. It has long been appreciated that SREBP-1 promotes the expression of many genes involved in lipogenesis that are up-regulated after alcohol consumption^[69]. However, the exact mechanism by which SREBP-1 leads to enhanced expression is not well-defined. One emerging hypothesis is that SREBP-1 acetylation plays an important role. SREBP-1 is known to be acetylated in its DNA binding domain, and that when acetylated, DNA binding is enhanced. SREBP-1 is also known to be ubiquitinated on the same lysine residues leading to its proteosomal degradation^[70]. Thus, in alcohol-treated hepatocytes the prediction is that hyperacetylation prevents SREBP-1 proteosomal degradation by displacing the ubiquitin while enhancing its DNA binding which leads to increased transcription of lipogenic enzymes.

A similar scenario is emerging for the transcriptional activator, PGC-1 α , but it is the deacetylated protein that up-regulates expression of genes regulating fatty acid β -oxidation^[22]. Therefore in ethanol-treated hepatocytes, the prediction is that the hyperacetylated PGC-1 α will be inactive. Based on this prediction and the one for SREBP-1, a straightforward scenario emerges. Alcohol-induced SREBP-1 hyperacetylation enhances lipid

synthesis by activating lipogenic enzyme transcription while PGC-1 α hyperacetylation impairs lipid catabolism by inhibiting transcription of enzymes involved in fatty acid oxidation. The altered transcriptional activation in either case leads to hepatic fatty acid accumulation that likely contributes to development of steatosis. Because many other components of the transcriptional machinery are known to be acetylated (Tables 3 and 4), it is likely that alcohol-induced hyperacetylation will have far-reaching effects on hepatic gene expression.

Ethanol-induced microtubule acetylation leads to impaired protein trafficking

Because microtubules are central to multiple cellular processes, changes in their dynamics will likely alter hepatic function. An active area of research has been aimed at understanding the relationship between protein trafficking and alterations in microtubule dynamics. Not only is protein trafficking microtubule-dependent, the trafficking of many hepatic proteins is also impaired by ethanol^[71-74]. Two transport pathways appear to be affected: transport of newly-synthesized secretory or membrane proteins from the Golgi to the basolateral membrane and receptor-mediated endocytosis from the sinusoidal surface (Figure 1). One attractive hypothesis is that the alcohol-induced defects in secretion and endocytosis can be explained by increased microtubule acetylation and stability.

To test this hypothesis, recent studies have examined the trafficking of selected proteins in WIF-B cells treated with ethanol or TSA, a potent inhibitor of HDAC6, the major tubulin deacetylase in liver and WIF-B cells^[39] (Table 3). Importantly, TSA induces increased microtubule acetylation and stability to the same extent as ethanol^[75]. As shown previously *in situ*, the endocytic trafficking of asialoglycoprotein-receptor (ASGP-R) was impaired in ethanol-treated WIF-B cells^[75] (Figure 1). This impairment required ethanol metabolism and was likely mediated by acetaldehyde^[75]. TSA also impaired ASGP-R endocytic trafficking, but to a lesser extent. Similarly, both ethanol and TSA impaired transcytosis of a single spanning apical resident, aminopeptidase (APN). For both ASGP-R and APN, and for both treatments, the block in trafficking was internalization from the basolateral membrane. Interestingly, no changes in transcytosis of the GPI-anchored protein, 5' nucleotidase (5'NT) (Figure 1), were observed suggesting that increased microtubule acetylation and stability differentially regulate internalization. It was further determined that albumin secretion was impaired in both ethanol- and TSA-treated cells^[75] indicating that increased microtubule acetylation and stability also disrupt this transport step. Thus, increased microtubule acetylation and stability explain, in part, the alcohol-induced defects in membrane trafficking.

There is evidence that suggests that different microtubule populations (and/or their modifications) support specific protein transport steps^[76]. Of particular interest are studies performed in WIF-B cells that used a novel microtubule depolymerizing drug, 201-F^[77]. This

drug specifically depolymerizes dynamic microtubules leaving only stable, acetylated polymers behind. In 201-F-treated cells, both secretion and transcytosis were impaired^[77]. Although the specific impaired transcytotic step was not identified, increased basolateral labeling of the apical proteins was observed. These results are remarkably consistent with the findings in ethanol or TSA treated cells where increased populations of stable microtubules were observed (presumably at the expense of dynamic microtubules) that correlated with impaired albumin secretion and basolateral internalization.

An unanswered question from these studies is why 5'NT distributions were not altered in treated cells. Furthermore, the internalization of cholera toxin B subunit (CTxB) (a known raft marker) was also not impaired by ethanol exposure (Figure 1). One possibility is that internalization mechanisms were differentially impaired by ethanol metabolism. There are at least three major internalization routes in mammalian cells: clathrin-mediated, caveolae/raft-mediated and non-clathrin/non-raft mediated^[78] that are characterized by specific molecular players, cargoes and regulators. In general, the receptors that displayed impaired endocytosis in ethanol-treated hepatocytes *in situ* (ASGP-R, EGF-R, and to a lesser extent, insulin *via* its receptor)^[71-73,79,80] and in WIF-B cells (ASGP-R, transferrin receptor (Tf-R) and polymeric IgA receptor (pIgA-R)^[75] (Figure 1) are internalized *via* clathrin-mediated pathways. Interestingly, the non-clathrin/non-raft-mediated fluid phase uptake of Lucifer Yellow was not changed in livers of ethanol-fed rats or WIF-B cells suggesting this pathway is not affected by ethanol metabolism^[81] (Figure 1). Thus, we propose that the molecular machinery that drives clathrin-mediated endocytosis is more prone to adduction (by acetaldehyde or other reactive metabolites) or covalent modification such that it is selectively impaired by alcohol treatment.

Another unanswered question from these studies is how the acetylation of lysine 40 specifically contributes to enhanced microtubule stability and trafficking defects. Although lysine 40 is thought to reside in the lumen of the microtubule^[82], it is possible that its acetylation may lead to altered tubulin conformation such that interactions with microtubule associated proteins and motors are altered. This hypothesis is supported by the findings that kinesin, dynein and dynactin preferentially bound acetylated microtubules in neuronal cells^[83-85]. This is further supported by the finding that vesicles recovered from livers of ethanol-fed rats have decreased motility *in vitro*^[86]. Clearly, further studies are needed to fully understand these results.

Mitochondrial dysfunction and protein hyperacetylation

Despite the large number of mitochondrial proteins known to be acetylated, little is known about the functional consequences of the modification on mitochondrial function. So far, only glutamate dehydrogenase and AceCS2 activities have been related to their acetylation states, and in both cases, increased acetylation correlated with decreased activity^[25]. Thus, the prediction is that in ethanol-treated hepatocytes,

the hyperacetylated enzymes would be inactivated leading to altered lipid metabolism that contributes to the development of steatosis as described above. Also, as described above, acetylation may function as an on/off switch for these and other mitochondrial metabolic enzymes such that alcohol-induced changes in this modification may have a large impact on hepatic metabolism.

However, recent results from knockout mice are not consistent with this conclusion^[48]. Striking levels of mitochondrial protein acetylation were observed in livers from SirT3 knockout mice whereas mitochondrial acetylation in SirT4 or SirT5 knockout mice was not changed. This suggests that SirT3 is the predominant mitochondrial deacetylase. Despite the high levels of mitochondrial protein acetylation, there was no discernible phenotype in SirT3 knockout mice. Specifically in liver, there was no change in morphology, no change in mitochondrial numbers and metabolism was not altered suggesting hyperacetylation does not affect mitochondrial function^[48]. However, it is possible that hyperacetylation is only detrimental under stressed conditions such that in the alcoholic liver, it leads to mitochondrial dysfunction.

This conclusion is consistent with a recent hypothesis that mitochondrial protein acetylation functions as a sensor for the overall cellular energy status^[23]. Kim *et al.*^[23] suggest that acetyl-CoA and NAD⁺ levels are the key indicators of energy status. Coincidentally, these two molecules serve as cofactors for HATs (acetyl-CoA) or sirtuins (NAD⁺). Furthermore, over 44% of mitochondrial dehydrogenases that require NAD⁺ for activity are known to be acetylated. Thus, one possibility is that lysine acetylation serves as a feedback mechanism for the regulation of dehydrogenases. For example, under NAD⁺-depleting conditions, sirtuins are less active resulting in higher protein acetylation and dehydrogenase activities. In contrast, when acetyl-CoA levels are limiting, HATs are inactivated leading to decreased protein acetylation and increased dehydrogenase activities. Thus, in the alcoholic liver where NAD⁺ is depleting, increased acetylation is predicted to correlate with impaired dehydrogenase activity and by extension, impaired mitochondrial function. Although it has been suggested that NAD⁺ levels recover after prolonged ethanol exposure, the finding that hyperacetylation remains long after chronic ethanol withdrawal^[13] suggests that this mechanism may have physiologic relevance. Clearly, this exciting hypothesis needs to be rigorously tested.

CONCLUSION

Chronic alcohol consumption leads to the hyperacetylation of numerous hepatic nuclear and nonnuclear proteins. Although many interesting and provocative mechanisms have been proposed that describe how hyperacetylation contributes to alcohol-induced hepatotoxicity, future work is clearly needed to test these hypotheses. New therapeutic strategies for

treating patients with chronic liver disease may be aimed at reducing protein acetylation. Currently, specific Sirt1 activators (e.g. resveratrol and SRT-501) are known to be well-tolerated in humans and are in clinical trials for treatment of various metabolic diseases including type 2 diabetes^[87]. Furthermore, resveratrol has been shown to attenuate fatty liver in alcohol-exposed mice^[88]. An exciting possibility is that this drug and other specific deacetylase activators or acetyltransferase inhibitors will be useful in treating alcoholic liver disease.

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Role of cathepsin B-mediated apoptosis in fulminant hepatic failure in mice

Bing-Zhu Yan, Wei Wang, Li-Yan Chen, Man-Ru Bi, Yan-Jie Lu, Bao-Xin Li, Bao-Shan Yang

Bing-Zhu Yan, Wei Wang, Li-Yan Chen, Man-Ru Bi, Bao-Shan Yang, Department of Infectious Diseases, the Second Clinical Hospital of Harbin Medical University, Harbin 150086, China

Yan-Jie Lu, Bao-Xin Li, Department of Pharmacology, Harbin Medical University, Harbin 150086, China

Author contributions: Yan BZ and Yang BS designed the study; Yan BZ, Wang W, Chen LY, Bi MR performed the experiment and analyzed the data; Yan BZ wrote the paper; Lu YJ, Li BX assisted in the experiment.

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Correspondence to: Bao-Shan Yang, Professor, Department of Infectious Diseases, the Second Clinical Hospital of Harbin Medical University, No. 246 Xuefu Road, Nangang District, Harbin 150086, China. baoshanyang@hotmail.com

Telephone: +86-451-86297509 Fax: +86-451-86605330

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Abstract

AIM: To investigate the pathogenic role of cathepsin B and the protective effect of a cathepsin B inhibitor (CA-074Me) in fulminant hepatic failure in mice.

METHODS: LPS/D-Gal N was injected into mice of the model group to induce fulminant hepatic failure; the protected group was administered CA-074me for 30 min before LPS/D-Gal N treatment; the normal group was given isochoric physiologic saline. Liver tissue histopathology was determined with HE at 2, 4, 6 and 8 h after Lps/D-Gal injection. Hepatocyte apoptosis was examined by TUNEL method. The expression of cathepsin B in liver tissues was investigated by immunohistochemistry, Western blot and RT-PCR.

RESULTS: Compared with the normal group, massive typical hepatocyte apoptosis occurred in the model group; the number of apoptotic cells reached a maximum 6 h after injection. The apoptosis index (AI) in the protected group was clearly reduced (30.4 ± 2.8 vs 18.1 ± 2.0 , $P < 0.01$). Cathepsin B activity was markedly increased in drug-treated mice compared with the normal group ($P < 0.01$). Incubation with LPS/D-Gal N at selected time points resulted in a time-dependent increase in cathepsin B activity, and reached

a maximum by 8 h. The expression of cathepsin B was significantly decreased in the protected group ($P < 0.01$).

CONCLUSION: Cathepsin B plays an essential role in the pathogenesis of fulminant hepatic failure, and the cathepsin B inhibitor CA-074me can attenuate apoptosis and liver injury.

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Key words: Fulminant hepatic failure; Hepatocyte apoptosis; Cathepsin B; CA-074me

Peer reviewer: Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

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INTRODUCTION

Fulminant hepatic failure is a rare, but severe, complication of acute hepatitis; it is associated with very high mortality. It has been reported that fulminant hepatic failure is an inflammatory process that causes the death of liver cells by necrosis or by triggering apoptosis^[1-3]. Cathepsins are a family of proteolytic enzymes, many of which, including cathepsin B, are cysteine proteinases. Recent evidence suggests that cathepsin B contributes to cell apoptosis^[4,5]. It is not known if cathepsin B-mediated hepatocyte apoptosis is involved in the pathogenesis of fulminant hepatic failure. The aim of the present study was to determine if fulminant hepatic failure contributes to a change in the expression of cathepsin B protein and mRNA. To ascertain its pathogenic role in hepatic failure, we examined the protective effect of a cathepsin B inhibitor, CA-074Me [N-(L-trans-propylcarbonyloxirane-2-carbonyl)-L-isoleucyl-L-proline] on fulminant hepatic failure in mice.

MATERIALS AND METHODS

Animals

Kunming mice (male, 18-20 g, 4 wk of age) were used. Animals were provided by the Animal Center of the First Clinical Hospital of Harbin Medical University. This study conformed to Harbin Medical University's guidelines for the care and use of laboratory animals.

Experimental groups

Seventy-two mice were randomized to three groups (normal control, model and protected). Galactosamine (D-Gal N) 800 mg/kg and lipopolysaccharide (LPS) 100 µg/kg were injected into the abdominal cavity of mice of the model group; mice in the protected group were administered CA-074me (10 mg/kg) for 30 min before LPS/D-Gal N treatment; and the normal control group was given isochoric physiologic saline. Six mice in each group were killed 2, 4, 6 and 8 h after injection. The liver was cut into small pieces and snap-frozen in liquid nitrogen and stored at -70°C, or fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C.

Materials

Goat anti-mouse cathepsin B polyclonal IgG and P-conjugated secondary antibodies for immunoblotting were purchased from Santa Cruz Biotechnology Inc., USA; D-Gal N, LPS and CA-074me from Sigma (USA); TUNEL reagent kit from Zhong Shan Biotechnical Ltd (Beijing, China); immunohistochemical kit from Zymed (series SP kits); ABC reagent and DAB kits from Wuhan Boster Biological Technology Co, Ltd (Wuhan, China); Trizol kits from Invitrogen (USA); and the reverse transcription-polymerase chain reaction (RT-PCR) kit from Promega (USA). Primers were synthesized by Shanghai Sangon Biological Engineering Technology Co, Ltd (Shanghai, China).

Histology and TUNEL assay

The liver was fixed in 4% paraformaldehyde for 48 h, and embedded in paraffin. Tissue sections were prepared with a microtome and placed on glass slides. Hematoxylin and eosin staining was done by standard methods. TUNEL assay was carried out with a commercially available kit according to the manufacturer's instructions. Hepatocyte apoptosis in liver sections was quantified by counting the number of TUNEL-positive cells in microscopic high-power fields.

Immunohistochemistry

Sections were incubated with goat anti-mice cathepsin B, which was pre-diluted by the manufacturer for staining formalin-fixed paraffin-embedded tissues. After washing the sections exhaustively, they were incubated for 45 min with biotin-conjugated anti-goat IgG antibody, and then with horseradish peroxidase (HRP)-conjugated streptavidin. Negative control slides were incubated with non-immune immunoglobulin under identical conditions. Liver cell endochylema or nucleus containing

yellow granulation served as a positive control, followed by semi-quantitative analysis using Image-plus 6.0 software.

RT-PCR

Total RNA was obtained from whole liver using trizol reagent. The RNA sample was reversely transcribed into cDNA according to manufacturer's instructions. The primers for the experiment were as follows: cathepsin B^[6], forward 5'-GAAGAAGCTGTGTGGCACTG-3', and reverse 5'-GTTTCGGTCAGAAATGGCTTC-3' (yielding a 198-bp product); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CTGCACCACCAACTGCTTAG-3', and reverse 5'-GTCTGGGATGGAAATT GTGA-3' (660-bp). GAPDH was used as a control for RNA integrity. Thermal cycling conditions were 15 seconds at 96°C, 62°C for 20 s, and 1 min at 70°C. Amplification was stopped after 34 cycles. Ten microliters of PCR products were obtained in each group, and confirmed by gel electrophoresis (coloration by EB, Ethidium Bromide). Gel electrophoresis photographs were taken; the band of Cathepsin B PCR products on electrophoresis gel were quantified using a DNA sequencer equipped with Quantity-One analysis software.

Western blotting

Samples were centrifuged at 10 000 × *g* for 20 min at 4°C to remove solid material. Supernatants were centrifuged at 100 000 × *g* for 1 h at 4°C. Cellular protein from each sample (50 µg) was mixed with sample buffer (0.25 mol/L Tris, pH 6.8, 8% SDS, 40% glycerol, 2.5% bromophenol blue, and 2% β-mercaptoethanol), heated for 3 min at 95°C, applied to a 12% acrylamide gel, separated by electrophoresis (SDS-polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h. Blots were incubated in cathepsin B polyclonal antibody at a dilution of 1: 200 for 2 h. After washed three times for 10 min with TBST, blots were incubated with HRP-conjugated anti-goat secondary antibody (1:5000) for 1 h. Following the secondary antibody incubations, blots were developed using an ECL-plus kit. Blots were visualized using the chemiluminescence detection system (Amersham Pharmaceuticals, Amersham, UK).

Statistical analyses

Data represent at least four independent experiments, and were expressed as the mean ± SD (unless otherwise indicated). The difference was determined using two-way analyses of variance (ANOVA) following SNK. Data were analyzed by SPSS software. *P* < 0.05 was considered to be statistically significant.

RESULTS

Histopathology of liver tissue under light microscopy

Liver histopathology was used to assess if different

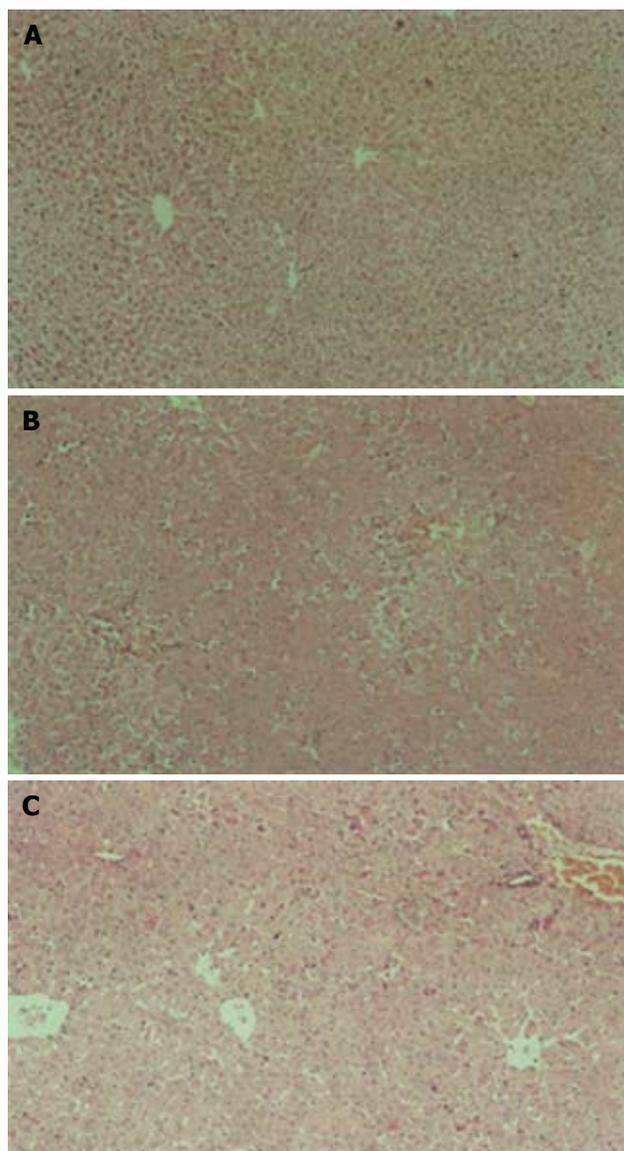


Figure 1 Histopathology of liver tissue at 6 h (HE, $\times 10$). A: Normal group; B: Model group; C: Protected group.

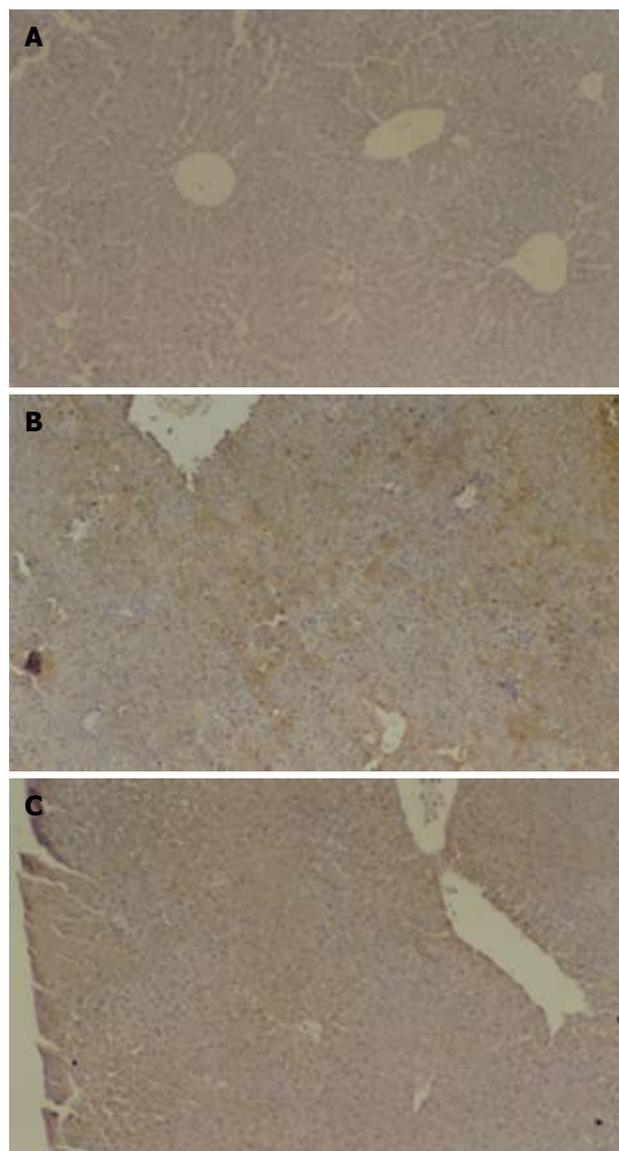


Figure 3 Immunohistochemical analysis of cathepsin B at 8 h (HE, $\times 10$). A: Normal group; B: Model group; C: Protected group.

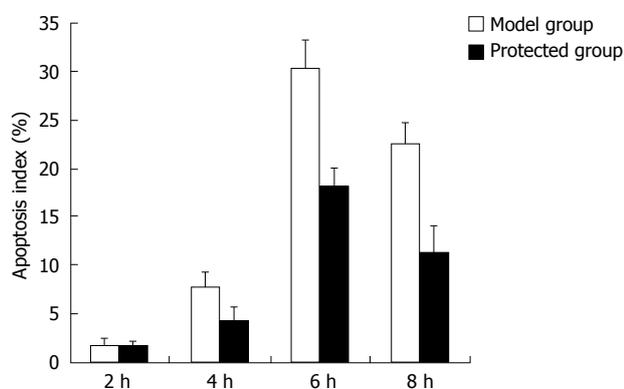


Figure 2 Apoptosis in the model group and protected group measured by TUNEL assay.

groups had different effects on liver injury (Figure 1). Liver histology was normal in the control group. After treatment with D-Gal N and LPS for 6 h, massive

hepatocyte apoptosis was detected in the model group; 8 h after injection, hepatocyte necrosis with massive infiltrates of neutrophils was widely spread. The protected group had far less apoptosis and necrosis, and little evidence of neutrophil accumulation, especially 6 and 8 h after injection.

TUNEL assay

Hepatocyte apoptosis was quantified using TUNEL assay (see Materials and Methods). None or few TUNEL-positive cells were observed in the normal group. In the model group, massive hepatocyte apoptosis occurred, and the number of apoptotic cells increased with time (Figure 2). The apoptosis index (AI) 2 h after injection of D-Gal N/LPS was (1.8 ± 0.7)%, (7.8 ± 1.5)% at 4 h, and reached a climax at 6 h (30.4 ± 2.8)%. After 8-h treatment, AI was lower than that of 6 h, and reached (22.6 ± 2.2)% at 8 h. Compared with the model group, the apoptotic cells in the protected group were

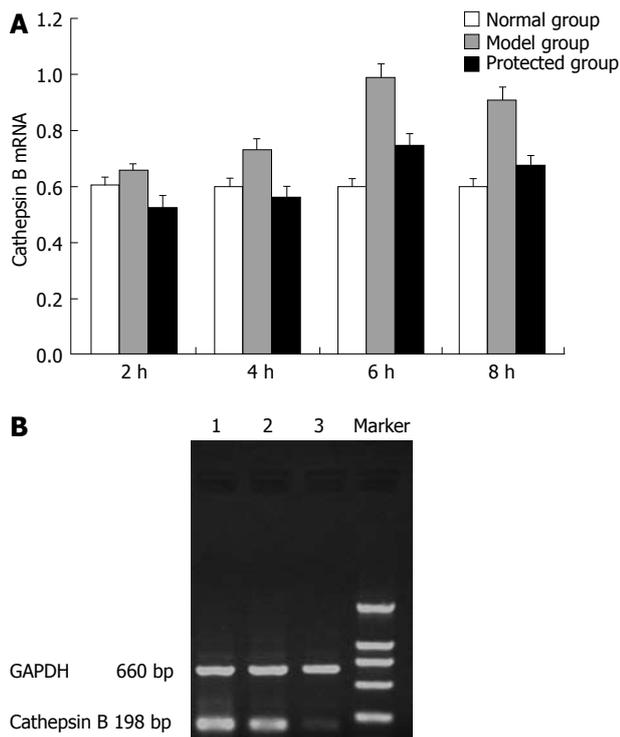


Figure 4 RT-PCR analysis of expression of cathepsin B mRNA in the normal, model and protected groups. A: Expression of cathepsin B mRNA in different groups; B: Images of agarose gel electrophoresis at 6 h; 1: Model group; 2: Protected group; 3: Normal group.

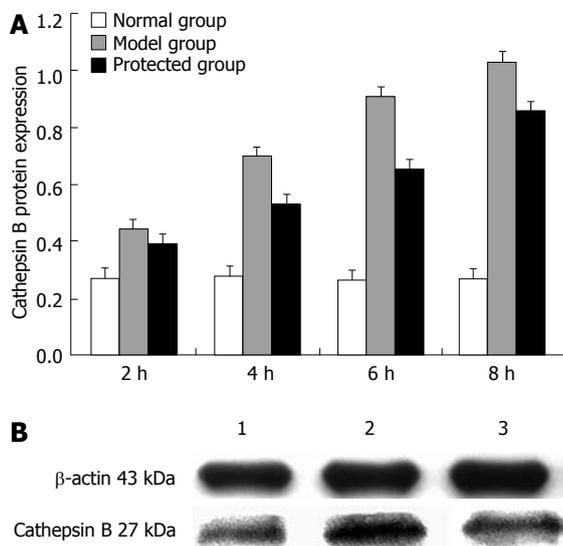


Figure 5 Western blot analysis of expression of cathepsin B protein in the normal, model and protected groups. A: Expression of cathepsin B Protein in different groups; B: Expression of cathepsin B protein at 8 h; 1: Normal group; 2: Model group; 3: Protected group.

obviously reduced at the same time points, particularly at 6 h ($18.1 \pm 2.0\%$) and 8 h ($11.4 \pm 2.6\%$) ($P < 0.01$).

Immunohistochemical analysis of cathepsin B

Immunohistochemistry for cathepsin B was done to confirm apoptosis (Figure 3). Strongly positive immunoreactivity for active cathepsin B was detected in mice injected with LPS/D-Gal N compared with

the normal group. After 6-h and 8-h treatment with LPS/D-Gal N, more expression of cathepsin B was found (0.251 ± 0.010 and 0.280 ± 0.011 , respectively). At the same time points, cathepsin B was found to be significantly less expressed in mice of the protected group (0.202 ± 0.008 and 0.241 ± 0.011 , respectively, $P < 0.01$).

Expression of cathepsin B mRNA

Semi-quantitative RT-PCR was used to assess the expression of cathepsin B mRNA. Using densitometric analysis in comparison with the housekeeping gene GAPDH, we found that the expression of cathepsin B mRNA significantly increased after treatment of LPS/D-Gal N compared with the normal group ($P < 0.01$). The expression of cathepsin B mRNA gradually increased 6 h and 8 h after injection with LPS/D-Gal N and reached a climax at 6 h. At the same time points, expression of cathepsin B mRNA in the protected group was significantly reduced compared with the model group, particularly at 6 h and 8 h (Figure 4).

Western blot analysis

Western blot analysis of cathepsin B protein expression is shown in Figure 5. A prominent band at a molecular size of about 27 kDa was detected, which represented the single-chain form of cathepsin B. Incubation with LPS/D-Gal N at selected time points resulted in a time-dependent increase in cathepsin B protein. The cathepsin B protein began to increase 2 h after treatment with LPS/D-Gal N and increased to maximum activity at 8 h compared with the normal group ($P < 0.01$). Expression of cathepsin B was significantly decreased in the protected group, particularly at 8 h.

DISCUSSION

The traditional view is that hepatocyte necrosis is the main feature of fulminant hepatic failure, but increasing evidence implicates a dominant role for hepatocyte apoptosis in this pathogenesis^[1,7]. The major objective of our study was to evaluate apoptosis-mediated fulminant hepatic failure in LPS/D-Gal N-induced liver injury.

Cathepsin B, a lysosomal cysteine protease, is a candidate for an apoptotic mediator originating from acidic vesicles. Cathepsin B is synthesized as a 38-kDa procathepsin B that undergoes sequential processing steps within lysosomes. First, a 30-kDa mature active form is generated by proteolytic cleavage^[8]. Further processing involves removal of the NH₂-terminal propeptide, cleavage of six residues from the COOH-terminus, and internal excision of residues 127 and 128 to generate a two-chain (a 27-kDa heavy chain and a 4-5-kDa light chain) form of the enzyme with inter-chain disulfide bonds. Pharmacologic inhibition of cathepsin B has been reported to block apoptosis induced by p53 and cytotoxic agents^[9]. Recent evidence suggests that cathepsin B contributes to tumor necrosis factor- (TNF- α)-induced apoptosis *in vitro* and *in vivo*^[10,11]. In cell culture systems, activation of caspase 8

is associated with the release of cathepsin B from acidic vesicles into the cytosol. In the cytosol, cathepsin B was found to induce mitochondrial release of cytochrome C^[12,13] and activate caspase 9 and 3^[14]. The importance of this pathway in TNF- α -mediated apoptosis *in vitro* was shown by demonstrating that hepatocytes isolated from cathepsin B knockout mice are resistant to TNF- α -induced apoptosis. More recently, some colleagues have demonstrated a dominant role for cathepsin B in TNF- α -mediated apoptosis. In a murine tumor cell line, caspase inhibition accentuated TNF- α -induced apoptosis by a cathepsin-B pathway. Inactivation of cathepsin B attenuates hepatocyte apoptosis and liver damage in liver reperfusion injury^[15,16] and cholestasis injury^[17]. CA-074me is a selective inhibitor of cathepsin B^[18]; it is highly cell-permeant and can decrease the expression or activity of cathepsin B. During the early stages of pancreatitis, trypsinogen activation in the pancreas is mediated by cathepsin B, indicating that pharmacological interventions that inhibit cathepsin B may prove useful in preventing acute pancreatitis or reducing its severity^[19].

In summary, our studies suggested a role for cathepsin B in fulminant hepatic failure. Compared with the normal group, massive hepatocyte apoptosis occurred in the model group, and the number of apoptotic cells increased to a maximum at 6 h. Incubation for longer periods did not lead to further increase in apoptosis and instead resulted in an increase of cell necrosis. The apoptosis index in the protected group was obviously reduced. Consistent with these data, the activity of cathepsin B was markedly increased in drug-treated mice compared with the normal group. Incubation with LPS/D-Gal N for selected time points resulted in a time-dependent increase in cathepsin B activity, and reached a maximum at 8 h. Cathepsin B expression was significantly decreased in the protected group. Collectively, these data suggest that LPS/D-Gal N-mediated cathepsin B expression initiates hepatocyte apoptosis in fulminant hepatic failure. Inhibition of cathepsin B attenuates apoptosis and liver injury, supporting a link between cathepsin B and fulminant hepatic failure. Inhibition of hepatocyte apoptosis with CA-074me seems to be a feasible therapeutic option for fulminant hepatic failure.

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COMMENTS

Background

Fulminant hepatic failure is a rare, but severe, complication of acute hepatitis; it is associated with very high mortality. The traditional view is that hepatocyte necrosis is the main feature of fulminant hepatic failure, but increasing evidence implicates a dominant role for hepatocyte apoptosis in this pathogenesis. Recent evidence suggests that cathepsin B contributes to cell apoptosis.

Research frontiers

It is not known if cathepsin B-mediated hepatocyte apoptosis is involved in the pathogenesis of fulminant hepatic failure.

Innovations and breakthroughs

The current study demonstrated that cathepsin B has an essential role in the pathogenesis of fulminant hepatic failure, and the cathepsin B inhibitor CA-074me could attenuate apoptosis and liver injury.

Applications

Inhibition of cathepsin B attenuates apoptosis and liver injury, and CA-074me seems to be a viable therapeutic option for fulminant hepatic failure.

Terminology

Cathepsin B, a lysosomal cysteine protease, is a candidate for an apoptotic mediator originating from acidic vesicles. CA-074me is a specific inhibitor of cathepsin B; it is highly cell-permeant and can decrease the expression or activity of cathepsin B.

Peer review

This is a well-designed, most part well-written paper, with important new data on the pathogenesis of fulminant hepatic failure. Authors have shown that apoptosis is important in the development and that the process can be partially reversed by specific cathepsin-B inhibitor.

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Clinical significance of "anti-HBc alone" in human immunodeficiency virus-positive patients

M^a Teresa Pérez-Rodríguez, Bernardo Sopeña, Manuel Crespo, Alberto Rivera, Teresa González del Blanco, Antonio Ocampo, César Martínez-Vázquez

M^a Teresa Pérez-Rodríguez, Bernardo Sopeña, Alberto Rivera, Antonio Ocampo, César Martínez-Vázquez, Infectious Diseases Unit, Internal Medicine Department, Xeral-Cies University Hospital, 36204 Vigo, Spain

Manuel Crespo, Infectious Diseases Department, Vall d'Hebron Hospital, 08035 Barcelona, Spain

Teresa González del Blanco, Microbiology Department, Xeral-Cies University Hospital, 36204 Vigo, Spain

César Martínez-Vázquez, Bernardo Sopeña, Faculty of Medicine, University of Santiago de Compostela, 15705 Santiago de Compostela, A Coruña, Spain

Authors contributions: Pérez-Rodríguez MT, Sopeña B, González del Blanco T, Ocampo A, and Martínez-Vázquez C contributed equally to this work; Crespo M and Rivera A analyzed data.

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Correspondence to: M^a Teresa Pérez-Rodríguez, Servicio de Medicina Interna, 11th Floor, Hospital Universitario Xeral-Cies de Vigo, C/Pizarro 22. 36204-Vigo, Pontevedra, Spain. maite_perez@yahoos.es

Telephone: +34-986816000 (Ext 216073) Fax: +34-986816029

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Abstract

AIM: To determine the prevalence and clinical relevance of isolated antibodies to hepatitis B core antigen as the only marker of infection ("anti-HBc alone") among human immunodeficiency virus (HIV) type-1 infected patients. Occult hepatitis B infection frequency was also evaluated.

METHODS: Three hundred and forty eight histories from 2388 HIV-positive patients were randomly reviewed. Patients with serological markers of hepatitis B virus (HBV) infection were classified into three groups: past hepatitis, "anti-HBc alone" and chronic hepatitis. Determination of DNA from HBV, and RNA and genotype from hepatitis C virus (HCV) were performed on "anti-HBc alone" patients.

RESULTS: One hundred and eighty seven (53.7%) HIV-positive patients had markers of HBV infection: 118 past infection (63.1%), 14 chronic hepatitis (7.5%) and 55 "anti-HBc alone" (29.4%). Younger age [2.3-fold higher per every 10 years younger; 95%

confidence intervals (CI) 1.33-4.00] and antibodies to HCV infection [odds ratio (OR) 2.87; 95% CI 1.10-7.48] were factors independently associated with the "anti-HBc alone" pattern. No differences in liver disease frequency were detected between both groups. Serum levels of anti-HBs were not associated with HCV infection (nor viral replication or HCV genotype), or with HIV replication or CD4 level. No "anti-HBc alone" patient tested positive for HBV DNA.

CONCLUSION: "Anti-HBc alone" prevalence in HIV-positive patients was similar to previously reported data and was associated with a younger age and with antibodies to HCV infection. In clinical practice, HBV DNA determination should be performed only in those patients with clinical or analytical signs of liver injury.

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Key words: Human immunodeficiency virus; "Anti-HBc alone"; Occult hepatitis; Hepatitis B virus DNA; Liver disease

Peer reviewer: Montiel-Jarquín Alvaro, MD, Hospital General de Zona No. 15, Tehuacan, Instituto Mexicano del Seguro Social, Puebla 72550, México

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INTRODUCTION

Infections by hepatitis B (HBV) and C (HCV) viruses are common in human immunodeficiency virus (HIV) infected patients, since nearly all these viruses share the same routes of transmission. HBV infection is present in between 49.2%-68% of HIV-positive patients^[1-3] and there is evidence that co-infection can modify the natural history of HBV^[4], involves potential consequences on morbidity and mortality and has implications in management of both infections^[5]. In fact, nowadays

HBV status is systematically and regularly assessed and systematic HBV vaccination is proposed in those patients without HBV markers.

Over recent years, numerous studies have been published about HBV-HIV co-infection, though some issues still remain unclear, such as clinical relevance and management of those patients with an isolated positive test for antibodies against hepatitis B core antigen (“anti-HBc alone” or defective immunological response)^[6,7]. This serological pattern is the second most frequent serological profile of HBV infection, occurring in about 30% of HBV infected patients^[1-3].

One of the most controversial questions is about the frequency of occult hepatitis, i.e. HBV DNA positive markers without HBV surface antigen (HBsAg), among patient with a defective immunological response. Some authors consider the “anti-HBc alone” pattern to be a marker of occult HBV infection^[1,3,8,9], whereas others have not been able to demonstrate HBV DNA in the sera of these patients^[10-13].

This work was carried out to establish the prevalence and clinical significance of the “anti-HBc alone” pattern among HIV-positive patients. The frequency of HBV occult infection, determined by standard assays commonly used in routine clinical practice, was also determined.

MATERIALS AND METHODS

Xeral-Cies University Hospital is a 700 bed Vigo University-affiliated Hospital which serves an urban population of about 400 000 inhabitants. From 2388 HIV-positive patients, who were followed-up in a specialised clinic of the hospital, 348 clinical histories were consecutively reviewed. Patients with some HBV markers were classified into three groups: chronic hepatitis [positive HBsAg and IgG anti-HBc, negative anti-HBs and IgM anti-HBc and positive or negative “e” antigen (HBeAg)]; past hepatitis (positive anti-HBs and IgG anti-HBc, negative HBsAg and IgM anti-HBc); and “anti-HBc alone” (positive IgG anti-HBc and negative HBsAg, anti-HBs, IgM anti-HBc and anti-HBe).

“Anti-HBc alone” patients were included only when a confirmatory test, performed two weeks later, showed a concurrent result. Patients that had been vaccinated against HBV were excluded.

In every “anti-HBc alone” patient and in those with past hepatitis epidemiologic characteristics (age, sex, risks conduct), co-morbidities (active infections, renal failure, diabetes, cancer, *etc*), antibodies against HCV, immunoglobulin levels and liver function tests were gathered. HCV RNA, HCV genotype and HIV viral load were tested and CD4 level was determined by flow cytometry. Also, abdominal imaging (ultrasound, CT, magnetic resonance) was performed to evaluate the presence of chronic liver disease (inhomogeneous hepatic texture or surface, rarefied hepatic central vein, an enlarged caudate lobe, splenomegaly or collateral veins)^[14]. In every case, intake of antiretroviral therapy

active against HBV, such as lamivudine or tenofovir, was recorded. Changes in the serological response after introduction of antiretroviral therapy or after an immunological improvement were evaluated. When it was possible, serum was extracted to determine HBV DNA.

Laboratory tests

HBs Ag, anti-HBe and anti-HBc were determined with 3rd generation microparticle enzyme immunoassays (MEIA) for qualitative detection of surface antigen, antibodies against “e antigen” and antibodies against “core antigen” of HBV [AxSYM HBsAg (V2) System, anti-HBe System y Core System. Abbott Laboratories, Chicago, IL, USA]. Anti-HBs was determined by 3rd generation MEIA for quantitative assessment of HBV surface antibodies (Abbott Laboratories, Chicago, IL, USA). Detection limits of the assay were 10-1000 IU/L. Serum HBV DNA levels were determined by an automated quantitative technique of molecular hybridation with genomic amplification; owned primers against the core region of the genome contained in a National “Reference Laboratory” were used. The lower detection limit of this assay was 10⁴ copies/mL.

Antibodies against HCV were measured with 3rd generation MEIA to qualitatively determine this antibody (AxSYM HCV version 3.0 System. Abbott Laboratories, Chicago, IL, USA). Serum HCV RNA was quantified by molecular hybridation using a branched DNA technique [Versant HCV-RNA 3.0 (bDNA) - Bayer Diagnostics]. This assay has a lower detection limit of 3200 copies/mL. HCV genotype was determined by automatic sequencing with a fluorescent marker.

Antibodies against HIV-type 1, HIV-type 2 and p24 antigen were determined at the same time by 3rd generation MEIA (AxSYM HIV Ag/Ab Combo System. Abbott Laboratories, Chicago, IL, USA). Positive test results were confirmed by Western-Blot (NEW LAV-BLOT I Bio-Rad. France). HIV viral load was quantified by the Amplicor HIV-1 Monitor (Roche Diagnostics). The lower detection limit of this assay was 40 copies/mL.

The study was reviewed and approved by the Research Ethical Committee of Vigo Hospitality University Complex. All patients gave informed consent to participate in the study.

Statistical analyses

Results were expressed as absolute values (percentage) and median (interquartile range, IQR) as appropriate. Baseline characteristics were compared by using χ^2 or Fisher exact test for categorical data and Mann-Whitney *U* test for continuous data.

When variables were significantly associated ($P < 0.05$) with a defective pattern in the univariate analysis, a backward logistic regression analysis was conducted to identify those factors independently associated with “anti-HBc alone”.

Table 1 Differences between HIV-positive patients with "anti-HBc alone" and past hepatitis B

	"anti-HBc alone" pattern (n = 55)	Past hepatitis B (n = 55)	P
Age, yr	38 (33-43)	45 (37-49)	0.001
Male, n (%)	38 (69.1)	38 (69.1)	1.000
Transmission mode n (%)			0.021
IDU	44 (80)	34 (61.8)	
Homosexual	4 (7.3)	15 (27.3)	
Heterosexual	7 (7.7)	6 (10.9)	
AST (IU/mL)	43 (23-65)	31 (22-47)	0.092
ALT (IU/mL)	39 (19-73)	29 (20-45)	0.301
Albumin (g/L)	42 (38.8-45.6)	43 (40.8-45.9)	0.261
Anti-HCV positive, n (%)	45 (81.8)	34 (61.8)	0.020
CDC C stage, n (%)	15 (27.3)	19 (34.5)	0.409
CD4 (cells/mm ³)	434 (325-714)	459 (303-636)	0.740
HIV DNA < 50 copies/mL	25 (47.2)	32 (59.3)	0.210

IDU: Intravenous drug user; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

RESULTS

From 348 clinical histories, 187 (53.7%) patients had positive tests against HBV; 118 past hepatitis (33.9%), 14 chronic hepatitis (4%), and 55 "anti-HBc alone" (15.8%). Among patients who had been infected by HBV, 29.4% developed an "anti-HBc alone" pattern and 63% showed a cured past infection. Fifty five patients with past hepatitis were randomly selected to be compared with "anti-HBc alone" patients. Epidemiologic characteristics of both groups are shown in Table 1.

The factors independently associated with a defective pattern were younger age [2.3-fold higher per every 10 years younger; 95% confidence intervals (CI) 1.33-4.00] and antibodies to HCV infection [odds ratio (OR) 2.87; 95% CI 1.10-7.48]. Intravenous drug users (IDU) were significantly more frequent in the "anti-HBc alone" group (80% *vs* 61.8%, $P = 0.021$).

Liver function tests, CD4 levels, HIV viral load or AIDS stages were not significantly different between the two groups. Ultrasound signs of chronic liver disease were only present in HCV co-infected patients ($P < 0.05$). Serum levels of anti-HBs were not associated with HCV infection (nor with viral replication or HCV genotype), and were not associated with HIV replication or CD4 level.

Serum HBV DNA was tested in 30 "anti-HBc alone" patients and no-one was positive. However, 10 patients were taking lamivudine or tenofovir when the tests were performed.

DISCUSSION

In the present study, as in other studies^[1-3], a high prevalence of HBV infection (53.7%) among HIV infected patients was found. Although diverse frequencies in the "anti-HBc alone" pattern have been reported according to different geographic areas or

selected populations^[15,16], the frequency data among HIV patients (24.5-37.8%^[1-3]) are fairly similar to data reported in this study (29.4%).

One of the independent factors related to the defective serological pattern was a younger age. This event has been previously reported in only one study^[17], in which a higher frequency of "anti-HBc alone" status was also found among women. Nevertheless, in our study the proportion of women in the "anti-HBc alone" group was the same as that in the past hepatitis group (30.9%).

The presence of HCV infection is another independent factor identified in our work which has been reported before^[1,3,9,16,18]. A study showed that "anti-HBc alone" phenotype was significantly more frequent in HCV-viraemic than in HCV-recovered patients^[18]. HCV replication could produce a down regulation in HBV replication, and this could be expressed as a defective serological pattern^[7,18].

IDU has also been reported previously as significantly more frequent in the "anti-HBc alone" group^[19] and it has been related to a higher frequency of HCV, as IDU is one of the strongest risk factors for HCV infection.

This is the first study that evaluates liver disease by abdominal imaging scan and no statistically significant differences were found between "anti-HBc alone" patients and past hepatitis patients. In both groups, signs of liver disease were only demonstrated in patients co-infected with HCV.

Occult hepatitis prevalence data reported in "anti-HBs alone" HIV-positive patients varies greatly from 0% to 89.5%^[1-3,9,12,17,20,21]. However, in those studies that have found viral replication, detected viral load was usually very low ($< 10^3$ copies/mL)^[3,20]. Ultra-sensitive PCR techniques (50-10² copies/mL) were broadly used in those experimental studies, but are not available in daily clinical practice. In our study, no case of occult hepatitis was proved by standard assays commonly employed in routine clinical practice that can detect 10⁴ copies/mL. Clinical relevance and management of this low viral replication is unclear because a higher incidence of hepatic damage was not found in these patients^[16]. On the other hand, current therapeutic guidelines do not recommend starting treatment if the viral load is lower than 10⁴-10⁵ copies/mL^[6,21-24]. More than 90% of doctors that attend HIV patients follow this practice^[22]. However, the latest recommendations in HIV-positive patients with the "anti-HBc alone" pattern advise to test for HBV DNA in every patient^[6,10,12,13,20]. We believe that HBV DNA testing should be performed only in those patients with an unexplained high level of alanine aminotransferase (ALT) or signs of liver disease.

The present study has some limitations. HBV DNA was not tested in every "anti-HBc alone" patient but non medical reasons prevented us from getting some serum specimens. Moreover, some tested patients were taking lamivudine or tenofovir. However, a study showed that the mean HBV load was similar among patients whether

or not they were treated with lamivudine, and that this was probably associated with an increasing number of resistance mutations^[2]. Furthermore, in another study in which one case of occult hepatitis was demonstrated the patient was on lamivudine^[11]. The present study displays a complete evaluation of HIV-positive patients with the “anti-HBc alone” pattern, since clinical, virological and radiological parameters have been considered in these patients.

In conclusion, in our population “anti-HBc alone” prevalence in HIV-positive subjects is similar to previously reported data and is associated with a younger age and with antibodies to HCV infection. After evaluating the results of the present study and others with similar results, HBV DNA determination should not be performed in every patient with the “anti-HBc alone” pattern, but only in those patients with unexplained clinical or analytical signs of liver injury.

COMMENTS

Background

Over recent years, numerous studies have been published about hepatitis B virus (HBV)-human immunodeficiency virus (HIV) co-infection, though some issues remain still unclear, such as clinical relevance and management of those patients with “anti-HBc alone” pattern or the frequency of occult hepatitis among patients with a defective immunological response. Some authors consider the “anti-HBc alone” pattern to be a marker of occult HBV infection, whereas others have not been able to demonstrate HBV DNA in the sera of these patients.

Research frontiers

The authors studied the prevalence and clinical significance of the “anti-HBc alone” pattern among HIV-positive patients and the frequency of HBV occult infection, determined by standard assays commonly used in routine clinical practice.

Innovations and breakthroughs

This is the first study that evaluates liver disease by abdominal imaging scan and no statistically significant differences were found between “anti-HBc alone” patients and past hepatitis patients. In both groups, signs of liver disease were only demonstrated in patients co-infected with hepatitis C virus (HCV). No single case of occult hepatitis was proved by standard assay commonly employed in routine clinical practice that can detect 10⁴ copies/mL.

Applications

Although the latest recommendations in HIV-positive patients with the “anti-HBc alone” pattern advise to test for HBV DNA in every patient, this and other studies show that viral load reported in these patients is usually low. Current therapeutic guidelines do not recommend starting treatment when viral load is lower than 10⁴-10⁵ copies/mL. The authors believe that HBV DNA testing should be performed only in those patients with an unexplained high level of alanine aminotransferase (ALT) or signs of liver disease.

Terminology

“Anti-HBc alone” pattern or defective immunological response: positive hepatitis B core antigen as the only marker of hepatitis B infection. Occult hepatitis B: HBV DNA positive without HBV surface antigen.

Peer review

The “anti-HBc alone” pattern is very common among HIV-positive patients and it is not associated with liver injury. However this serological pattern can be associated with occult hepatitis B, usually with a very low viral load of HBV. The authors recommend testing DNA HBV only in those patients with the “anti-HBc alone” pattern and unexplained high levels of ALT or signs of liver disease.

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BRIEF ARTICLES

Prognostic factors for 5-year survival after local excision of rectal cancer

Dong-Bing Zhao, Yong-Kai Wu, Yong-Fu Shao, Cheng-Feng Wang, Jian-Qiang Cai

Dong-Bing Zhao, Yong-Kai Wu, Yong-Fu Shao, Cheng-Feng Wang, Jian-Qiang Cai, Cancer Institute (Hospital), Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Author contributions: Zhao DB, Shao YF and Cai JQ contributed equally to this work; Zhao DB, Shao YF, Wang CF and Cai JQ performed the research; Wu YK and Zhao DB analyzed the data; Zhao DB wrote the paper.

Correspondence to: Dong-Bing Zhao, Cancer Institute (Hospital), Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China. dbzhao2003@sina.com

Telephone: +86-10-87787120 Fax: +86-10-67730386

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recurrence, active postoperative follow-up, and administration of salvage therapy are the effective methods to increase the efficacy of local excision of rectal cancer.

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Zhao DB, Wu YK, Shao YF, Wang CF, Cai JQ. Prognostic factors for 5-year survival after local excision of rectal cancer. *World J Gastroenterol* 2009; 15(10): 1242-1245 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1242.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1242>

Abstract

AIM: To evaluate the prognostic factors for 5-year survival after local excision of rectal cancer, and to examine the therapeutic efficacy and surgical indications for this procedure.

METHODS: Clinical data, obtained from 106 local rectal cancer excisions performed between January 1980 and December 2005, were retrospectively analyzed. Survival analysis was performed using the Kaplan-Meier method, statistical comparisons were performed using the log-rank test, and multivariate analysis was performed using the Cox proportional hazards model.

RESULTS: Transanal, transsacral, and transvaginal excisions were performed in 92, 12, and 2 cases, respectively. The rate of complication, local recurrence, and 5-year survival was 6.6%, 17.0%, and 86.7%, respectively. Univariate analysis showed that T stage, vascular invasion, and local recurrence were related to the prognosis of the cases ($P < 0.05$). Multivariate analysis showed that T stage [$P = 0.011$, 95% confidence interval (CI) = 1.194-3.878] and local recurrence ($P = 0.022$, 95% CI = 1.194-10.160) were the major prognostic factors for 5-year survival of cases after local excision of rectal cancer.

CONCLUSION: Local rectal cancer excision is associated with few complications, and suitable for stages Tis and T1 rectal cancer. Prevention of local

INTRODUCTION

In China, unlike in Western countries, rectal cancer accounts for approximately 70% of colorectal cancers. Both abdominoperineal resection and anterior resection of rectal cancer can result in postoperative urinary impairment and sexual dysfunction, and the presence of a permanent colostomy significantly impacts the quality of life. With the advancement of imaging techniques, the accuracy of preoperative rectal cancer staging has increased dramatically, and the preservation of physical function in rectal cancer patients has become a very important aim of research. Local tumor excision preserves anal, urinary, and sexual function in patients with low rectal cancer. Some patients with early tumor have suitable indications for local tumor excision^[1-5]. This study was to determine the prognostic factors for survival after local excision of rectal cancer.

MATERIALS AND METHODS

Clinical data

This study included 106 low rectal cancer patients at the age of 26-81 years (60 males, 46 females, with a median age of 60 years) who underwent local tumor excision at our hospital between January 1980 and December 2005. The main symptom on presentation was rectal bleeding

(95 patients). Other symptoms included passage of mucus, altered bowel habits. Tumors were located at the anterior wall in 45 patients, posterior wall in 35 patients, and lateral wall in 21 patients, none located in 5 patients. The diameter of the tumor was ≤ 3.0 cm in 70.5% (80/106) patients and > 3.0 cm in 17.9% (19/106) patients. Colonoscopy or barium enema was performed to exclude multiple primary tumors, while ultrasound, chest X-ray, or computed tomography (CT) was conducted to exclude distant metastases, thus a pathological diagnosis was made before operation.

Treatment method

Transanal excision (TAE), transsacral excision (TSE), and transvaginal excision (TVE) were performed in 92, 12, and 2 patients, respectively. The margin was excised 1-2 cm from the tumor, and postoperative pathology reports indicated negative margins in all cases.

Four patients received preoperative radiotherapy and 41 patients underwent postoperative radiotherapy at the dose of 14-75 Gy (mean 53.5 Gy).

Follow-up

Of the 106 patients, 104 received follow-up (by outpatient appointments, telephone, or mail), and the follow-up rate was 98.1%. Follow-up was performed between 11 mo and 20 years after operation, and the median follow-up time was 72 mo. During the follow-up period, 14 patients died. Of them, 9 died of tumor metastasis, 4 of other conditions, and 1 of a second primary esophageal cancer, respectively.

Statistical analysis

SPSS 13.0 software was used for statistical processing. Survival analysis was performed using the Kaplan-Meier method, univariate analysis of prognostic factors was performed using the log-rank test, and multivariate analysis was performed using the Cox proportional hazards model. $P < 0.05$ was considered statistically significant.

RESULTS

Complications

The complication rate was 6.6% (7/106). Postoperative bleeding occurred in 1 case and wound breakdown was observed in 1 case after TSE. Four cases had anastomotic leakage after TAE, 1 of them was accompanied with bleeding. One case had rectovaginal fistulation after TVE. No death occurred during surgery.

Local recurrence and related factors

Of the 106 patients, 18 (17%) had local recurrence 4-174 mo (mean 48.3 mo) after operation. The local recurrence rate for Tis-, T1-, and T2-stage tumors was 7.1%, 18.7%, and 20.8%, respectively ($P = 0.331$), while the local recurrence rate for tumors with or without vascular invasion was 66.7% and 15.5%, respectively ($P = 0.02$). Surgical treatment was reattempted for 12 patients after recurrence. Local excision was

performed in 5 patients. Of them, 1 died after 93 mo and the other 4 survived. Abdominoperineal resection was performed in 6 patients, and Park's procedure was performed in 1 patient. Of these 7 patients, 2 died after 36 and 40 mo, respectively, the other 5 survived. Distal metastases were present in 9 patients, who were then treated with chemotherapy, radiotherapy, or interventional therapy. Four T3 stage patients received preoperative radiotherapy, and local excision was performed after down staging. Tumors recurred in 2 patients with a recurrence rate of 50%.

Prognosis and influencing factors

The overall 5-year survival rate for our patients was 86.7%. The 5-year survival rate for Tis-, T1-, and T2-stage tumors was 100%, 88.8%, and 67.9%, respectively. Univariate analysis showed that infiltration depth, vascular invasion, and local recurrence were the prognostic factors for tumors ($P < 0.05$, Figure 1). Multivariate analysis showed that T stage [$P = 0.011$, OR = 2.152, 95% confidence interval (CI) = 1.194-3.878] and local recurrence ($P = 0.022$, OR = 3.483, 95% CI = 1.194-10.160) were the major prognostic factors for tumors.

DISCUSSION

Although radical resection is an effective treatment for rectal cancer, it can lead to anal, urinary, and reproductive function impairment as well as surgical complications and death. Morson *et al*^[6] have reported a 5-year survival rate of 82% in patients after local excision of early rectal cancer. Subsequent studies demonstrated that local excision of early stage low rectal cancer can produce a good outcome and preserve anal, urinary, and reproductive function^[3,7-10], but a higher local recurrence rate of tumor (5%-27%) has limited its wide application. Nevertheless, the 5-year survival rate after local tumor excision reported in the literature is 72%-95%^[3,7-10]. In our study, the local recurrence rate was 17.0% and the 5-year survival rate was 86.7%, respectively, which are similar to the reported data^[3,7-10].

Risk factors affecting low rectal cancer prognosis after excision include the age and sex of patients, treatment methods, and vascular invasion and tumor stage. In our study, the age and sex of patients were unrelated to the prognosis of low rectal cancer. Vascular invasion and tumor stage affected rectal cancer recurrence and prognosis after local excision. Bouvet *et al*^[11] found that T stage is the most important pathological factor for local rectal cancer recurrence after excision. In our patients, excision of Tis, T1, and T2 tumors was associated with the 5-year survival rate ($P < 0.05$). The local recurrence and distant metastasis rates were significantly increased when the tumor was poorly differentiated, macroscopically ulcerative, or larger than 3.0 cm in diameter. However, because the number of patients was small in our study, the differences were not statistically significant. It was reported that rectal cancer patients also have a higher rate of lymphatic spread^[12,13], and therefore local excision should be recommended

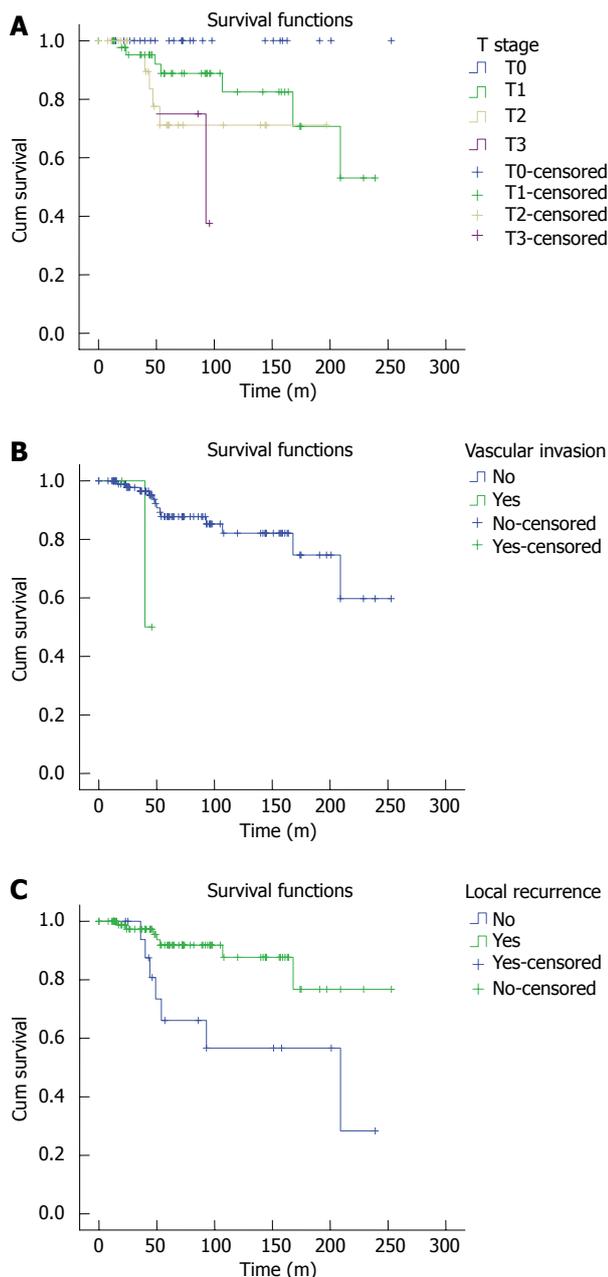


Figure 1 Survival curves for patients with different tumor T stages (A), different vascular invasions (B), and different local recurrence rates (C). The survival rate for the patients with T0 ($n = 28$), T1 ($n = 48$), T2 ($n = 26$), T3 ($n = 4$) varied significantly ($P = 0.022$). Patients without vascular invasion ($n = 103$) had a higher survival rate than those with vascular invasion. ($n = 3$) ($P = 0.01$). Patients without local recurrence ($n = 88$) had a higher survival rate than those with local recurrence ($n = 18$) ($P = 0.005$).

with caution for such patients.

The excision method of local tumor may affect its prognosis. If R0 tumor excision is achieved, the surgical method used does not affect its local recurrence rate or the survival rate of patients. In our patients, all margins on pathological examination were negative. It was reported that if tumor margin is positive, the recurrence rate of tumor will be high and its prognosis is poor^[14]. Therefore, if the margin is positive or borderline positive, the excision margins should be widened, otherwise, local tumor excision should be abandoned. Frozen section examination of margins during surgery

ensures margin negativity, which is essential for complete local tumor excision. It was reported that adjuvant chemotherapy and radiotherapy for tumor after local excision can reduce its local recurrence rate^[15]. Our data show that radiotherapy was unrelated to local recurrence and prognosis of tumor, which may be due to the different indications for radiotherapy, and the standardized treatment modalities. It is generally believed that rectal cancer patients should undergo adjuvant chemotherapy and radiotherapy after local excision of T2 rectal tumor and poorly-differentiated or high-risk T1 rectal tumor^[16].

Salvage therapy is of great importance in the treatment of recurrent rectal tumor after local excision and can be performed when rectal wall involvement is significantly greater than extramural or pelvic cavity involvement. Therefore, the frequency of second surgery is higher in patients treated with local excision than in patients treated with radical resection. Mellgren *et al*^[3] reported 17 cases (68%) of recurrent rectal cancer in the rectal wall and 8 cases (32%) of recurrent rectal cancer in extramural. Sengupta *et al*^[4] found that 40%-100% of recurrent rectal cancer patients could be cured. Friel *et al*^[7] used salvage therapy for 29 T1 or T2 tumor patients with recurrence after local excision (90% involving the rectal wall and 10% completely extramural), and found no residual tumor in 17 survivors after a mean follow-up time of 39 mo. Of the 18 patients with rectal tumor recurrence in our study, 12 were retreated surgically. Of the 5 patients treated with local excision, 1 died after 93 mo and 4 survived. In the 6 patients treated with abdominoperineal resection and 1 patient treated with Park's procedure, 2 died after 36 and 40 mo, respectively, and the other 5 survived, suggesting that salvage therapy is effective against early recurrent rectal cancer.

In rectal cancer patients who are unsuitable for or refuse abdominoperineal resection, local excision can be performed after the tumor is down staged^[17]. Kim *et al*^[18] showed that preoperative adjuvant therapy can effectively down stage T2 and T3 rectal cancer. In their study, 25 patients who were staged before operation by endorectal ultrasound (ERUS) received radiotherapy (45 Gy, 25 cycles) and chemotherapy (5-fluorouracil, 300 mg/m² per day, 5 d/week). Partial pathological response was achieved in 4 patients and complete response in 22 patients. In the 22 patients with complete-response, no recurrent rectal cancer occurred after local excision. In our 4 patients with preoperative stage T3 rectal cancer treated with local excision after chemotherapy, the cancer recurred in 2 cases with a recurrence rate of 50%. Since T3 and T4 tumors have a higher recurrent rate, they are unsuitable for local excision in patients who cannot tolerate or refuse abdominoperineal resection.

Local rectal cancer can be treated with a variety of methods, including TAE, TSE, TVE, and minimally invasive endoscopic transanal excision^[19]. Irrespective of the surgical method, R0 excision is the most important procedure. TAE, a minimally invasive treatment modality, has few complications^[20]. In our 92 patients, TAE had a low complication rate of 2.2%. Preoperative pelvic

CT and ultrasound examination can provide important information for surgical planning. Kwok *et al.*^[21] analyzed data on the accuracy, sensitivity, and specificity of preoperative CT, magnetic resonance imaging (MRI), MRI-endorectal coil (MRI-ERC), and ERUS for rectal cancer staging, and found that the accuracy of CT, MRI, MRI-ERC, and ERUS is 80%, 74%, 81%, and 84%, respectively, with a higher accuracy for T1 tumors. At present, local excision is a generally accepted treatment modality for stage Tis and T1 rectal cancer. However, close follow-up is required. Early detection and salvage therapy can prolong the survival time of rectal cancer patients.

COMMENTS

Background

Both abdominoperineal resection and anterior resection of rectal cancer can result in postoperative urinary impairment and sexual dysfunction, and the presence of a permanent colostomy significantly impacts the quality of life. With the advancement of imaging techniques, the accuracy of preoperative rectal cancer staging has increased dramatically, and the preservation of physical function in rectal cancer patients has become a very important aim of research. Local tumor excision preserves anal, urinary, and sexual function in patients with low rectal cancer. Therapeutic efficacy and surgical indications for this procedure are controversial.

Research frontiers

Some studies have demonstrated that local tumor excision of early stage low rectal cancer can produce a good outcome and preserve anal, urinary, and reproductive function. However, a higher local recurrence rate (5%-27%) of this procedure has limited its use in clinical practice. Nevertheless, the 5-year survival rate after local tumor excision is 72%-95%. Risk factors affecting low rectal cancer prognosis after local excision include age and sex of patients, treatment modalities, and vascular invasion and tumor stage.

Innovations and breakthroughs

In this study, age and sex of the patients were unrelated to their prognosis. Vascular invasion and tumor stage affected rectal cancer recurrence and prognosis after local excision, and local recurrence and distance metastasis rates increased significantly when the tumor was poorly differentiated, macroscopically ulcerative, or larger than 3.0 cm in diameter. Therefore local excision should be recommended with caution for such patients. Frozen section examination of margins during surgery ensures margin negativity, which is essential for complete local tumor excision. Salvage therapy is of great importance in the treatment of recurrent rectal cancer after local excision.

Applications

Local rectal cancer excision has few complications, and is suitable for stages Tis and T1 rectal cancer. Prevention of local recurrence, active postoperative follow-up, and salvage therapy can greatly increase the efficacy of local excision.

Terminology

Local rectal cancer can be treated with a variety of methods including transanal excision (TAE), transsacral excision (TSE), transvaginal excision (TVE), transsphincter local excision, and minimally invasive endoscopic transanal excision.

Peer review

The study is well designed and its results have confirmed the efficiency of different methods for rectal cancer and the discussion is reasonable.

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BRIEF ARTICLES

Morphology and ontogeny of dendritic cells in rats at different development periods

Yi-Jun Zhou, Juan Gao, Hua-Mei Yang, Jian-Xin Zhu, Tong-Xin Chen, Zhen-Juan He

Yi-Jun Zhou, Hua-Mei Yang, Jian-Xin Zhu, Tong-Xin Chen, Zhen-Juan He, Xinhua Hospital Affiliated to Medical College, Shanghai Jiaotong University, Shanghai 200092, China
Juan Gao, Shanghai Sixth People's Hospital Affiliated to Medical College, Shanghai Jiaotong University, Shanghai 200233, China; Xinhua Hospital Affiliated to Medical College, Shanghai Jiaotong University, Shanghai 200092, China

Author contributions: Zhou YJ and He ZJ designed the research; Zhou YJ, Gao J, Yang HM performed the research; Zhu JX and Chen TX contributed new reagents; Zhou YJ, Gao J and He ZJ analyzed data; Zhou YJ wrote the paper; All authors are involved in the writing, critically reviewed and approved the paper.

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Correspondence to: Zhen-Juan He, Xinhua Hospital Affiliated to Medical College, Shanghai Jiaotong University, Shanghai 200092, China. hezhenjuan@gmail.com

Telephone: +86-21-65790000 Fax: +86-21-65795173

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Abstract

AIM: To study the morphology and ontogeny of dendritic cells of Peyer's patches in rats at different development periods.

METHODS: The morphometric and flow cytometric analyses were performed to detect all the parameters of villous-crypts axis and the number of OX62⁺DC, OX62⁺CD4⁺SIRP⁺DC, and OX62⁺CD4⁺SIRP⁻DC in the small intestine in different groups of rats. The relationship between the parameters of villous-axis and the number of DC and DC subtype were analyzed.

RESULTS: All morphometric parameters changed significantly with the development of pups in the different age groups ($F = 10.751, 12.374, 16.527, 5.291, 3.486; P = 0.000, 0.000, 0.000, 0.001, 0.015$). Villous height levels were unstable and increased from 115.24 μm to 140.43 μm as early as 3 wk postpartum. Villous area increased significantly between 5 and 7 wk postpartum, peaked up to 13817.60 μm^2 at 7 wk postpartum. Villous height and crypt depth ratios were relatively stable and increased significantly from 2.80 ± 1.01 to 4.54 ± 1.56 , 9-11 wk postpartum. The expression of OX62⁺DC increased from 33.30% \pm 5.80% to 80% \pm 17.30%, 3-11 wk postpartum ($F =$

5.536, $P = 0.0013$). OX62⁺CD4⁺SIRP⁺DC subset levels detected in single-cell suspensions of rat total Peyer's patch dendritic cells (PP-DCs) increased significantly from 30.73% \pm 5.16% to 35.50% \pm 4.08%, 5-7 wk postpartum and from 34.20% \pm 1.35% to 43.60% \pm 2.07% 9-11 wk postpartum ($F = 7.216, P = 0.005$).

CONCLUSION: This study confirms the age-related changes in villous-crypt axis differentiation in the small intestine. Simultaneously, there are also development and maturation in rat PP-DCs phenotypic expression. Furthermore, the morphological changes of intestinal mucosa and the development of immune cells (especially DC) peaked at 9-11 wk postpartum, indicating that the intestinal mucosae reached a relatively mature state at 11 wk postpartum.

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Key words: Intestinal mucosa; Dendritic cell; Peyer's patches; Intestinal development

Peer reviewer: Atsushi Mizoguchi, Assistant Professor, Experimental Pathology, Massachusetts General Hospital, Simches 8234, 185 Cambridge Street, Boston, MA 02114, United States

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INTRODUCTION

Different development periods have been suggested to play a role in controlling the development of gastrointestinal mucosal immune responses, which induce the intestinal mucosal immunity reflected differently over different development periods in children. As the lymphoid tissue is the primary site for the induction of mucosal immune responses. The morphometry of the villous-crypt axis in the small intestine reflects the function and adaptation of intestinal mucosal barriers. It was therefore of interest to investigate the potentially disparate phenotypic expression of dendritic cells (DCs) and the morphology of intestinal mucosa

found at different periods as a basis for determining the mechanisms that are apparently critical in intestinal mucosal immunity.

DCs were first identified in 1973 by Steinman and Cohn^[1,2] and were found in two locations in the intestinal mucosa: the Peyer's patches (PPs) and lamina propria (LP). PP-DCs were isolated by Spalding in 1983^[3]. It is now clear that PP-DCs may be unique in their ability to induce the differentiation of T cells that produce important cytokines such as IL-4 and IL-10 and other cytokines, including TGF- β , which is important for B cell differentiation and bystander suppression after oral antigen feeding^[4,5]. PP-DCs primarily perform two important tasks: (1) uptake of antigen after its transcytosis across the follicle-associated epithelium (FAE), which is mediated by immature DCs located largely in the subepithelial dome (SED); (2) T and B cell activation by mature DCs, which are found in the SED. T and B cells activated in PPs are "imprinted" and back to the gut due to the unique ability of PP-DCs to induce lymphocytes to regulate immunity^[6-9].

DC studies to date have been primarily carried out in mice *in vitro*. Little literature exists about rat DCs and of DCs *in vivo*, as current techniques for DC isolation are likely to induce phenotypic changes in DC. However, rats are of great value as experimental animals, especially in the field of nutrition. Additionally, some important findings about DC have been achieved through animal experiments. We therefore chose the rat model as the experimental object to detect the statement *in vivo*. In previous studies, DCs in mice had been divided into two subsets: CD8 $\alpha\alpha$ + and CD8 $\alpha\alpha$ -DC^[10]. CD8 $\alpha\alpha$ + and CD8 $\alpha\alpha$ -DC preferentially activate T cells toward Th1 and Th2 differentiation, respectively^[11]. CD8 $\alpha\alpha$ +DC constitutively cross-presents antigens to T cells, while CD8 $\alpha\alpha$ -DC does so upon their activation^[12]. Kelsall *et al.*^[13] also identified two distinct subsets of DCs in 6 to 8-wk-old murine PP. One population of DCs was positioned to capture antigens transported by overlying M cells, while the other subset activated native T cells to become effector cells. Recently, four different subsets of DCs have been described in mouse PPs^[14,15]. Conversely, progress in the studies of rat DC is slow because of the relative shortage of reagents. CD11C, MHC-class II, and $\alpha\epsilon$ -intergin (OX62 antigen) expression is routinely detected in rats to define DC in peripheral and lymphoid tissues. $\alpha\epsilon$ -intergin expression is the strongest in mucosa-associated DC^[16,17]. Additionally, as early as in 1980, rat DCs were subdivided on the basis of differential expression of CD4 and a member of the SIRP (signal inhibitory regulatory protein) family of molecules (detected by OX41)^[18,19]. The existence of CD4⁻/SIRP⁻ and CD4⁺/SIRP⁺DC subsets from the small intestine has recently been described^[20]. Some reports^[20] have shown that 12 to 16-wk-old rat intestinal and hepatic lymph DC is αE_2 intergin^{hi} (OX-62) and includes two subsets: (1) signal regulatory protein α (SIRP α)^{hi/low} and (2) CD4^{hi/low}, which most likely represents murine CD8 $\alpha\alpha$ -/+DC. In both lymph and the spleen, SIRP⁺/CD4⁺ DCs are more potent than

SIRP⁻/CD4⁻ DCs in the activation of allogeneic CD4⁺ and CD8⁺ T cells, naïve Ag-specific CD4⁺ T cells *in vivo*, and sensitized Ag-specific CD4⁺ T cells *in vitro*. To date, few studies have been conducted in rat PP-DCs at different development periods, our work focused on the morphometry of the villous-crypt axis in the small intestine as well as PP-DC differentiation and maturation in rats during ontogeny. Flow cytometry was used with emphasis laid on the implications of PP-DC interactions with intestinal mucosal immunity.

Our current study was to gain a better understanding of the expression and activation of PP-DCs in Sprague-Dawley rats. Single-cell suspension from the PP of the small bowel was isolated via collagenase A digestion. By collecting and manipulating these cells at 4°C, we maintained the cell activity. Using this model, we ascertained two phenotypically distinct subsets of PP-DC (both OX62⁺) that were distinguished by the existence or nonexistence of CD4 and SIRP coexpression. We also explored whether age-related development of DC ontogeny and morphological change occurred simultaneously. Our results demonstrate that morphological change is associated with DC ontogeny, suggesting that the intestinal mucosal immune system continuously changes as young rats mature.

MATERIALS AND METHODS

Animals

SD rats were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China. Rats were held in plastic cages in temperature- and humidity-controlled animal quarters under a 12 h light/dark cycle and were fed a standard diet (rodent rat chow) *ad libitum* with free access to tap water. All procedures were approved by the Institutional Animal Care Committee.

Reagents

Abs mouse anti-rat OX62:RPE, mouse anti-rat CD172a: FITC (OX41), and mouse anti-rat CD4:APC (Serotec) were purchased from Shanghai Jingmei Corporation.

Tissue samples

SD rats were bred and maintained under specific pathogen-free conditions at Xinhua Hospital Affiliated to the Medical College, Shanghai Jiaotong University, Shanghai, China. Animals, aged 3 wk and weighing 52-58 g, were divided into 5 groups of equal size and approximately equal mean body weight (54 g), namely groups of 3, 5, 7, 9 and 11 wk. Rats were killed after a postpartum period of 21, 35, 49, 63 and 77 d. Abdominal cavities were opened by horizontal incision along the midsection and guts were excised. Central ileum tissue samples (0.5 cm) were taken. All PP tissue samples were taken from the small bowel.

HE staining

Immediately after collection, ileum tissue samples were washed 3 times in cold phosphate buffered saline (PBS)

Table 1 Morphological changes of intestinal mucosa at different development periods in SD rats (mean \pm SD)

	3 wk	5 wk	7 wk	9 wk	11 wk	F	P
Villous height (μm)	115.24 \pm 21.82	140.43 \pm 22.30	210.71 \pm 59.47	145.43 \pm 34.21	205.14 \pm 51.31	10.751	0.000
Villous width (μm)	34.41 \pm 8.67	60.57 \pm 20.61	93.06 \pm 35.03	66.91 \pm 21.28	38.82 \pm 8.96	12.374	0.000
Villous areas (μm^2)	3334.46 \pm 1134.11	5451.92 \pm 2029.10	13817.60 \pm 5236.52	7495.52 \pm 3059.18	7209.91 \pm 2087.77	16.527	0.000
Crypt depth (μm)	30.06 \pm 6.61	44.12 \pm 9.25	58.76 \pm 14.16	58.70 \pm 29.04	48.29 \pm 12.94	5.291	0.001
Villous height/crypt depth	3.92 \pm 0.76	3.30 \pm 0.79	3.74 \pm 1.22	2.80 \pm 1.01	4.54 \pm 1.56	3.486	0.015

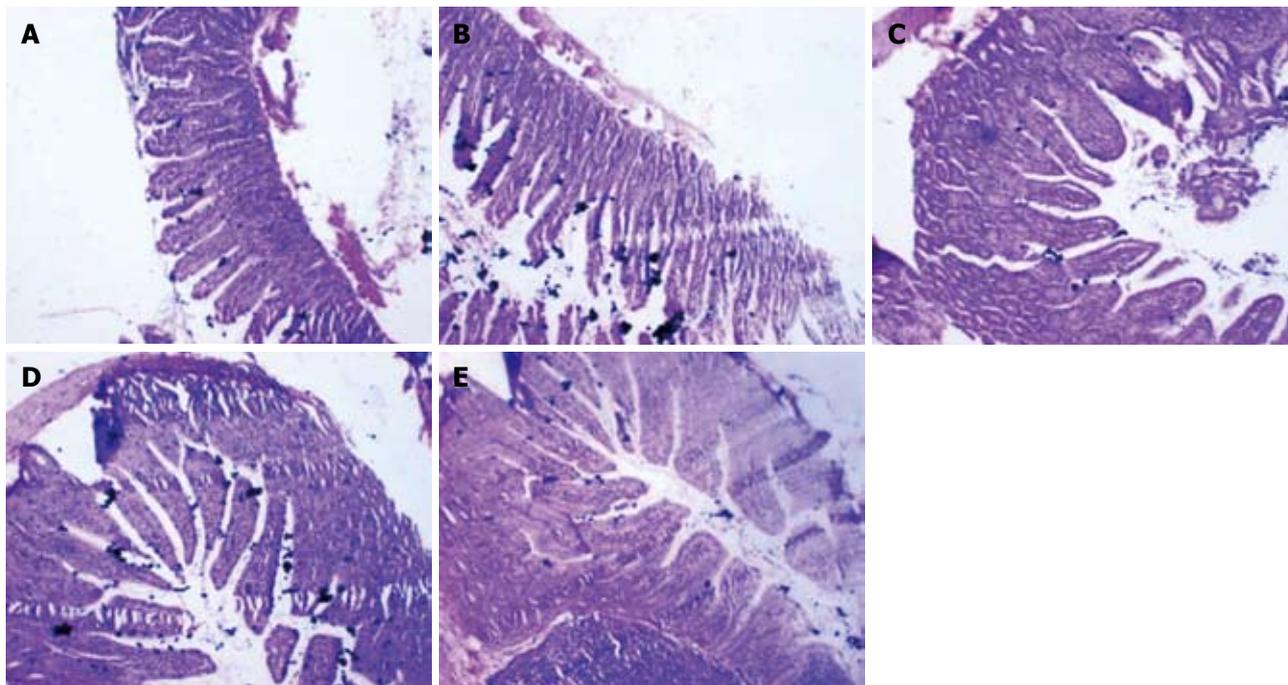


Figure 1 Comparison of morphological changes of small intestinal mucosa (including villous height, villous width, villous area, crypt depth, and ratio of villous height to crypt depth) at different development periods in SD rats (HE, \times 100). A: Group of 3 wk; B: Group of 5 wk; C: Group of 7 wk; D: Group of 9 wk; E: group of 11 wk.

and fixed for 48 h in 4% formalin solution. After fixation, specimens were dehydrated and embedded in paraffin. Sections from each sample were cut at a thickness of 4 μm and stained with hematoxylin and eosin (HE).

Determination of staining results

Sections were examined under a light microscope. Villous height, villous width, and crypt depth in all tissues were determined using the image analysis system. Villous height was measured from the top of the villi to the Lamina muscularis mucosae. Villous width was defined as the distance from one crypt-villi junction to the next. The villous area (height \times width) was calculated out of these 2 parameters. PP areas in the ileum were imaged and measured in triplicate using a digital camera and software. PP outlines were performed by hand.

Flow cytometry

PP tissue samples were washed extensively 3 times in cold PBS. They were then cut into small segments and placed in cold PBS (4°C). After centrifugation at 800 rpm for 5 min at 4°C, the supernatant was removed and the remaining tissues were digested with 0.75 mg/mL collagenase A for 45 min at 37°C with periodic agitation.

Undigested stromal material was removed by passing over membrane filtration. Single-cell suspension was prepared and cells were incubated with mouse anti-rat OX62:RPE, mouse anti-rat CD172a:FITC (OX41) and mouse anti-rat CD4:APC for 30 min.

Statistical analysis

SPSS 11.0 statistical software was used for data analysis. Results were expressed as mean values with standard deviation (SD). Statistical analysis was completed using one-way ANOVA. Tukey's test was used to determine the significance of the difference between groups when the ANOVA test indicated a significant effect. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Morphological analysis of villous-crypt axis

The morphological parameters of the villous-crypt axis in the small intestine included villous height, villous width, villous areas, crypt depth, and the ratio of villous height to crypt depth. As shown in Table 1, Figure 1 and Figure 2A-E, all morphometric parameters changed significantly with the development of pups in

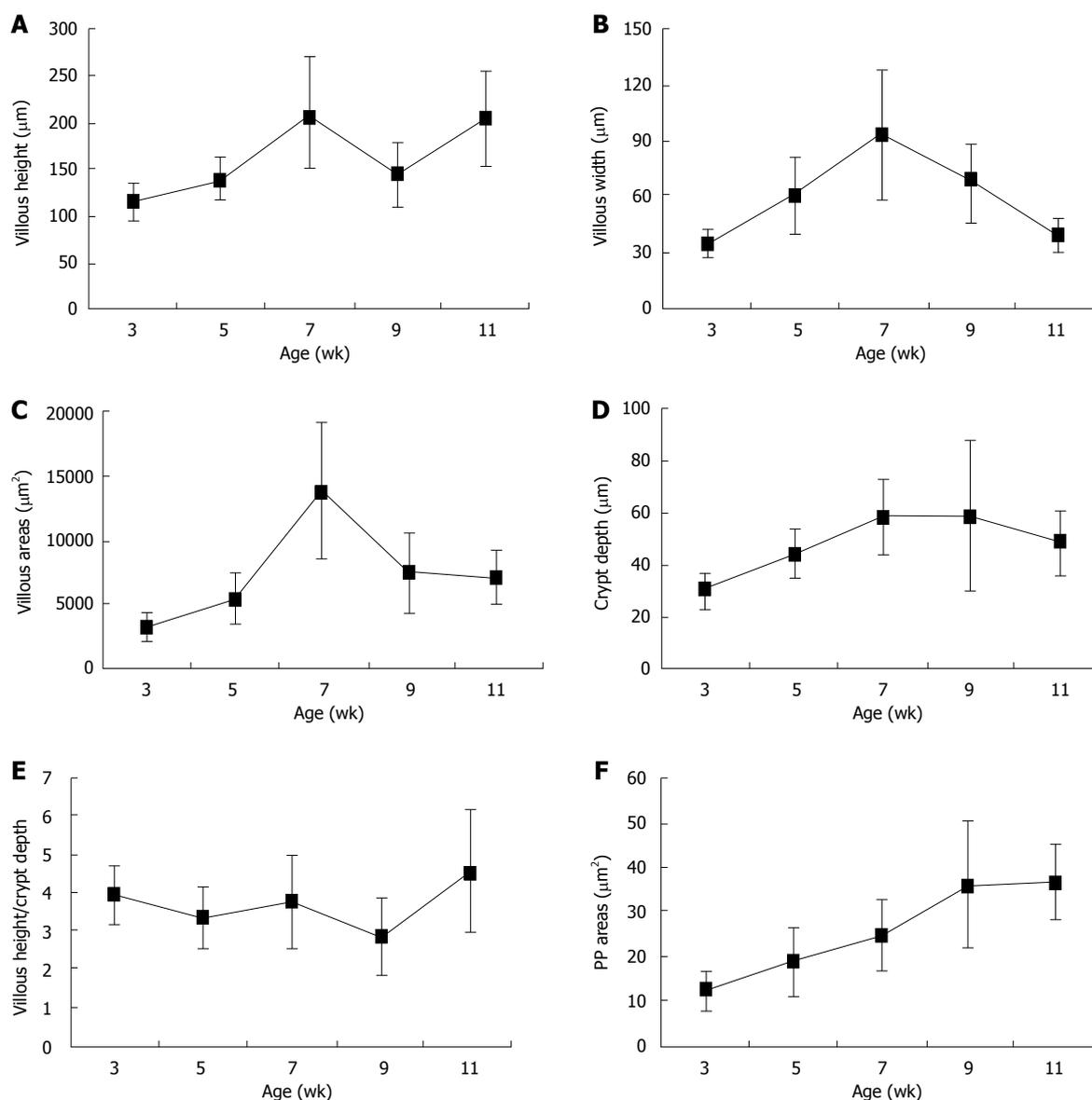


Figure 2 Morphological analysis of villous-crypt axis: All morphological parameters matured as age increased. The results were presented as mean \pm SD from 5 rats. A: Villous height increased at 3 wk postpartum, decreased 7 to 9 wk postpartum, and increased again after 9 wk postpartum; B: Villous width increased at 3 wk postpartum, peaked at 7 wk postpartum; C: Villous area increased significantly between 5 and 7 wk postpartum, peaked at 7 wk postpartum; D: Crypt depth increased from 3 to 7 wk postpartum and decreased slightly at 9 wk postpartum; E: Ratio of villous height to crypt depth were relatively stable and increased significantly from 9 to 11 wk postpartum; F: PP increased from 3 to 11 wk postpartum.

the different age groups ($F = 10.751, 12.374, 16.527, 5.291, 3.486; P < 0.05$). Villous height was unstable and increased from $115.24 \pm 21.82 \mu\text{m}$ to $140.43 \pm 22.30 \mu\text{m}$ as early as 3 wk postpartum, decreased from $210.71 \pm 59.47 \mu\text{m}$ to $145.43 \pm 34.21 \mu\text{m}$ at 7-9 wk, and then increased again after 9 wk. Villous width increased from 3 wk postpartum, peaked at 7 wk up to $93.06 \pm 35.03 \mu\text{m}$, and decreased thereafter. Villous areas increased significantly between 5 wk and 7 wk postpartum from $5451.92 \pm 2029.10 \mu\text{m}^2$ to $13817.60 \pm 5236.52 \mu\text{m}^2$, peaked at 7 wk, and decreased from then on, whereas few differences were found between 9 and 11 wk postpartum. Crypt depth increased from 3 wk to 7 wk postpartum, and decreased slightly at 9 wk. Few age-related differences were evident between 7 wk and 9 wk postpartum. Villous height and crypt depth ratio was

relatively stable and increased significantly from 2.8 ± 1.01 to 4.54 ± 1.56 at 9-11 wk postpartum.

Morphological analysis of PPs

PPs located in the small intestine showed a highly significant increase in size and number with age ($P < 0.05$), and the degree of increase in different age groups varied from a few to a moderate number (from $12.25 \pm 4.69 \mu\text{m}^2$ to $37.12 \pm 8.20 \mu\text{m}^2$ at 3-11 wk postpartum) (Table 2, Figure 2F).

Ontogeny analysis of PP-DCs

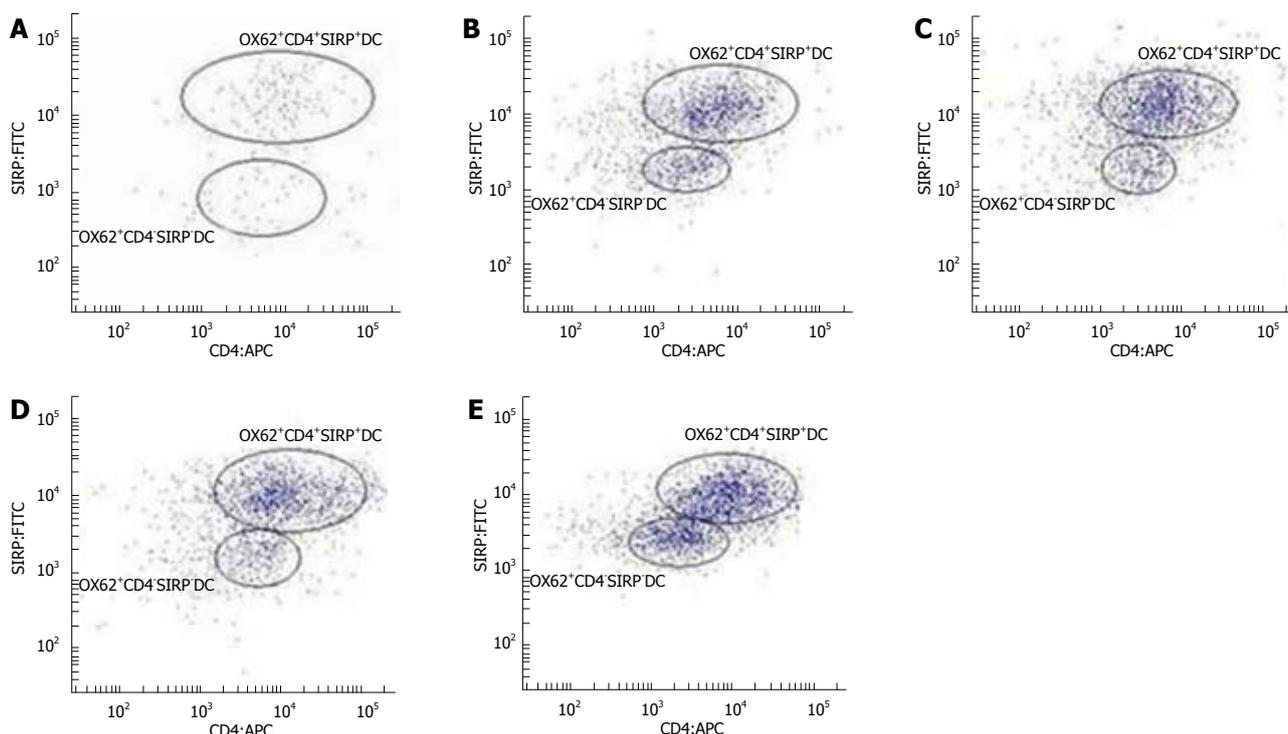
Single-cell suspensions of the total PP-DCs in rats were identified by immunolabelling for OX62, which contained two populations distinguished by co-expression of CD4 and SIRP α or not. As shown in Table 3, Figures

Table 2 Changes of the areas of Peyer's patches (PP) at different development periods in SD rats (mean \pm SD)

	3 wk	5 wk	7 wk	9 wk	11 wk	F	P
PP areas (μm^2)	12.25 \pm 4.69	18.91 \pm 7.73	24.68 \pm 7.73	36.13 \pm 14.59	37.12 \pm 8.20	7.474	0.000

Table 3 Changes in expression of OX62⁺DC, OX62⁺CD4⁺SIRP⁺DC and OX62⁺CD4⁻SIRP⁻DC subsets with development of SD rats (mean \pm SD)

Age	3 wk	5 wk	7 wk	9 wk	11 wk	F	P
OX62 ⁺ DC/total cells in PP (%)	0.333 \pm 0.058	0.367 \pm 0.115	0.467 \pm 0.153	0.533 \pm 0.153	0.8 \pm 0.173	5.536	0.013
OX62 ⁺ CD4 ⁺ SIRP ⁺ DC/OX62 ⁺ DC (%)	31.87 \pm 1.99	30.73 \pm 5.16	35.50 \pm 4.08	34.20 \pm 1.35	43.60 \pm 2.07	7.216	0.005
OX62 ⁺ CD4 ⁻ SIRP ⁻ DC/OX62 ⁺ DC (%)	33.27 \pm 5.71	25.33 \pm 2.82	24.53 \pm 2.64	28.97 \pm 1.89	28.43 \pm 1.94	3.242	0.060

**Figure 3** FCM of expression of OX62⁺CD4⁺SIRP⁺DC and OX62⁺CD4⁻SIRP⁻DC subsets at different development periods in SD rats. A: Group of 3 wk; B: Group of 5 wk; C: Group of 7 wk; D: Group of 9 wk; E: Group of 11 wk.

3 and 4, the expression of OX62⁺DC (i.e. the total PP-DC) increased from 33.3% \pm 5.8% to 80% \pm 17.3% at 3-11 wk postpartum. Moreover, age-related changes in total PP-DCs occurred as early as 3-5 wk postpartum and became more pronounced 9-11 wk postpartum. OX62⁺CD4⁺SIRP⁺DC subset levels detected in single-cell suspensions of rat total PP-DCs increased significantly from 30.73% \pm 5.16% to 35.5% \pm 4.08% at 3-5 wk postpartum and from 34.2% \pm 1.35% to 43.6% \pm 2.07% at 7-9 wk postpartum, whereas few age-related differences were found 9-11 wk postpartum. OX62⁺CD4⁻SIRP⁻DC levels varied in different age groups, whose trends were downward on the whole from 33.27% \pm 5.71% to 28.43% \pm 1.49% at 3-11 wk, although small fluctuations were observed 7-9 wk postpartum.

DISCUSSION

PPs^[21] are typical gut-associated lymphoid tissues located

along the small intestine wall and serve as the major sites for generation of immunity to intestinal antigens. PPs contain large amounts of immunocytes, of which DCs are the most potent antigen-presenting cell for activation of further immune response. Moreover, intestinal DC results in immune response to the intestinal mucosa, and therefore will not stimulate the immune response system. Thus, intestinal DC^[22], one of the best characters representing the maturation and activation of intestinal mucosal immunity, can exist in different levels of maturation and activation that are reflected in different ways, including antigen capture and process, effector cell activation, and cytokine networking.

It is well known that rats at 21-28 d, 28-60 d, and > 60 d postpartum approximate to the human weaning stage, childhood period, and adult period, respectively. Therefore, in our study we selected rats at the ages of 3, 5, 7, 9 and 11 wk postpartum to simulate the different human development periods. We found that DCs

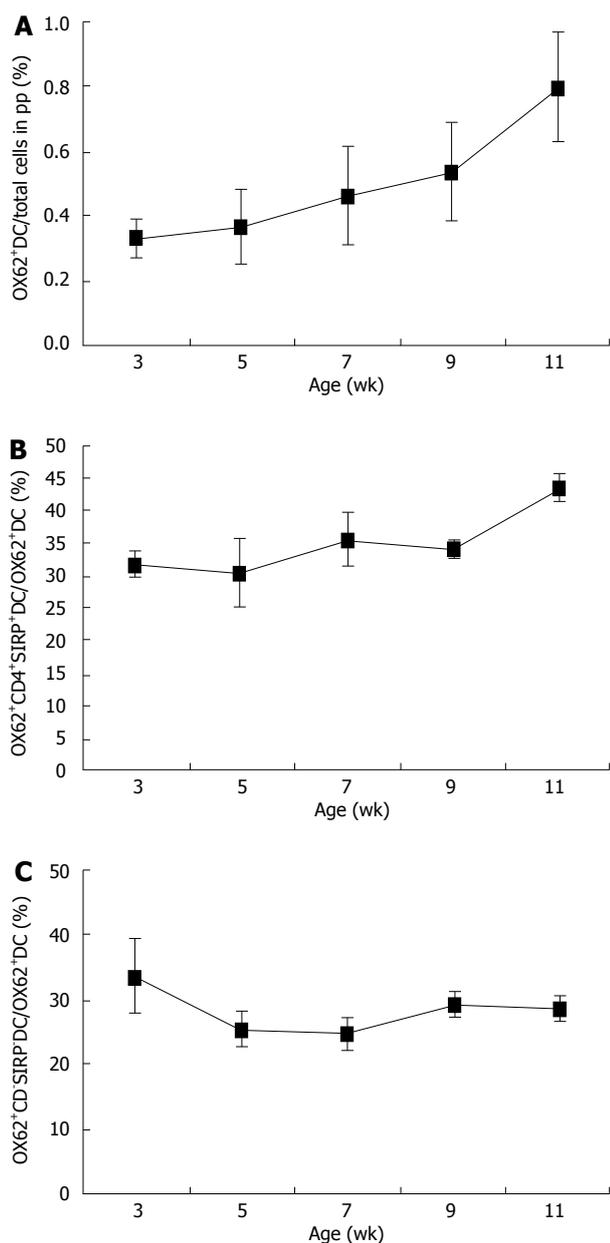


Figure 4 Flow cytometric analyses of OX62⁺CD4⁺SIRP⁺ and OX62⁺CD4⁻SIRP⁻ PP DCs *in vivo* at 3, 5, 7, 9 and 11 wk postpartum. The results were presented as mean \pm SD from 5 rats. A: Significant growth occurred in different age groups for the number of OX62⁺DCs; B: Levels of OX62⁺CD4⁺OX41⁺DC subsets increased significantly at 3-5 wk postpartum and 7-9 wk postpartum; C: OX62⁺CD4⁻SIRP⁻DC levels declined on the whole, with small fluctuation from 7 to 9 wk postpartum.

isolated from single-cell suspensions of rat PP revealed OX62 expression and were subdivided based on the differential expression of CD4 and a member of the signal inhibitory regulatory protein (SIRP) family of molecules (detected by the OX41). These results concur with a previous study^[17,20]. Trinite *et al.*^[23] have proposed that CD4 and SIRP expression levels may be related to DC developmental stages or reflect differential induction in response to the nature of the microenvironment and their expression is entirely tissue-specific and related to the function of DC in these tissues. DCs that coexpress CD4 and OX41, a member of the SIRP family, have functional properties typical of mature DCs. In contrast,

CD4⁺OX41⁺DCs are weak APCs for specific Ag, and in the allogeneic MLR survive poorly in cultures, contain large cytoplasmic inclusions, and are very strongly nonspecific esterase (NSE).

The current study revealed that development of the rat intestinal mucosa varies among different age groups, and the morphological parameters of the villous-crypt axis in the small intestine reflected proliferation and differentiation with age. Additionally, the total number of PP-DCs as well as the DC subpopulation trends varied significantly. Zaph *et al.*^[24] have also proposed that immune cells could promote the development of intestinal mucosa, and the mature immune system could promote the development of intestinal morphology. Our results therefore indicate that the development changes consisting of the proliferation and differentiation of intestinal morphology and the activation and maturity of intestinal immune cells (particularly DC) occurred simultaneously and not accidentally. The small intestine morphometric parameters and the total PP-DC increased with age and were more pronounced 9-11 wk postpartum, which indicates that the function of the intestinal mucosal barrier and mucosal immune system of rats gradually matured. The trend of CD4⁺SIRP⁺ DC subpopulations was upward at the same time that CD4⁻SIRP⁻ DC subpopulations were downward, which indicates that the immune system matured with age. The number of PP-DC subpopulations changed notably in the early post-weaning period (3-5 wk postpartum), which may be related to the change in diet from milk to solid feeding as well as intestinal adaptive adjustment induced by an increase in the number of antigens. All morphometric parameters detected in the small intestine increased promptly in rats aged 5-7 wk postpartum compared with rats at other age periods. It has been reported that the phenomenon of villous atrophy in mammals occurs after diet changes; this mechanism is related to a reduction in the rate of cell update and an increase in the rate of cell loss. Moreover, villous atrophy can also result in the intestinal immune function decline; therefore, we speculate that the phenomenon of delayed development of villous parameters may correspond to the diet change in the early post-weaning period (3-5 wk postpartum), which further impacted intestinal mucosal immune function in the late post-weaning period (5-11 wk postpartum). Finally, in the late post-weaning period, the development rate of PP-DCs decreased and the PP-DCs were in a relatively stable state. Additionally, the villous height/crypt depth ratio is a useful criterion for estimating digestive capacity in the small intestine^[25]. Although a reduction in villous height occurred 7-11 wk postpartum, maintenance of the villous height/crypt depth ratio 7-11 wk postpartum suggested that a reduction in villous height was less deleterious when it was not accompanied by increased crypt depth. These results coincide with those of Montagne *et al.*^[25].

In conclusion, results from the current study indicate that the intestinal mucosal immune system is continuously changing as young rats mature. This

study confirmed the age-related changes in villous-crypt axis proliferation and differentiation in the small intestine. Simultaneously, there are also developments and maturations in rat PP-DCs phenotypic expression. Furthermore, the morphological changes of intestinal mucosa and the development of immune cells (especially DC) peaked at 9-11 wk postpartum, indicating that the intestinal mucosae reached a relatively mature state at 11 wk postpartum. In the early weaning period, the number and the phenotypic expression of PP-DCs as well as the morphology changed rapidly due to ingestion of solid food, however, its mechanism requires further studies.

COMMENTS

Background

Recent evidences indicated that the different development period plays a role in controlling the development of gastrointestinal mucosal immunity. Peyer's patches (PPs) were the primary sites for the induction of mucosal immune responses. Dendritic cell (DC) populations play a major role in regulating gastrointestinal mucosal immune responses. The morphometry of the villous-crypt axis in the small intestine reflects the function and adaptation of intestinal mucosal barriers. It is therefore of interest to investigate the potentially disparate phenotypic expression of DC and the morphology of intestinal mucosa found in different periods.

Research frontiers

PPs are typical gut-associated lymphoid tissues, which contain large amounts of immunocytes. The PP-DC can result in immune response to the intestinal mucosa, and therefore will not stimulate the immune response system. However, the study on DC was most thorough *in vitro* or in serum, because the techniques for DC isolation from mucosal lymphoid tissue such as PP are relatively rare. Therefore, this research investigated the PP-DCs and the morphology of intestinal mucosa at different periods as a basis for determining the mechanism by which it may initiate regulatory events that are apparently critical in intestinal mucosal immunity.

Innovations and breakthrough

The authors evaluated, perhaps for the first time, the morphological and ontogeny of dendritic cells at different development periods. The analysis was carried out in 5 groups. As a result, all morphometric parameters (including villous height, villous width, villous areas, crypt depth, and villous height and crypt depth ratios) changed significantly with the development of pups at the different age groups ($F = 10.751, 12.374, 16.527, 5.291, 3.486; P < 0.05$). The OX62⁺CD4⁺SIRP⁺DC subset detected in single-cell suspensions of the rat total PP-DCs increased significantly at 3-11 wk postpartum, while the OX62⁺CD4⁺SIRP⁺DC levels varied in different age groups.

Applications

This analysis of morphometric parameters and ontogeny of dendritic cells at different development periods may help further study the gastrointestinal mucosal immunity.

Peer review

The measurement of morphometric parameters and ontogeny of immunity cells in gastrointestinal mucosa is useful for studies of gastrointestinal mucosal immunity. Although further studies are required, this study indicates the novel possibility for investigating the mucosal immunity cells in gastrointestinal.

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BRIEF ARTICLES

Meta-analysis of capsule endoscopy in patients diagnosed or suspected with esophageal varices

Yi Lu, Rui Gao, Zhuan Liao, Liang-Hao Hu, Zhao-Shen Li

Yi Lu, Rui Gao, Zhuan Liao, Liang-Hao Hu, Zhao-Shen Li, Department of Gastroenterology, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, China

Author contributions: Liao Z, Gao R and Lu Y contributed equally to this work; Li ZS and Liao Z designed the research; Lu Y, Gao R and Hu LH performed the research; Liao Z and Gao R collected data; Lu Y and Hu LH analyzed data; Lu Y and Gao R wrote the paper.

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Correspondence to: Zhao-Shen Li, Professor, Department of Gastroenterology, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, China. zhaoshenli@hotmail.com

Telephone: +86-21-25070552 Fax: +86-21-55621735

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Abstract

AIM: To review the literature on capsule endoscopy (CE) for detecting esophageal varices using conventional esophagogastroduodenoscopy (EGD) as the standard.

METHODS: A strict literature search of studies comparing the yield of CE and EGD in patients diagnosed or suspected as having esophageal varices was conducted by both computer search and manual search. Data were extracted to estimate the pooled diagnostic sensitivity and specificity.

RESULTS: There were seven studies appropriate for meta-analysis in our study, involving 446 patients. The pooled sensitivity and specificity of CE for detecting esophageal varices were 85.8% and 80.5%, respectively. In subgroup analysis, the pooled sensitivity and specificity were 82.7% and 54.8% in screened patients, and 87.3% and 84.7% in the screened/patients under surveillance, respectively.

CONCLUSION: CE appears to have acceptable sensitivity and specificity in detecting esophageal varices. However, data are insufficient to determine the accurate diagnostic value of CE in the screen/surveillance of patients alone.

INTRODUCTION

Variceal bleeding is a significant contributor to the morbidity and mortality associated with cirrhosis and portal hypertension. It has been estimated that up to 90% of patients with cirrhosis will ultimately develop esophageal varices^[1]. Once the varices develop, the occurrence of subsequent variceal bleeding in the next 24 mo is 25%-35%, and the risk of the patient dying as a result of the index hemorrhage is up to 50%^[2,3]. Esophagogastroduodenoscopy (EGD) is the most effective method of evaluating patients with portal hypertension and cirrhosis^[4]. However, the procedure is unpleasant, and still associated with a small but potential risk of complications^[5]. In addition, it is often carried out with the patients under sedation, which may bring additional complications in patients with cirrhosis^[6].

The PillCam ESO (Given Imaging, Israel) (esophageal capsule endoscopy, ECE) is a novel, wireless endoscope, similar in size to that of the standard PillCam small-bowel capsule endoscope (SBCE), which acquires video-images from both ends of the device during passage through the esophagus, and it has been approved by the United States Food and Drug Administration^[7]. ECE provides a potentially less invasive diagnostic alternative in evaluating diseases of the esophagus such as esophageal varices, Barrett's esophagus, and gastroesophageal reflux disease (GERD)/erosive esophagitis^[8-11]. Furthermore, Ramirez *et al*^[12] attached a string to SBCE in the middle of the device which can allow the capsule to move up and down in the esophagus by swallowing the capsule and pulling the string. This

improvement can extend the retention time of CE in the esophagus and provide adequate information about esophageal details.

Recent years, a number of studies have been performed for evaluating CE in diagnosing esophageal disease, especially for Barrett's esophagus and esophageal varices, and the results varied^[8-10]. If CE is a definitive diagnostic tool for esophageal varices, it is necessary to evaluate whether CE is sufficiently accurate for this purpose. In order to estimate the sensitivity and specificity of CE in the diagnosis of esophageal varices, a systematic review and meta-analysis of studies using CE for detecting esophageal varices with EGD as the standard was conducted.

MATERIALS AND METHODS

Study selection

A search in MEDLINE, EMBASE and ENBASE reviews (CDSR, ACP Journal Club, DARE, CCTR, CLCMR, CLHTA and CLEED) was conducted in May 2008. We did not confine our search to English language publications. Thorough literature retrieval for studies comparing CE and EGD for detecting esophageal varices in patients diagnosed or suspected as having esophageal varices was conducted along with a search of reference lists.

An additional search of abstracts presented at the proceedings of Digestive Disease Week (DDW) from 2004 to 2008 and international conference on CE through 2004 to 2007 was performed. If multiple updates of the same data were found, we selected only the most recent version or the most complete data for analysis. The search strategy employed is shown in Figure 1.

Data extraction

Predefined criteria had to be met for the studies to be included. Studies comparing CE diagnostic accuracy in patients diagnosed or suspected as having esophageal varices with EGD as standard were included. CE frames were assessed by an investigator who was blinded to patient's EGD findings. The studies which met the following one or more criteria were excluded: (1) esophageal varices were detected by CE but were performed in obscure gastrointestinal bleeding patients or for assessment of small bowel diseases or in miscellaneous patients with esophageal diseases; (2) string CE was used to detect esophageal varices; and (3) studies with patient number less than 10.

The study parameters were extracted first independently and subsequently in consensus if there was a disagreement between the reviewers (Liao Z, Gao R) concerning the numeric value of a parameter. Data were extracted based on the previous reported standards. The absolute number of true-positive, false-positive, true-negative and false-negative was retrieved or calculated with Bayes theorem if values for sensitivity and specificity and predictive values were reported. These were also performed separately by the two

reviewers and subsequently checked for an agreement. The full text of papers (if available) of all relevant studies were obtained.

Statistical analysis

Meta-analysis was undertaken using MetaDiSc statistical software (Meta-DiSc, version 1.4, Madrid, Spain) to estimate the overall sensitivity and specificity. χ^2 test was then performed to test for heterogeneity between studies, *P* value less than 0.1 was considered significant for heterogeneity. Wherever zero counts occurred for study data, a continuity correction of 0.5 was added to each value for that study. In order to define the calculation of sensitivity and specificity, fixed effect model was used when *P* value was more than 0.1 for heterogeneity test and random effect model used for *P* value was less than 0.1^[13].

RESULTS

Eighty-one articles were selected through original searches in MEDLINE, EMBASE and ENBASE reviews (CDSR, ACP Journal Club, DARE, CCTR, CLCMR, CLHTA and CLEED) databases. Fifty-one articles were excluded after review of the titles and abstracts, leaving 30 articles for detailed evaluation by two independent reviewers. Of these, five met our inclusion criteria^[10,14-17]. Two studies were identified by hand search in DDW 2008 meeting abstracts and reference lists respectively^[18,19]. In total, seven studies, involving 446 patients were appropriate for meta-analysis. In the seven studies, CE type was PillCam ESO without string and they were all published in English. One study was performed in western Europe^[14], one in Australia^[15], two were international multi-center studies^[10,17] and three in the USA^[16,18,19]. The patient inclusion/exclusion criteria were all reported in these studies. All patients were for clinically indicated screening (suspected) or surveillance (diagnosed) of esophageal varices. EGD was performed after CE in the same day for most patients, and all patients in these studies served as their own controls. All endoscopists who assessed the CE images were blinded to the EGD diagnoses. CE transit time was variable from 134.5 s (median) to 251 s (Table 1).

Forrest plots for sensitivity and specificity are shown in Figures 2 and 3. Reported sensitivity ranged from 68.4% to 100% in the individual studies, while specificity ranged from 8.3% to 100%. The pooled estimate of sensitivity was 85.8% (95% CI: 80.5%-90.1%) and the pooled estimate of specificity was 80.5% (95% CI: 74.7%-85.5%). However, both estimates were subject to significant heterogeneity (*P* = 0.010 and *P* < 0.001, respectively). In the presence of such heterogeneity, pooled estimates should be interpreted with caution.

Studies included in our analysis were further classified into two subgroups: screening group and screening/surveillance group. There were four studies including 106 patients in the screening group, no heterogeneity (*P* = 0.166) was found for the analysis of sensitivity, and the pooled sensitivity was 82.7% (95% CI: 72.2%-90.4%),

Table 1 Summary of studies included for meta-analysis

Author and year of publication	Patients (screening)	Country	Article type	Study design	Setting	Patients inclusion/exclusion criteria reported	Two methods performed time	Transit time in esophagus
Lapalus, 2006	20 (20)	France	O	P, B	S	Yes	EGD after ECE on the same day	213 s
Eisen, 2006	32 (10)	International	O	P, B	3 centers	Yes	EGD after ECE on the same day	134.5 s (median)
Smith, 2007	15 (15)	Australia	A	NR, B	S	Yes	EGD within 1-2 h after ECE	NR
Groce, 2007	21 (21)	USA	A	P, B	S	Yes	EGD immediately after ECE	251 s
Pena, 2008	20 (8)	USA	O	P, B	S	Yes	EGD within 1h after ECE	NR
Jensen, 2008	50 (50)	USA	O	P, B	S	Yes	NR	NR
de Franchis, 2008	288 (195)	International	O	P, B	11 centers	Yes	EGD within 48 h after ECE	NR

O: Original paper; A: Abstract; P: Prospective; B: Investigator who reviewed ECE results was blinded to EGD results; NR: Not reported; S: Single center.

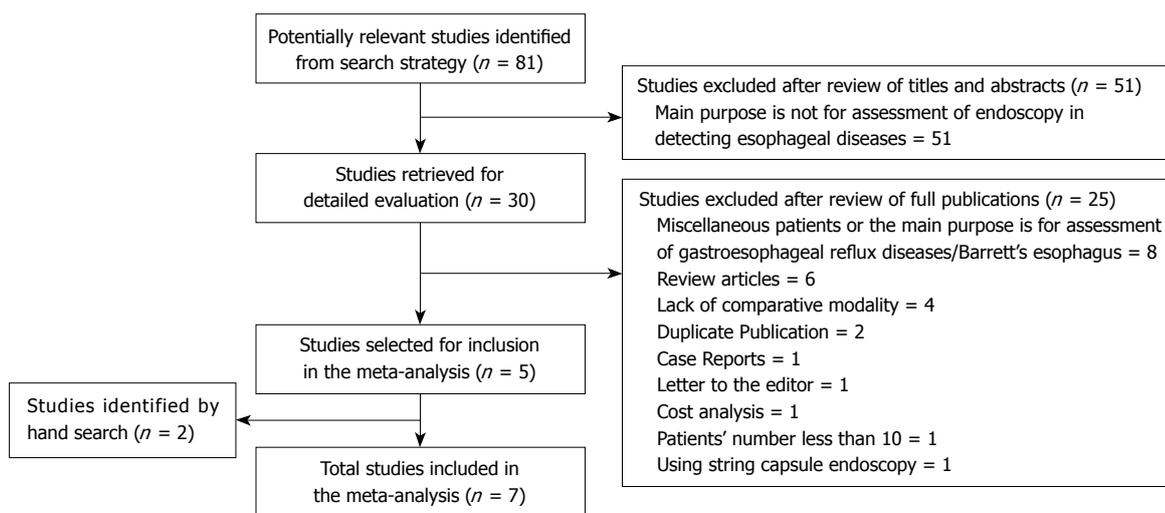


Figure 1 Search flow for Meta-analysis.

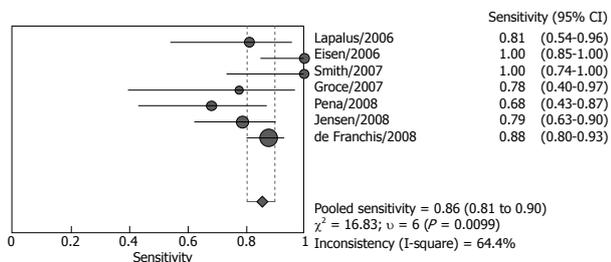


Figure 2 Pooled sensitivity of total studies included for meta-analysis.

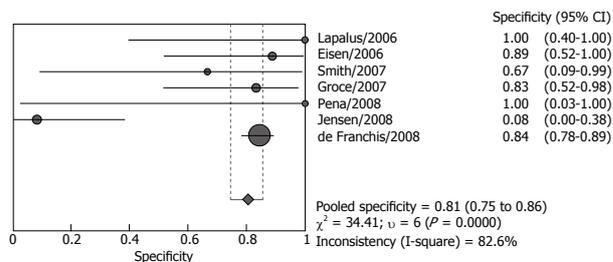


Figure 3 Pooled specificity of total studies included for meta-analysis.

however, heterogeneity was found significant for specificity ($P < 0.001$), the pooled result was 54.8% (95% CI: 36.0%-72.7%) (Figures 4 and 5). Both screened patients and patients under surveillance were included in the other three studies, involving 340 patients, and the detailed endoscopic results cannot be independently extracted for screen patients or surveillance patients. The pooled sensitivity was 87.3% (95% CI: 80.9%-92.2%) in a random effect model ($P = 0.004$) and pooled specificity was 84.7% (95% CI: 78.8%-89.5%) in a fixed effect model ($P = 0.789$). (Figures 6 and 7)

DISCUSSION

Meta-analysis has been performed for SBCE in detecting small bowel disease comparing with other methods. It

is reported SBCE was superior when compared with push enteroscopy and small bowel barium radiography for OGIB ($P < 0.00001$)^[20]. In detecting Crohn's disease, SBCE was also superior to small bowel barium radiography ($P < 0.001$), colonoscopy with ileoscopy ($P = 0.02$) and CT ($P = 0.001$)^[21]. Furthermore, the yield of SBCE for small bowel diseases was similar to double-balloon enteroscopy in combination with oral and anal approaches^[22].

In the largest study performed in 11 centers (288 patients), the sensitivity and specificity were 88.0% and 84.4%, respectively, and ECE had good agreement with EGD^[17]. The present meta-analysis indicates that CE appears to have acceptable sensitivity and specificity for esophageal varices with EGD as the standard. However, the limitations of these data need to be appreciated.

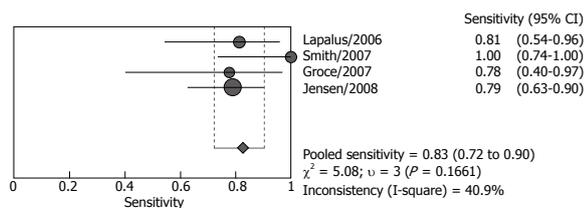


Figure 4 Pooled sensitivity of studies for screening patients.

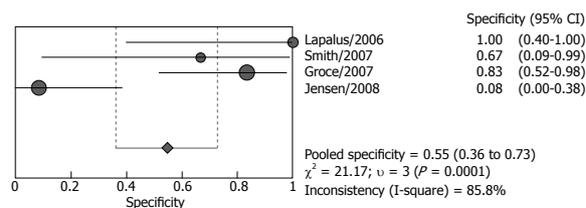


Figure 5 Pooled specificity of studies for screening patients.

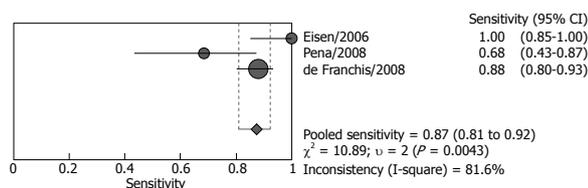


Figure 6 Pooled sensitivity of studies for screening/surveillance patients.

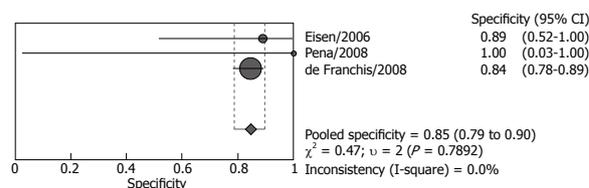


Figure 7 Pooled specificity of studies for screening/surveillance patients.

Firstly, because this new device has not been widely used for detecting esophageal varices, and the number of studies was small. Secondly, sample sizes of different studies were significantly different, ranging from 15 to 288 patients. So we considered the P value less than 0.1 as significant for heterogeneity in this analysis. At last, patients underwent CE including both screened patients and patients under surveillance, in 3 studies the detailed endoscopic results can not be extracted for these patients, and the accurate pooled diagnostic value of CE for patients under surveillance was not known.

There was considerable heterogeneity between the studies. In Pena's and Groce's studies, the sensitivity of ECE in detecting esophageal varices was only 68.4% and 77.8% respectively^[16,18]. In Eisen's and Smith's studies, the sensitivity was both as high as 100%^[10,15]. The diagnostic specificity of these seven studies varied significantly, the specificity was lowest in Jensen's study, only 8.3%, and the accuracy of capsule for detecting esophageal varices was modest^[19]. However, in Lapalus's^[14] and Pena's^[16] studies, the specificity was both 100%. There was no heterogeneity in the analysis of sensitivity and specificity for the screening group and the screening/surveillance group, respectively. Although the sample was too small to undertake meta-regression to identify the cause of the heterogeneity, some potential reasons for heterogeneity may be identified. Except for the small sample size of most studies, the most possible causes for the existence of heterogeneity may be related to the experience of the endoscopists and the number of endoscopists who read the capsule images. Although the ECE investigators were all blinded to EGD findings, their experience was not described in detail. Only in Pena's study, the endoscopists were those with more than 5 years of experience in reading SBCE but no experience in reading ECE^[16]. In two studies, ECE images were reviewed by two independent investigators^[14,19], and one investigator in another five studies^[10,19].

The pooled sensitivity and specificity of the screening/surveillance group was higher than the screening group. The pooled data of screened patients

may be confused by Jensen's study, which had the largest number of patients in the screening studies and with a specificity of 8.3%^[19]. In studies with screening/surveillance patients, the detailed results cannot be extracted, so the pooled data of surveillance patients cannot be obtained. One defect in the three studies was the percentages of screening or surveillance patients that were variable (Figure 1).

In summary, CE appears to have acceptable sensitivity and specificity in detecting esophageal varices, however, it seems inaccurate in screening patients based on the present data. There was insufficient data to determine the accurate diagnostic value of CE in patients under surveillance alone. Also, further researches with large numbers of patients are needed.

COMMENTS

Background

Capsule endoscopy (CE) has been reported to play an important role in detecting esophageal diseases, especially for esophageal varices. There are concerns about whether CE is sufficiently accurate for evaluating this disease.

Research frontiers

Data concerning CE in detecting esophageal varices with esophagogastroduodenoscopy (EGD) results as the standard were derived from published original papers or conference abstracts. A meta-analysis was conducted to estimate the sensitivity and specificity of CE in diagnosis of esophageal varices.

Innovations and breakthroughs

The current study demonstrated that CE appears to have acceptable sensitivity and specificity in detecting esophageal varices. However, it seems inaccurate in screening patients.

Applications

The diagnostic accuracy of CE was low in screening patients in this study. Studies for further evaluating CE diagnostic accuracy in screening patients alone were needed.

Terminology

Esophageal capsule endoscope (ECE) is a novel, wireless endoscope, similar in size to small-bowel capsule endoscope, which acquires video-images from both ends of the device during passage through the esophagus.

Peer review

This meta-analysis was performed based on five peer reviewed papers and 2 in abstract form, involving about 500 patients. This study was well done, however, the missing rate of the small-sized varices is high and this tool is suitable for medium- and large-sized varices.

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Antisense expression of PKC α improved sensitivity of SGC7901/VCR cells to doxorubicin

Da-Long Wu, Feng-Ying Sui, Cheng Du, Cheng-Wen Zhang, Bin Hui, Shui-Ling Xu, Huan-Zhang Lu, Guo-Jie Song

Da-Long Wu, Feng-Ying Sui, Cheng-Wen Zhang, Bin Hui, Guo-Jie Song, Department of Pharmacology, School of Medicine, Jiaxing College, Jiaxing 314001, Zhejiang Province, China
Cheng Du, Traditional Chinese Medicine Hospital of Jiaxing City, Jiaxing 314001, Zhejiang Province, China
Shui-Ling Xu, Department of Microbiology, School of Medicine, Jiaxing College, Jiaxing 314001, Zhejiang Province, China
Huan-Zhang Lu, Department of Clinical Pharmacology, Affiliated Hospital, Chinese Academy of Military Medical Sciences, Beijing 100039, China

Author contributions: Wu DL, Zhang CW and Hui B performed the research; Sui FY, Du C, Xu SL and Song GJ gave some advice; Wu DL and Lu HZ designed the research; Wu DL wrote the paper.

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Correspondence to: Da-Long Wu, PhD, Associate Professor, Department of Pharmacology, School of Medicine, Jiaxing College, Jiaxing 314001, Zhejiang Province, China. wudalong66@sina.com

Telephone: +86-573-83643836 **Fax:** +86-573-83643497

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Abstract

AIM: To explore whether antisense blocking of protein kinase C alpha (PKC α) would reverse multi-drug resistance (MDR) in the vincristine (VCR)-resistant human gastric cancer cell line SGC7901/VCR.

METHODS: SGC7901/VCR cells expressing antisense PKC α , SGC7901/VCR/aPKC, were established by transfection with a recombinant plasmid reversely inserted with PKC α cDNA. Empty vector (PCI-neo)-transfected cell clones, SGC7901/VCR/neo, served as the control. Western blot method was used to detect PKC α content in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells, using PKC α -specific antibody. The sensitivity of SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells to doxorubicin (DOX) *in vitro* was determined by MTT assay. The uptake of DOX in these cells was detected with fluorescence spectrophotometer.

RESULTS: Western blot analysis showed that the PKC α

protein level was about 8.7-fold higher in SGC7901/VCR cells than that in SGC7901 cells, whereas the protein expression of PKC α was reduced by 78% in SGC7901/VCR/aPKC cells when compared with the SGC7901/VCR cells. SGC7901/VCR/aPKC cells had a 4.2-fold increase in DOX cytotoxicity, accompanied by a 1.7-fold increase of DOX accumulation in comparison with SGC7901/VCR cells.

CONCLUSION: PKC α positively regulates MDR in SGC7901 cells, and inhibition of PKC α can partially attenuate MDR in human gastric cancer cells.

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Key words: Multi-drug resistance; Protein kinase C alpha; SGC7901; Gastric cancer

Peer reviewer: Shannon S Glaser, PhD, Department of Internal Medicine, Scott & White Hospital, 702 SW HK Dodgen Loop, Medical Research Building, Temple 76504, United States

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INTRODUCTION

Resistance of cancer cells to chemotherapeutic drugs is a major problem in clinical treatment of cancer. Multi-drug resistance (MDR) is the most important form of drug resistance characterized by decreased cellular sensitivity to a broad range of chemotherapeutic drugs, including anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes, antibiotics and some of the new topo- I inhibitors^[1,2]. Classical MDR is mainly caused by overexpression of P-glycoprotein (Pgp), which is coded by the MDR1 gene and functions as an ATP-dependent drug-efflux membrane transporter that rapidly extrudes a variety of hydrophobic anticancer drugs from exerting cytotoxic effect^[3,4]. Additionally, studies have shown that MDR is also accompanied by changes in the activity of protein kinase C (PKC).

Activation of PKC results in phosphorylation of Pgp and a decrease in drug accumulation, while inhibition of PKC can partially reverse the MDR phenotype^[5,6].

PKC comprises a family of at least 12 distinct serine/threonine kinase isoenzymes which are found in varying ratios in the cytosolic and membrane fractions of cells, depending on the type of issue and its physiological state^[6,7]. The family of PKC is composed of three subclasses: classical, novel and atypical PKC, each having different isoforms. The members of the classical PKCs (α , β I, β II and γ) bind phorbol esters and are Ca^{2+} dependent. The novel PKCs (δ , ϵ and η) do not depend on Ca^{2+} but bind phorbol esters. The third subfamily includes the atypical PKCs (τ , ζ , λ and μ), which do not bind to either Ca^{2+} or phorbol ester. It is likely that each isoform has a specific role in a given cell^[5,7]. PKC regulates numerous cell processes including proliferation, apoptosis and differentiation by phosphorylating proteins in response to transmembrane signals from hormones, growth factors, neuro-transmitters and pharmacological agents^[5]. Recently accumulated evidence indicated that PKC, especially classical PKC, plays a significant role in the formation of cancer MDR^[5,6]. The isoenzymes of PKC possess distinct differences in localization in different cells, and research on distinct function of isoforms in cancer MDR has important significance^[5,6].

Among those isoforms of PKC, PKC α is likely to play a decisive role in maintaining MDR phenotypes in some cancer cells, and may therefore represent potential novel targets for the treatment of cancers^[6,8-12]. In an effort to see whether down-regulation of a single isoform of PKC could affect drug resistance, vincristine (VCR)-resistant human gastric carcinoma cell line SGC7901/VCR was transfected with an expression vector containing the cDNA for PKC α in the antisense orientation, and their expression of PKC α , doxorubicin (DOX) sensitivity and DOX accumulation were determined.

MATERIALS AND METHODS

Materials

MTT and DOX were purchased from Sigma, VCR from the Twelfth Shanghai Pharmaceutical Product Factory, Lipofectamine 2000 from Invitrogen, nitrocellulose membranes and 3', 3'-diaminobenzidine (DAB) from Sigma, G418 and rabbit polyclonal anti-PKC α from Gibco-BRL. Plasmid pSP64-PKC α was kindly provided by Dr. PJ Parker (Imperial Cancer Foundation, England). The eukaryotic expression vector, plasmid PCI-neo, was kindly provided by Dr. Jun-Jie Xu (Institute of Microbiology and Epidemiology, Chinese Academy of Military Medical Sciences). The human gastric cancer cell line SGC7901, and its VCR-resistant counterpart SGC7901/VCR selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR, were purchased from Wuhan University Type Culture Collection (Wuhan, China).

Cell culture

Both SGC7901 and SGC7901/VCR cells were grown in

RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO₂. The SGC7901/VCR cells were cultured in the presence of 0.8 $\mu\text{mol/L}$ VCR and grown in drug-free medium 2 wk before the experiments.

Plasmid construction

The full-length cDNA encoding PKC α cDNA (2.3 kb) was recovered from Sal I sites in pSP64-PKC α plasmid and subcloned into Sal I sites of PCI-neo plasmid. The recombinant plasmid with the PKC α cDNA in the antisense orientation was confirmed by BamHI and/or Sal I restriction digestion, and designated PCI-neo-aPKC α .

Cloning of cells transfected with antisense vector PCI-neo-aPKC α

The process was performed as described by Wang *et al.*^[13]. Briefly, SGC7901/VCR cells were seeded in six-well plates to 70%-80% confluence. Empty vector PCI-neo and antisense vector PCI-neo-aPKC α were transfected into SGC7901/VCR cells *via* Lipofectamine 2000. The two kinds of transfected cells were named SGC7901/VCR/neo and SGC7901/VCR/aPKC respectively. After two days of transfection, cells were selected by culture medium containing G418 (400 mg/L) for 2 wk. The single clone was picked out using limiting dilution method and was expanded and maintained in medium containing 400 mg/L G418 until 1 wk prior to experiments.

Western blot analysis

The whole cell lysates were extracted with lysis buffer containing 1% Triton-100, 50 mmol/L NaCl, 50 mmol/L sodium fluoride, 20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium vanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5% NP40. An aliquot was taken for protein determination, and the remainder was mixed (1:1) with 2 \times SDS sample buffer and boiled for 5 min. Samples were run on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After blocking, the membranes were incubated with rabbit polyclonal antibody against PKC α isoform at a dilution of 1:1000. Blots were washed three times with PBST to block nonspecific binding sites and incubated with secondary antiserum (goat anti-rabbit IgG conjugated to horseradish peroxidase) for 1 h at 37°C, then washed three times with 50 mmol/L Tris-HCl (pH 6.8). Color was developed with DAB as the substrate.

Cytotoxicity assay

The cells in exponential growth were seeded in 96-well plates at a density of 2×10^4 cells per well and 24 h later graded DOX (at the concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10 $\mu\text{mol/L}$) were added, respectively. The total medium volume of each well was 200 μL . Three days after drug addition, 10 μL MTT (5.0 g/L in PBS) was added. After 4 h of incubation, supernatants were removed and replaced by 150 μL DMSO. After formazan solubilization, the absorbance at 570 nm

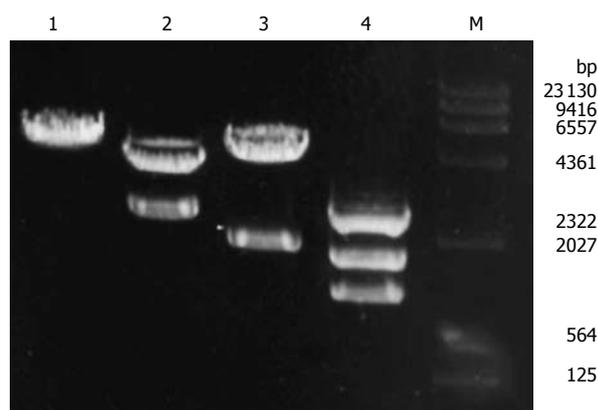


Figure 1 Restriction map of recombinant plasmid PCI-neo-aPKC α . Lane 1: PCI-neo digested with *Sal* I *Eco*R1; Lane 2: PCI-neo-aPKC α digested with *Bam*H1; Lane 3: PCI-neo-aPKC α digested with *Sa* I; Lane 4: PCI-neo-aPKC α digested with *Bam*H1 + *Sal* I; Lane M: λ DNA *Hind*III Marker.

was recorded using an automated microplate reader. IC₅₀ (concentration resulting in 50% inhibition of cell growth) values for DOX were calculated as 100% from plotted results using untreated cells.

Intracellular DOX accumulation

The assay was performed as described by our previous report^[2]. Briefly, 1.0×10^6 cells were exposed to 4.0 μ mol/L DOX for 60 min. Following incubation, the cells were washed twice with ice-cold PBS, then suspended in 6.0 mL of 50% ethanol -0.3 mol/L hydrochloride, extracted for 2 h, centrifugated at $100 \times g$ for 10 min, and the supernatant was collected. DOX-associated mean fluorescence intensity (MFI) was measured by fluorescence spectrophotometer at an excitation wavelength of 488 nm and an emission wavelength of 590 nm. The DOX standard solution (0.1 - 1.0×10^3 nmol/L) was prepared, and a standard work curve with related quotient was constructed.

Statistical analysis

Data were expressed as mean \pm SD. Student's *t* test was used to assess statistical significance of differences. *P* < 0.05 was considered statistically significant.

RESULTS

Establishment of SGC7901/VCR cells expressing antisense PKC α

To construct the PKC α antisense expression plasmid, the full-length cDNA of PKC α (2.3 kb) recovered from *Sal* I sites in pSP64-PKC α plasmid was subcloned into *Sal* I sites of the eukaryotic expression vector PCI-neo (5.5 kb). A recombinant plasmid with PKC α cDNA in the antisense orientation was obtained and designated PCI-neo-aPKC α , which contains the neomycin resistance gene neo for drug selection. Two fragments were obtained by digesting PCI-neo-aPKC α with *Sal* I, one was PKC α cDNA fragment about 2.3 kb and the other was PCI-neo cDNA fragment about 5.5 kb (Figure 1). And the inserted fragment was verified by sequencing (data not shown).

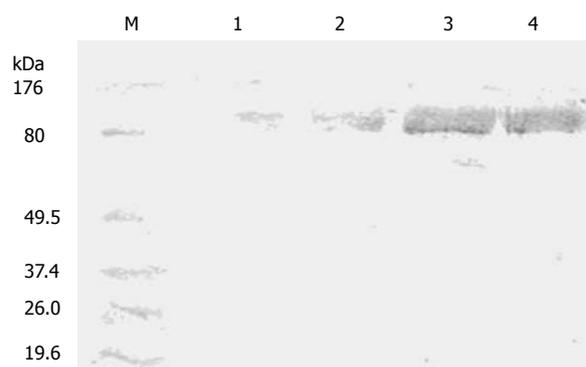


Figure 2 Western blot identification of PKC α protein in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells. Lane M: Benchmark™ Marker; Lane 1: SGC7901 cells; Lane 2: SGC7901/VCR/aPKC cells; Lane 3: SGC7901/VCR/neo cells; Lane 4: SGC7901/VCR cells.

SGC7901/VCR cells were transfected with either PCI-neo or PCI-neo-aPKC α , respectively, and G418-resistant clones were isolated and amplified after G418 selection. Empty vector-transfected cell clones were named SGC7901/VCR/neo, which together with the parental cell line served as the controls for these experiments. Clones transfected with the PKC α antisense expression plasmid (PCI-neo-aPKC α) were named SGC7901/VCR/aPKC.

Western blot analysis was performed to assess protein abundance of PKC α in SGC7901, SGC7901/VCR, SGC7901/VCR/aPKC and SGC7901/VCR/neo cells. As shown in Figure 2, the PKC α protein level was about 8.7-fold higher in SGC7901/VCR cells than that in SGC7901 cells. Transfection of SGC7901/VCR cells with the control vector did not alter PKC α expression, whereas the protein expression of PKC α was reduced by 78% in SGC7901/VCR/aPKC cells when compared with the SGC7901/VCR cells.

Modulating effect of antisense expression of PkC α on MDR

Using the MTT assay, the *in vitro* cytotoxicity of DOX in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells was examined. As shown in Table 1, SGC7901/VCR cells were 23.5 times more resistant to DOX in comparison with SGC7901 cells. The IC₅₀ in SGC7901/VCR/aPKC was 4.2-fold lower than that in SGC7901/VCR cells, indicating that antisense PkC α could partially reverse resistance to DOX in multi-drug resistant SGC7901 cells.

Effect of antisense expression of protein kinase C α on intracellular accumulation of DOX in multi-drug resistant SGC7901 cells

DOX accumulation in SGC7901, SGC7901/VCR, SGC7901/VCR/neo cells and SGC7901/VCR/aPKC was measured by fluorescence spectrophotometry after incubation of cells with 4.0 μ mol/L DOX for 60 min. As shown in Figure 3, SGC7901/VCR cells accumulated 4.3-fold less DOX than SGC7901 cells, whereas SGC7901/VCR/aPKC cells showed 1.7-fold more drug retention than SGC7901/VCR cells.

Table 1 Modulating effect of antisense expression of protein kinase C α on resistance to DOX in multi-drug resistant SGC7901 cells

Cell lines	IC ₅₀ of DOX ($\mu\text{mol/L}$)	Resistance index (RI)
SGC7901	0.076 \pm 0.0074	1.0
SGC7901/VCR	2.4 \pm 0.14 ^b	31.6
SGC7901/VCR/neo	2.3 \pm 0.19 ^b	30.3
SGC7901/VCR/aPKC	0.58 \pm 0.076 ^b	7.6

^b $P < 0.01$ vs IC₅₀ of SGC7901. The data are presented as mean \pm SD.

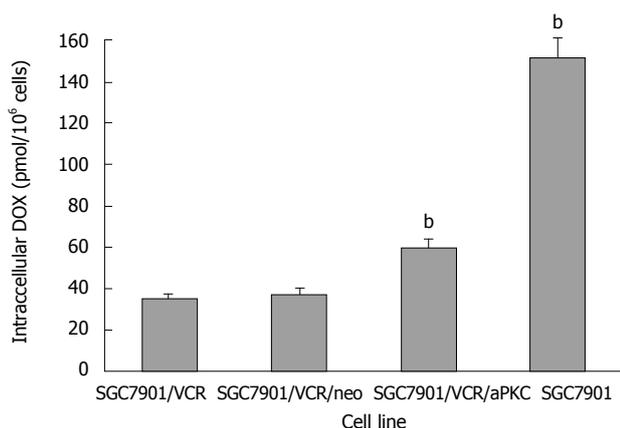


Figure 3 Accumulation of intracellular DOX in SGC7901, SGC7901/VCR, SGC7901/VCR/neo and SGC7901/VCR/aPKC cells. Cells were incubated with 4.0 $\mu\text{mol/L}$ DOX for 60 min. Each point represents the mean \pm SD from three experiments. ^b $P < 0.01$ vs SGC7901/VCR cells.

DISCUSSION

MDR to cancer treatment has been studied for more than 20 years. No useful method of reversing MDR suitable for clinical use has yet emerged from this large quantity of work. The reason could be complicated mechanisms involved. There are several ways for cancer cells to develop MDR^[4]. The most investigated mechanisms with known clinical significance are: (1) activation of transmembrane proteins effluxing different chemical substances from the cells, in which Pgp is the best known efflux pump; (2) activation of the enzymes of the glutathione detoxification system; (3) alterations of the genes and the proteins involved in the control of apoptosis (especially p53 and Bcl-2)^[10]. Recent studies have indicated a role for PKC in the regulation of the MDR phenotype. A number of studies have demonstrated that PKC activity was elevated in MDR-selected cell lines, and several PKC inhibitors are able to partially reverse MDR and inhibit Pgp phosphorylation^[5,6]. Pgp is phosphorylated by PKC and that phosphorylation positively modulates its transport function^[14]. The PKC family consists of at least 12 isoforms, and different isoforms of PKC possess distinct differences in expression and function in different MDR cells^[12-15]. The elevated PKC activity level of the MDR cancer cells was frequently a result of increased PKC α expression in most cancers^[8-12], but in some cancers it was due to enhanced expression of other isoforms, such as PKC β I, PKC γ or PKC η ^[15-18].

SGC7901/VCR is an established VCR-resistant cell line selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR. Previous reports have proved that the SGC7901/VCR cells possess the characteristics of classical MDR with overexpression of Pgp, and the SGC7901/VCR cell line has been successfully used as an *in vitro* MDR reversal model by several study groups, including ours^[19-23]. The recent report showed that the expression of PKC α was significantly higher in SGC7901/VCR cells than in SGC7901 cells, and there was no significant difference in the expression of PKC β I, PKC β II and PKC γ between SGC7901/VCR and SGC7901 cells^[10].

In the present study, we observed that the expression of PKC α was significantly higher in SGC7901/VCR cells than in SGC7901 cells, which was consistent with the previous report by Han *et al*^[10]. To investigate the possible effect of PKC α on MDR for chemotherapeutic drug in human gastric cancer cells, we established SGC7901/VCR cells stably expressing antisense PKC α . We demonstrated that down-regulation of the predominant PKC α isoform expressed in SGC7901/VCR cells by the expression of antisense PKC α led to greater DOX accumulation and partial reversal of resistance to DOX. These data support the thesis that MDR in this cell line is modulated, in part, by PKC α . These results suggest that a more effective means of reducing PKC α activity, possibly with RNA interference, might be a more efficient way of increasing drug sensitivity in this MDR cell line.

COMMENTS

Background

Protein kinase C (PKC) constitutes a family of closely related protein serine/threonine kinase which are sub-grouped into classical (α , β I, β II, and γ), novel (δ , ϵ , η , and θ), and atypical (ι and ζ) isoforms. The members of the PKC family are involved in the regulation of numerous cell processes including proliferation, apoptosis, and differentiation. It is likely that each isoform has a specific role in a given cell.

Research frontiers

A recent study confirmed that PKC α , PKC β I, PKC β II and PKC γ were expressed in both human gastric carcinoma cell line SGC7901 and vincristine (VCR)-resistant human gastric carcinoma cell line SGC7901/VCR, but the expression of PKC α was significantly higher in SGC7901/VCR cells than that in SGC7901 cells. There was no significant difference in the expression of PKC β I, PKC β II and PKC γ between SGC7901/VCR cells and SGC7901 cells. In this report, the authors transfected SGC7901/VCR cells with an expression vector containing the cDNA for PKC α in the antisense orientation to see whether down-regulation of PKC α could affect drug resistance in SGC7901/VCR cells.

Innovations and breakthroughs

In the present study the authors observed that down-regulation of the predominant PKC α isoform expressed in SGC7901/VCR cells by the expression of antisense PKC α led to greater doxorubicin accumulation and partial reversal of resistance to doxorubicin. These data support the thesis that MDR in this cell line is modulated in part by PKC α .

Applications

This study provided a new target against MDR in human gastric carcinoma, suggesting that a more effective means of reducing PKC α activity, possibly with RNA interference, might be a more efficient way of increasing drug sensitivity in this MDR cell line.

Peer review

The manuscript entitled "Antisense expression of PKC α improved sensitivity of SGC7901/VCR cells to doxorubicin" by Wu *et al* examines the effects of anti-

sense inhibition of PKC α expression on the MDR phenotype of gastric cancer cells. The authors used antisense technology to establish a derivative of the multi-drug/vincristine-resistant SGC7901-VCR cell line with reduced expression of PKC α . Using this isogenic pair of cell lines, the authors conclude that PKC α deficiency leads to increased intracellular accumulation of DOX and partially restores sensitivity to the drug, thus reversing the MDR phenotype.

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CASE REPORT

Cannabinoid hyperemesis syndrome: Clinical diagnosis of an underrecognised manifestation of chronic cannabis abuse

Siva P Sontineni, Sanjay Chaudhary, Vijaya Sontineni, Stephen J Lanspa

Siva P Sontineni, Sanjay Chaudhary, Vijaya Sontineni, Stephen J Lanspa, Department of Medicine, Creighton University, Suite 5850, 601 N 30th Street, Omaha, NE 68131, United States

Author contributions: Sontineni SP provided the patient's data, organized, conceptualized and contributed to the manuscript writing and final approval; Chaudhary S collected the patient data, reviewed the literature and contributed to the manuscript writing; Sontineni V reviewed the literature and compiled the references; Lanspa SJ supervised, provided critical review and obtained financial support from the division; All authors approved the final manuscript.

Correspondence to: Siva P Sontineni, MD, Department of Internal Medicine, 601 N 30th St Suite 5850, Creighton University Medical Centre, Omaha, NE 68131, United States. ssontineni@gmail.com

Telephone: +1-402-4158319 Fax: +1-402-2804220

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Abstract

Cannabis is a common drug of abuse that is associated with various long-term and short-term adverse effects. The nature of its association with vomiting after chronic abuse is obscure and is underrecognised by clinicians. In some patients this vomiting can take on a pattern similar to cyclic vomiting syndrome with a peculiar compulsive hot bathing pattern, which relieves intense feelings of nausea and accompanying symptoms. In this case report, we describe a twenty-two year-old-male with a history of chronic cannabis abuse presenting with recurrent vomiting, intense nausea and abdominal pain. In addition, the patient reported that the hot baths improved his symptoms during these episodes. Abstinence from cannabis led to resolution of the vomiting symptoms and abdominal pain. We conclude that in the setting of chronic cannabis abuse, patients presenting with chronic severe nausea and vomiting that can sometimes be accompanied by abdominal pain and compulsive hot bathing behaviour, in the absence of other obvious causes, a diagnosis of cannabinoid hyperemesis syndrome should be considered.

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Key words: Cannabinoid; Cannabis; Cyclic vomiting; Hyperemesis; Marijuana; Vomiting

Peer reviewer: Yvette Taché, PhD, Digestive Diseases Research Center and Center for Neurovisceral Sciences and Women's Health, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, University of California, Los Angeles and VA Greater Los Angeles Healthcare System; 11301 Wilshire Boulevard, CURE Building 115, Room 117, Los Angeles, CA, 90073, United States

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INTRODUCTION

Cannabis has been used recreationally for millennia and is the third most commonly used drug after tobacco and alcohol^[1,2]. Research into the neurobiology of the compound has led to the discovery of an endogenous cannabinoid system. The therapeutic potential of cannabinoids has been recognized and these compounds are utilized as anti-emetics^[3-5]. Recently, a distinct syndrome in chronic cannabis abusers characterized by recurrent vomiting associated with abdominal pain and a tendency to take hot showers has been increasingly recognised. This clinical manifestation is paradoxical to the previously identified therapeutic role of cannabinoids as anti-emetics. We describe the case of a young male seeking repeated emergency room care with recurrent nausea and vomiting.

CASE REPORT

A 22-year male presented with recurrent episodes of nausea, refractory vomiting, and colicky epigastric pain for one week. The symptoms were characterized by treatment-resistant nausea in the morning, continuous vomiting, and colicky epigastric abdominal pain. Each episode lasted 2 to 3 h and increased with food intake. He often had two or more episodes a day during the symptomatic period. He had been treated for the severe nausea and vomiting in the emergency room on two occasions in the preceding two months. He also reported having learned to help himself by taking a hot bath each time the symptoms appeared, which dramatically

improved his symptoms. This habit had become a compulsion for him for symptom relief with each episode of hyperemesis. On physical examination his mucous membranes were dry, his pulse rate was 102/min and blood pressure was 140/100 with positive orthostasis. The remainder of the physical examination was unremarkable. His complete blood count and comprehensive metabolic panel were unremarkable. In addition, serum amylase and lipase levels were within the normal range. His urine drug screen was positive for tetrahydrocannabinol (THC). Abdominal X-ray series and ultrasonography were within normal limits.

Oesophagogastroduodenoscopy revealed Grade 2 distal oesophagitis and hiatal hernia. On further interviewing, he admitted to consistent marijuana abuse for the past 6 years, often smoking cannabis every hour or two on a daily basis. The patient and his mother did not recall any significant past illnesses or recurrent vomiting when he was a child. He was treated with intravenous fluids with steady improvement in symptoms, and metoclopramide, pantoprazole and morphine for the abdominal pain. It was explained that marijuana was the cause of his symptoms and he was advised not to resume marijuana abuse. On subsequent follow-up, he had abstained from cannabis and remained symptom-free.

DISCUSSION

Cannabis is one of the most commonly abused drugs worldwide. Over the past decade, marijuana has remained the most commonly used illicit substance with close to 50% of high school seniors admitting use at some time^[1]. It is estimated that each year 2.6 million individuals in the USA become new users and most are younger than 19 years of age^[6].

The long-term and short-term toxicity of cannabis abuse is associated with pathological and behavioural effects. However, cannabis has also been suggested to have therapeutic properties with anticonvulsive, analgesic, antianxiety and anti-emetic activities. Cannabis has also been used to treat anorexia in patients with acquired immunodeficiency syndrome^[3-5]. The actions of cannabis are mediated by specific cannabinoid receptors. The first of the cannabinoid receptors-CB-1-was identified in 1990 and this finding revolutionized the study of cannabinoid biology. Since then, a multitude of roles for the endogenous cannabinoid system has been proposed. A large number of endogenous cannabinoid neurotransmitters or endocannabinoids have been identified, and the CB-1 and CB-2 cannabinoid receptors have been characterized^[7]. The CB-1 receptors exert a neuromodulatory role in the central nervous system and enteric plexus^[8]. Cannabinoid type 2 receptors have an immunomodulatory effect and are located on tissues such as microglia^[5]. The presence of other receptors, transporters, and enzymes responsible for the synthesis or metabolism of endocannabinoids are being recognised at an extraordinary pace. Cannabinoids have a wide variety of effects on the body systems and physiologic states (Table 1) due to their actions on the

Table 1 Harmful effects of cannabinoids on body systems^[1,6,7]

Cognitive and mental health
Impaired memory
Impaired attention, organization and integration of complex information
Association with schizophrenia
Increased risk for depression
Pulmonary
Carcinogenic effect
Obstructive lung disease
Increased propensity toward infections
Acute and chronic bronchitis
Behavioural
Weapon possession and physical fighting
Unwanted and unprotected sexual encounters
Unwanted pregnancies
School dropout
Amotivational syndrome
Impairment of driving skill and coordination
Endocrine
Decreased testosterone, sperm motility and production, disruption of ovulatory cycle
Pregnancy
Low birth weight
Problems with attention, memory and higher cognitive function
Cardiovascular
Stroke
Dose-dependent increase in HR
Orthostasis
Decreased exercise tolerance
Precipitation of angina or myocardial infarction

receptors as well as direct toxic effects.

The anti-emetic effect of cannabinoids is largely mediated by CB-1 receptors in the brain and the intestinal tract, although some of their effect may also be receptor-independent. However, in this report, we were presented with the paradoxical effect of hyperemesis in a susceptible chronic cannabis abuser. Such a paradoxical response has previously only been demonstrated following acute toxicity to an intravenous injection of crude marijuana extract^[9]. Proposed mechanisms of cannabinoid hyperemesis include toxicity due to marijuana's long half-life, fat solubility, delayed gastric emptying, and thermoregulatory and autonomic disequilibrium *via* the limbic system^[10]. Cannabinoids are known to impair peristalsis in a dose-dependent manner^[11,12], which can theoretically override the centrally mediated anti-emetic effects, thus leading to hyperemesis. It is not known why the hyperemesis syndrome surfaces after several years of cannabis abuse. The effects of cannabinoids on the functions of the thermoregulatory and autonomic mechanisms of the brain can lead to behavioural changes^[10]. Such effects might be the underlying mechanism for the compulsive hot bathing behaviour. There is also a supposition that the syndrome could represent a type of cyclic vomiting. Cyclic vomiting syndrome (CVS) in adults is now very well recognized, and it has been proposed that marijuana contributes to CVS^[13]. However, unlike the other forms of CVS, patients with cannabinoid hyperemesis are not likely to have a history of migraine or other psychosocial stressors and the peculiar behaviour of hot showers is

Table 2 Clinical diagnosis of cannabinoid hyperemesis

Essential for diagnosis:

History of regular cannabis use for years

Major clinical features of syndrome

Severe nausea and vomiting

Vomiting that recurs in a cyclic pattern over months

Resolution of symptoms after stopping cannabis use

Supportive features

Compulsive hot baths with symptom relief

Colicky abdominal pain

No evidence of gall bladder or pancreatic inflammation

unique to this syndrome.

Based on the published research and case reports^[10,14-16], we propose the set of clinical characteristics for the diagnosis of cannabinoid hyperemesis syndrome shown in Table 2. Allen *et al*^[10] first noted this condition in a group of nineteen patients from Australia with chronic cannabis abuse and cyclical vomiting illness. An earlier case report by de Moore *et al*^[17] described a chronic cannabis abuser with psychogenic vomiting, which was complicated by spontaneous pneumomediastinum. Subsequent reports have identified similar clinical presentations^[7-9,18]. Given the high prevalence of chronic cannabis abuse worldwide and the paucity of reports in the literature, clinicians need to be more attentive to the clinical features of this underrecognised condition.

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Liver cell adenoma showing sequential alteration of radiological findings suggestive of well-differentiated hepatocellular carcinoma

Takayuki Kogure, Yoshiyuki Ueno, Satoshi Sekiguchi, Kazuyuki Ishida, Takehiko Igarashi, Yuta Wakui, Takao Iwasaki, Tooru Shimosegawa

Takayuki Kogure, Yoshiyuki Ueno, Yuta Wakui, Takao Iwasaki, Tooru Shimosegawa, Division of Gastroenterology, Graduate School of Medicine, Tohoku University, Sendai 980-8574, Japan

Satoshi Sekiguchi, Division of Advanced Surgical Science and Technology, Graduate School of Medicine, Tohoku University, Sendai 980-8574, Japan

Kazuyuki Ishida, Department of Pathology, Tohoku University Hospital, Sendai 980-8574, Japan

Takehiko Igarashi, Division of Gastroenterology Osaki Citizen Hospital, Furukawa 989-6183, Japan

Author contributions: Kogure T, Ueno Y and Wakui Y participated in writing of the manuscript; Kogure T, Ueno Y, Iwasaki T, Shimosegawa T, Sekiguchi S, Ishida K, and Igarashi T participated in treatment of the patient.

Correspondence to: Yoshiyuki Ueno, MD, PhD, Division of Gastroenterology, Graduate School of Medicine, Tohoku University, 1-1 Seiryō, Aoba-ku, Sendai 980-8574, Japan. yueno@mail.tains.tohoku.ac.jp

Telephone: +81-22-7177171 Fax: +81-22-7177177

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Abstract

A liver tumor 35 mm in diameter was found incidentally in a 40-year-old woman who had no history of liver diseases or the use of oral contraceptives. Radiological diagnostics showed the typical findings of liver cell adenoma (LCA). Dynamic computed tomography revealed that the tumor showed a homogenous enhancement in the arterial phase and almost the same enhancement as the surrounding liver parenchyma in the delayed phase. The tumor was found to contain fat on magnetic resonance imaging. A benign fat containing liver tumor was suggested. However, radiological findings altered, which caused us to suspect that a well-differentiated hepatocellular carcinoma (HCC) containing fat was becoming dedifferentiated. Partial hepatectomy was performed and the pathological findings showed the typical findings of LCA. This case was an extremely rare LCA, which had no background of risk for LCA and developed the sequential alteration of the radiological findings to suspect well-differentiated HCC.

INTRODUCTION

Liver cell adenoma (LCA) is a benign tumor of the liver parenchyma that is associated with the use of oral contraceptives or with glycogen-storage disease^[1]. The occurrence of LCA in patients without such backgrounds is extremely rare^[2]. We report a case of LCA found in a 40-year-old woman without a history of oral contraceptive use in which the sequential alteration of the radiological findings suggested well-differentiated hepatocellular carcinoma (HCC).

CASE REPORT

In November 2006, a 40-year-old woman developed lower abdominal pain and was admitted to a hospital. The patient had no history of liver disease, alcohol consumption, oral contraceptive use, nor the use of any other medication. The family history of the patient was not noteworthy. The patient was diagnosed as having a left tubo-ovarian abscess and tubo-ovariectomy was performed. The resected ovary and oviduct showed the findings of endometriosis with bacterial infection, but there was no indication of neoplastic lesion. At this time, a space-occupying lesion (SOL) 35 mm in diameter was found in the liver by abdominal ultrasonography and computed tomography (CT). In the CT, the lesion in

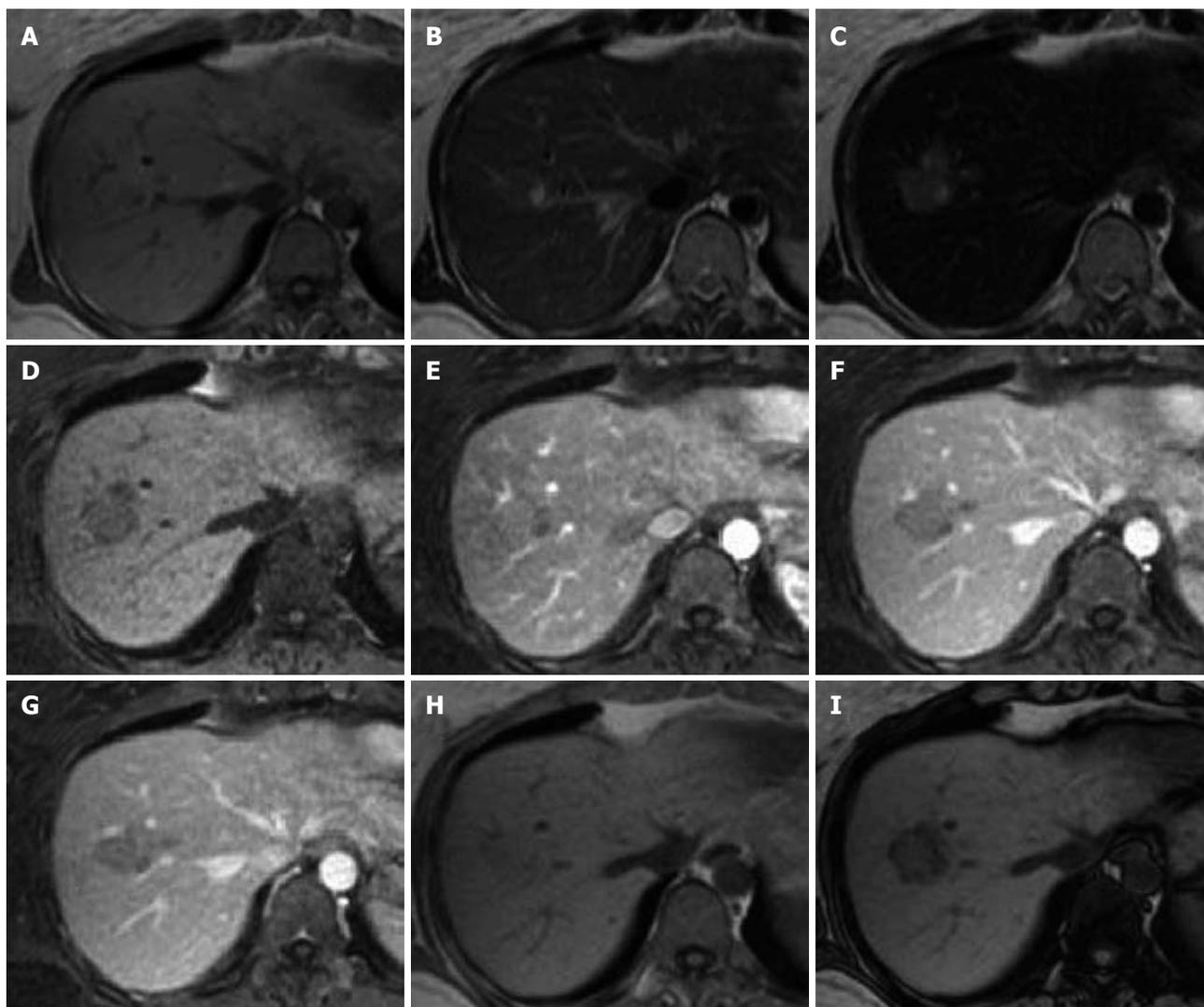


Figure 1 Magnetic resonance imaging (MRI). A: T1; B: T2; C: T2-SPIO; D: Plain; E: Arterial phase; F: Portal phase; G: Delayed phase; H: T1 in phase; I: T1 opposed phase.

the liver (segment 8) showed slight low density by plain examination and an enhancing effect by the contrast agent. In the magnetic resonance imaging (MRI), the SOL showed almost isointensity on T1 and slightly high intensity on T2 (Figure 1A-B). Superparamagnetic iron-oxide (SPIO)-MRI indicated that the SOL did not show most of the SPIO uptake (Figure 1C). On the gadolinium-enhanced T1, the SOL indicated a slight homogenous enhancement in the arterial phase, and almost the same enhancement as the surrounding liver parenchyma in the delayed phase (Figure 1D-G). The SOL showed almost isointensity on the in-phase-T1 and slightly low intensity on the opposed-phase-T1, which indicated that the SOL contained a fat component (Figure 1-H, 1-I). A capsule or scars were not detected in the SOL. A benign, fat containing tumor was suggested. The dynamic CT in March 2007 showed that the SOL had not changed in size (Figure 2). The SOL indicated low density by plain examination, showed a slight homogenous enhancing effect in the arterial phase, and, an enhancing effect that was slightly lower than the surrounding liver in the delayed phase (Figure 2). In the

dynamic CT after four months, the density of the SOL on plain examination was elevated and the enhancing effect in the arterial phase increased slightly, although the size and the form of the SOL did not show apparent changes (Figure 3). Because of the alteration of the findings in this CT, we suspected that the findings were suggestive of well-differentiated HCC. In August 2007, the patient was referred to our hospital for further examination and treatment. The physical examination of the patient showed no abnormal findings. The blood test indicated no abnormalities including liver related enzymes except slight iron deficiency anemia (Table 1). All the hepatitis virus markers were negative. There were no abnormal findings suggestive of autoimmune liver disease or metabolic liver disease including glycogen-storage disease. Hepatic arteriography was performed and the nodule presented with a slight tumor stain in the arterial phase and the hepatic parenchymal phase, and it did not show an apparent drainage vein (Figure 4). Positron emission tomography showed no difference in accumulation between the nodule and the surrounding hepatic parenchyma and no abnormal accumulation

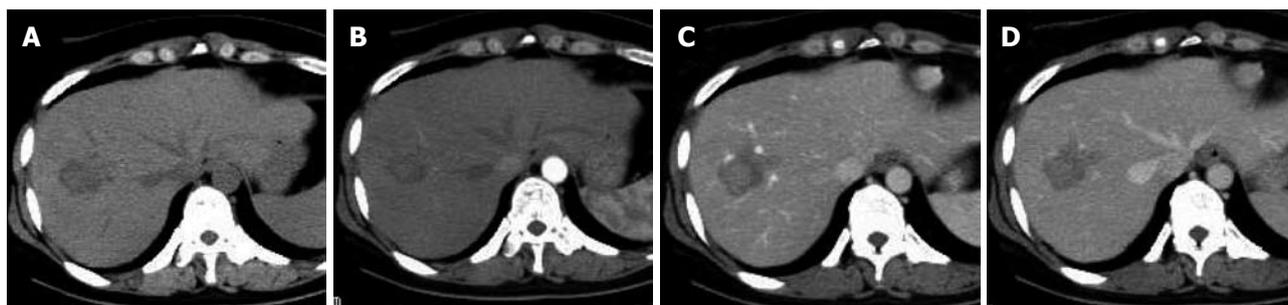


Figure 2 Computed tomography (CT) in March 2007. A: Plain; B: Arterial phase; C: Portal phase; D: Delayed phase.

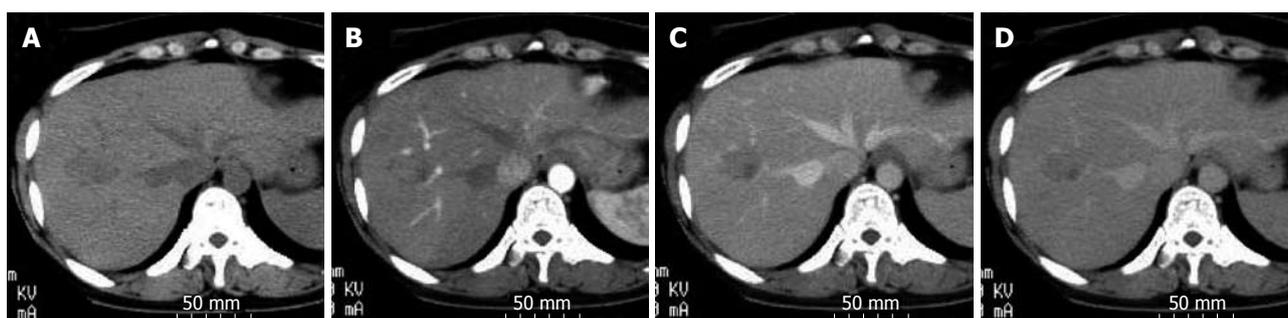


Figure 3 CT in July 2007. A: Plain; B: Arterial phase; C: Portal phase; D: Delayed phase.

Table 1 Laboratory data

Indicators	Value
White blood cell	3980/ μ L
Hemoglobin	9.8 g/dL
Platelet	232×10^3 / μ L
Prothrombin time	105%
Activated partial thromboplastin time	26.7 s
Aspartate aminotransferase	17 IU/L
Alanine aminotransferase	15 IU/L
Lactate dehydrogenase	146 IU/L
Alkaline phosphatase	175 IU/L
Gamma glutamyl transpeptidase	23 IU/L
Cholinesterase	257 IU/L
Total bilirubin	0.6 mg/dL
Direct bilirubin	0.1 mg/dL
Total protein	6.9 g/dL
Albumin	4.2 g/dL
Blood urea nitrogen	7.5 mg/dL
Creatinine	0.6 mg/dL
Uric acid	4.0 mg/dL
Na	142 mEq/L
K	4.2 mEq/L
Cl	106 mEq/L
Total cholesterol	202 mg/dL
Triglyceride	77 mg/dL
Glucose	101 mg/dL
C-reactive protein	0.03 mg/dL
IgG	1157 mg/dL
IgA	206 mg/dL
IgM	124 mg/dL
HBs-Ag	Negative
HBs-Ab	Negative
HCV-Ab	Negative
α -fetoprotein	5.1 ng/mL
Des- γ -carboxy prothrombin	34 mAU/mL
Anti-nuclear antibody	Negative
Anti-smooth muscle antibody	Negative
Anti-mitochondrial antibody	Negative

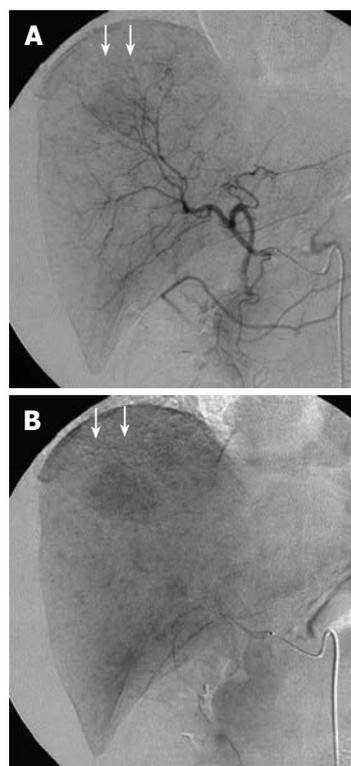


Figure 4 Digital subtraction angiography (DSA). A: Arterial phase; B: Liver parenchymal phase. Arrow: Tumor stain.

suspicious of malignant tumors in other organs. No abnormal findings were found suggesting a primary tumor in other organs in whole-body CT. Digestive tract endoscopy showed no abnormal findings. Taken together, the above-mentioned findings suggested the

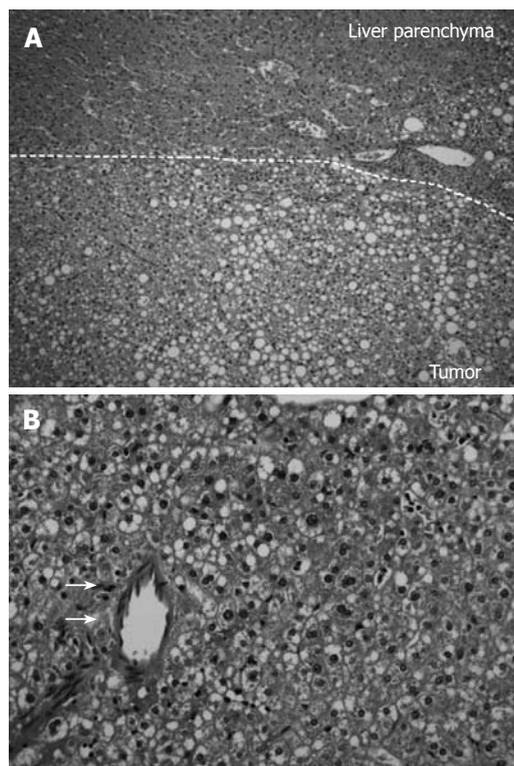


Figure 5 Pathological findings. A: Hematoxylin and eosin staining, original magnification, $\times 50$; B: Hematoxylin and eosin staining, original magnification, $\times 200$. Arrow: Myogenic artery.

possibility that the nodule was a well-differentiated HCC that was dedifferentiating. After receiving informed consent, partial hepatectomy was performed on August 28, 2007.

The pathological finding (Figure 5) revealed the following. Hepatocytes more lucent than that of the surrounding hepatic parenchyma had proliferated thickly in the nodule. Hepatocytes did not show severe cellular atypia. Exclusion of the surrounding hepatic parenchyma was apparent, but direct invasion to the surrounding was not seen. The nodule did not contain the portal tracts and bile ducts. Myogenic arteries, large lipid droplets, hemorrhage and degeneration were seen in the nodule. The surrounding hepatic parenchyma had no abnormal findings except fatty change with large lipid droplets around the nodule. Thus, we diagnosed the nodule as a LCA.

DISCUSSION

LCA is considered a rare benign tumor of the liver and is distinguished from solitary tumors and adenomatosis^[3,4]. LCA was a very rare tumor until an association with oral contraceptives was reported. Before 1954, only two cases were detected among 5000 autopsies in 36 years^[5]. The association with oral contraceptives was thoroughly described in the 1970s^[6,7], and many cases of LCA have been reported since^[8-10].

Due to the progress of diagnostic imaging techniques, the typical findings of LCA have been identified^[4,11]. A typical LCA shows low density or isodensity on plain

CT, presents a homogenous contrasting effect on arterial phase and does not show an apparent wash out on delayed phase. A typical LCA in MRI shows almost the same signal intensity as the surrounding parenchyma on T1 and T2 and shows high intensity on fat suppression T2. LCA may often present difficulties in a differential diagnosis with well-differentiated HCC and focal nodular hyperplasia^[12-14]. The pathological findings of a typical LCA include the following^[2,10,15-17]: (1) the tumor consists of hepatocytes with almost normal nuclei and cytoplasm presenting a homogenous increase, (2) and does not include the portal area and bile duct, (3) hepatocytes form hepatic cords, but sinusoids are pressed, and hepatic lobule structure is absent, (4) the tumor includes macrovesicular fatty changes, hemorrhage, degeneration, and myogenic arteries.

LCA can be divided into three categories: (1) LCA associated with oral administration of medicines including oral contraceptives and steroids^[8-10], (2) LCA developing as a complication of glycogen-storage diseases^[18-20], (3) adenomatosis^[3,4,15]. Solitary LCAs are most frequently caused by oral contraceptives^[7,21]. The occurrence of adenomatosis is unrelated to the use of oral contraceptives and the frequency of development is not related to sex^[15]. It is reported that LCA occurs in patients of glycogen-storage disease type I^[19]. In addition to oral contraceptives, oral administrations of such medications as clomiphene^[22], barbituric acid^[23] or androgen^[24,25] are reported as risk factors for LCA. Also, it is reported that LCAs associated with oral contraceptives show regression after discontinuing the drug^[26,27].

The main complications of LCA include tumor hemorrhage and malignant transformation. It is reported that hemorrhage is found in about one half of LCAs and can result in death^[9,16,28]. Malignant transformation of LCA is considered to be rare. However, in several reported cases, LCA associated with glycogen-storage diseases and glucocorticosteroids developed HCC^[29-32]. In another reported case, hepatocarcinogenesis occurred several years after discontinuation of oral contraceptives^[32].

Although LCA is a benign liver tumor, treatment is often conducted to avoid hemorrhage and malignant transformation^[3,4]. Surgical treatments such as lobectomy^[21,33], enucleation^[34] and liver transplantation^[35] are performed for LCA. Percutaneous ethanol injection^[20] and transcatheter arterial embolization^[33] are established therapies for low invasive treatment of HCC, and were reported to be performed for LCA. However, precise pathological examination is not possible by these treatments. Since a definitive diagnosis of LCA by diagnostic imaging alone or by needle biopsy is difficult^[11], tumor resection is often the most suitable approach. Our case was found by chance during treatment of the endometriosis complicated with bacterial infection. The patient did not have a history of using oral contraceptives or glucocorticosteroids which are known risk factors for LCA. Nor did the patient have metabolic liver disease including glycogen-storage

disease. By MRI, the tumor was found to contain fat. In dynamic studies, the tumor showed a homogenous enhancement in the arterial phase and almost the same enhancement as the surrounding liver parenchyma in the delayed phase. These findings were typical for LCA. However, the tumor showed an elevation of the density on plain CT and an increase of the early enhancement in the arterial phase, which caused us to suspect that well-differentiated HCC containing fat was becoming dedifferentiated. Accordingly, hepatic resection was performed.

In a prospective study of 48 LCA cases reported by van der Windt *et al*^[27], serial observation was considered appropriate for tumors with the typical image findings of LCA if the diameter was less than 5 cm. Five of the 48 cases of LCA were resected because the radiological findings changed during serial observations and three of the five cases had well-differentiated HCC, while in the remaining two cases, it was difficult to distinguish between well-differentiated HCC and LCA pathologically. The present case showed the typical radiological findings for LCA and was 35 mm in diameter without an increasing tendency. However, diagnosis only by radiological findings is difficult in patients without a background supporting a diagnosis of LCA such as a history of receiving the above mentioned drugs or glycogen-storage disease. Resection of the tumor in the present case was therefore considered appropriate because of the risk of hemorrhage and possible malignant transformation.

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Intraductal papillary mucinous neoplasm in chronic calcifying pancreatitis: Egg or hen?

Evangelos Kalaitzakis, Barbara Braden, Palak Trivedi, Yalda Sharifi, Roger Chapman

Evangelos Kalaitzakis, Barbara Braden, Palak Trivedi, Yalda Sharifi, Roger Chapman, Department of Gastroenterology, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Evangelos Kalaitzakis, Section of Gastroenterology and Hepatology, Department of Internal Medicine, Sahlgrenska University Hospital, 41345 Gothenburg, Sweden

Author contributions: All the authors contributed to the idea conception, writing of manuscript, and approval of final manuscript.

Correspondence to: Evangelos Kalaitzakis, MD, PhD, Section of Gastroenterology and Hepatology, Department of Internal Medicine, Sahlgrenska University Hospital, 41345 Gothenburg, Sweden. evangelos.kalaitzakis@vgregion.se

Telephone: +46-31-3421000 Fax: +46-31-822152

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TO THE EDITOR

Intraductal papillary mucinous neoplasm (IPMN) is an increasingly reported entity, representing up to one third of all pancreatic cystic neoplasms^[1]. IPMNs typically present with cystic dilatation of the pancreatic duct associated with mucin production and variable cellular atypia. Although extensive pancreatic calcification is generally considered to be a pathognomonic sign of chronic pancreatitis, it may also occur simultaneously with IPMN which may lead to diagnostic confusion^[2,3]. We report a case of a patient who was initially diagnosed with chronic calcifying pancreatitis and was later shown to have an IPMN.

An 81-year-old man was referred to the gastroenterology clinic due to weight loss, diarrhea, and anemia. He did not drink any alcohol and got quit of smoking pipe 50 years ago. Medical history was unremarkable apart from chronic obstructive pulmonary disease and type 2 diabetes mellitus diagnosed one year prior to presentation. Laboratory tests revealed mild normocytic normochromic anemia with 11.3 g/L hemoglobin (reference 13.0-17.0 g/L) but essentially normal white cell and platelet counts as well as normal electrolytes, creatinine, albumin, liver function tests, amylase, endomysial antibodies, plasma protein electrophoresis, and thyroid function tests.

Esophagogastroduodenoscopy was normal. A CT-colonography showed normal colonic appearances but there was evidence of a grossly atrophic calcific pancreas with a dilated pancreatic duct. No mass lesion was seen. The patient did not have a history of acute pancreatitis, abdominal radiotherapy or trauma to the epigastric region. His serum calcium and triglycerides were normal. Fecal elastase was < 15 µg/g of stool (reference > 500 µg/g of stools). Therefore, exocrine pancreatic insufficiency due to idiopathic chronic calcifying pancreatitis was diagnosed and pancreatic enzyme substitution was started.

Despite some initial improvements, the patient reported further weight loss six months after initial

Abstract

Intraductal papillary mucinous neoplasm (IPMN) is an increasingly reported entity. Extensive pancreatic calcification is generally thought to be a sign of chronic pancreatitis, but it may occur simultaneously with IPMN leading to diagnostic difficulties. We report a case of a patient initially diagnosed with chronic calcifying pancreatitis who was later shown to have a malignant IPMN. This case illustrates potential pitfalls in the diagnosis of IPMN in the case of extensive pancreatic calcification as well as clues that may lead the clinician to suspecting the diagnosis. The possible mechanisms of the relation between pancreatic calcification and IPMN are also reviewed.

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Key words: Intraductal papillary mucinous neoplasm; Endoscopic ultrasound; Calcifying pancreatitis; Carcinoembryonic antigen; Endoscopic retrograde cholangiopancreatography

Peer reviewer: Wei Tang, MD, EngD, Assistant Professor, H-B-P Surgery Division, Artificial Organ and Transplantation Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

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Figure 1 Abdominal computed tomography image showing gross pancreatic duct dilatation and extensive pancreatic calcification as well as bile duct dilatation. No distinct mass was seen.



Figure 2 Endoscopic ultrasound revealing a dilated pancreatic duct (> 10 mm in diameter) with mural irregularities, calculi within the pancreatic duct, and an atrophic pancreas.

presentation. Laboratory testing showed that his alkaline phosphatase was 2629 IU/L (reference 95-320 IU/L), bilirubin was 42 $\mu\text{mol/L}$ (reference 3-17 $\mu\text{mol/L}$), and alanine aminotransferase was 347 IU/L (reference 10-45 IU/L). A pancreatic protocol CT demonstrated gross pancreatic atrophy and calcification, dilated pancreatic as well as intrahepatic and common bile ducts. No mass lesion was seen (Figure 1). Endoscopic ultrasound confirmed these findings and also showed an enlarged heterogeneous pancreatic head with dilated side branches of the pancreatic duct as well as an area of hypoechoogenicity, but did not show any distinct mass. The main pancreatic duct in the head was dilated with its diameter > 10 mm and with wall irregularities (Figure 2). Mucus extrusion from a protruding gaping major papilla was observed. Endoscopic retrograde cholangiopancreatography was attempted in which opacification of the main pancreatic duct showed multiple filling defects suggestive of intraductal mucin. Common bile duct cannulation failed and biliary drainage was achieved by means of a percutaneous transhepatic cholangiography. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels in mucin aspirated from the pancreatic duct were 24132 ng/mL and 60840 U/mL, respectively. On the basis of the imaging findings, the fact that mucus extrusion was observed from the papilla, and the high levels of CEA and CA 19-9 in pancreatic duct aspirates, a malignant IPMN was diagnosed. Due to his clinical condition, the patient was judged not to be a candidate for surgery and was offered palliative therapy.

Twelve to sixty percent of patients with IPMN have a history leading to a diagnosis of chronic pancreatitis^[4,5] and roughly 2% of all diagnoses of chronic pancreatitis are associated with IPMN^[5]. However, few reports of patients with concomitant calcifying pancreatitis and IPMN are available^[2,3]. In a cohort study of 473 patients suffering from chronic pancreatitis, only 6 were found to have IPMN during follow-up but none had calcifications^[5]. Of the 16 cases of patients with IPMN and pancreatic calcification published thus far, 8 had diffuse pancreatic calcification which did not involve the tumor itself in most of the cases^[2,3]. The pathogenesis

of calcification in IPMN is unknown. Apart from the unlikely possibility that the two entities may occur coincidentally, it is possible that IPMN may be a complication of chronic calcifying pancreatitis, but no report of a patient with this condition developing IPMN in the course of the disease is available. It has also been proposed that IPMN may be responsible for pancreatic calcification due to chronic partial ductal obstruction by mucin plugs^[2,3]. In any case, the concomitant presence of pancreatic cystic lesions, duct dilatation, and calcification should raise the suspicion of an IPMN. Particular care should be taken when diagnosing chronic pancreatitis in patients who do not present typical characteristics of the disease, namely age over 50 years, moderate alcohol intake, and non-smokers, which may be suggestive of IPMN^[5]. In our case, the patient was 81 years of age and did not consume any alcohol as verified by the patient himself and his relatives.

Although CEA and CA 19-9 measurements are frequently performed during the diagnostic work-up of patients with pancreatic cystic lesions, it has been suggested that cyst fluid or pancreatic duct CEA or CA 19-9 is not helpful in the differentiation between benign and malignant IPMNs^[6] but published data in the literature are not unanimous^[7]. A CEA > 800 ng/mL has been reported to be helpful in differentiating mucinous cystadenocarcinomas from mucinous cystadenomas^[8]. Although only 5/16 patients with concomitant calcifying pancreatitis and IPMN previously reported were shown to have a malignant lesion^[2,3], in the present case the fact that both CEA and CA 19-9 in the pancreatic duct aspirate were extremely high was felt to be highly suggestive of malignancy. A main pancreatic duct > 6 mm in diameter and protrusion of the papilla of Vater are also considered signs predicting malignancy^[9]. Thus, endoscopic ultrasound is not just a technique to obtain fluid from cystic pancreatic lesions for tumor marker analysis, but it also provides invaluable morphological information on mural pancreatic duct irregularities, presence of solid lesions or septa in cysts. Compared to other imaging methods, it achieves the highest detail resolution.

In summary, concomitant presence of pancreatic

cystic lesions, duct dilatation, and calcification should raise the suspicion of an IPMN, especially when patients do not present typical characteristics of chronic pancreatitis, namely age over 50 years, moderate alcohol intake, and non-smokers. Inspection of the ampulla with a duodenoscope and aspiration of fluid from the pancreatic duct or endoscopic ultrasound-guided fine needle aspiration from a cystic pancreatic lesion for analysis of CEA and CA 19-9 may be useful in the diagnosis of such patients.

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Rudi Beyaert, Professor

Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology and Ghent University Technologiepark 927, B-9052 Gent, Belgium

Edmund J Bini, Professor

VA New York Harbor Healthcare System, Division of Gastroenterology (111D), 423 East 23rd Street, New York, NY 10010, United States

Reinhard Buettner, Professor

Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany

Michael F Byrne, MD, Clinical Associate Professor

Division of Gastroenterology Vancouver General Hospital, 100-2647 Willow Street Vancouver BC V5Z 3P1, Canada

Ramsey Chi-man Cheung, MD, Professor

Division of GI & Hepatology, VAPAHCS (154C), 3801 Miranda Ave, Stanford University School of Medicine, Palo Alto, CA 94304, United States

George N Dalekos, MD, PhD, Associate Professor Medicine

Department of Medicine, Academic Liver Unit & Research Lab of Internal Medicine, Medical School, University of Thessaly, University Hospital of Larissa, PO Box 1425, 41110, Larissa, Greece

Giovanni D De Palma, Professor

Department of Surgery and Advanced Technologies, University of Naples Federico II, School of Medicine, Naples 80131, Italy

Conor P Delaney, MD, MCh, PhD, FRCSI, FACS, Professor of Surgery Case Western Reserve University, Chief

Division of Colorectal Surgery, Vice-Chairman, Department of Surgery, Director, Institute for Surgery and Innovation, University Hospitals, Case Medical Center, 11100 Euclid Avenue Cleveland, OH 44106-5047, United States

Dong Jin Suh, MD

Departments of Gastroenterology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

Marko Duvnjak, MD

Department of Gastroenterology and Hepatology, Sestre milosrdnice University Hospital, Vinogradska cesta 29, 10000 Zagreb, Croatia

Robert JL Fraser, Associate Professor

Investigations and Procedures Unit, Repatriation General Hospital, Daw Park, Australia

Mark D Gorrell, PhD, Professor

Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

Yik-Hong Ho, Professor

Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Sabine Mihm, Professor

Department of Gastroenterology, Georg-August-Universität, Robert-Koch-Str.40, Göttingen D-37099, Germany

Kenji Miki, MD

Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

Chris JJ Mulder, Professor

Department of Gastroenterology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands

Carlos J Pirola, PhD, FAHA

Instituto de Investigaciones Medicas A Lanari, Combatientes de Malvinas 3150, Buenos Aires-1427, Argentina

Marco Romano, MD, Professor

Dipartimento di Internistica Clinica e Sperimentale-Gastroenterologia, II Policlinico, Edificio 3, II piano, Via Pansini 5, 80131 Napoli, Italy

Ned Snyder, MD, FACP, AGAF

Professor of Medicine, Chief of Clinical Gastroenterology and Hepatology, Department of Internal Medicine, The University of Texas Medical Branch, 301 University Blvd., Galveston, Texas 77555-0764, United States

Dr. Kevin J Spring

Conjoint Gastroenterology Laboratory, The Queensland Institute of Medical Research, the Bancroft Centre, rm H07, PO Royal Brisbane Hospital, Herston, QLD 4029, Australia

Christer S von Holstein, Associate professor

Department of Surgery, Lund University Hospital, SE-221 85 Lund, Sweden

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.apdwc.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
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Gastro 2009 UEGW/World Congress of Gastroenterology
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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 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]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

^[3]Passed away on June 14, 2008



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Post-pyloric feeding

Eva Niv, Zvi Fireman, Nachum Vaisman

Eva Niv, Zvi Fireman, Department of Gastroenterology, Hillel Yaffe Medical Center, Hadera 38100, Israel

Eva Niv, Nachum Vaisman, The Unit of Clinical Nutrition, Tel Aviv Sourasky Medical Center, Tel Aviv, 6974444, Israel

Author contributions: Niv E, Fireman Z and Vaisman N performed the literature review and wrote the paper.

Correspondence to: Eva Niv, MD, Department of Gastroenterology, Hillel Yaffe Medical Center, PO Box 169, Hadera 38100, Israel. niv_em@netvision.net.il

Telephone: +972-4-6304480 Fax: +972-4-6304408

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Abstract

Postpyloric feeding is an important and promising alternative to parenteral nutrition. The indications for this kind of feeding are increasing and include a variety of clinical conditions, such as gastroparesis, acute pancreatitis, gastric outlet stenosis, hyperemesis (including gravida), recurrent aspiration, tracheoesophageal fistula and stenosis in gastroenterostomy. This review discusses the differences between pre- and postpyloric feeding, indications and contraindications, advantages and disadvantages, and provides an overview of the techniques of placement of various postpyloric devices.

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Key words: Postpyloric feeding; Nasojejunal feeding; Nasojejunal tube; Jejunostomy; Nasoenteric tube; Percutaneous endoscopic gastrostomy-jejunostomy tube; Percutaneous endoscopic jejunostomy

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INTRODUCTION

According to both European and American guidelines for enteral and parenteral nutrition, enteral feeding is

the preferred method of nutritional support in patients who have a functioning gastrointestinal (GI) tract but cannot maintain an adequate oral intake^[1,2]. Enteral nutrition prevents GI mucosal atrophy, keeps intestinal integrity and prevents bacterial translocation from the GI lumen to the rest of the body, by maintaining normal permeability of the GI mucosal barrier^[3-6]. In addition, it is less expensive and has significantly fewer complications than parenteral nutrition^[1,2].

The enteral route traditionally delivered nutrition directly into the stomach *via* a nasogastric tube or gastrostomy (prepyloric feeding). The concept of postpyloric feeding has been developed over the past few decades and has become a part of the routine practice of nutritional teams in many countries. A wide variety of postpyloric nutrition devices are currently available, including different types of nasoduodenal and nasojejunal tubes and jejunostomies.

What are the differences between pre- and postpyloric feeding approaches? Why is the location of the tip of a nutritional device before or after the pylorus so important? The current review will discuss the major differences between these two methods of enteral nutrition in order to provide essential information for every nutritionist and gastroenterologist in making the right choice for every specific case. This review will provide a comprehensive overview of accepted indications and contraindications of postpyloric feeding, based on existing studies and guidelines. In addition, various devices for postpyloric feeding, as well as different techniques for their insertion, their advantages and disadvantages will be discussed.

PHYSIOLOGICAL DIFFERENCES BETWEEN PRE- AND POSTPYLORIC FEEDING

There are several physiological differences between pre- and postpyloric feeding. The first major difference is a mechanical one. Postpyloric delivery of food significantly reduces the likelihood of aspiration/vomiting caused by gastroesophageal reflux, especially in the case of intrajejunal and not intraduodenal feeding. The second major difference is the neurohormonal effect of food that is supplied directly to the small intestine or the duodenum, compared to intragastric supply. It has different effects on pancreatico-biliary secretions and on small bowel and gallbladder motility. Ledebøer *et al*^[7] have demonstrated that intraduodenal feeding causes a stronger GI response than intragastric feeding. It stimulates gallbladder

Table 1 Differences between gastric and jejunal feeding

	Nasogastric tube	Nasojejunal tube
Indications	Anorexia, dysphagia, odynophagia	Gastroparesis, gastric outlet obstruction, recurrent aspirations, severe pancreatitis, hyperemesis gravidarum, proximal enteric fistula, postoperative anastomotic gastroenteric stenosis
Insertion technique	Easy access, no need for endoscopic or radiological study or medication	Needs endoscope or prokinetic agents
Costs	Much cheaper because: 1. low cost of the tube; 2. may be inserted by a nurse	More expensive because: 1. costly equipment; 2. requires insertion by physician
Physiology	More physiological, keep normal motility and hormonal profile	Less controlled motility and hormonal control. Less pancreatic stimulation if inserted after the Trietz ligament
Feeding mode	Bolus or continuous. Pump is not mandatory	Continuous only. Pump is mandatory in most cases
Risk of aspiration	High in patients with GER and swallowing impairments	Less frequent but not absolutely prevented
Clogging rate	Rare thanks to larger diameter of tube	Frequent

contractions, accelerates small bowel transit time, and increases cholecystokinin and pancreatic polypeptide release^[7]. Intrajejunal feeding has a completely different effect^[8,9]. The classic work in a canine model by Ragins *et al*^[10] has demonstrated that jejunal feeding does not stimulate pancreatic secretion, as is seen in intragastric or intraduodenal delivery of food, which increases the volume and changes the content of pancreatic secretions. These results have been supported by animal and human studies on models of acute pancreatitis^[11-14].

Unfortunately, almost all these studies have focused on the influence of intrajejunal feeding on the pancreas and failed to address the intriguing issue of its impact on small bowel function. Data on the changes in the levels of relevant hormones and changes in the motor pattern of the small and large intestine are scarce.

Table 1 demonstrates differences between gastric and jejunal feeding.

ACCESS ROUTES FOR POSTPYLORIC FEEDING

The access routes for postpyloric feeding include nasoduodenal and nasojejunal tubes and jejunostomy. Nasoenteric tubes may be placed manually or with endoscopic, radiological or surgical guidance. Nasoenteric tubes are a good choice for short-term feeding but have many drawbacks for long-term management. They tend to recoil into the stomach, become clogged, cause nasal pressure sores, and can be pushed out of place accidentally. As such, jejunostomy is the preferred option for long-term postpyloric feeding. Feeding by jejunostomy generally requires surgical placement, although endoscopic or fluoroscopic placement for jejunostomy has been successful in some medical centers with adequate experience^[15,16]. In cases for which a surgical jejunostomy is considered, the benefits and tolerability of surgical jejunostomy and postpyloric feeding can be assessed by temporarily placing a nasojejunal tube or by inserting a jejunal extension of a percutaneous endoscopic gastrostomy-jejunostomy (PEG-J).

There are several kinds of nasoenteric tubes made from various materials (e.g. polyurethane and polyvinylchloride), that have different diameters (8-12 French), with and without guide wires, and with and

without weight at their tips. Unweighted tubes of smaller diameter (8 French) are used for endoscopic insertion to ensure a proper passage through a working channel of the endoscope. The length of a nasoenteric tube ranges from 140 cm to 220 cm.

Nasoduodenal tubes and duodenostomies have been in common use in the past, but cumulative experience has shown that the duodenal route is very problematic because of the tendency of a nasoduodenal tube to recoil back into the stomach, as well as the strong stimulatory effect of intraduodenal feeding on pancreatic secretions^[7]. In addition, a feeding formula tends to flow to the stomach because of duodenogastric reflux^[17-19]. Thus, intraduodenal feeding is contraindicated in the setting of recurrent aspiration and severe pancreatitis, which are the most common indications for postpyloric feeding. As a result, intraduodenal feeding is no longer routinely used in most medical centers and will not be discussed further.

INDICATIONS FOR POSTPYLORIC FEEDING

There are several clinical situations in which postpyloric feeding is preferable to the intragastric route. One of the most common indications is gastroparesis that is not responsive to prokinetics^[20,21]. This situation is most frequently encountered in the early postoperative setting or in critical care patients. Theoretically, postpyloric feeding would appear to be an attractive option in critically ill patients because of the frequently present problems of gastroparesis and aspirations^[22-25]. It is, however, associated with significant cost and risks of nasojejunal tube insertion. It has been accepted widely in the past that every critically ill patient should be fed postpylorically, and this approach has been investigated in many studies and meta-analyses^[26-29]. One of the most interesting of these was by Montejo *et al*^[30], who conducted a multicenter, prospective, randomized, single-blind study on 110 patients with similar characteristics who were randomized to be fed pre- or postpylorically. The authors concluded that the nutritional results were similar in both groups, and therefore, the routine use of a nasojejunal tube in critically ill patients is not justified. It may, however, have a role in selected patients with high gastric residuals on nasogastric feeding, or

with various conditions of the GI tract, such as severe pancreatitis. Boulton-Jones *et al.*^[31] have investigated postpyloric feeding in selected groups of 138 critically ill patients who suffered from burn injury, severe pancreatitis, sepsis, postoperative gastroparesis, and vomiting induced by bone marrow transplantation and chemotherapy. The results of that study demonstrated good nutritional results in all the patients. On the basis of these and additional studies that have been published since then, the current European and American guidelines for enteral and parenteral nutrition^[1,2] support nasojejunal feeding only in selected groups of critically ill patients with one of indications mentioned in this section (i.e. gastroparesis, recurrent aspirations, severe hyperemesis, and severe acute pancreatitis).

Another common indication for postpyloric feeding is recurrent aspiration caused by severe gastroesophageal reflux disease (GERD) in bedridden patients^[22,23]. One of the classic studies on this subject was by Montecalvo *et al.*^[22]. Thirty-eight patients were randomly assigned to feeding by nasogastric or nasojejunal tube. There were no documented aspirations in the postpyloric feeding group compared to two aspirations in the nasojejunal tube group. It is important to define whether episodes of aspiration are truly caused by GERD or are the result of disorders in swallowing.

Nasojejunal tube insertion has become routine practice in many hospitals in cases of severe pancreatitis. This kind of feeding enables the provision of enteral nutrition with less stimulation of pancreatic secretions and less exacerbation of inflammation in the pancreas. There are four phases of pancreatic secretions: (1) basal - very little pancreatic secretion during fasting; (2) cephalic - mildly increased secretion when the individual looks at food; (3) gastric - increased pancreatic secretion initiated by gastric distention with food and mediated by gastrin and acid; and (4) duodenal - extensive stimulation of pancreatic secretions initiated by the entry of chyme and acid into the duodenum, and mediated by secretin and cholecystokinin. The classic work of Ragins *et al.*^[10] in a canine model has demonstrated that intragastric or intraduodenal delivery of food increases the volume and changes the content of pancreatic secretions. In contrast, jejunal feeding does not stimulate pancreatic secretions^[11,32]. Since it is very important to provide pancreatic rest during acute pancreatitis, the idea of intrajejunal feeding has become very attractive and it has been investigated in animal models of acute pancreatitis^[33] and in several prospective randomized controlled human studies that have compared nasojejunal feeding with total parenteral nutrition (TPN)^[12,13,34]. The consensus is that nasojejunal feeding has a good and even better clinical outcome and time of recovery from severe pancreatitis than those with TPN^[32]. Moreover, TPN is more expensive and associated with more complications than intrajejunal feeding, giving further advantage to the latter. There have been only a few studies on the formula of choice for nasojejunal feeding in severe pancreatitis^[35-37]. Most of these studies support polymeric formulas, but some show advantages for elemental or semi-elemental formulas. Polymeric formulas are preferred in most centers because of their lower cost.

A rare but important indication is a proximal enteric fistula. For example, if a fistula is located in the esophagus/stomach/duodenum (usually tracheo-esophageal fistula), a nasojejunal tube will supply food more distally and make it possible to provide food enterally as an alternative to parenteral nutrition.

A relatively newly defined indication is hyperemesis gravida. Parenteral nutrition had previously been indicated in some cases of severe hyperemesis gravida with significant weight loss. Two small studies have described the possibility of nasoenteric tube feeding in these women^[38,39]. A pioneer study by Vaisman *et al.*^[40] has examined the feasibility and efficacy of nasojejunal feeding in 11 pregnant women with severe hyperemesis gravida that persisted despite in-hospital anti-emetic treatment. The nasojejunal feeding approach proved to be effective, reducing vomiting within the first 48 h, with complete resolution after 5 d in most of the women. More prospective studies are needed to validate this promising method.

Postpyloric feeding is the only route for enteral feeding in pyloric or duodenal outlet stenosis. This condition is common in malnourished oncological patients with gastric or pancreatic cancers who are waiting for definitive or palliative surgery, and who are required to improve their nutritional status prior to undergoing surgery.

Another common situation is the postoperative setting after Bilroth II or Whipple procedures. Postoperative transient edema in a gastroenteric anastomosis might create a significant problem in gastric emptying. Temporary insertion of a nasojejunal tube below the anastomosis will provide an enteral feeding route for these patients until the edema resolves^[41]. In some cases of difficult GI anastomosis, the preventive intraoperative insertion of a nasojejunal tube is recommended to enable early postoperative enteral feeding.

CONTRAINDICATIONS FOR POSTPYLORIC FEEDING

The major contraindication for postpyloric feeding is an obstruction in different parts of the GI tract (esophagus, gastric outlet or intestine). An endoscopic nasojejunal tube or an endoscopic jejunostomy are contraindicated in some clinical scenarios because of the inability of inserting the gastroscope postpylorically, but surgical jejunostomy may still be indicated, as in the case of complete obstruction of the esophagus/stomach/duodenum. Endoscopic nasojejunal tube insertion may nevertheless be an option in some cases of partial obstruction of the upper GI tract because it is possible to push the tip of the tube far beyond the location of the endoscope, and the procedure might even be done blindly beyond a visible stricture. The feasibility of inserting an endoscopic nasojejunal tube depends on the degree of stenosis. Even a pinpoint passage that is sufficient for passage of a guide wire permits the insertion of a nasojejunal tube. Of course, surgical jejunostomy does not require any passage of an endoscope through the GI tract, which provides more

possibilities for applying this kind of technique.

The most important absolute contraindication for all kinds of postpyloric feeding is bowel obstruction or perforation/leakage. Therefore, exact information about the GI tract's mechanical problems, previous GI tract surgery, imaging of the GI tract and verification of GI tract patency must be obtained before postpyloric feeding can be considered.

Contraindications for jejunostomy, but not for a nasojeunal tube, are significant ascites, coagulopathy, peritoneal dialysis, and peritoneal metastasis. For endoscopic insertion of jejunostomy, there are additional contraindications, such as morbid obesity and the inability to transilluminate through the abdominal wall or to see a digital imprint.

TECHNIQUES OF INSERTION

Nasoenteric tube placement

Nasoenteric tubes may be placed by using manual (blind) techniques or with the aid of fluoroscopy or endoscopy^[42]. Nasojeunal tubes for surgical patients may be placed during laparotomy. There are several manual techniques for nasojeunal tube placement. Usually, a nasoenteric tube (8-9 French) is inserted with a guide wire and a weighted tip is inserted into the stomach using the usual technique for nasogastric tube insertion. The patient is then asked to change his/her position to right lateral decubitus and the tube is pushed through the pylorus. The guide wire should be removed at the end of the procedure^[43]. Several techniques have been developed to facilitate the passage of the tube through the pylorus, among them air insufflation of the stomach^[44,45], pH-sensor feeding tube guidance^[46,47], and prokinetic agents, such as intravenous erythromycin (250-500 mg)^[48-51] or 10 mg metoclopramide^[52-54]. For example, a very interesting randomized, double-blind, placebo-controlled study has been published by Griffith *et al*^[51]. Thirty-six critical ill patients were randomized to receive a single bolus of intravenous erythromycin (500 mg) or saline before placement of 10-French feeding tubes, using a standardized active bedside protocol. The conclusion of the study was very impressive, with a 93% success rate in the erythromycin group *versus* 55% in the placebo group. In contrast, a study by Gharpure *et al*^[50], with a similar design, on a group of critically ill children demonstrated no clinical advantage with intravenous erythromycin (10 mg/kg) *versus* saline in facilitation of transpyloric passage of nasojeunal tubes.

There is no consensus on the best technique of manual insertion of a nasojeunal tube because of the great variety of success rates (30%-95%) reported in many studies carried out in different centers^[44,46,48,52]. The advantage of weighted over unweighted tubes is uncertain^[55], although it is a widely accepted belief.

The nasojeunal feeding tube is commonly placed endoscopically, which allows placement under direct vision^[56-59]. Its major disadvantage is the requirement of a complete gastroscopy, which increases the cost and duration of the procedure, the risks related to intravenous sedation, and the number of possible complications associated with gastroscopy, such as perforation and

dental injury. The high success rate of this procedure (93%-98%), however, makes it very attractive^[56-59]. The technique is simple: after a gastroscope is placed deeply in the duodenum, a flexible unweighted 8-French nasojeunal tube with a guide wire is advanced through a working channel of the endoscope and pushed deep into the jejunum, beyond the tip of the endoscope during simultaneous withdrawal of the endoscope. When the procedure has been completed, the guide wire is removed and a feeding tube is passed from the mouth to the nose by means of a plastic device. Some centers also use a drag technique in which a suture is tied to the end of a feeding tube, which is then passed into the stomach *via* the nasopharynx. This suture is dragged with the endoscope snare or forceps from the stomach to the duodenum. Once the tube is in position, the suture is released and the endoscope is withdrawn. This procedure is less successful because the feeding tube frequently moves back into the stomach when the endoscope is removed.

A new technique of nasoenteric tube insertion has become very popular. It involves a transnasal thin endoscope that is inserted transnasally into the stomach and then into the duodenum^[60-62]. A thin guide wire is inserted through a working channel while the endoscope is removed, after which a feeding tube is placed over the guide wire, which is then removed.

Fluoroscopic techniques of nasoenteric tube placement require skilled radiological support and exposure to radiation. In addition, they necessitate changes in the patient's position that may not be feasible for the critically ill. The success rate of radiological placement varies from 40% to 94%, depending on the local expertise of the staff in different medical centers^[57,63-65].

Whatever the technique that is used for nasojeunal tube placement, proper position of the nasoenteric feeding tubes must be verified radiographically before the feeding is initiated^[66]. Clinicians should not rely on the accepted ways of checking nasogastric tube position, because it is impossible to adequately hear the entrance of air injected through the tube into the jejunum, and to distinguish its erroneous placement in the stomach/esophagus/lungs. In addition, air insufflation of the jejunum is unsafe.

Jejunostomy placement

Most jejunostomies are placed at least 20 cm beyond the ligament of Treitz (a point of transition of the duodenum to the jejunum) because of the increased rate of complications of duodenostomy compared with jejunostomy. A jejunostomy may be inserted with endoscopic assistance (percutaneous endoscopic jejunostomy; PEJ) or surgically (surgical jejunostomy). A PEJ may be inserted indirectly *via* a previously placed gastrostomy (PEG-J)^[16,67,68] or directly^[15,69-71].

For the placement of a PEG-J, a feeding tube long enough to pass beyond the pylorus is inserted through an existing PEG tube. The tip of the feeding tube is then grasped with the biopsy forceps of the endoscope and the tube is pushed as far as possible into the duodenum. Extra tubing length is left within the stomach to allow peristalsis to pull the tip of the feeding tube past the

ligament of Treitz. Although this procedure is simple, its major disadvantage is the tendency of the feeding tube to return back into the stomach during the withdrawal of the gastroscope. In addition, the feeding tube tends to dislodge from the outer gastrostomy.

An enteroscope or colonoscope should be placed into the proximal jejunum for direct PEJ placement^[15,69-71]. One of the most common techniques^[70] includes the insertion of a 19-gauge needle into the jejunal lumen at the site of the transillumination or a finger indentation marking the jejunal loop that is closest to the abdominal wall. The needle should be snared tightly, fixing the small bowel against the abdominal wall. The plastic sheath with stylet should then be inserted adjacent to the 19-gauge needle and snared by a wire loop that has been removed from the needle. An insertion wire should then be passed through the plastic sheath and grasped with a snare. The rest of the procedure is similar to the PEG's pull technique: the gastroscope together with a wire is pulled out through the duodenum, stomach, esophagus and mouth. The insertion wire is then secured to the loop at the end of the feeding tube with an internal jejunal bolster and the assembly is pulled through the mouth all the way to the duodenum. The tube is pulled through an incision in the abdominal wall, sufficiently tight to compress the jejunal wall against the anterior abdominal wall. Intrajejunal tube placement is then verified by a second gastroscopy. Finally, a skin disk is secured to the outside portion of the feeding tube to ensure the creation of a tract between the skin and jejunal lumen. It is important to avoid excess tension when approximating the jejunum to the abdominal wall, so as to prevent pressure sores of the skin or jejunal mucosa.

For patients in whom endoscopy is contraindicated, jejunal feeding tubes can be placed with radiological guidance^[72]. Access is obtained at a previous gastrostomy site^[73] or by direct jejunal punctures^[74]. With this method, the stomach and the jejunum are insufflated with air *via* a nasogastric or nasojejunal tube, and the location of internal organs is identified by means of ultrasound or fluoroscopy to ensure that no organs lie between the jejunum and the abdominal wall. A needle is inserted through the abdominal wall into the jejunal lumen and a guide wire is inserted through the needle. The needle is removed, the tract is dilated, and a feeding tube is placed over the guide wire and secured.

Surgical placement of a jejunostomy can be performed by a needle catheter or by Witzel techniques. A needle catheter jejunostomy is placed during laparotomy for surgical patients who need short-term enteral support^[75,76]. A purse-string suture is placed in the bowel wall, through which a large-bore needle is tunneled subserosally for several centimeters before entering the bowel lumen. A 5-, 7-, or 9-French feeding catheter with a flexible stylet is inserted through this needle and advanced distally into the bowel. The needle is removed and the purse string is tied. Next, a 3-5 cm Witzel tunnel is created in the abdominal wall proximal to the catheter insertion. A second large-bore needle is inserted through the abdominal wall and the feeding catheter and stylet are passed through the needle to the skin. The needle and stylet are then removed and the intestine is fastened to the anterior abdominal wall

to prevent leakage.

The Witzel jejunostomy is another open-surgery method. A tube is placed through an incision in the anterior abdominal wall and a tunneled incision is made in the jejunal wall. The adherence of jejunum to the abdominal wall is ensured by sutures^[77].

Some centers perform laparoscopic jejunostomy. Duh *et al.*^[78] have used this technique in 36 patients who could not undergo gastrostomy, with a good rate of success.

COMPLICATIONS AND DISADVANTAGES OF POSTPYLORIC FEEDING

There are various complications of postpyloric feeding. Some of them are specific to a specific device (nasojejunal tube *versus* jejunostomy) and others are universal for all kinds of postpyloric feeding techniques. Tables 2 and 3 specify common and uncommon complications of nasojejunal and jejunostomy feedings. The common complications of nasojejunal tubes are as follows: failure of nasojejunal tube placement (the rate depends on the technique of insertion), displacement of the tube, clogging of the tube, mild transient epistaxis, nasal mucosal irritation, feeding-related diarrhea, abdominal cramping, and hyperglycemia^[79]. The common complications of jejunostomy include pain and infection at the jejunostomy site, displacement of the jejunostomy, clogging, feeding-related diarrhea, abdominal cramping, hyperglycemia, transient pneumoperitoneum immediately after the insertion (in most cases, without any clinical significance), and leakage around the jejunostomy site^[80,81]. It is essential to take into account any existing risks of intravenous sedation and gastroscopy as well as the risks of anesthesia and surgery. There is a possibility that the patient will experience abdominal cramping, hyperperistalsis and diarrhea whatever device is used for this kind of feeding. The considerable costs of postpyloric devices compared to prepyloric ones need to be taken into account as well^[82].

Although the list of possible complications is a long one, most of them might be successfully avoided by using proper techniques of placement and management of the post-pyloric devices. For example, a misplacement of a nasojejunal tube and subsequent aspiration may be detected and avoided by radiological verification of the tube's location before feeding is started^[66]. The displacement of a nasojejunal tube may be prevented by proper fixation. Nasoenteric tubes tend to be blocked because they are usually longer and of finer bore. They are especially susceptible to being obstructed by crushed medications, viscous feeds and inadequate flushing. Therefore, these tubes should be flushed every 4-6 h, always before and after usage, and dense feeds and medications should be avoided. In the event of clogging, a tube can usually be unblocked by flushing it with hot water, coca-cola or pancreatic enzymes. The sudden influx of a hyperosmotic formula is likely to lead to abdominal cramping, hyperperistalsis and diarrhea since the jejunum relies on controlled delivery of isotonic substrates. An intrajejunal feeding is less physiological compared with an intragastric one. The ability of the

Table 2 Potential complications of nasojejunal tube feeding

Common (> 10%)
Failure of placement ¹
Displacement
Clogging of the tube
Mild transient epistaxis
Irritation of nasal, pharyngeal or esophageal areas
Feeding-related diarrhea
Abdominal cramping
Metabolic complication, such as hyperglycemia
Uncommon (< 10%)
Otitis media
Nasal mucosal pressure sores
Esophageal ulcers
Risks of intravenous sedation and gastroscopy
Sinusitis
Misplacement (pulmonary or intracranial intubation)
Dumping-like symptoms

¹Depends on the technique of insertion.

stomach to distend and contain a large amount of food all at once is a great advantage compared to the limited distension capability of the jejunum. Some patients who are fed postpylorically may develop symptoms similar to dumping syndrome, i.e. faintness, palpitations, sweating, tachycardia, rebound hypoglycemia, and diarrhea. Therefore, intrajejunal feeding should always be carried out continuously by pump and not by boluses^[42]. The recommended actions for cases of diarrhea are to exclude other possible causes, to decrease the rate of feeding, and to consider a change in formula to a less osmotic one and one that contains fibers.

FORMULAS FOR POSTPYLORIC FEEDING

The mode of administration, the appropriate formula and the rate of administration are important features for successful postpyloric feeding. The preferable kind of formula has yet to be determined, and there are few studies that have addressed this issue^[35,36,83,84]. Some of them advocate elemental and semi-elemental feeds and others support polymeric solutions. Lacking sufficient data, each medical center develops its own protocol.

Postpyloric feeds for children have traditionally been elemental or hydrolyzed and less viscous because of the narrow lumen of the tubes needed to pass the pylorus, although polymeric feeds have also been tolerated^[85]. For adults, polymeric formulas are usually chosen except for patients with malabsorptive disorders or lymph duct problems.

As mentioned earlier, the sudden influx of a hyperosmotic feed is likely to lead to abdominal cramping, hyperperistalsis, diarrhea and symptoms similar to dumping syndrome, since the jejunum relies on a controlled delivery of isotonic substrates. It is worth repeating that postpyloric feeds should be administered continuously by pump. The initial rate of administration should be slow and increased gradually. Parenteral support is sometimes used as caloric intake is gradually increased until the target caloric intake has been reached.

Table 3 Potential complications of jejunostomy feeding

Common (> 10%)
Pain at the jejunostomy site
Skin infection of the jejunostomy site
Feeding-related diarrhea
Abdominal cramping
Clogging of tube
Transient pneumoperitoneum immediately after the insertion (but it has no clinical significance in most cases)
Metabolic complication, such as hyperglycemia
Displacement of jejunostomy
Leakage around the jejunostomy
Uncommon (< 10%)
Failure of placement
Misplacement
Gastric hemorrhage
Perforation of internal organs during the placement and peritonitis
Colocutaneous fistula
Persistent jejunocutaneous fistula after the removal of jejunostomy
Risks of intravenous sedation and gastroscopy or risks of anesthesia and surgery
Hemorrhage at jejunostomy site
Pressure sore due to skin disk of jejunostomy
Dumping-like symptoms

CONCLUSION

The postpyloric route is a promising method of enteral feeding. In some cases, it is the only feasible way of maintaining enteral input and avoiding parenteral nutrition. Knowledge on the indications, contraindications, advantages and disadvantages and experience with the placement and replacement of different kinds of postpyloric devices should be an essential part of training of gastroenterologists and nutritionists. Further research on the physiological differences between intragastric and intra-jejunal food supply, including hormonal and enzymatic changes, is warranted.

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Dr. Shahid A Khan, *Series Editor*

Imaging of liver cancer

Ben Ariff, Claire R Lloyd, Sameer Khan, Mohamed Shariff, Andrew V Thillainayagam, Devinder S Bansi, Shahid A Khan, Simon D Taylor-Robinson, Adrian KP Lim

Ben Ariff, Claire R Lloyd, Sameer Khan, Adrian KP Lim, Department of Imaging, Imperial College Healthcare NHS Trust, Charing Cross Hospital Campus, Fulham Palace Road, London W6 8RF, United Kingdom

Mohamed Shariff, Andrew V Thillainayagam, Devinder S Bansi, Shahid A Khan, Simon D Taylor-Robinson, Department of Hepatology and Gastroenterology, Division of Medicine, Imperial College London, St Mary's Hospital Campus, South Wharf Road, London W2 1NY, United Kingdom
Author contributions: Ariff B, Lloyd CR and Khan S contributed equally to this work; Shariff M, Thillainayagam AV, Bansi DS, Khan SA and Taylor-Robinson SD all contributed to the clinical aspects of the paper; Lim AKP is the senior author and edited the final draft, advised on the content and provided the images.

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Correspondence to: Dr. Adrian KP Lim, Department of Imaging, Charing Cross Hospital, London W6 8RF, United Kingdom. a.lim@imperial.ac.uk

Telephone: +44-208-8461858 Fax: +44-208-846186

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Abstract

Improvements in imaging technology allow exploitation of the dual blood supply of the liver to aid in the identification and characterisation of both malignant and benign liver lesions. Imaging techniques available include contrast enhanced ultrasound, computed tomography and magnetic resonance imaging. This review discusses the application of several imaging techniques in the diagnosis and staging of both hepatocellular carcinoma and cholangiocarcinoma and outlines certain characteristics of benign liver lesions. The advantages of each imaging technique are highlighted, while underscoring the potential pitfalls and limitations of each imaging modality.

Key words: Hepatic cell carcinoma; Cholangiocarcinoma; Contrast enhanced ultrasound; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Although metastatic tumours are the commonest malignant lesions affecting the liver, the incidence of 'primary' liver malignancies has significantly increased over the last 20 years. This is particularly true for hepatocellular carcinoma (HCC), which is globally the commonest liver primary, and for cholangiocarcinoma, the second commonest primary liver tumour^[1]. HCC is now one of the commonest causes of cancer death worldwide and is the fifth most common cancer worldwide^[2]. Cholangiocarcinoma accounts for 3% of all gastrointestinal cancers^[3] but has increased relatively rapidly worldwide^[4]. Mesenchymal liver tumours are rare, but include hepatic angiosarcoma and primary hepatic lymphoma.

Improvements in imaging technology have allowed exploitation of the dual blood supply of the liver by both the hepatic artery (25%-30%) and portal vein (70%-75%), and the fact that many benign lesions demonstrate characteristic contrast enhancement, due to their vascular supply. Imaging of the liver is often achieved in three distinct phases following intravenous contrast enhancement - the arterial phase, portal venous phase and a late phase.

For the purposes of this review, while we concentrate on the diagnosis and staging of both HCC and cholangiocarcinoma, we also discuss certain characteristics of benign liver lesions. We aim to highlight the advantages of each imaging technique, as well as underscoring potential pitfalls and limitations.

IMAGING MODALITIES USED IN THE ASSESSMENT OF LIVER CANCER

Ultrasound

B mode ultrasound (US) is often the first line investigation in liver disease and its use is outlined in the British Society of Gastroenterologist (BSG) guidelines for diagnosis of both HCC and cholangiocarcinoma in adults^[5,6].

Contrast-enhanced ultrasound (CEUS): Progress in both technical advances by ultrasound manufacturers and in the development of ultrasound contrast agents (UCAs) has allowed the role of UCAs to change from Doppler rescue agents to diagnostic agents, providing an assessment of contrast enhancement patterns of liver lesions in real-time.

UCAs used in diagnostic US are characterized by a microbubble structure, consisting of gas bubbles stabilized by a shell^[7]. Current generation microbubbles are based on perfluorocarbons with a phospholipid membrane, providing low solubility and favorable resonance behavior at low acoustic pressures. Microbubble sizes typically range from 3 to 5 μm and on intravenous injection remain in the vascular compartment for several minutes, being small enough to avoid filtration by the lungs and too large to enter the interstitial fluid. These compounds demonstrate strong non-linear harmonic responses when insonated with low acoustic pressure and generate specific signals without being destroyed when insonated at low mechanical index (MI), thus allowing continuous real-time imaging^[7].

UCAs act as blood pool agents allowing the definition and visualization of three overlapping vascular phases—the arterial phase, portal venous phase and late phase, which last until there is clearance of the UCA from the hepatic parenchyma. This late phase differs from the equilibrium phase of extracellular computed tomography (CT) and magnetic resonance imaging (MRI) agents and may reflect sinusoid pooling and reticulo-endothelial system (RES) or Kupffer cell uptake^[8,9].

Minor adverse effects of UCAs are reported in less than 5% of subjects and typically include transient discomfort at the injection site, taste aberrations and vasovagal attacks. UCAs are not nephrotoxic and it is not necessary to check renal function prior to their administration. The incidence of severe hypersensitivity or allergic reaction is lower than current X-ray contrast agents and comparable to that of MR contrast agents^[10].

CT

The development of multidetector row helical computed tomography (MDCT), with its superior spatial and temporal resolution, has resulted in improved detection and characterization of focal liver lesions^[11]. The acquisition of multiple data sets with each rotation of the x-ray tube in MDCT means the entire liver can be imaged in 10 s or less, compared with 25-30 s for single slice helical CT technology. The short time needed to image the liver allows multiple passes through the liver in different vascular phases following bolus

contrast injection and thin-section collimation produces volume data sets with isotropic or near-isotropic voxel dimensions, resulting in superior spatial resolution and the capability to display data in multiple planes.

With single-slice helical CT, a 'dual-phase' technique is commonly employed with image acquisition in the hepatic arterial-dominant phase and in the portal venous-dominant phase. The 'triple-phase' technique includes an early arterial phase, imaged 18-25 s following bolus injection of contrast^[12]. Using multiplanar reconstructions, a 3D CT hepatic-mesenteric angiogram can be obtained.

Hypervascular liver lesions are best appreciated in the late arterial phase as they show maximal enhancement relative to the background liver parenchyma. In the portal venous-dominant phase there is maximal parenchymal enhancement with opacification of the hepatic veins. This phase is extended to include the entire abdomen and, depending on the clinical indication, the pelvis. A delayed or equilibrium phase performed 3-5 min following contrast administration may be helpful in further characterizing focal liver lesions.

MRI

Although MRI is often viewed as the most sensitive and specific technique for evaluating the liver, this is probably debatable, given the recent revolution in multi-detector CT technology^[13,14]. Nevertheless, lesion/liver contrast is higher for MRI than with CT and the flexibility and range of pulse sequences available in MRI provide a significant advantage over CT.

Hepatobiliary MRI uses several magnetic resonance pulse sequences, each of which produces images that provide unique information about the liver and the biliary tree. Most examinations include a T₁-weighted in-phase/out-of-phase spoiled gradient echo sequence and one or more T₂-weighted sequences. Combining these sequences with extracellular intravenous contrast agents, usually with a fat-saturated spoiled gradient echo sequence, also allows patterns of tumour enhancement to be determined. In addition, use of tissue-specific contrast agents such as super paramagnetic iron oxide, allows improved detection and characterisation of liver tumours^[15-17]. Contraindications to MRI include pacemakers, implantable cardiac defibrillators, cochlear implants and metallic orbital foreign bodies.

Intravenous MR contrast agents: Categories of clinically available liver contrast agents include non-specific extracellular contrast; liver-specific categories of hepatocyte selective; RES-specific; and agents with combined early blood pool and late RES-specific properties.

Extracellular gadolinium chelates are used extensively for liver MRI. Following intravenous injection of a gadolinium-based agent, typically three phases of contrast enhancement are imaged: the arterial, portal venous phase and the equilibrium phase^[15]. During the arterial phase, most of the liver does not enhance as the majority of the liver's blood supply is *via* the portal vein^[15]. Enhancement patterns of liver lesions are similar

to those demonstrated on CEUS and contrast-enhanced CT. The equilibrium phase or delayed phase is useful for identifying late enhancement of liver lesions. In addition, washout of contrast from HCC and peripheral or heterogeneous washout from liver metastases are characteristic findings on delayed imaging^[15]. Recent concern about nephrogenic systemic sclerosis in renal impairment following intravenous gadolinium may require modifications in imaging protocols^[18,19].

Liver-specific contrast agents

Hepatocyte-selective contrast agents: Hepatocyte-selective contrast agents undergo uptake by hepatocytes and are eliminated through renal and biliary excretion^[20]. All are T₁-relaxation enhancing agents and increase the signal intensity in normal liver tissue and hepatocyte containing tumors. Non-hepatocyte containing tumors, such as hemangiomas and metastases, do not take up these agents and are rendered more conspicuous by the increase in signal of the background liver on delayed imaging. Agents such as Gd-BOPTA (Multihance) also exhibit early perfusional information, similar to gadolinium chelates.

Reticuloendothelial agents: Reticuloendothelial agents target the RES, particularly the liver and spleen and reflect the number of functioning macrophages^[21]. Reticuloendothelial agents currently in clinical use include superparamagnetic iron oxide (SPIO) particles. SPIO particles act as a negative contrast agent and can be used alone or in combination with gadolinium^[22,23]. Most liver tumors, whether benign or malignant are deficient in Kupffer cells and do not exhibit SPIO particle uptake^[24,25]. Hence, most liver tumors appear relatively hyperintense, because the background liver darkens preferentially following SPIO administration. Combining gadolinium and SPIO-enhanced imaging in a 'dual contrast' MRI could be the most accurate technique for the detection of liver tumors^[26].

Diffusion-weighted MRI: Diffusion-weighted imaging (DWI) uses pulse sequence techniques that are sensitive to the very small scale motion of water protons at a microscopic level and improves the conspicuity of many hepatic and extrahepatic tumors^[27].

Positron emission tomography (PET)

The advent of molecular imaging with PET has revolutionized the concept of functional imaging in the management of disease, particularly in the field of oncology, which accounts for 90% of PET applications. Imaging with PET rather than gamma camera SPECT radiopharmaceuticals allows higher spatial resolution and good image quality, with better detection of even small lesions^[28]. PET has the advantage over cross-sectional anatomical imaging of providing whole body imaging, allowing the detection of multifocal and metastatic disease. There are many radiopharmaceuticals based on labeled short-lived positron emitters, of which, the most widely used is the fluorinated glucose derivative

¹⁸F-fluorodeoxyglucose (¹⁸F-FDG). The use of other nuclear medicine techniques in the imaging of liver malignancy, e.g. colloid scintigraphy, has been rendered largely obsolete by improvements in other cross-sectional imaging techniques, principally MRI and US^[29].

HCC

The majority of HCC cases develop in the cirrhotic liver. Cirrhosis is the strongest predisposing factor for HCC, with chronic viral hepatitis the commonest underlying etiological cause worldwide^[30]. Curative resection, ablation or liver transplantation is possible if the tumor is detected at an early stage. Early detection of HCC is thus critical to achieve effective treatment and prolong survival^[31]. However, it is often difficult to diagnose small HCCs, particularly in the presence of cirrhosis. In Europe, patients with chronic liver disease undergo regular surveillance, usually involving both alpha-fetoprotein (AFP) determination and conventional B-mode US examination of the liver^[32]. Alpha-fetoprotein, the most commonly used serum tumor marker, is unfortunately not a dependable biomarker for diagnostic and prognostic purposes, having poor sensitivity, specificity, and positive predictive value, emphasizing the importance of high quality imaging techniques^[33].

Hepatocarcinogenesis is considered a multi-step process characterized by the development of a spectrum of nodules from benign regenerative nodules (RNs) and dysplastic nodules (DNs) to overt malignant HCC. Although conventional US can reveal the different types of nodules in cirrhosis, the ability to distinguish RNs from DN or malignant HCC is limited as this necessitates an understanding of hemodynamic changes: The vascular supply of RNs is similar to that of the liver parenchyma^[34], whereas DN demonstrate a more complex vascular supply with a degree of capillarization ranging from that similar to RNs to the arterial hypervascularization typical of HCC. Thus, a reduction of both portal vein and normal hepatic artery branches with a progressive increase in abnormal hepatic arteries are considered histological features of malignant transformation^[34]. It is this process of tumor neoangiogenesis that gives rise to the characteristic appearances of HCC on several different contrast-enhanced imaging techniques (CEUS, contrast-enhanced CT and MR with gadolinium enhancement) and facilitates its differentiation from other benign causes of focal liver lesions.

In patients with liver cirrhosis, the diagnosis of HCC can be based on clinical, laboratory and imaging techniques with an accuracy of up to 99%^[35]. If a mass detected on US is ≥ 2 cm in diameter, there is a greater than 95% chance that the lesion is HCC and biopsy is not indicated.

Characteristic appearances of HCC

Ultrasound: HCC appearances on B-mode US are variable. Small lesions are usually hypochoic, but larger le-



Figure 1 HCC. B-mode US demonstrates a heterogeneous hypoechoic solid liver lesion (arrow) in segment IV, confirmed to be HCC at histological examination. Note the background of an echogenic liver with an irregular surface.



Figure 3 HCC. Contrast enhanced CT demonstrates heterogeneous arterial phase enhancement of a focal liver lesion (arrow) adjacent to a low attenuation area representing a previous radiofrequency ablation site (arrowhead).

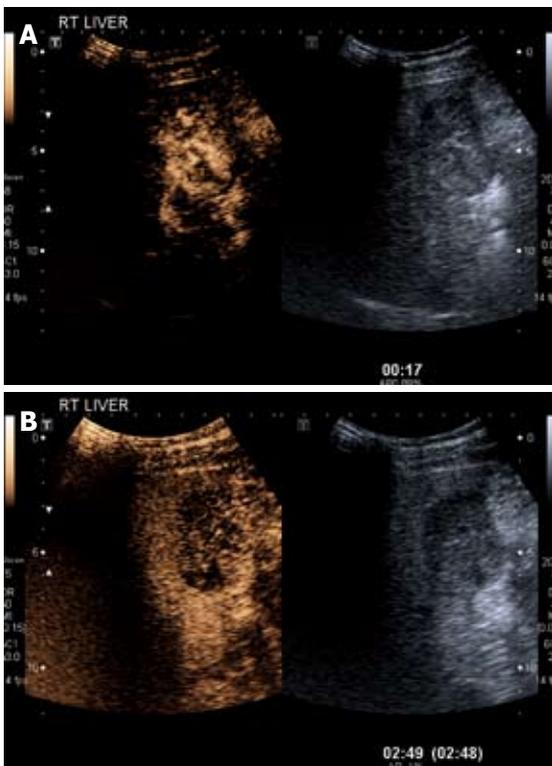


Figure 2 HCC. A: CEUS in the arterial phase (17 s following injection) demonstrates arterial phase enhancement of the solid lesion (corresponding grey scale image of the lesion is shown on the right hand side; software known as "twin view" which helps the sonologist track the lesion through the phases of enhancement). Note the "basket weave" pattern of angiogenic vessels with haphazard enhancement of this lesion; B: Twin view of the same segment IV lesion in the delayed phase (2 min 49 s following injection) of the same lesion as Figure 2A demonstrates contrast wash-out compared to the surrounding liver parenchyma which are features of a malignant lesion.

sions may demonstrate heterogeneous echotexture due to necrosis and fibrosis (Figure 1).

The most common feature of HCC using CEUS is the presence of early, intense and homogenous intra-tumoural enhancement (Figure 2A). Real-time imaging can demonstrate the 'basket pattern' of blood flow with peripheral vessels encircling and internally penetrating the tumor. After the arterial hyperenhancement, HCC show, 'wash-out', resulting in an isoechoic or hypoechoic

appearance in the portal-venous and delayed phase (Figure 2B). In most cases, this 'wash-out' is slower than with other malignant lesions, such as metastases. The degree of late-phase enhancement is determined by the degree of similarity of the nodule to normal liver parenchyma^[36]. RNs usually have a hypoechoic or isoechoic appearance in the arterial phase and an isoechoic appearance in the portal and late phases^[37]; and the detection of arterial hypervascularization reflects transformation to HCC. Some caution needs to be exercised, however, as up to 30% of HCCs, particularly the small, well-differentiated variety, may be isoechoic rather than hypoechoic on the late phase of imaging and thus be mistaken for a regenerative nodule or dysplastic nodule.

CT: On contrast-enhanced MDCT, the typical HCC demonstrates similar enhancement patterns to those seen on CEUS, with intense inhomogeneous enhancement seen in the hepatic arterial-dominant phase and contrast wash-out in the late portal venous phase^[38] (Figure 3). A minority of HCCs are hypovascular tumors and do not demonstrate arterial enhancement, appearing as hypoattenuating lesions relative to the liver parenchyma on the portal venous-dominant phase.

HCC arising in the non-cirrhotic liver is often large, due to its long asymptomatic course and late presentation^[39,40]. This contrasts with the multi-focal tumors more commonly found in patients with other forms of chronic liver disease. Large HCCs may demonstrate a number of characteristic appearances on MDCT which make differentiation from other causes of focal liver masses relatively straightforward. A mosaic appearance may be seen in large tumors, with fibrous septa separating areas of variable attenuation which represent internal regions of hemorrhage, necrosis, fatty degeneration and fibrosis. Characteristic satellite tumor nodules close to the margins of large tumors may be seen. Well-defined, lobulated margins and a distinct fibrous capsule are also features of large HCCs. The fibrous capsule has low attenuation on unenhanced images and does not enhance in the arterial phase but begins to enhance in the late portal venous phase. Retention of contrast within the capsule

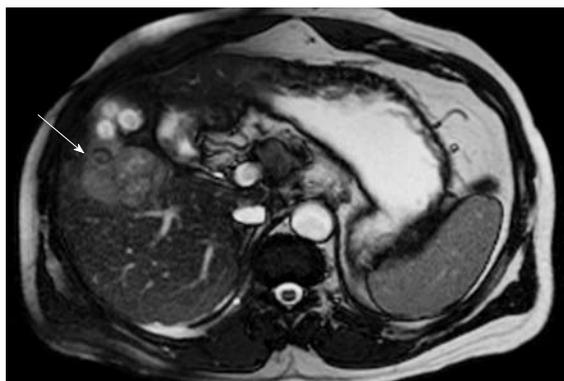


Figure 4 HCC. T₂-weighted gradient echo sequence shows a solid lesion in segment V (arrow) of the liver which is of intermediate signal hyperintensity compared to the surrounding liver parenchyma.

increases its conspicuity in the equilibrium phase. HCC has a propensity for early invasion of the portal venous system and biliary tree. The portal vein may be expanded by tumor thrombus that can be differentiated from bland tumor thrombus by the demonstration of arterial enhancement, either diffuse or streaky^[41].

A number of benign lesions, including hemangiomas, focal confluent fibrosis, peliosis, benign regenerative nodules and transient hepatic attenuation difference (THAD), can simulate small HCC lesions on CT, and an awareness of these lesions and their imaging characteristics is vital for the radiologist interpreting multi-phasic hepatic CT studies. THAD describes a focal increase in hepatic arterial flow that, in the context of cirrhosis, is most commonly due to arterial-portal shunting, but THAD can also result from intrahepatic thrombosis of hepatic or portal veins. The typical appearance of THAD is a peripheral, often wedge-shaped, area of arterial phase enhancement^[42]. In a study of a large screening population with cirrhosis, Brancatelli and colleagues found an 8% false-positive rate upon comparison of pre-transplant CT studies with pathological examination of explanted livers^[43]. Both hypo- and hyper-attenuating nodules were incorrectly diagnosed as HCC on CT but most of the lesions were small (< 1.5 cm diameter).

The use of both double arterial phase imaging and delayed phase imaging improve detection of HCC, particularly of small (≤ 2 cm) nodules^[44]. However, with radiation dose considerations in mind, most centers adopt a pragmatic approach in combining late arterial phase and portal venous phase imaging to evaluate HCC. Double arterial phase imaging is often reserved for patients who are candidates for surgical resection or chemoembolization, in whom accurate delineation of hepatic arterial anatomy is vital. The addition of an unenhanced phase does not improve detection of HCC in the cirrhotic liver^[45], but is used in some centres to differentiate high attenuation siderotic regenerative nodules from arterially-enhancing nodules. Delayed CT, following intra-arterial injection of iodized oil (Lipiodol®, Guerbet, Paris, France), may improve detection of HCC nodules and facilitate targeted biopsy due to the uptake and retention of contrast within the hypervascular tumor nodules^[46,47].

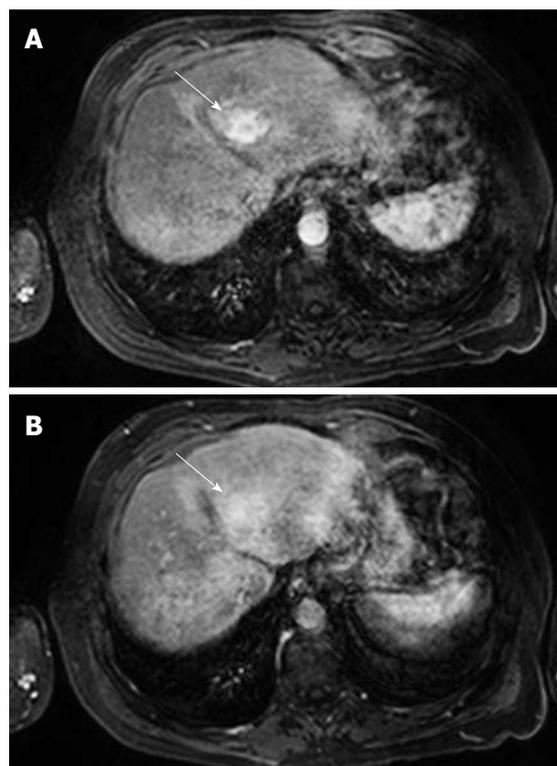


Figure 5 HCC. A: T₁-weighted fat-suppressed sequence following gadolinium intravenous injection shows arterial phase enhancement of a focal liver lesion in segment IV (arrow); B: HCC: The same lesion as shown in Figure 5A becomes relatively less conspicuous on the portal phase images (arrow) as the surrounding liver parenchyma begins to enhance.

MRI: HCC can have a variable appearance on unenhanced T₁-weighted images and typically shows increased signal on T₂-weighted images^[48] (Figure 4). Following gadolinium administration, HCC demonstrates characteristic early enhancement on arterial phase imaging^[49] and washout on the delayed images - resulting in a hypointense lesion compared to the surrounding parenchyma^[15] (Figure 5A and B).

Fibrolamellar HCC, a rare tumor found in young patients without a history of pre-existing liver disease, is usually seen as a large, well-circumscribed focal lesion with low signal intensity on T₁-weighted MR images and high signal on T₂-weighted images. There is usually early heterogeneous contrast enhancement and a central radiating scar is seen in 80% of cases, which is usually hypointense relative to the remainder of the tumor^[50].

Diffusion-weighted MRI may aid detection and characterization of focal liver lesions. Liver parenchyma is dark on DWI, whereas liver tumors (both benign and malignant) are depicted as high signal intensity masses^[27,51-53], although malignant liver tumors have lower apparent diffusion coefficients (ADCs) than benign cysts and hemangiomas^[51,53].

PET

¹⁸F-fluorodeoxyglucose (¹⁸F-FDG), the most widely used PET radiopharmaceutical in oncological imaging, can demonstrate the presence of malignant cells, because tumor cells utilize more glucose than normal

tissue. FDG is phosphorylated *via* hexokinase to FDG 6-phosphate, which has a much slower rate of dephosphorylation compared to glucose 6-phosphate, and is progressively trapped inside metabolically active cells. The rate of FDG trapping in the cell is therefore proportional to the rate of glycolysis. However, differentiated hepatocytes have a relatively high glucose-6-phosphatase activity which allows dephosphorylation of FDG and subsequent leakage of the tracer from cells. Trojan and colleagues reported a sensitivity of 50% in detecting HCC, with increased FDG uptake associated with moderate or poor differentiation, high serum levels of AFP and large tumor burden^[54]. A study by Khan and colleagues demonstrated a similar sensitivity of 55%^[55]. Therefore, ¹⁸F-FDG is of very limited value in diagnosing HCC. This has led to the search for other PET tracers which may be used to screen for the presence of HCC. In a study by Ho and colleagues, dual imaging using ¹¹C-acetate and ¹⁸F-FDG was assessed^[56]. These authors found that the poorly differentiated HCCs were detected by ¹⁸F-FDG and the well-differentiated HCCs were detected by ¹¹C-acetate, leading to a 100% sensitivity using both tracers. In addition, other liver malignancies such as cholangiocarcinomas and liver metastases did not demonstrate abnormal ¹¹C-acetate uptake, although focal nodular hyperplasia did show mild increased uptake.

HCC detection

Despite technical improvements in all modalities used in the imaging of HCC, difficulties remain in detecting and characterizing small (≤ 2 cm) lesions in the cirrhotic liver.

A recent prospective study comparing imaging findings with pathological examination of the explanted liver in a pre-transplant population, found that US, MRI and CT had similar sensitivities for HCC detection on a lesion-by-lesion basis, although US performed slightly better on a patient-by-patient basis^[15]. All three modalities missed small lesions. PET failed to detect any of the pathologically proven HCC lesions.

Currently, B-mode US is recommended in the screening of patients at risk of HCC, including patients with hepatitis B and cirrhosis^[5]. In experienced hands, B-mode US can detect 80%-95% of lesions 3-5 cm in diameter and has 60%-80% sensitivity in the detection of lesions of 1 cm^[57,58]. CEUS can further improve the detection of lesions, even if < 2 cm^[59-62], having the same sensitivity as helical CT^[59]. Furthermore, the presence of 'washout' improves the specificity of CEUS in the detection of HCC by allowing the distinction from hemangiomas or hypervascular DNAs and pseudonodules that can mimic HCC due to the presence of homogeneous arterial enhancement. In cases of elevated AFP with no nodule visible on US, CT is recommended to investigate the possibility of infiltrative HCC.

In a prospective study comparing CT findings and pathological examination of the explanted liver in a large pre-transplant population with cirrhosis, CT detected tumor in only 44% of patients in whom HCC was found at pathological examination^[63]. However, a variety of

CT protocols were used and if only triphasic helical CT (comprising non-contrast, hepatic arterial and portal venous phases) was considered, prospective tumor detection improved to 59%. This study employed single slice helical CT technology and current use of MDCT technology is likely to have improved tumor detection rate.

The sensitivity of MRI in the detection of HCC decreases in cases with advanced cirrhosis^[14], and the presence of ascites can generate significant artifacts. Additional difficulties can arise in distinguishing well-differentiated HCC from regenerative and DNAs^[49]. Frequently, patients with cirrhosis have transient foci of enhancement on arterial phase imaging that cannot be visualized on any other pulse sequence^[64,65]. While these are usually benign and likely to represent small arteriovenous shunts or dysplastic nodules, small HCCs can exhibit an identical appearance in up to 13%^[66]. Although some advocate the use of SPIO contrast agents to improve sensitivity and specificity, this has had mixed results^[13]. HCCs show variable enhancement with SPIO depending on the degree of differentiation and number of functioning Kupffer cells^[15], and often in the context of cirrhosis and small tumors (≤ 1.5 cm) gadolinium enhanced imaging is preferred^[67]. The combination of gadolinium and SPIO, however, provides a dual contrast assessment of HCC that is more effective than either contrast agent alone^[22,68]. Given the rapid doubling time of most HCCs, close follow-up of cases with transient foci of enhancement may provide an additional strategy^[69].

CHOLANGIOCARCINOMA

Cholangiocarcinomas are generally classified as intrahepatic, hilar or extrahepatic. Most cholangiocarcinomas, up to 60%, occur at the liver hilus^[6]. Hilar and extrahepatic cholangiocarcinomas typically present with biliary duct obstruction. A multi-modality imaging approach, including endoscopic retrograde cholangiopancreatography (ERCP), US, CT, MRI and magnetic resonance cholangiopancreatography (MRCP), is used in patients with a suspected malignant cause for biliary obstruction.

Although the identification of a mass with B-mode US varies from 37% to 87%^[70,71], B-mode US is a highly sensitive method for confirming biliary duct dilatation, localization of the site of obstruction and excluding gallstones^[72]. Peripheral cholangiocarcinomas can appear as a hyper- or hypoechoic mass similar to HCC, and distal lesions can present with intra- and extra-hepatic duct dilatation^[73]. On CEUS, a cholangiocarcinoma can behave in the same way as a hypovascular metastasis and result in an area of hypoenhancement in the delayed phase following contrast administration.

Dual phase (arterial dominant and portal venous dominant) contrast-enhanced CT may be helpful in the assessment of cholangiocarcinoma. Small central tumors are often difficult to detect because of their size, infiltrating nature and iso- or subtle hypo-attenuation



Figure 6 Cholangiocarcinoma. Contrast enhanced CT in the portal venous-dominant phase demonstrates a poorly-enhancing central liver lesion. The hypodensity appears to follow some of the biliary radicals.

compared to adjacent liver parenchyma, and marked dilatation of the intrahepatic biliary tree may be the only evidence of disease on CT. Visualized central tumors are usually hypoattenuating masses that enhance poorly in the arterial and portal venous phases (Figure 6). Enhancement may be seen on delayed images, thought to be due to retention of contrast within the dense fibrous stroma^[74]. The peripheral form of cholangiocarcinoma may appear as a nodular, hypoattenuating lesion surrounded by dilated biliary ducts. Previous studies have shown that CT tends to underestimate disease extent, accurately assessing tumor resectability in only 60% of patients in one study^[75]. However, a more recent study by Aloia and colleagues showed that high resolution helical CT with 2.5 mm slice reconstructions correctly predicted tumor resectability in 17 of 18 patients who underwent laparotomy for cholangiocarcinoma^[76].

The appearance of intrahepatic cholangiocarcinoma on MRI is non-specific, typically appearing hypointense on T₁-weighted and mildly hyperintense on T₂-weighted sequences. More than 50% of patients will have satellite or remote intrahepatic tumor nodules^[77]. Following gadolinium chelate contrast, peripheral enhancement can be seen initially and delayed equilibrium enhancement can show dense contrast retention adding to the detection and characterisation of these lesions^[78]. A central scar may also be evident.

Extrahepatic cholangiocarcinomas have similar signal characteristics to intrahepatic tumors on MRI. The majority of extrahepatic cholangiocarcinomas show heterogeneous enhancement following gadolinium with a gradual increase to a peak on delayed imaging^[79]. Peripheral early enhancement is rarely seen. Dynamic contrast-enhanced MRI is comparable to angiography in the assessment of the portal vasculature invasion in patients with cholangiocarcinoma^[80].

MRCP is the non-invasive imaging study of choice to investigate the biliary tree. MRCP has an excellent overall sensitivity and specificity for demonstrating the level and the presence of biliary obstruction, with slightly lower sensitivity for detecting stones or differentiating malignant from benign obstruction than ERCP^[81]. Evaluation of

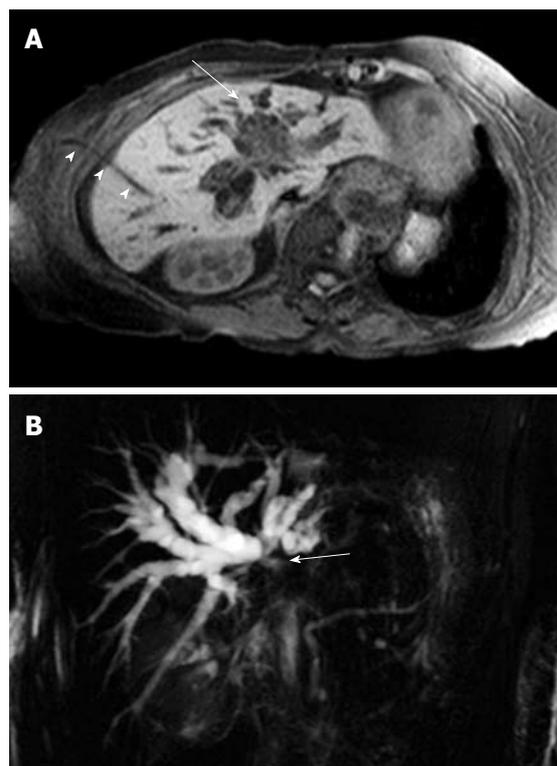


Figure 7 Cholangiocarcinoma. A: Contrast-enhanced T₁-weighted magnetic resonance sequence shows irregular central liver mass (arrow) which enhances poorly in comparison to the adjacent liver parenchyma. There is intrahepatic bile duct dilatation and an external biliary drain (arrowheads); B: Maximum intensity projection image of the MRCP study of the same case as shown in Figure 7A demonstrates marked intrahepatic bile duct dilatation and abrupt cut-off at the liver hilum (due to obstructing tumour) (arrow) with non-visualisation of the extrahepatic ducts.

non-dilated biliary ducts, however, remains problematic as ductal distension is likely an important factor in visualizing subtle biliary abnormalities, particularly given the relatively limited spatial resolution of MRCP techniques.

Cholangiocarcinoma can present as a stenosing/sclerosing process resulting in ductal stricturing, usually with shouldered margins. Cholangiocarcinomas can also result in focal duct wall thickening or nodule formation or as an intraductal papillary lesion.

MRCP permits visualization of ductal irregularity and narrowing as well as intraluminal tumor extent, although bile duct involvement can be underestimated with MRCP^[82]. Compared to ERCP, however, MRCP can more accurately determine the suprahilar tumor extension^[82]. Furthermore, the combination of MRI and MRCP imaging allows the evaluation of the presence and size of extraluminal extension of the tumor and liver parenchyma invasion, leading to a greater accuracy in preoperative staging (Figure 7A and B).

There are few data regarding the use of ¹⁸F-FDG PET in the diagnosis of cholangiocarcinomas. Anderson and colleagues reported a sensitivity of 85% for the nodular morphological type of cholangiocarcinoma but only 18% for the infiltrating type^[83]. In addition, sensitivity for metastatic disease was 65%. All of these metastases were unsuspected on other imaging and led to a change



Figure 8 Hepatic metastases. Delayed phase CEUS (3 min 49 s following contrast injection) demonstrates two hypoechoic liver metastases following contrast washout with a residual peripheral rim of enhancement (arrows). Note that the adjacent image demonstrates that these lesions are echogenic on grey-scale and are poorly defined. The margins are better defined with contrast enhancement (right hand image).



Figure 9 Hepatic metastases. CEUS obtained in the arterial phase (17 s following contrast injection) demonstrates enhancement of a hypervascular liver metastasis from a colorectal primary tumor. It is the washout with peripheral rim enhancement in the delayed phase (see Figure 8) which helps confirm this to be a metastasis.

in management in all of the patients. Studies have shown that tumors of the tubular pathological type with high cell density and limited mucin production^[84], and peripheral^[85], nodular^[83] lesions are better detected than hilar cholangiocarcinomas, infiltrating lesions and mucinous tumours. Dynamic ¹⁸F-FDG PET may have a useful role in the detection of cholangiocarcinoma in patients with primary sclerosing cholangitis where other modalities such as CT, MRI and US are of limited value^[86].

LIVER METASTASES

20%-25% of patients with known solid malignant tumors have hepatic metastases at the time of diagnosis. The incidence of solid benign liver tumors is around 20%^[87], thus in patients with known malignancy, 20%-25% of lesions under 2 cm are benign^[88]. The most frequent benign lesion is hemangioma with a prevalence of 7%-21%, followed by focal nodular hyperplasia (FNH) with a prevalence of up to 3%^[87]; other benign lesions are far rarer. Hence, imaging techniques of the liver in patients with malignancy not only require high sensitivity, but also the ability to reliably differentiate malignant from benign tumors.

CEUS improves the sensitivity of US in the detection of individual lesions by about 20% in comparison to baseline, with a resultant sensitivity of 82%-86%, comparable to contrast-enhanced CT and MRI with non-specific gadolinium chelates^[89-92]. Although metastases show characteristic features in the three phases after contrast injection, the hypoenhancement of solid lesions or 'wash-out' in late phase is the key to distinguishing malignant from benign lesions. Benign lesions demonstrate sustained enhancement in the portal and late phases (Figure 8). The appearance of metastases in the arterial phase of enhancement depends on the extent of arterial perfusion. For example, the hypervascular metastases in neuroendocrine carcinomas demonstrate homogenous enhancement, whereas hypovascular metastases, commonly arising from breast, lung, colonic

or pancreatic primaries, may only demonstrate rim enhancement (Figure 9). For benign lesions, the arterial phase is of particular use in the further characterisation of the lesion: hemangiomas characteristically demonstrate peripheral nodular enhancement with gradual filling in of the lesion in the portal venous phase, whereas FNH may demonstrate the typical 'spoke-wheel' arterial pattern with centrifugal filling early in the arterial phase *via* a dominant filling artery^[93].

Pseudo liver tumors, such as focal fatty infiltration or focal fatty sparing, exhibit the same characteristics as the surrounding liver parenchyma and thus remain isoechoic to normal liver tissue^[93].

Appearances of hypovascular liver metastases on MDCT are similar to those seen on CEUS. Lesions are typically rounded and uniformly hypoattenuating on portal venous phase CT imaging. They may demonstrate peripheral rim enhancement on late arterial phase images^[94]. Hypervascular hepatic metastases typically demonstrate homogeneous late arterial enhancement on MDCT although inhomogeneous enhancement may be seen due to areas of necrosis or hemorrhage^[95].

MRI typically demonstrates liver metastases as hypointense on T₁-weighted images and hyperintense on T₂-weighted images^[48]. Most liver metastases show restricted water diffusion on DW images and therefore appear as hyperintense masses^[52]. While it is generally accepted that CE MRI increases the sensitivity for detecting metastases, the sensitivity of unenhanced MRI for liver metastases being in the region of 70% compared to 90% following contrast enhancement and being comparable to, if not better than, CT^[26], there is some debate on which contrast agent to use^[13], and indeed, on whether detection rates remain comparable to CT with 64 plus multi-detector CT^[13].

SPIO-enhanced MRI can be most useful in patients with colorectal carcinoma when being considered for hepatic resection on the basis of limited metastatic disease^[96]. In addition, combining gadolinium with SPIO provides advantages for the depiction of liver metastases: hypovascular metastases are better identified

on SPIO, whereas hypervascular metastases are better depicted and characterized with gadolinium^[97].

TUMOUR STAGING

HCC

Staging of HCC depends on several factors including location of the lesion, presence of satellite nodules, biliary extension and vascular invasion. CT and MR remain the imaging techniques of choice for evaluating the liver parenchyma and the presence or absence of distant spread, although CEUS may also be useful in the assessment of vascular invasion.

A lesion detected on US examination in the cirrhotic liver with a diameter ≥ 2 cm has a greater than 95% chance of representing an HCC in the presence of a raised serum AFP level. Current BSG guidelines recommend further imaging (CT or MRI) to assess local and distant disease extent and enable planning of suitable therapy^[5,6]. Common sites of extrahepatic disease include the peritoneum, abdominal lymph nodes, lungs and bone, and staging CT examination should include the chest. If the AFP level is normal, a confident diagnosis can usually be established without the need for needle biopsy if characteristic CT or MRI appearances (arterial enhancement, late washout) are demonstrated. Biopsy should be reserved for lesions where imaging appearances are equivocal and AFP level normal.

Intra-operative US (IOUS) of the liver is being used with increasing frequency as an aid for surgical planning and provides real-time information that can affect the surgical decision-making^[98,99]. IOUS has been shown to change the clinical management in up to 50% of patients undergoing hepatic resection for malignancy^[100-102], detecting more lesions than the pre-operative conventional B-mode US, CT or angiography^[103].

Cholangiocarcinoma

In patients in whom a diagnosis of cholangiocarcinoma is suspected, current BSG guidelines^[6] recommend MRI and MRCP as the optimal investigations to assess liver and biliary anatomy, local tumor extent (including duct and vascular involvement) and the presence of hepatic metastases. Invasive cholangiography (*via* either the endoscopic or percutaneous transhepatic routes) is usually reserved for tissue diagnosis and cases where therapeutic biliary decompression is required due to cholangitis. Endoscopic US may also be helpful in allowing visualization of the distal extrahepatic biliary tree, gallbladder, regional lymph nodes and vasculature as well as facilitating the use of fine needle aspiration or biopsy of lesions. Chest radiography is recommended to look for lung metastases and abdominal CT examination to screen for intra-abdominal metastatic disease if MRI has not been performed.

CONCLUSION

Recent advances in imaging techniques, particularly in the development of CEUS, MDCT and MRI contrast

agents, have improved detection and characterization of focal liver lesions and enabled accurate staging and appropriate treatment planning in both hepatocellular carcinoma and cholangiocarcinoma. Challenges remain in imaging evaluation of the cirrhotic liver, particularly in the detection of small hepatocellular carcinomas and their differentiation from a number of benign liver pathologies.

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Dr. Shahid A Khan, Series Editor

Diagnosis of hepatocellular carcinoma

Asmaa I Gomaa, Shahid A Khan, Edward LS Leen, Imam Waked, Simon D Taylor-Robinson

Asmaa I Gomaa, Shahid A Khan, Simon D Taylor-Robinson, Department of Hepatology and Gastroenterology, Division of Medicine, Imperial College London, St Mary's Hospital Campus, South Wharf Road, London W2 1NY, United Kingdom

Edward LS Leen, Imaging Sciences Department, MRC Clinical Sciences Centre, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0HS, United Kingdom

Asmaa I Gomaa, Imam Waked, National Liver Institute, Menoufiya University, Shebeen ElKom, 32111, Egypt

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Correspondence to: Dr. Shahid A Khan, Department of Hepatology and Gastroenterology, Division of Medicine, Imperial College London, St Mary's Hospital Campus, South Wharf Road, London W2 1NY,

United Kingdom. shahid.khan@imperial.ac.uk

Telephone: +44-207-8866454 Fax: +44-207-7249369

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Abstract

Hepatocellular carcinoma (HCC) is one of the commonest cancers worldwide, particularly in parts of the developing world, and is increasing in incidence. This article reviews the current modalities employed for the diagnosis of HCC, including serum markers, radiological techniques and histological evaluation, and summarises international guidelines for the diagnostic approach to HCC.

Key words: Diagnosis; Hepatocellular carcinoma; Imaging; Serum markers

Peer reviewer: Dr. Paolo Del Poggio, Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the commonest cancers worldwide. It is a major health problem and its incidence is increasing^[1]. The presence of cirrhosis of the liver is the major risk factor and worldwide this is largely due to chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) infection. The diagnostic modalities, especially with respect to hepatic imaging, have improved in recent years. This, along with HCC surveillance in patients with cirrhosis, has led to the detection of HCC at an earlier stage, when curative therapy is likely to be more successful.

The major diagnostic techniques for HCC include serum markers, various imaging modalities and histological analysis.

SERUM MARKERS FOR HCC

A number of serum markers have been proposed and several are currently used in commonplace clinical practice as a method for detecting HCC (Table 1).

Alpha-1 fetoprotein (AFP)

Under physiological conditions, AFP is a fetal-specific glycoprotein with a molecular weight of around 70 kDa. It is synthesized primarily by the embryonic liver, by cells of the vitelline sac and by the fetal intestinal tract in the first trimester of pregnancy. The serum concentration of AFP declines rapidly after birth and its expression is repressed in adults. Pathologically, patients with chronic liver disease, particularly those associated with a high degree of hepatocyte regeneration, can express

Table 1 Serum markers for HCC

Alpha-1 fetoprotein
Lens culinaris agglutinin-reactive AFP (AFP-L3)
Des-gamma carboxyprothrombin (DCP)
α -L-Fucosidase
Glypican-3
Squamous cell carcinoma antigen (SCCA)
Golgi protein 73 (GP73)
Hepatocyte growth factor (HGF)
Transforming growth factor- β 1 (TGF- β 1)
Vascular endothelial growth factor (VEGF)
Serum proteomics

AFP in the absence of cancer. Also, AFP is elevated in hepatocarcinogenesis, embryonic carcinomas^[2-7] and in gastric^[8] and lung cancer^[9].

AFP has been used as a serum marker for HCC for many years. It was first described by Abelev *et al*^[10] in the 1960s. The first quantitative serum assays for AFP were established by Ruoshlati and Seppala^[11]. AFP is not elevated in all patients with HCC. Some patients with cirrhosis and/or hepatic inflammation can have an elevated AFP, even without the presence of a tumor. The test had a sensitivity of 39%-65%, a specificity of 76%-94%, and a positive predictive value of 9%-50% for the presence of HCC in previously published studies^[12]. The variation in sensitivity and specificity of AFP in the studies performed may be due to the diversity of patient populations examined, varying study designs and differing cut-off values for normality.

There is a debate in defining the AFP cut-off level for the diagnosis of HCC. An AFP value above 400-500 ng/mL has been considered to be diagnostic for HCC in patients with cirrhosis. However, such a cut-off value is problematic in absolute diagnostic terms, since high levels of this magnitude are not as common in the presence of smaller tumors (< 5 cm) and furthermore, only 30% of HCC patients have levels higher than 100 ng/mL in this context^[3,13].

A case-control study of 170 HCC patients and 170 matched patients with chronic liver disease was conducted in Italy. The authors defined an AFP level of 16 ng/mL as the threshold that maximized sensitivity and specificity for the diagnosis of HCC. Using an AFP level of 20 ng/mL (the upper normal range) as the cut-off yielded equivalent sensitivity (60.0% *vs* 62.4%) and specificity (90.6% *vs* 89.4%). The positive and negative predictive values were 85% and 70%, respectively. The positive predictive value increased to 100% for patients without chronic hepatitis B or hepatitis C infection^[14]. However, up to 42% of patients with HCC present with serum AFP levels within normal values^[3,15].

In another study, conducted in 290 Chinese patients with chronic hepatitis B, 44 patients were found to have an elevated AFP (> 20 ng/mL)^[16]. Of these, only six (14%) had HCC and the remaining 38 patients had an elevated AFP due to hepatitis B viral flares ($n = 18$), or due to unknown causes ($n = 20$). It is clear that using an AFP level of just above normal as a cut-off level gives a very low positive predictive value^[17]. Moreover, patients

with chronic hepatitis B or C with reactivation were found to have AFP levels > 500 ng/mL^[18].

AFP seems to be of prognostic value at the time of tumor diagnosis. A high AFP concentration (≥ 400 ng/mL) in HCC patients is associated with greater tumour size, bilobar involvement, portal vein invasion, and a lower median survival rate^[7]. According to a recent study, patients with serum AFP greater than 1000 ng/mL have a higher incidence of vascular invasion (61%) compared to patients with an AFP level less than or equal to 1000 ng/mL (32%)^[19]. This may relate to the finding that well-differentiated tumours express lower levels of AFP^[20].

In addition, AFP can be used as a marker for detecting tumour progression in patients with AFP-producing HCC. After treatment of the tumour, complete response is likely if the pre-treatment elevated AFP levels decline to and remain at normal levels during subsequent follow-up measurements. Reduction of AFP levels after palliative treatment, such as transarterial chemoembolization, usually indicates a favorable response to treatment. In addition, AFP is an excellent marker for detection of *de novo* HCC after treatment, if the new lesion is of the AFP-secreting variety^[17].

A large multicenter study, based on both retrospective and prospective data collection, was carried out by Farinati *et al*^[21] over a consecutive series of more than 1000 HCC patients. Only 18% of the studied patients had an AFP level of > 400 ng/mL. Moreover, patients with high AFP had poor survival. In this study, AFP was not a sensitive marker to detect the presence of HCC. Also, the prognostic value of AFP is limited, but it is correlated with the overall survival in untreated patients, or in those treated by liver transplantation or locoregional therapies^[21].

Lens culinaris agglutinin-reactive AFP

There are three different AFP variants, differing in their sugar chains (AFP-L1, AFP-L2, AFP-L3). AFP-L1, the non-LCA-bound fraction, is the main glycoform of AFP in the serum of patients with non-malignant chronic liver disease. In contrast, Lens culinaris-reactive AFP, also known as AFP-L3, is the main glycoform of AFP in the serum of HCC patients and it can be detected in approximately one third of patients with small HCC (< 3 cm), when cut-off values of 10% to 15% are used^[22]. Its sensitivity and specificity ranges from 75% to 96.90% and 90% to 92%, respectively at the cut-off level of 15%^[22-24].

In a study conducted in HCC patients with lesions less than 2 cm in size, using a cut-off level of 10% was diagnostic for the presence of HCC. AFP-L3 was found to be associated with poorly differentiated and advanced HCC. Higher AFP-L3 levels were found in hypervascular HCC, compared to iso- or hypovascular HCC^[25]. Elevated levels of AFP-L3 were associated with a shorter tumor doubling time in comparison with those with low levels of AFP-L3.

Moreover, AFP-L3 acts as a marker for clearance of HCC after treatment and as a predictor of recurrence as

failure to decline to the normal level indicates residual disease. Recurrence of HCC is expected when AFP-L3 levels increase to > 10% or rise after normalization with treatment^[17]. Another study reported that AFP-L3 > 15% is a significant predictor for HCC recurrence^[26].

AFP-L3 levels were found to be related to progression from moderately differentiated to poorly differentiated tumors. HCC patients with AFP-L3 > 10% had a higher frequency of poorly differentiated tumors^[27]. Thus, this biomarker may be able to predict advanced tumor stage and a worse prognosis.

It is reported that an AFP-L3 level of 15% or more is correlated with HCC-associated portal vein invasion^[28], both total serum AFP and AFP-L3 can be measured simultaneously^[29], and estimating the AFP-L3/AFP ratio is helpful in diagnosis and prognosis of HCC.

Des-gamma carboxyprothrombin (DCP)

DCP is an abnormal prothrombin protein that is found in the serum of patients with HCC and in patients with vitamin K-deficiency or on warfarin therapy. It is a “so-called” protein induced by vitamin K absence or antagonist- II (PIVKA- II)^[29,30].

DCP is produced as a result of an acquired defect in the post-translational carboxylation of the prothrombin precursor (the 10 glutamic acid residues at the N terminus) in malignant cells^[31]. The reduced activity of gamma-carboxylase was attributed to defective gene expression in HCC patients^[7]. A DCP level of 40 mAU/mL is commonly used as a cut-off level, at which the rate of early detection of small HCC is improved^[17]. Serum DCP was found to have a sensitivity of 48% to 62%, a specificity of 81% to 98%, and a diagnostic accuracy of 59% to 84% in diagnosing HCC in several large case-controlled studies^[7,32].

DCP is a well-recognized tumor marker used for the diagnosis of HCC. Its diagnostic accuracy has been investigated in multiple studies, with conflicting results. DCP has been reported to be more sensitive and specific than AFP in the diagnosis of HCC, especially in Eastern Asian countries and in North America^[3,7,33]. Conversely, in Europe, studies have not shown these results. These discrepant results were related not only to racial factors but also to different etiological factors in liver disease. Even with these results, the measurement of both markers is suggested to increase diagnostic efficacy^[3].

A recent study compared the performance characteristics of AFP, DCP and AFP-L3 in the diagnosis of HCC. DCP was significantly better than the other markers in differentiating HCC from cirrhosis, with a sensitivity of 86% and a specificity of 93%^[34]. However, tumor size can affect the sensitivity and specificity of DCP in detecting HCC. According to a study by Nakamura and colleagues^[35], the efficacy of DCP was lower than that of AFP in the diagnosis of small HCC tumors, although higher than that of AFP for large tumors.

Multiple studies have shown that DCP can be a useful indicator of vascular invasion in HCC patients^[27,28,36]. Moreover, it was found to be a helpful marker for

monitoring the effectiveness of treatment and the recurrence of HCC after treatment^[17,37].

Alpha-l-fucosidase (AFU)

AFU is a glycosidase found in all mammalian cell lysosomes and is concerned with the degradation of a variety of fucose-containing fucoglyco-conjugates^[38]. The activity of this lysosomal enzyme is detectable in the sera of healthy subjects. Increased activities are found in patients with HCC compared to healthy individuals and patients with chronic liver disease. Using a cut-off value of 870 nmol/mL, the sensitivity and specificity was 81.7% and 70.7%, respectively. Simultaneous determination of both AFP and AFU can increase the sensitivity to 82.6%. AFU activity was correlated with tumor size in patients with HCC^[7,39,40]. According to another study conducted in 884 Chinese subjects^[41], the AFU activity was significantly higher in HCC patients compared to patients with cirrhosis, chronic hepatitis, other malignant tumors, other diseases and healthy individuals. The sensitivity for AFU was 81.5% and the specificity was 85.4%. Furthermore, the persistently elevated AFU level in patients with cirrhosis adds to the detection of HCC at an earlier stage^[7,22,38], owing to elevated activity of AFU at least 6 mo before the detection of HCC by ultrasonography in 85% of patients^[39].

However, it should be noted that the prolonged storage of serum samples affects the enzyme activities over time^[42]. Furthermore, AFU activity is not only elevated in primary HCC, but also in cases of colorectal cancer^[43] and ovarian cancer^[44], in addition to some non-malignant extrahepatic diseases, such as diabetes, pancreatitis, and hypothyroidism^[29].

Glypican-3 (GPC3)

GPC3 is a cell-surface glycoprotein which is a member of the glypican family of glycosyl-phosphatidylinositol-anchored cell-surface heparin-sulfate proteoglycans^[45]. GPC3 mRNA and protein are not detectable in normal tissues, except placenta and fetal liver, and they are expressed in the majority of HCCs^[46,47]. Normally, GPC3 has a role in regulating cell proliferation and survival during embryonic development by modulating the activity of various growth factors. Also, it functions as a tumor suppressor^[29,48,49].

GPC3 was expressed in 72% of HCC tissues, while it was lacking in hepatocytes from normal liver and non-malignant hepatic diseases. In addition, GPC3 was detected in sera from 53% of HCC patients, and it was not detected in the serum of patients with chronic hepatitis, or healthy individuals. No correlation was found between GPC3 and total AFP levels in patients with HCC. However, simultaneous measurement of GPC3 and total AFP increased the sensitivity without affecting the specificity^[50].

Hippo and colleagues^[51] found that serum levels of soluble GPC3 (sGPC3), the NH₂-terminal portion of GPC3, were significantly higher in HCC patients, compared to patients with cirrhosis and healthy controls.

sGPC3 was better than AFP in detecting well- or moderately-differentiated HCC and the combination of both markers improved overall sensitivity from 50% to 72%^[22,51].

A recent study evaluated the level of GPC3 in 49 fine needle aspiration biopsies, using immunocytochemical staining. For the diagnosis of HCC in the cytological material, the sensitivity of GPC3 was 83.3%, the specificity 96%, and the positive predictive value and the negative predictive value were 95% and 85.7%, respectively. This high sensitivity and specificity enabled the delineation of HCC, distinct from other benign and malignant hepatic lesions, and from most metastatic lesions^[52]. Recently, it has been suggested that GPC3 can induce oncogenesis through activation of the insulin-like growth factor II (IGF-II) signalling pathway^[53].

Squamous cell carcinoma antigen (SCCA)

SCCA, a member of the serpin (serine protease inhibitor) family, is physiologically expressed in the skin and other squamous epithelial cells^[54]. High levels have been reported in tissues of head and neck cancer and other epithelial cancers^[55]. It has also been reported to be over-expressed in HCC tissue and in serum from patients with HCC^[56].

Giannelli and colleagues measured serum SCCA in three patient groups: 120 patients with HCC, 90 with cirrhosis, and 41 healthy subjects. SCCA levels were significantly elevated in HCC patients, compared to patients with cirrhosis only or normal subjects. The sensitivity and specificity for SCCA were 84% and 49%, respectively, at the optimum cut-off value of 0.37 ng/mL^[56]. The authors suggested that combination of the SCCA (high sensitivity/low specificity) and AFP (low sensitivity/high specificity) markers may be beneficial. According to a recent study, SCCA was found to be expressed in pre-malignant dysplastic nodules as well as malignant lesions^[57].

It has been reported recently that both AFP and SCCA can react with the IgM class of immunoglobulins to form the immunocomplexes AFPIC and SCCAIC, respectively. Both of these can be detected in the serum of HCC patients^[54,58,59].

Between 2001 and 2005, 961 consecutive patients with HCC ($n = 499$) and those with cirrhosis uncomplicated by malignancy ($n = 462$) were studied in multiple centers in Italy and France^[54]. AFP, SCCA, AFPIC and SCCAIC were measured using ELISA tests in all patients. SCCA levels were inversely correlated with tumor size. The combined use of AFPIC, SCCA and SCCAIC in patients with low levels of AFP (< 20 IU/mL) detected 25.6% of HCCs (186/725). There was no correlation found between AFP and the other markers investigated. The authors suggested, perhaps optimistically, that each marker was related to a different aspect of HCC, so that the use of all of these markers in combination in clinical practice would provide a non-invasive and relatively simple

series of tests that could improve the accuracy of HCC diagnosis^[54]. However, it is unlikely that this suggestion will be taken up widely, as many of these tests have restricted availability and there are cost implications that will need further evaluation.

Golgi protein 73 (GP73, also known as Golp2)

GP73 is a resident Golgi-specific membrane protein expressed by biliary epithelial cells in normal liver. Hepatocyte expression of GP73 is up-regulated in patients with acute hepatitis, cirrhosis and HCC, while in published studies, there is no considerable difference in biliary epithelial cell expression of this marker^[60,61].

It has been reported that GP73 is superior to AFP for the detection of early HCC in patients with cirrhosis. According to a study of 352 patients, measurement of serum GP73 based on immunoblots revealed that HCC patients had significantly higher levels than patients with cirrhosis^[62]. At the optimal cut-off (10 relative units), the sensitivity and specificity were 69% and 75%, respectively. For the diagnosis of early HCC, this marker had a significantly higher sensitivity (62%) than AFP (25%)^[62]. Interestingly, serum GP73 levels were elevated in 57% of patients with HCC associated with normal AFP levels.

Hepatocyte growth factor (HGF)

HGF is a multi-functional factor that is produced in various body organs and can affect mitogenesis, cell motility, matrix invasion, and epithelial carcinogenesis^[29,63,64]. Increased HGF serum levels have been reported in patients with squamous cell carcinoma of the esophagus^[65] and lymphomas, in addition to non-malignant diseases, such as aortic dissection, pulmonary thromboembolism^[66], coronary syndrome^[67], cerebral infarction^[68] and sepsis^[69,70]. In a prospective study, blood samples were collected from 99 patients with chronic hepatitis, cirrhosis, and HCC^[71]. Serum HGF levels were significantly elevated in HCC patients compared to patients with cirrhosis or chronic hepatitis but no malignancy. All patients with a serum HGF concentration of greater than 0.6 ng/mL had HCC, irrespective of the AFP or DCP levels.

HGF has been used as a prognostic marker in HCC. Serum HGF levels greater than or equal to 1.0 ng/mL have been associated with poor survival in HCC patients^[72]. A recent study conducted in HCC patients who underwent hepatic resection associated high HGF levels in peripheral and portal blood with adverse prognosis^[73]. The authors postulated that HGF induces proliferation and invasiveness of HCC cells through expression of its receptor, the c-met receptor. Also, the persistent elevated level of serum HGF with intensive expression of c-met protein after partial hepatectomy were found to predict early tumor recurrence and metastasis^[74]. This may be explained by the role of HGF in initiating proliferation of normal and malignant hepatocytes after partial hepatectomy.

Transforming growth factor-beta 1 (TGF- β 1)

TGF- β 1, a multifunctional factor, has a vital role in the regulation of growth and differentiation of normal and transformed cells, angiogenesis, extracellular matrix formation, immunosuppression and carcinogenesis^[7,75]. It has been reported that TGF- β 1 and TGF- β 1 mRNA levels were significantly higher in the serum of patients with HCC compared to patients with non-malignant chronic liver diseases^[7,75,76]. Using a cut-off level of 1.2 μ g/L for the diagnosis of HCC, the sensitivity was 89.5% and the specificity was 94%. Interestingly, expression of TGF- β 1 in liver tissues was related to the degree of HCC differentiation. Hence, this biomarker might find a role as a prognostic marker in HCC. Simultaneous detection of TGF- β 1 level and serum AFP yielded a higher detection rate of 97.4%^[75].

It has also been reported that TGF- β 1 levels might increase in patients with cirrhosis, owing to decreased hepatic clearance in such patients. In addition, this biomarker is up-regulated in extra-hepatic tumors, wound healing, angiogenesis and fibrosis, indicating lack of disease-specificity^[29,76].

Vascular endothelial growth factor (VEGF)

VEGF is an endothelial cell mitogen that initiates and promotes neovascularization and endothelial cell proliferation, and it was initially identified as a vascular permeability factor. VEGF has a major effect in regulating angiogenesis, and its expression has been shown to correlate with carcinogenesis^[7]. In a study by Poon and colleagues, conducted in 108 patients with HCC and 20 healthy controls^[77], serum VEGF levels in HCC patients were significantly higher, compared to control individuals, and was correlated with venous invasion and advanced tumour stage. In this study, a serum VEGF level of 245 pg/mL or more was associated with poor overall survival.

The expression of VEGF in HCC tissues was correlated with AFP, DCP tumor size and histological grade of the tumor^[78]. Furthermore, this biomarker was related to invasiveness and metastasis of HCC^[17,79]. The expression of VEGF in HCC patients with microscopic venous invasion was significantly higher than that in HCC patients without microscopic venous invasion^[80].

Serum proteomics

Recently, surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF) has been used to identify specific serum protein fragments. Paradis and colleagues conducted a SELDI-based study in 82 French patients and identified a six-peak panel that distinguished HCC and non-HCC patients in 90% of the cases^[81]. The C-terminal fragment of vitronectin was identified as the highest discriminating peak. Identification of this fragment as a marker for HCC is possible, as it could be generated *in vitro* by cleavage of the intact vitronectin molecule by a metalloprotease^[29].

A more recent study compared the sensitivity and specificity of SELDI-TOF MS with AFP, AFP-L3,

and prothrombin induced by vitamin K absence-II (PIVKA-II) for the detection of established HCC^[82]. For AFP, the sensitivity and specificity were 73% and 71%, respectively, using a cut-off level of 20 ng/mL. With AFP-L3, a cut-off level of 10% gave a sensitivity and specificity of 63% and 94%, respectively. Using the PIVKA-II cut-off of 125 mAU, the sensitivity and specificity were 84% and 69%, respectively. The sensitivity and specificity of SELDI-TOF MS were 79% and 86%, respectively. The authors concluded that SELDI-TOF MS analysis is more accurate than other conventional means of biomarker assessment in detecting small tumors.

RADIOLOGICAL DIAGNOSIS OF HCC

(FIGURE 1)

Imaging modalities employed in HCC diagnosis can be divided into two main groups: those routinely used such as ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI); and those that are more invasive, including iodized oil-CT, CT during hepatic arteriography (CTHA), CT arterial portography (CTAP) and conventional hepatic angiography^[83].

Ultrasound scanning (US)

Currently, US is the technique of choice for screening focal hepatic lesions. On US, lesions may appear hyperechoic, hypoechoic or show a 'target lesion' appearance, but none of these is specific^[84,85]. Any mass detected on US in a cirrhotic liver is suspicious of HCC, particularly if it is > 1 cm in size. As a screening test, US has a sensitivity of 65%-80% and has a specificity of > 90%^[84-86]. US permits the detection of smaller sized tumors (1 cm) in early carcinogenesis^[3,13,87]. However, the detection of small HCC in a cirrhotic liver by US is much more difficult than the detection of metastases in a normal liver, owing to disturbed parenchymal architecture^[88,89].

The ultrasonographic characteristics of HCC depend on nodule characteristics and tumor size. Most small HCC nodules (less than 3 cm) are well defined, homogeneous and hypoechoic without posterior echo-enhancement. These features are non-specific and may be indifferent from the echo pattern of regeneration nodules in cirrhosis^[88,89]. As the tumor grows in size, it becomes non-homogeneous and more hyperechoic or isoechoic, owing to fatty degeneration or coagulation necrosis. Alternatively, it may show a heterogeneous (mosaic) pattern with a star-shaped central hypoechoic area, owing to the presence of fibrous septae. There is another, less common type of nodular HCC that is homogeneous and diffusely hyperechoic, owing to fatty changes or dilated sinusoids. These features do not differ as the tumor grows^[88].

In addition, US can be used to assess the vascular structures and the presence of hilar adenopathies associated with advanced tumor stage^[3]. The presence

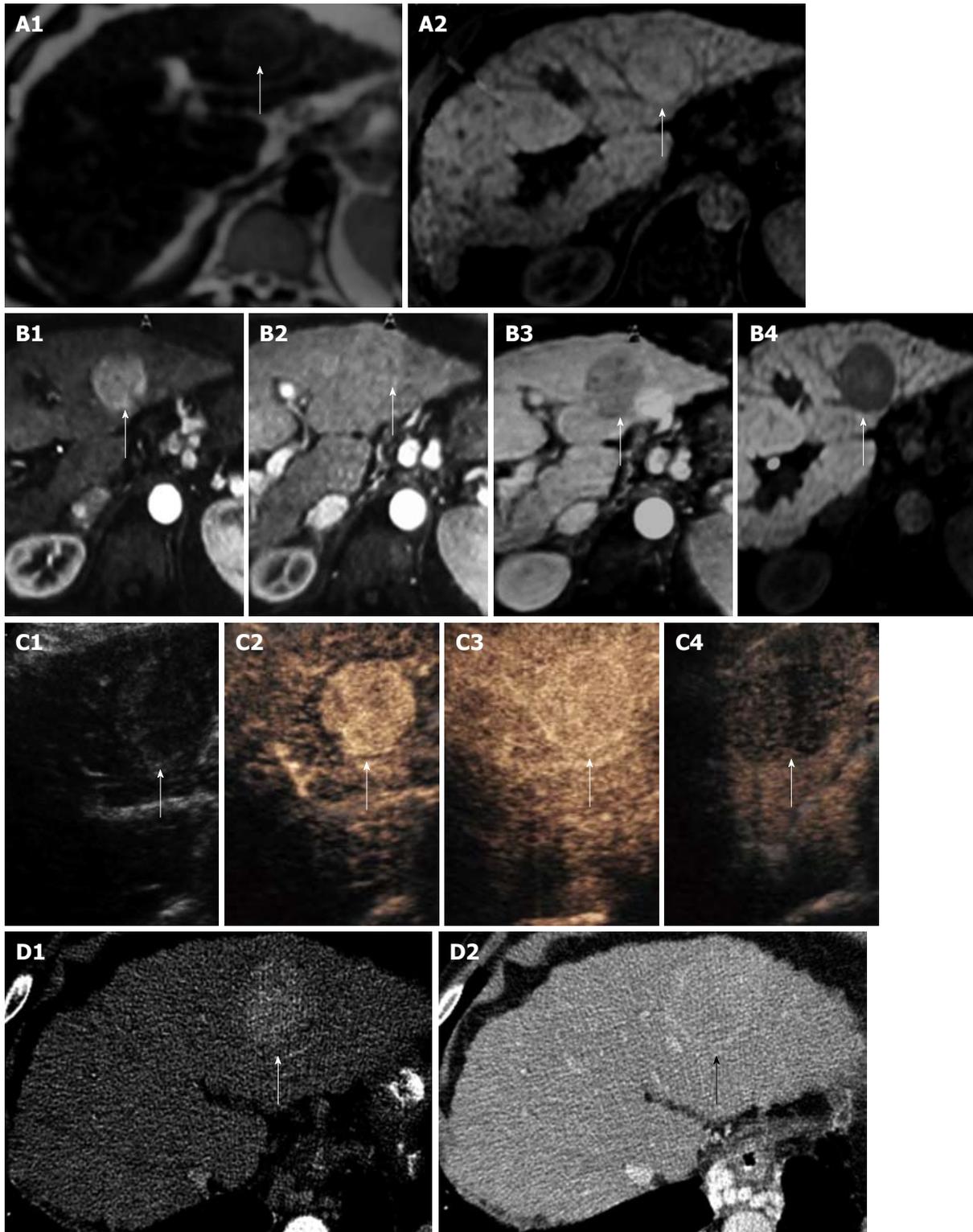


Figure 1 There is a large HCC in the left lobe of the liver with a pseudocapsule, hyper-enhancing on arterial phase and showing washout on late phases on MR and CEUS and is iso-intense in portal phases on CT and CEUS. The pseudo-capsule enhances in the portal phase on all modalities. A1: T₂ weighted scan showing slightly higher intensity HCC (arrow); A2: T₁ weighted scan shows same HCC which is iso-intense (arrow); B1: MultiHance enhanced T₁ weighted scan in the arterial phase showing enhancement of the HCC (arrow); B2: MultiHance enhanced T₁ weighted scan in the portal phase showing iso-enhancement of the HCC (arrow); B3: MultiHance enhanced T₁ weighted scan at 2 min showing contrast wash-out in the HCC (arrow); B4: MultiHance enhanced T₁ weighted scan at 40 min showing hypointense HCC (arrow); C1: Baseline ultrasound shows iso-echoic HCC (arrow); C2: SonoVue enhanced ultrasound shows hyper-enhancing HCC (arrow) in the arterial phase; C3: SonoVue enhanced ultrasound shows iso-enhancing HCC (arrow) in the portal phase with enhancement of the pseudocapsule; C4: SonoVue enhanced ultrasound shows wash-out of the HCC (arrow) in the late phase; D1: Contrast-enhanced CT scan shows enhancement of the HCC (arrow) in the arterial phase; D2: Contrast-enhanced CT scan shows iso-enhancement of the HCC (arrow) in the portal phase with enhancement of the pseudocapsule.

of intra-hepatic venous thrombosis, a mass protruding from the hepatic surface or a dilated intra-hepatic bile

duct offer indirect evidence that raise the suspicion of a liver tumor, even in the absence of a definite finding of

a liver mass on US^[88].

Tumor size has been found to affect the sensitivity of US in detecting HCC. Kim and colleagues (2001) assessed the performance of grey-scale US in pre-transplant patients. The sensitivity for HCC nodules greater than 2 cm was 38% and for lesions less than 2 cm, it was 30%^[83]. Other studies showed the sensitivity for tumors smaller than 1 cm to be about 42%^[3,90,91] compared to 95% for tumors of larger size^[92].

It has been found that US had low sensitivity and high specificity in detecting HCC and DN in patients with end-stage liver disease requiring liver transplantation^[89]. According to a retrospective study of 200 patients with liver failure who underwent liver transplantation within 90 d of US scanning, correlating the US findings with explanted livers showed a sensitivity of 75% for large lesions (> 5 cm), but for small lesions (1-5 cm), the sensitivity ranged from 13.6 to 50%^[93].

Colour Doppler US

Colour Doppler US gives an approximation of the mean velocity of blood flow within a vessel by color coding the flow and displaying it superimposed on the grey-scale image, while power Doppler assesses the amplitude of signals^[89]. The diagnostic performance of US in the identification of tumor portal thrombosis in patients with HCC can be increased when combining Doppler with US: the sensitivity reaches up to 92% and the specificity virtually 100%. When US-Doppler reveals permeability of the portal system, portal thrombosis is ruled out and hepatic arteriography should be avoided^[3,94].

Patients with tumors treated with percutaneous ethanol injection may develop chemical thrombosis. Such benign thrombi can be differentiated from tumoral thrombosis using US Doppler, based on the presence of blood flow in the thrombus. The presence of a hepatofugal pulsatile flow inside the thrombus confirms the presence of tumoral invasion of portal vessels^[3,95].

Contrast enhanced ultrasound (CEUS)

CEUS using non-linear imaging modes has been used to improve sonographic visualization of hepatic tumor vascularity^[96,97]. CEUS can give information about the nature of liver lesions that are not characterized with baseline US, and every lesion detected during US surveillance in patients with chronic liver disease, or in patients with past history of malignancy^[96,98]. CEUS is safe and well tolerated. The contrast study can be performed once a focal lesion is detected on standard US, providing immediate information about the vascular characteristics of the nodule^[97].

US contrast agents consist of microbubbles of low-solubility gas surrounded by a protein, lipid or polymer shell. The microbubbles are 1 to 10 μm , which are too large to pass through the vascular endothelium and, as such, they are considered pure blood pool agents^[99,100]. In the liver, the microbubbles dissolve several minutes later in the circulation leading to exhalation of the gas

and metabolism of the shell^[99,101]. The microbubbles change their size when subjected to an US wave. On the other hand, soft tissues express minor changes. The bubbles are highly reflective, even when they are present in a small concentration. Furthermore, the expansion of these bubbles during the rarefaction phase exceeds their contraction during the pressure phase leading to the production of a returning signal (echo) that contains harmonics^[99,102].

The characterization of a hepatic lesion with microbubbles depends on all phases of contrast enhancement, i.e. the hepatic arterial phase (starting from 10-20 s after injection of contrast agent and lasting for about 10-15 s), portal venous phase (up to 120 s post-injection) and late parenchymal phase (up to 4-6 min after injection)^[103]. The arterial phase helps in predicting the degree and pattern of vascularity, while the portal and late phases are helpful in determining the nature of a lesion, as most malignant lesions are hypo-enhancing in contrast with the benign lesions which are iso- or hyper-enhancing^[103].

The majority of HCCs are characterized by arterial phase enhancement and washout of the contrast during the late phase, so as to appear as a defect. However, well-differentiated HCCs may not show this washout. Moreover, it has been observed that the more differentiated a lesion, the more gradually it is likely to washout^[99,103,104]. Recently, it was reported by Pompili and colleagues that CEUS and multidetector row CT have a similar sensitivity (up to 87%), unaffected by nodule size (< 2 cm *vs* 2-3 cm)^[97]. Most hypervascular nodules (85.2%) were exactly identified by both methods, while CT was slightly more sensitive in detecting arterial vascularity. The authors concluded that CEUS is a dependable imaging tool for vascular characterization of small nodules (less than 2 cm) in patients with cirrhosis.

CEUS gives equivalent accuracy to CT and MRI in the characterisation of focal liver lesions^[98] and is probably the best alternative when there are contraindications to CT or MRI^[97]. However, the performance of CEUS compared with CT or MRI, is highly affected by operator skill and experience, patient-related factors, such as body habitus and cooperativeness, and tumor-related factors, such as nodule location. Also, it is inappropriate for panoramic detection and staging of HCC^[97].

CT

Multiphasic helical CT (MPCT) is deemed the imaging technique of choice for the detection and staging of HCC^[88,105,106]. MPCT includes four phases: pre-contrast, hepatic arterial, portal venous and delayed phases. A high-speed single detector spiral scanner is used to achieve the images. Images are obtained after contrast injection at a delay of 25 s (arterial phase), 70 s (portal venous phase) and 300 s (equilibrium phase). HCCs appear hypervascular during the hepatic arterial phase, owing to the fact that the hepatic artery provides the main blood supply, and it appears rather hypodense during the delayed phase, which is attributed to the early wash-out of contrast. It was found that delayed phase images can aid in the diagnosis of HCC in 14% of

patients^[88,107]. Typically, HCC lesions are heterogenous on CT and the appearance of satellite nodules around the lesion is characteristic^[108]. Combining the hepatic arterial and portal venous phases improves the detection of small malignant tumors^[88,109].

It has been reported that the presence of intraluminal low attenuation with distension of the occluded venous segment would be indicative of tumor thrombus^[88]. Tumor thrombosis can be differentiated from benign thrombosis during the arterial phase. Since tumor thrombosis enhances, such enhancement can be detected either as “diffuse”, which is typical of HCC, or as streaks of tumor vessels inside the thrombus^[89,110]. Several studies have assessed the performance of spiral CT in the diagnosis of HCC. One study on 41 patients who underwent transplantation within 100 d of imaging, revealed a sensitivity and specificity for HCC of 80% and 96%, respectively^[111].

The diagnostic accuracy of CT is affected by technical factors, such as the injection of contrast, and intrinsic factors related to the tumor, such as tumor size and vascularity. It was reported that the diagnostic efficacy of CT is diminished in small tumors (less than 2 cm) owing to the hypo-vascularization of small-sized tumors^[3,112,113]. The sensitivity of four phase CT in detecting HCC was up to 100% for tumors greater than 2 cm in size, 93% for tumours 1-2 cm in size, and for tumors less than 1 cm in size, it was 60%^[114].

Spiral CT is the standard imaging technique for detecting the response to loco-regional treatment of HCC^[115]. It has been reported to be more effective than combining US scanning and AFP level estimation in the detection of early HCC recurrence after successful treatment^[116].

More recently, a study to evaluate the diagnostic efficacy of contrast-enhanced helical computed tomography (CECT) and CEUS has been conducted in patients with small hepatic nodules, previously detected by surveillance programs^[117]. The sensitivity, specificity and diagnostic accuracy were 91.1%, 87.2%, and 89.3%, respectively, for CEUS. For CECT, the sensitivity, specificity and diagnostic accuracy were 80.4%, 97.9%, and 88.4%, respectively. The authors found no significant difference between CEUS and CECT in characterising small (1-2 cm) hepatic nodules.

Multidetector helical CT (MDCT)

Recently, MDCT has allowed collection of early (18-28 s after injection of contrast) and late or so-called early parenchymal (35-45 s) arterial phase images. The early arterial images illustrate vessels optimally needed for treatment planning in patients who are likely to undergo surgery, while the late arterial phase images demonstrate the lesions better than the early arterial phase^[118]. Evaluation of both early and late arterial phase images results in better sensitivity and positive predictive values^[89,119]. MDCT with two arterial phases carry the risk of increased radiation exposure thus, limiting its use^[89]. MDCT has been shown to have a higher sensitivity in the detection of HCC in cirrhotic liver, owing to the

high speed and flexibility leading to achievement of high quality, thin section imaging and three dimensional capabilities^[108]. In addition, vascular tumors can appear hypodense, relative to liver parenchyma during the equilibrium phase (3-5 min post-injection). It is reported that tumors measuring less than 2 cm can be best detected in this phase, owing to the more rapid washout of contrast from the tumor than from the normal liver parenchyma^[89,120].

Recently, it was reported that MDCT scanning is useful in early detection and the effective treatment of small HCCs, especially during follow-up of patients with chronic hepatitis and cirrhosis. A study compared the efficiency of gadolinium-enhanced multiphase dynamic MRI with MDCT scanning in the detection of small HCC^[121]. The detection rate of small HCC on MDCT was 97.5%-97.6% and it was 90.7%-94.7% on MRI, according to tumor size. For very small HCC \leq 1 cm, the sensitivity of detection on MDCT was higher compared to MRI (90.0%-95.0% and 70.0%-85.0%, respectively). The authors concluded that MDCT scanning was better than MRI for early detection of small HCC during the follow-up of patients with chronic hepatitis and cirrhosis^[121].

MRI

MRI has been used to improve detection and characterization of hepatic malignant lesions^[3]. HCC appears hyper-intense on T₂-weighted images with variable signal intensity on T₁-weighted images. Usually, there is no signal drop-out on the in- and out-of-phase images, because of a low incidence of fatty change. With dynamic gadolinium-enhanced imaging, the lesion enhances in the arterial phase then becomes isointense in the portal phase then becomes hypo-intense in the delayed phase^[88,122].

MRI was found to be more accurate than CT or US in detecting HCC and estimating the actual tumor size^[123]. Moreover, MRI was more effective than spiral CT in detecting HCC and dysplastic nodules in patients with cirrhotic liver. The sensitivity of MRI for characterising HCC was 61% while the sensitivity of CT was 52%^[124].

The sensitivity of MRI in detecting HCC depends on tumor size. It is about 95% in tumors larger than 2 cm, while in tumors less than 2 cm the sensitivity is reduced to 30%^[125]. MRI is also very good at delineating the internal architecture of the tumor, the tumoral margins and intrahepatic vascular invasion^[125]. Hence, MRI is deemed the best tool in differentiating HCC from hepatic hemangioma^[3,13]. It has also been suggested that liver function may affect hepatic parenchymal signal intensity leading to the appearance of observed liver-to-lesion contrast on delayed images^[89,126].

Angiography

Owing to the hypervascular nature of HCC, the arterial supply to the tumor is often dilated, tortuous, distorted and displaced. An intense tumor stain, vascular lakes and venous pools are commonly observed^[88]. It is thought that the diagnostic efficacy of hepatic arteriography

Table 2 EASL consensus diagnostic criteria for HCC (adapted from Bruix *et al.*^[115], 2001)

Cyto-histological criteria
Non-invasive criteria (restricted to patients with cirrhosis)
Radiological criteria: two coincident imaging techniques
Focal lesion > 2 cm with arterial hypervascularisation
Combined criteria: one imaging technique associated with elevated serum AFP levels
Focal lesion > 2 cm with arterial hypervascularisation
AFP levels > 400 ng/mL

is related to tumor size and vascularization^[112]. The sensitivity, specificity and diagnostic accuracy of angiography in the detection of HCC smaller than 5 cm has been reported as 82%-93%, 73% and 89%, respectively. When tumor size was smaller than 2 cm, these values were reduced^[3,127,128]. Currently, angiography is often used to delineate hepatic anatomy before resection or as guidance for transarterial chemoembolization of HCC lesions^[108].

Histological diagnosis of HCC

Cytological examination of a suspected lesion can be achieved by fine needle aspiration biopsy (FNAB). FNAB diagnostic efficacy varies from 60% to 90% according to the size of the lesion, the diameter of the puncturing needle and level of operator training. The specificity and positive predictive value of this technique are from 90% to 100%. It is a safe technique with minimal risk of complications^[3,13,129,130].

Histopathological examination is considered the chief method for a sure diagnosis of HCC. It is mandatory to study non-tumoral liver tissue to exclude or to confirm the presence of liver cirrhosis, which affects the treatment modality^[3,129]. Complications associated with liver biopsy are low with mortality rates of 0.006%-0.3%. The risk of tumor seeding along the needle tract has been estimated at up to 3%^[108,131,140,144].

A combination of the two pathological techniques can improve diagnostic performance^[3,129]. One study reported that the sensitivity of cytological and histological examination was about 80% for each one separately. When combining the two methods, sensitivity reached 89%^[132].

Microscopically, HCC cells have an elevated nuclear to cytoplasmic ratio, trabecular architecture, atypical naked nuclei, and peripheral endothelial wrapping^[108,133]. Pathological findings range from almost normal-appearing hepatocytes in well differentiated tumors to the largely anaplastic multinucleate giant cells in poorly differentiated HCC^[108,134].

DIAGNOSTIC APPROACH

The European Association for the Study of the Liver (EASL) has formulated a consensus statement to regulate the diagnostic approach in HCC patients, based on histological and radiological criteria for identifying HCC in patients with cirrhosis. Recommendations were

Table 3 Diagnostic criteria for HCC (adapted from Bruix *et al.*^[85], 2006)

Cyto-histological criteria
Non-invasive criteria (cirrhotic patients)
Focal lesion ≤ 2 cm. Two imaging techniques with arterial hypervascularisation and venous washout
Focal lesion > 2 cm. One imaging technique with arterial hypervascularisation and venous washout

considered based on the size of the lesion (Table 2)^[115,135].

HCC lesions of greater than 2 cm in diameter can be diagnosed non-invasively, based on radiographic criteria in patients with cirrhosis^[115,135]. Detection of nodules with arterial hypervascularization in two imaging modalities, or in only a single imaging modality associated with an AFP level ≥ 400 ng/mL in the cirrhotic liver, is considered diagnostic of HCC. EASL recommended evaluating the vascularity of hepatic nodules using US, contrast-enhanced CT or MRI, with formal angiography used in cases of diagnostic uncertainty. Histological confirmation by biopsy was not mandatory owing to the excellent diagnostic accuracy of imaging criteria and the 10%-20% false-negative rate from histological samples^[136-138].

Focal hepatic lesions of less than 1 cm in size were found to be non-malignant in 50% of cases^[135,141-143]. The EASL consensus statement recommended repeated ultrasound scanning every 3 mo until the lesion grows to 1 cm in diameter. Nodules from 1-2 cm in size are more likely to be HCC and pathological confirmation was recommended using fine-needle aspiration or biopsy or both for the diagnosis of these nodules. However, it carries a hazard for tumor seeding with a 30%-40% false-negative diagnostic rate^[115,135,139].

Recently, the American Association for the Study of Liver Disease (AASLD, 2005) issued guidelines which also proposed a diagnostic approach for HCC (Table 3). AFP of 200 ng/mL should lead to diagnostic suspicion of HCC requiring further investigation. In terms of imaging, nodules less than 1 cm in diameter should be repeatedly imaged for up to 2 years, due to uncertainty in the current diagnostic techniques in establishing a firm diagnosis. Nodules between 1 and 2 cm should be investigated with two dynamic imaging techniques such as CT scan, CE US or MRI^[85]. If they show hypervascularity with washout in the portal venous phase, the lesion can be diagnosed as HCC. Nodules greater than 2 cm in size that reveal typical features of HCC on dynamic profile (arterial hypervascularity with wash-out in the early or delayed venous phase) can be diagnosed as HCC by using a single imaging modality. Histological diagnosis was recommended if the vascular pattern is not characteristic for HCC on imaging modalities, to establish the diagnosis^[84,85].

CONCLUSION

The incidence of HCC is increasing around the world.

Although international consensus exists on a diagnostic pathway, there is no ideal screening modality. AFP serum level is the most commonly used serum test, while US is the most commonly used imaging test. Future research may delineate more specific and sensitive markers using proteomic or metabolomic approaches to screening blood or other biofluids such as urine.

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Hugh James Freeman, MD, FRCPC, FACP, Series Editor

Long-term natural history of Crohn's disease

Hugh James Freeman

Hugh James Freeman, Department of Medicine (Gastroenterology), University of British Columbia, Vancouver V6T 1W5, Canada

Author contributions: Freeman HJ contributed all to this paper.
Correspondence to: Dr. Hugh James Freeman, MD, FRCPC, FACP, Department of Medicine (Gastroenterology), University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver V6T 1W5, Canada. hugfree@shaw.ca

Telephone: +1-604-8227216 Fax: +1-604-8227236

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Abstract

Crohn's disease is a chronic inflammatory granulomatous process that usually involves different sites in the intestinal tract. Genetic and environmental factors are thought to play a role in its etiology and pathogenesis. The disorder has a heterogeneous clinical expression and data from tertiary care settings have documented its female predominance, occasional familial nature, and high rate of stricture formation and penetrating disease. It may appear from early childhood to late adulthood, although over 80% are currently diagnosed before age 40 years, usually with terminal ileal and colonic involvement. Several studies have now shown differences in phenotypic clinical expression depending on the initial age at diagnosis, with pediatric-onset disease being more severe and more extensive with more involvement of the upper gastrointestinal tract compared to adult-onset disease. In addition, long-term studies from these tertiary care settings have documented that the disorder may evolve with time into a more complex disease with stricture formation and penetrating disease complications (i.e. fistula and abscess). Although prolonged remission with no evidence of inflammatory disease may occur, discrete periods of symptomatic and active granulomatous inflammatory disease may re-appear over many decades. Long-term studies on the natural history have also suggested that discrete events (or agents) may precipitate this granulomatous inflammatory process.

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Key words: Natural history; Crohn's disease; Age-dependent phenotypes; Clinical behavior; Granulomatous inflammation

Peer reviewer: Dr. Cosimo Prantera, Chief, Head of Gastroenterology Unit Azienda Ospedaliera, S.Camillo-Forlanini, Circonvallazione Gianicolense 87, Roma 00152, Italy

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INTRODUCTION

Crohn's disease is a chronic inflammatory granulomatous disorder that usually involves different sites along the length of the intestinal tract. Genetic and environmental factors are believed to play a role in its etiology and pathogenesis. As Crohn's disease is very heterogeneous in its clinical expression, working groups at the World Congress of Gastroenterology, held initially in Vienna and later in Montreal, have developed an evolving classification scheme^[1,2]. An important goal has been to enumerate different phenotypic characteristics so that more homogeneous subgroups can be explored. Application to a specialist care clinical database noted a female predominance, occasional familial nature, and a high rate of stricture formation and penetrating disease^[3-5].

The disorder seems to be life-long and may appear at almost any time from early childhood to late adulthood^[1-3]. However, for most, the actual onset of the disease, or more precisely the age at diagnosis, is usually during the late teens and early twenties, and now, during the past two or three decades, over 80% of patients with Crohn's disease are diagnosed before the age of 40 years^[3]. Moreover, the majority have involvement primarily of the ileum and colon, at least based on the most sensitive and modern imaging methods^[1-3]. Finally, most specialists usually see late complicated disease, while early disease without stricture or fistula development may not be seen as often, particularly in specialist or tertiary-care centers.

DISEASE ONSET AND AGE AT DIAGNOSIS

Clearly, an appreciation for the natural history of Crohn's disease must depend, not on the age at diagnosis

defined by these classifications, but the actual time of onset of the disorder. These are obviously very different. Symptoms that eventually lead the patient to specialist referral include diarrhea, abdominal pain and weight loss, but these are not always universally present. Occasionally, only a single symptom, possibly abdominal pain, may be evident. In some, detection of an unexpected ileal inflammatory process at laparotomy for suspected appendicitis may be the first presentation. For others, extra-intestinal findings (e.g. arthropathic process or a skin disorder, such as erythema nodosum) may be present without significant intestinal symptoms. In some, symptoms have often been present for variable periods of time, even months or years, before a diagnosis is established. Also, in these, an event (or possibly an infectious agent) that initiated this destructive inflammatory process may no longer be detectable, having long left the patient. Indeed, multiple events (or agents) can conceivably generate this insidious inflammatory cascade that causes clinical symptoms and the appearance of a common end-pathological change labeled Crohn's disease. Even hypothetical genetic, microbiological and immunological factors that permit ongoing progression of this frustrating clinical disorder still require elucidation.

DISEASE LOCALIZATION, EXTENT AND BEHAVIOR

Most intriguing is the apparent predilection of the disease for the distal small intestine and proximal colon^[3]. More extensive involvement of the small intestine and colon may occur^[6], and the old adage that Crohn's disease can potentially involve any site "from mouth to anus (and probably elsewhere in the case of "metastatic" disease)" still holds true. Moreover, genetic factors may play a role in localization of the disease to different intestinal sites^[7,8]. Further information, however, is clearly required on basic luminal and intestinal factors that play a role in localization of the disorder to specific sites along the gastrointestinal tract. Crohn's disease can occasionally occur in the upper gastrointestinal tract, usually with disease elsewhere in the ileum, colon, or both. The disorder may also occur only in the upper gastrointestinal tract without disease involvement elsewhere, albeit rarely^[2,3]. Also, extensive jejunoileal involvement, evaluated over the long-term, illustrates a special subgroup of Crohn's disease that historically responds poorly to medication, which often leads to surgical treatment and long-term nutritional support^[6]. New and developing biological treatment paradigms largely focused on reducing numerical indices in ileocolonic disease may have little impact on this subgroup, unless the severity and extent of the inflammatory process can be reduced.

Crohn's disease is a chronic, persistent and destructive disorder with distinct forms of clinical behavior that may also, in part, be genetically-based^[9,10]. It also seems progressive, although the rate of progression may be

altered or slowed, by the use of some therapy, such as steroids, antibiotics, or resective surgery, at least for a period of time. It appears that the disease begins as an inflammatory process that progressively develops over time to a more complex disease with stricture and fistula formation^[11-13]. Once initiated, it is likely that numerous genetic and environmental factors play a role in regulating the rate of progression, but these are poorly understood. Moreover, the progression itself may not be linear but occur in a step-wise fashion with prolonged symptom-free periods over many decades^[14].

Crohn's disease may also initially present as an already advanced and clinically complex disease with extensive or multiple jejunoileal strictures^[6], sometimes even with free perforation of the small intestine, or alternatively, with large intra-abdominal inflammatory masses, sometimes with a deeply penetrating fistula (e.g. ileosigmoid fistula). In some, it has been hypothesized that recurrent disease may occur as a patterned clinical response, possibly related to specific genetic regulatory factors. For instance, recurrent stenotic events may result in a localized ileocecal resection, new erosions and ulcers in the neo-terminal ileum, and further stricture formation, recurrent obstructive symptoms and another resection^[15]. Or, recurrent penetrating events with fistula and abscess formation may occur^[15]. Classifying clinical behavior in Crohn's disease is difficult and may not be truly reflective of natural history as the rates of development of a complication, such as a stricture formation, may differ markedly, not only among patients, but even in the same patient during the disease course. Some may have either a rapidly progressive inflammatory process, or alternatively, a low grade sub-clinical process, possibly present for months, that suddenly becomes clinically expressed.

AGE-RELATED PHENOTYPIC EXPRESSION

Early historical studies have suggested that the phenotypic clinical expression of Crohn's disease differs substantially, depending on the age of initial diagnosis^[16-20]. This age-dependent phenotypic clinical expression probably reflects the dynamic nature of the disorder^[21]. Disease developing earlier in children and adolescents tends to be much more severe, often resulting in significant disease complications, including strictures or fistulae, or both^[22-25]. It is also more extensive, often involving multiple sites in the small and large intestine, with a higher frequency of involvement of the upper gastrointestinal tract^[22-25]. Comparative studies also show significant differences in clinical expression for children and adults^[21,22], as well as the elderly^[26]. Others have defined a difference in some immunoreactive characteristics of early-onset compared to late-onset Crohn's disease^[27,28]. The hypothesis that a dysregulated immune response, likely affected by aging *per se* and leading to different phenotypic disease expressions of Crohn's disease, needs to be further elucidated.

CLINICOPATHOLOGICAL CORRELATIONS

Clinical and pathological correlations have been explored during the long-term clinical course of Crohn's disease. Some early historical descriptions have tended to avoid the possible temporal sequence of progression of pathological lesions and have focused on their prognostic significance^[29,30]. However, more recent studies of their chronological sequence have suggested early small ulcers or granulomas with ongoing progression to later sinuses and strictures complicating large ulcers^[31]. Later studies have also confirmed that this granulomatous inflammation may be a histopathological marker for an early phase of the inflammatory process in Crohn's disease, at least prior to development of fibrotic strictures and fistulous tracts^[31]. Granulomatous inflammatory change has been documented in multiple biopsy or surgically resected specimens obtained over many decades^[14]. Often, long intervals of relatively symptom-free disease are also evident^[14]. This temporal pattern may indicate multiple initiating events in Crohn's disease with different rates of progression, or alternatively, granulomas may reflect ongoing active inflammation, even as subclinical or asymptomatic disease.

These studies have also suggested a geographic change in Crohn's disease with extended periods of time^[32]. For instance, granulomatous ileal involvement was defined many years after initial colonic disease, and gastroduodenal disease occurred after ileocolic resection. If there were a single event that initiated this granulomatous inflammatory process, then different tissues along the length of the gastrointestinal tract may develop a granulomatous response at different rates, or alternatively, there might be multiple or recurring initiating events. Different gastrointestinal sites may differ in sensitivity to a possible initiating event (or infectious agent). This could result from a site-specific differential in intestinal permeability or differing immunological responses along the length of the gastrointestinal tract^[33,34].

HETEROGENEOUS DISEASE

Crohn's disease remains an intriguing disorder characterized by a granulomatous inflammatory process. The phenotypic clinical expression of Crohn's disease is clearly age-onset-dependent, as most children and adolescents suffer more severe, extensive and complicated disease than most adults and the elderly. If evaluated over a long period of time, the disease appears to be progressive, but only intermittently active, and at a rate that may be very heterogeneous, with some appearing to have prolonged periods of sub-clinical disease and others expressing complex disease with stricture formation and penetrating complications, even at the time of initial clinical presentation. Although the precise cause of Crohn's disease remains a mystery, an increasing appreciation for the long-term natural history of Crohn's disease may permit development of more effective treatment regimens.

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Sonography of the small intestine

Kim Nylund, Svein Ødegaard, Trygve Hausken, Geir Folvik, Gülen Arslan Lied, Ivan Viola, Helwig Hauser, Odd-Helge Gilja

Kim Nylund, Trygve Hausken, Svein Ødegaard, Odd-Helge Gilja, National Centre for Ultrasound in Gastroenterology, Department of Medicine, Haukeland University Hospital, 5021 Bergen, Norway

Geir Folvik, Gülen Arslan Lied, Department of Medicine, Haukeland University Hospital, 5021 Bergen, Norway

Ivan Viola, Helwig Hauser, Institute of Informatics, University of Bergen, 5008 Bergen, Norway

Kim Nylund, Trygve Hausken, Svein Ødegaard, Odd-Helge Gilja, Institute of Medicine, University of Bergen, 5021 Bergen, Norway

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Correspondence to: Kim Nylund, Institute of Medicine, University of Bergen, Haukeland University Hospital, Jonas Lies vei 65, 5021 Bergen, Norway. kim.nylund@med.uib.no
Telephone: +47-55-973075 Fax: +47-55-972950

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have enabled more anatomical and physiological changes in the small intestine to be observed. Accordingly, ultrasound of the small intestine is an attractive clinical tool to study patients with a range of diseases.

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Peer reviewer: Dr. Andreas G Schreyer, Department of Radiology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

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Abstract

In the last two decades, there has been substantial development in the diagnostic possibilities for examining the small intestine. Compared with computerized tomography, magnetic resonance imaging, capsule endoscopy and double-balloon endoscopy, ultrasonography has the advantage of being cheap, portable, flexible and user- and patient-friendly, while at the same time providing the clinician with image data of high temporal and spatial resolution. The method has limitations with penetration in obesity and with intestinal air impairing image quality. The flexibility ultrasonography offers the examiner also implies that a systematic approach during scanning is needed. This paper reviews the basic scanning techniques and new modalities such as contrast-enhanced ultrasound, elastography, strain rate imaging, hydrosonography, allergosonography, endoscopic sonography and nutritional imaging, and the literature on disease-specific findings in the small intestine. Some of these methods have shown clinical benefit, while others are under research and development to establish their role in the diagnostic repertoire. However, along with improved overall image quality of new ultrasound scanners, these methods

INTRODUCTION

The small intestine is the most difficult part to examine of the gastrointestinal (GI) tract because of its length and tortuous course. The traditional investigations with small bowel enteroclysis and small bowel follow-through reveal information sparingly, and unfortunately involve radiation exposure of the patient. Although it is an organ that is spared from frequent disease, more precise and patient-friendly methods are needed. In the last three decades, new imaging techniques have been developed that have proven useful. Computerized tomography (CT), magnetic resonance imaging (MRI), wireless capsule endoscopy and double-balloon endoscopy are all relatively new additions to the diagnostic armamentarium.

Compared with these methods, transabdominal bowel sonography (TABS), has the advantage of being cheap, portable, flexible and user- and patient-friendly. There are challenges with depth penetration and intestinal air precluding optimal image quality, and the flexibility of ultrasonography (US) warrants a systematic approach by the examiner. However, the development of improved

scanner technology and high-resolution transducers has provided the clinician with image data of high temporal and spatial resolution, thus making it a useful tool in the diagnosis of small intestinal diseases^[1-3]. This paper presents an overview of established US techniques for examining the small bowel and a description of new, promising scanning techniques.

When using US frequencies in the range of 7.5 to 14 MHz, the wall of the small intestine usually exhibits five different layers that correspond well to the histological layers. When looking at the mesenteric side of the intestine transabdominally, the first layer that is observed is the serosa/subserosa. It can be difficult to define exactly, as it is an interface echo between the surrounding structures (peritoneal wall/intestine/fat) and the serosa. The interface echo is thicker than the actual serosa and extends into the muscularis propria. Thus, the first layer that is clearly defined is echo-poor and corresponds to the muscularis propria. Next, is an echo-rich layer that corresponds to the submucosa, and an interface echo between the submucosa and the mucosa. Subsequently, there is an echo-poor layer followed by an echo-rich layer that corresponds to the mucosa and the interface echo between the mucosa and the luminal content. The wall layers as seen from the luminal side are described in the section on endoscopic sonography. The GI wall has a normal stratification if five US layers are visible, and there is loss of stratification if one or more US layers are missing^[4,5].

BASIC SCANNING TECHNIQUES

Preparations

The patient should be examined in the supine position and usually the examination requires no special preparation. Fasting for > 6 h, however, leads to smaller amounts of fluid and air in the intestine and reduces motility. The intake of fluids orally or through a feeding tube reduces the air content in the intestine and makes it easier to separate the lumen from the wall and different bowel loops from each other. Furthermore, the mesenteric wall of the intestine, which is often hidden behind pockets of air, can be more easily examined with these preparations^[6]. Another technique for dealing with the problem of air is graded compression, for which the examiner uses the US transducer to squeeze the air away from the region of interest^[7].

High frequency B-mode

It is recommended that the examiner starts performing a regular scan of the abdomen with a 3.5-5 MHz transducer. This might give additional information, a better overview, and larger lesions in the intestine can be imaged completely. For a detailed transabdominal examination of the small intestine, a curved or linear transducer with frequencies in the range 7.5-14 MHz should be used. If pathology is detected, wall thickness, stratification, luminal patency, degree of stenosis or dilatation, and motility pattern should be determined.

Doppler modalities

Duplex scanning of the blood velocity in the superior mesenteric artery (SMA) provides several quantifiable parameters. The peak systolic (PV) and end diastolic velocity (EDV) can be used to calculate the resistive index (RI) = (PV-EDV)/PV, and the estimated mean velocity together with the inner diameter of the SMA is used to calculate the mean blood flow (MBF). The SMA is examined in the long axis. The best place to position the sample area is 2 cm after the SMA branches off from the aorta, and as it runs parallel to the aorta. The examiner should tilt the probe towards the epigastrium to obtain an angle < 60°. Intra- and interobserver agreements are good when the examiners are well trained^[8]. Color and power Doppler gives information about the blood flow in smaller vessels that are difficult to find with duplex scanning. The information obtained from these images is qualitative. Triplex scanning can however be used for the estimation of RI in the GI wall.

DISEASE-SPECIFIC FINDINGS

Crohn's disease (CD)

CD is the most common reason for performing TABS of the small intestine. The typical US findings can be divided into bowel wall changes, focal reactions and complications of the disease. The wall changes are discontinuous and consist of wall thickening, ulcerations, infiltrations, flow alterations, and changes in stratification^[9-11]. Around the bowel loops, enlarged lymph nodes and fatty infiltration of the mesentery can be found^[12,13]. Several studies have also shown that complications such as fistulas, abscesses and stenosis with signs of obstruction can be found using TABS^[14,15].

The main finding in CD is a marked thickening of the intestinal wall of up to 15 mm. Fraquelli *et al*^[16] have shown in a meta-analysis that, with a cut off of 3-mm, there was 88% sensitivity and 93% specificity for determining CD, while with a cut off of 4 mm, it was 75% and 97% respectively. The conclusion from this analysis should be that when used in primary diagnostics, 4 mm is a sensible lower limit, while in follow-up, a wall thickness > 3 mm should be considered as a sign of disease. Increased wall thickness is also associated with increased risk for surgery and for recurrence of disease after surgery^[17,18]. Wall thickening is, however, not specific for CD and the final diagnosis should rest on endoscopic and histological findings if possible. In a thickened intestinal segment, loss of stratification is associated with inflammation, while retained stratification suggests fibrosis^[11,19,20].

CD is associated with hypervascularity of the bowel wall during active disease. This has been the rationale for performing Doppler studies of regional and local blood flow. Studies with duplex scanning have shown a correlation between disease activity, increased PV, MBF and decreased RI in the SMA^[21-23]. Other studies looking at the difference in MBF, pulsatility index and RI before and after a meal have also found a significant

association^[24,25]. Most of these studies have compared groups of less than 15 patients and have not been confirmed in larger studies. The measurements in the SMA are also influenced by age, presence of atherosclerosis, and the length of the intestinal segment that is affected^[26,27]. The most promising parameters, such as MBF, are difficult to assess and have found little use in clinical practice. Other studies have compared disease activity with semi-quantitative and quantitative estimates of vessel density in the GI wall with color and power Doppler. An association has been found in some studies^[28,29], but the results have been inconclusive in others^[30,31]. The reason for these conflicting findings might be that the clinical assessment of disease activity with the CD activity index (CDAI) does not reflect vascularity. This theory might be supported by studies using US contrast in combination with power Doppler or harmonic imaging. Several studies have found the expected increase of contrast enhancement in patients with an elevated CDAI, but a subgroup of patients with contrast enhancement with low CDAI^[32-34]. There is strong reason to believe that there is a correlation between vascularity and contrast intensity^[35]. The hypervascularity in the GI wall of these patients may be a presentation of subclinical disease and it should be followed in prospective studies. There have been some recent studies with Doppler imaging that have investigated risk of relapse and treatment effects, which have indicated that detection of increased flow to and in the small intestine gives prognostic information^[25,36].

Celiac disease

The associated findings of TABS are fluid-filled distended intestinal loops and continuous peristalsis in the small bowel segments during fasting^[3,37-39]. Furthermore, a reduction in the number of mucosal folds in the jejunum and an increase in the ileum is another typical finding^[37,39]. Some authors have also found intermittent invaginations^[37,38]. Less specific findings are free fluid, enlarged mesenteric lymph nodes, increased gall bladder size and a slight wall thickening. The studies performed have been mostly of an explorative nature, but in a prospective cohort study in patients with chronic diarrhea, iron deficiency or dyspepsia, dilated fluid-filled bowel loops were the most sensitive finding with a sensitivity of 92% and a specificity of 77%. In combination with increased gall bladder size, thickened small bowel wall (> 3 mm), increased fasting peristalsis, enlarged mesenteric lymph nodes and free abdominal fluid, the sensitivity was reduced to 33%, but specificity rose to 99%^[3]. In untreated patients with celiac disease, increased peak velocity, mean velocity and MBF was found, while the RI was decreased as expected^[40-42]. These findings disappeared with therapy^[42] but reappeared after exposure to gluten^[41].

Intussusception

In children with intussusception or intestinal invagination, the typical finding on TABS is a multilayered lesion with

concentric circles (onion sign) in the right fossa, when seen in the transversal plane. When the mesentery is involved, this forms an echo-rich crescent open towards the ante-mesenteric side. This is called the crescent in the donut sign. When seen longitudinally, the mesentery is seen as an echo-rich layer between two multilayered structures, the sandwich sign. Using these criteria, a sensitivity of nearly 100% and a specificity around 90% have been found in prospective studies^[1,43]. TABS can also be used as guide in treatment procedures with hydrostatic reduction^[44]. Color Doppler examination of children with intussusception shows that absence of flow in the wall of the invaginated intestine makes reduction more difficult, but does not necessarily mean the intestine is necrotic^[45].

In adults, TABS has proven useful as a primary diagnostic method, but since intestinal invagination is a rare cause of bowel obstruction in adults, it is often found by other means. The TABS appearance is similar to that in children, but there is often other pathology present that is the pathological lead point of the invagination^[46,47]. As a result of the low number of incidents, there have been no prospective studies in the literature. Transient intestinal invaginations occur in children and adults, and mostly without symptoms. If there are no pathological lead points, the discovery is incidental, and if the intestinal segment is shorter than 3.5 cm, they are considered harmless^[48,49].

Malignant tumors

Malignant tumors in the small intestine represent only 2% of GI cancers, and it is mainly the malignant tumors that are diagnosed as result of symptoms. The most frequent is adenocarcinoma (30%-50%), followed by carcinoids (25%-30%) and lymphoma (15%-20%)^[50]. Less frequent are mesenchymal tumors of the GI tract, such as malignant GI stromal tumors and leiomyosarcomas^[51]. The distribution in the small intestine is mainly duodenal adenocarcinoma, mesenchymal tumor in the jejunum, and lymphoma and carcinoids in the ileum^[52]. As a result of the low number of patients, there have been few studies confirming the transabdominal echo characteristics of small bowel tumors. However, explorative studies and case series suggest some echo characteristics. Lymphoma is associated with target lesions caused by gross wall thickening with loss of stratification and sometimes aneurysmal dilatation of the intestine in the affected area^[53,54]. Carcinoids have been shown to be intraluminal, oval and smoothly shaped. Furthermore they are echo-poor, and homogeneous, with an interruption of submucosa and a thickening of the adjacent muscularis propria^[55]. Mesenchymal tumors appear as echo-poor, exophytic or dumbbell-shaped, with little contact with the bowel wall. If the bowel wall is involved, the submucosa is intact over the tumor and the muscularis propria adjacent to the tumor is unaffected. A typical feature might also be a peripheral, echo-rich or anechoic crescent-shaped area that represents necrosis^[56].

Ischemic disease

In chronic ischemia of the small bowel, stenotic or occlusive lesions in the celiac and/or mesenteric arteries are found, and the patients typically have postprandial epigastric pain and weight loss. It is considered evidence of significant stenosis if duplex scanning of the celiac artery gives PV > 200 cm/s and EVD > 55 cm/s, and if the SMA PV is > 275-300 cm/s and EDV > 45 cm/s. If the patients have typical symptoms, it is indicative of ischemia, but still angiography must be performed for a definite diagnosis^[57-59]. Duplex scanning is not the method of choice for diagnosing acute ischemia of the small bowel. If the ischemia has lasted a few hours, dilated bowel loops and a thickened bowel wall can be seen, but these signs are unspecific, and the examination is often made difficult by increasing amounts of intraluminal air. An arterial occlusion can be found, but the method is unlikely to show distal embolization and has not been studied with non-occlusive disease^[60].

CONTRAST ENHANCED ULTRASOUND (CEUS)

Ultrasound contrast agents consist of micro-bubbles (1-7 μm), often made of a phospholipid shell with a gaseous content. They are given intravenously and excreted through the lungs. The use of CEUS has led to several new clinical applications^[61]. As a result of their size, they stay in the circulation and because of their shape and gaseous content, they produce a non-linear, harmonic response that can be separated from the tissue signal using contrast harmonic US. When examining the intestine, it is preferable to use frequencies above 7.5 MHz, so that the different wall layers can be separated. Since there are fewer micro-bubbles that resonate at these high frequencies, higher contrast doses should be used. When a bolus of contrast is injected it will travel from the peripheral vein *via* the pulmonary bed and arteries before reaching capillaries in the intestinal wall. This lasts about 10-15 s and the moment contrast can be observed in the capillaries is the time of arrival. The concentration will continue to increase further as the contrast enters the capillaries and reaches its maximum concentration after approximately 30 s (Figure 1). This is the peak intensity, and the period leading up to this is called the arterial phase. In the intestine, it is followed by the venous phase in which the contrast has been distributed to the whole capillary bed and the concentration decreases as result of excretion of the contrast agent in the lungs^[62].

The simplest way of applying US contrast is to examine the intestinal wall 30 s after injection with power Doppler. This will enhance the Doppler signal, but also destroy all the micro-bubbles in the region of interest, which gives only a momentary flash of enhancement. Using contrast harmonic US with a low mechanical index (MI), the destruction of micro-bubbles is minimal and the arterial and venous phases can be observed continuously. The temporal dimension

to this examination gives quantifiable parameters that are independent of attenuation.

For quantification, there are currently two different methods (Figure 2). First, bolus tracking is a method in which the examiner simply injects a standard dose of contrast for a standardized injection time and registers the arterial and late phases. From the time intensity curve (TIC), time of arrival, peak intensity, time to peak and area under the curve can all be estimated. The problem with this method is that one needs to correct for attenuation, and the parameters are dependent on the injection speed and dose. Second, the burst replenishment method circumvents the problem of injection speed by giving a constant infusion of contrast agent. To observe how the capillary bed fills up in a region of interest, the micro-bubbles are simply destroyed by a burst of high-MI US, and then the replenishment is observed. Time to peak replenishment and the slope of increase are both quantifiable parameters that can be derived from a TIC. The burst replenishment method can however only provide indirect measurements of perfusion, while bolus-tracking potentially can be used for estimation of true perfusion when corrected for the arterial input function and attenuation.

STRAIN IMAGING AND ELASTOGRAPHY

Occasionally an intestinal tumor or stenotic intestinal section in patients with CD can be palpated trans-abdominally. Palpation is informative since different tissues have different elastic properties. Elastography or strain imaging are US methods using this information by detecting the elasticity or stiffness of a tissue and providing a visual display^[63-65]. The basis of every strain imaging technique is to measure tissue deformation caused by an external stimulus. The derivative of the tissue displacement is called strain, and can be calculated by cross-correlating the radio-frequency data before and after compression. The strain value in each point is color-coded and displayed in an elastogram. This elastogram can then be combined with the B-mode image to display the elastic properties of the tissue to the examiner through color information. Equipment using a quasi-static method of producing strain in the tissue through external compression with the US probe is now commercially available for clinical application^[66].

The quasi-static application of elastography of the small intestine represents special difficulties, as the pressure from the US transducer can cause several changes. When adding pressure, one not only deforms the GI wall, but also displaces the luminal content and the intestine itself. The intestinal content can be displaced using a preload, and in some areas, the small intestine can be fixed against the abdominal wall. The terminal ileum can be compressed against the psoas muscle in the right lower quadrant and this may prove useful in CD patients, who often have stenosis in this area^[67]. To the best of our knowledge, there have been no human studies using the quasi-static method or other

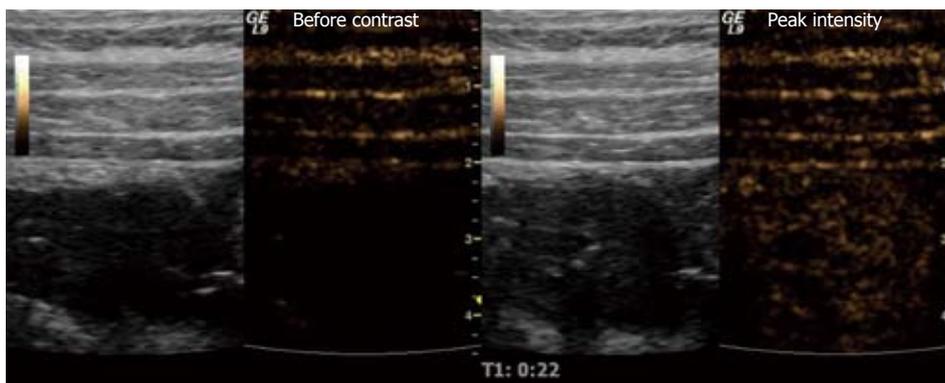


Figure 1 Stenosis of the terminal ileum in a patient with CD examined with ultrasound contrast. The grey-toned images are the B-mode scouts while the brown-toned images portray the harmonic signals that are almost exclusively from micro-bubbles at a low MI. The right image shows the situation before contrast injection, while the left picture is taken at peak intensity.

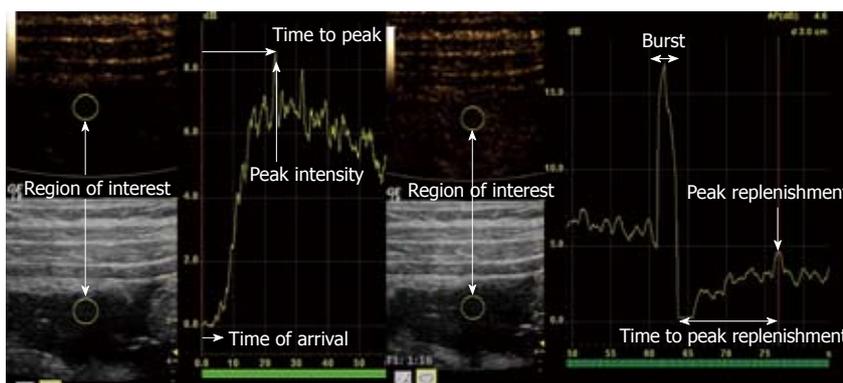


Figure 2 Stenosis of the terminal ileum in a patient with CD. The right image shows a TIC using bolus tracking. The left image shows burst replenishment without constant infusion in the same patient. When the micro-bubbles are hit by ultrasound with a high MI, this causes them to burst, which produces high-intensity images (Burst). Quantifiable parameters such as time of arrival, peak intensity, time to peak, peak replenishment and time to peak replenishment are shown in the illustration.

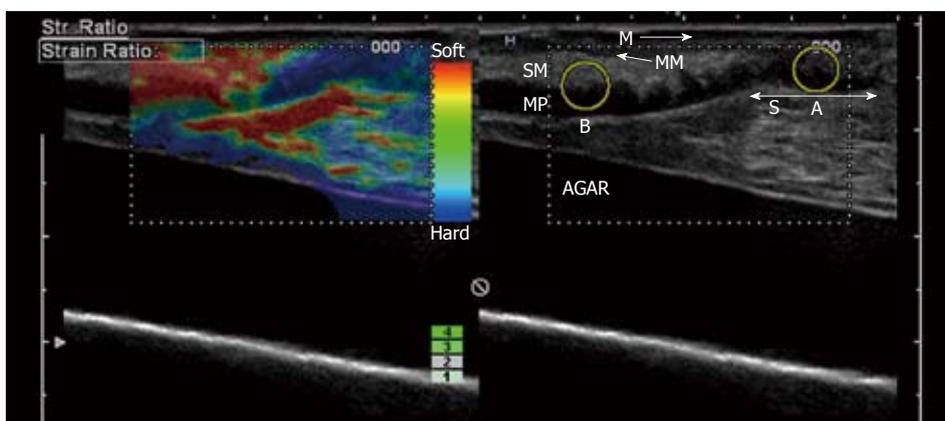


Figure 3 *In vitro* examination of a stenotic terminal ileum in a patient with CD. The intestine has been cut open longitudinally and is resting on agar. The image was acquired with a 13-MHz linear probe (Hitachi Medical Systems Europe Holding AG, Zug, Switzerland) applied directly to the mucosa. The left image is an ordinary B-mode image showing the thickened GI wall (> 1 cm *in vitro*) with the muscularis propria (MP), submucosa (SM), mucosa (M) and a thickened muscularis mucosa (MM). Towards the stenosis (S), the stratification is lost. In the right corresponding elastography image, there is a clear transition between the blue (hard) stenotic area and the red (soft) pre-stenotic area. The strain measured in region A and B (yellow circles) differ with a ratio of 3.77 (A: 0.27%; B: 1.02%; B/A: 3.77), which indicates a distinct difference in elasticity. Histology confirmed a fibrotic stenosis (Bar indicates 0.5 cm/unit).

elastography techniques transabdominally on the small intestine, but it has been applied with some success in the endosonographic detection of malignant lymph nodes^[68,69].

CD with stenotic obstruction is a potential application for elastography. We present two cases with stenosis of the terminal ileum. In one patient, the resection specimen was examined *ex vivo* (Figure 3), and in the other, the stenosis was examined transabdominally with elastography before surgery (Figure 4). In both patients, elastography indicated harder tissue in the stenotic area compared to the surrounding tissue. The

pathologist found a fibrotic stenosis in both surgical specimens.

HYDROSONOGRAPHY

High-resolution transabdominal ultrasound for imaging the small intestine is often hampered by intestinal gas. The lumen of the small bowel is collapsed frequently and significant intraluminal pathology can be missed. Moreover, separation of different bowel loops can be difficult. Hydrosonography of the small intestine is a method in which an echo-poor contrast liquid is used

for distending the bowel and removing intestinal air, and thereby improving imaging. Most often, an isotonic polyethylene glycol (PEG) solution is used (e.g. Laxabon®). The PEG solution is indigestible and non-absorbable, therefore, it gives a predictable amount of luminal fluid and intestinal distension. In principle, any echo-poor fluid can be used. The amount of PEG solution used in different studies varies between 200 and 2000 mL. Oral intake of PEG solution (small intestine contrast ultrasonography) is the least invasive way of giving the contrast liquid, and probably the preferred method of most patients^[70]. However, in patients having trouble drinking large amounts of PEG solution, a nasojejunal feeding tube can be used. The tube is placed at the duodenojejunal flexure by endoscopy, fluoroscopy or ultrasound guidance. These procedures are more time consuming than a regular transabdominal ultrasound examination, and the average examination may vary between 30 and 40 min (range 10-90 min). Examination time is very much dependent on the passage of contrast fluid to the terminal ileum. Sensitivity for diagnosing a suspected disorder in the small intestine in patients referred for a barium study varies as much as 64%-100%, while generally, a near 100% specificity is found^[6,71-73]. In patients with known CD, the sensitivity for detecting a lesion in the small intestine varies between 96% and 100%^[73-75]. Hydrosoneography of the small intestine is safe. As opposed to enteroclysis and CT, the method can be used for examining the small intestine in patients in whom radiation should be avoided. Particularly, it is useful in the follow-up of patients with inflammatory bowel disease where repeated examinations often are necessary.

ALLERGOSONOGRAPHY

Food hypersensitivity reactions, including GI reactions due to allergy against food items, can be visualized by advanced imaging and visualization modalities such as endosonography^[76], TABS and MRI. In Western countries, the rate of perceived food hypersensitivity is around 25% in the general population^[77]. Conversely, a diagnosis of true food allergy in adults is confirmed in only 1%-4% of the cases. The difficulty is to diagnose the reactions, which occur by non-allergic mechanisms, or local reactions in the GI tract. They usually have negative allergic test results and when they reach the doctor, the symptoms are already gone. Therefore, a provocation test is often necessary to determine the reactions and symptoms.

Objective tests for diagnosing of food allergy are missing. In previous studies, double-blind placebo-controlled food challenge (DBPCFC) has been considered the gold standard for many years, but the latest literature has criticized the method because the procedure is labor-intensive and time-consuming, and the assessment is based on subjective symptoms such as abdominal discomfort and bloating^[78]. In practice, therefore, the diagnosis is based on history and skin prick tests, and DBPCFC is seldom performed.

We have applied US to establish whether food hypersensitivity reactions can be objectively visualized. Not only mucosal swelling, but also luminal fluid can be visualized by TABS. Therefore, we wanted to explore the feasibility of using US to monitor the response of the intestine to direct luminal provocation in 32 patients with food hypersensitivity^[79]. A nasoduodenal feeding tube was placed with its tip in the duodenum, aided by a gastroscope. The intestinal mucosa was challenged with the suspected food item dissolved in 10 mL water or saline given through the nasoduodenal tube over a 3-min period. Sonography was performed before (-5 min) and 5, 20, 30 and 60 min after the provocation. The following sonographic features were explored: wall thickness and diameter of the duodenal bulb, wall thickness and diameter of the pars descendens of the duodenum, wall thickness and diameter of the proximal jejunum, number of fluid-filled jejunal loops, air in the small intestine, and peristalsis of the small bowel. Sonographic changes were observed after challenge in 14 (44%) of the 32 patients. A positive sonographic response (increased wall thickness, diameter, peristalsis and/or luminal fluid) was significantly related to positive skin prick test ($P = 0.008$) and positive DBPCFC ($P = 0.03$). A significant correlation was found between provocation-induced symptoms and wall thickness of the duodenal bulb ($r = 0.50$, $P = 0.004$) or jejunum ($r = 0.42$, $P = 0.02$). Intra- and interobserver variation of the tracing procedure revealed low values.

Sonography appeared to be the most sensitive test applied, but the specificity of the new test is not yet known. Based on our findings, we conclude that responses of the proximal small intestines to direct provocation (swelling of the wall and exudation of fluid into the lumen) can be visualized by TABS, and that this new provocation test may become helpful in the evaluation of patients with food hypersensitivity.

ENDOSCOPIC SONOGRAPHY

Intraluminal US (endosonography) is the common denomination of US examination using intracorporal transducers that are inserted under endoscopic guidance or blindly into the GI tract. Thus, the visceral wall and adjacent structures can be imaged in detail. Endoscopic US (EUS) has had an impact on the clinical management of several GI disorders and has become a valuable tool for investigating mucosal and deeper lesions of the GI wall, as well as biomechanical and motility abnormalities^[80,81].

The small intestine may be examined by relatively new endoscopic techniques such as wireless capsule endoscopy or double-balloon enteroscopy (DBE). DBE also allows therapeutic procedures to be performed and biopsies to be obtained. EUS of the duodenum and terminal ileum has been performed previously, but other parts of the small intestine have been regarded less accessible. However, when transducers are placed in the stomach or colon, it is possible to image loops and limited areas of the small intestine^[82].

The normal wall of the small intestine is thin and

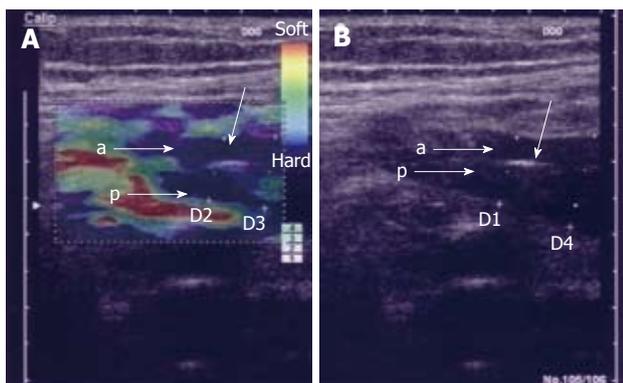


Figure 4 Elastography image acquired transabdominally in a patient with CD and stenosis of the terminal ileum. In the left image, the bowel wall is thickened without stratification and the anterior (A) and posterior (B) bowel wall is separated by luminal air (unmarked arrow). In the right elastography image, the stenotic area is colored blue relative to the surrounding tissue, which indicates that it is hard. The patient was operated upon and the histology confirmed a fibrotic stenosis. D1: 9.5 mm; D2: 8.8 mm; D3: 10.0 mm; D4: 13.0 mm.



Figure 7 Scan of the transpyloric area with velocity curve of gastric emptying episode (left) and duodenogastric reflux episode (right). Calibration bars indicate cm/s and 0.2 s on the y-axis and x-axis, respectively.

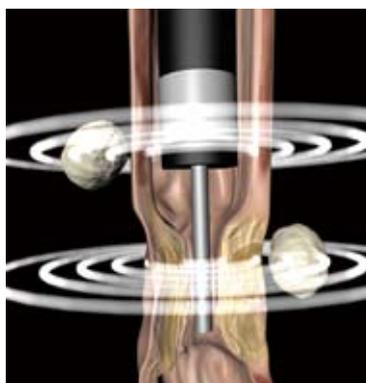


Figure 5 Illustration of EUS performed with an echoendoscope and an ultrasound miniprobe simultaneously. The echoendoscope can not pass a stenotic area which, however, can be further examined with a miniprobe. Lymph nodes are seen outside the GI wall.

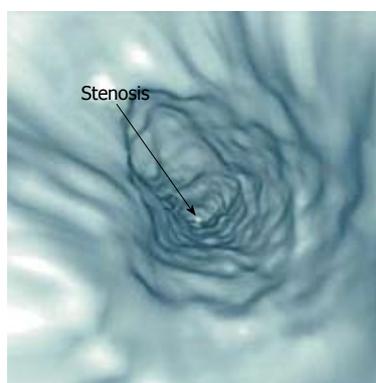


Figure 8 VE of the ileum demonstrating stenosis of the intestinal lumen (arrow) (Rogalla *et al* [90]).

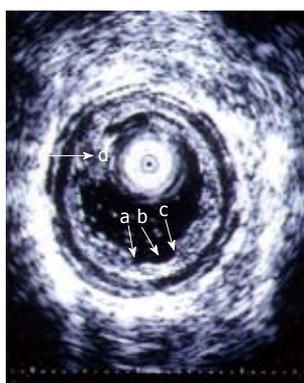


Figure 6 An ultrasound miniprobe (20 MHz) is placed in the ascending part of the partly water-filled jejunal lumen in a patient with CD. The layers of the wall are seen and also an erosion/superficial ulcer (b) can be observed. The edge of the lesion is indicated (a, c). A mucosal polypoid elevation is also seen (d).

folded and is displayed by high-frequency US as a layered structure usually consisting of a 5-layer structure but sometimes 3-9 layers are seen. When the visceral wall is imaged with an endoluminal transducer, the interface between the luminal content and mucosa accounts for the first, echo-rich layer. The rest of the mucosa appears as a second, echo-poor layer. The third, echo-rich layer usually includes the interface between the lamina propria and muscularis mucosae, the submucosa, and the interface at the border of the muscularis propria. In a five-layer structure, the rest of muscularis propria is imaged as an echo-poor fourth layer. The fifth, echo-

rich layer represents the outer wall delineation and is an interface echo at the serosal level^[4,83].

New and longer miniprobes that can be inserted through the working channel of enteroscopes are now available. These miniprobes may be helpful in the examination of small bowel lesions (e.g. CD, lymphoma, ischemic disease, subepithelial masses, tumors, strictures, stenoses and bleeding spots) when used in combination with DBE. Forward-viewing optic echoendoscopes can be combined with transendoscopic miniprobes, which allow endosonographic imaging with both instruments in the same procedure. Thus, imaging of stenotic areas not traversable for echoendoscopes, or other areas not accessible with echoendoscopes (e.g. parts of the jejunum) can be performed (Figure 5). Small bowel ulcers may exhibit a typical EUS pattern that consists of three separate elements: the ulcer crater, the echo-rich ulcer base, and frequently an echo-poor inflammation zone, which can extend beyond the thickness of the wall and more widely than the endoscopic changes (Figure 6). Enlarged vessels running into the ulcerated area can sometimes be demonstrated by EUS and may indicate an increased bleeding risk.

EUS has the potential to image biomechanical changes in all or individual GI wall layers as well as in the surrounding tissue. This information may improve our understanding of pathophysiological mechanisms of gut pathology. Several studies have been performed that combine miniprobe US with manometry to

describe the functional role of muscle layers of the GI wall. Recently, a multimodal device that combines bag distension, manometry, high frequency intraluminal US, laser Doppler flowmetry and symptom registration was applied to the esophagus, and demonstrated that these modalities can be used in combination without technical influence on each other^[84].

NUTRITIONAL IMAGING

Nutritional imaging is US imaging to study the effects of nutrition such as fat, proteins and glucose on the function of the GI tract. Information concerning movement of luminal contents in humans can be obtained by fluoroscopy, scintigraphy, MRI, and impedance and duplex sonography. Studies based on scintigraphy and standard US of the stomach and duodenum indirectly measure overall rates of gastric emptying, but these methods do not have the temporal resolution to assess the rapid changes of transpyloric flow.

Hausken *et al*^[85] have shown that using pulsed Doppler combined with real-time US (duplex sonography), it is possible to visualize antro-duodenal motility and transpyloric flow simultaneously. Antegrade and retrograde transpyloric flow is visualized using bidirectional velocity curves. Most contractions of the proximal duodenal bulb precede closure of the pylorus (and the terminal antrum), and duodenal bulb contraction is often accompanied by a short burst of duodenogastric reflux that occurs immediately before closure of the pylorus (Figure 7).

Patients with functional dyspepsia often experience early satiety and discomfort after a meal. Using duplex sonography, it is possible to relate timing of symptoms and early postprandial emptying in patients with functional dyspepsia^[86]. Meal-related discomfort is experienced after commencement of transpyloric emptying. An inverse relationship has been found between the duration of the tasting period and symptom intensity, which suggests that the time allowed for duodenal tasting might be too short in patients with functional dyspepsia.

The effects of intraduodenal glucose, fat and protein on blood pressure, heart rate and SMA blood flow have been determined in healthy older subjects. Intraduodenal glucose, fat and protein decrease systolic BP in healthy older subjects, but the onset of the hypotensive response is earlier after glucose, and the effect of protein on SMA blood flow is less than that of the other nutrients^[87].

ADVANCED COMPUTATIONAL VISUALIZATION

When a 3D volume data set of the small intestine is acquired using 3D US, CT or MRI, a diagnostic procedure known as virtual endoscopy (VE)^[88] can be utilized. VE has been considered to be of interest for several years, mostly because of its potential to (at least

partially) substitute standard colonoscopic examinations (and the associated risks). Endoscopic views in the US examinations are nowadays incorporated into some medical workstations^[89].

While the colon is especially suitable for VE examinations since it is rather easy to inflate with air, and has a large diameter and a predictable position, the small intestine offer more of a challenge. Previous studies with liquid contrast and special procedures to minimize the presence of air have suggested that VE of the small intestine can be carried out^[90,91]. Contrasting the interior of the small intestine enables segmentation and the diagnosis of certain pathologies using VE. Figure 8 shows an example of a diagnosis of intestinal stenosis using VE of the small intestine.

VE generally aims at examining all relevant parts of an inspected tubular structure, such as the colon or small intestine, or areas outside the GI tract, such as the bronchi or, in case of high precision scans, even thin vascular structures. The viewpoint navigation of a complex tubular shape can introduce an unnecessary burden on the examiner's side. Therefore, an advanced VE approach should compute and suggest a camera path along the tube's center line to the examiner. The examiner can then easily navigate throughout the entire structure, and if a more fine-tuned viewpoint is needed, detach from the pre-computed path and navigate in freehand mode. In most cases, the computation of the path is based on a skeletonization of segmented tubular structures^[92]. The skeleton is a topological structure that is equidistant to the borders of given object. In medical imaging and visualization, it can be computed for particular organs using morphological operators or the distance transform.

Selective visualization techniques

VE does not display the inspected tissue in its original color, and this information is acquired by optical endoscopy only. The advantage of VE, however, is the ability to see structures that are in close vicinity to the tube walls from outside. A tumor, for example, which develops on the outside wall of the examined structure, can be investigated in this way. Using semi-transparency for rendering the inspected organ walls can help with revealing external, potentially pathological structures. This can be achieved, for example, with a two-step first-hit ray-casting approach^[93] or possibly with opacity peeling^[94] and volume rendering. First-hit ray-casting usually stops ray traversal when a selected density value is reached and displays this boundary as an iso-surface. The two-step extension means that there are two locations where the viewing ray intersects the iso-surface. The inner one is represented with a semi-transparent surface, whereas the second, outer surface is fully opaque. In the case of opacity peeling, the ray traversal accumulates the optical representatives of the density values in the same way as the common direct volume rendering, i.e. using the over operator. The difference is that when the accumulation during the ray traversal reaches nearly

full opacity, the accumulation is reset to zero and ray traversal continues until the accumulation reaches full opacity again. This means that the second level of accumulation is shown. When selecting an appropriate transfer function, the tube wall can be peeled away to see structures behind the wall upon the examiner's request.

VE is essentially a computerized form of the optical screening approach that shares the same basic principles of pursuing a walk-through along the tubular structure. Alternatively, there are other new approaches that aim to present the whole structure in a single image. In some sense, this is analogous to representing the earth on a sphere and by a conformal map projection of the world into a 2D plane. One of these techniques, usually referred to as colon unfolding^[95], simulates a cut and a consecutive virtual opening of the tube, eventually flattening it into two dimensions. This technique requires high reconstruction quality during unfolding and good design for the cut (to not dissect a polyp structure, for example). The advantage of easing the complete visual inspection comes with a certain risk of deforming structures of interest, e.g. polyps, which may lead to errors in the interpretation. This issue can be addressed by another technique, originally developed for vascular investigation and recently applied for virtual colonoscopy^[96], known as curved planar reformation (CPR). The principle of CPR^[97] is that a curved plane that includes the center line of the tubular structure cuts the tube in two halves. Then, both halves can be inspected separately or the plane can be rotated to redefine the cutting geometry. The center line that defines the plane can be estimated from the organ skeletonization^[92], for example. For particularly curved tubular structures, as in the case of the small intestine, the plane can be stretched or completely straightened to give a clear view on inside of the tube.

An alternative to the CPR technique is view-dependent section-view rendering such as the VesselGlyph focus + context technique^[98]. To view the internal part of the walls, the section is cut starting at the center line, with opening towards the viewer. All these representations, for which all structures are visible on a single image, or the outside-in view is incorporated, can be effectively combined with endoscopy for easy navigation and better and faster diagnosis.

CONCLUSION

In this review, we have presented different scanning techniques and US modalities for examining the small bowel. While TABS of the small intestine is standard in many tertiary centres for GI diseases around the world, some of the modalities reviewed here have not been implemented widely in clinical routine.

The strength of the US examination is high flexibility, repeatability, low patient risk and price, in combination with good spatial and excellent temporal resolution. If the initial US examination is not fully adequate, the operator has the opportunity to continue with more advanced methods, like CEUS or hydrosoneography.

However, further studies are needed to establish the methods' effectiveness at detecting small lesions in the small bowel. In patients in whom an invasive procedure is necessary, EUS with biopsy or even local resection is possible. Although the procedure is technically challenging, invasive and time-consuming, these factors are outweighed by the risk of an exploratory laparotomy or laparoscopy.

US can provide a wide range of physiological and pathophysiological parameters from the small bowel. Doppler techniques give information about the speed and direction of blood flow, and also provide indirect measurements of perfusion. The measurements can be difficult to reproduce and the perfusion estimates should be interpreted with care as detection of blood flow in small vessels is a great challenge. However, CEUS enhances the sensitivity of flow signal detection remarkably and enables calculation of true perfusion in a region of interest. This information is important in the characterization of small bowel tumors and lesions of CD.

Some of the techniques presented in this review have shown their potential in explorative studies, but need to be further tested to determine their ultimate clinical utility. Indeed, imaging of the small intestine is a great challenge to the clinician. In future, the clinician should be able to switch easily between the different modalities using the same scanner and with the help of improved visualization techniques, and have all relevant information presented simultaneously. Accordingly, the advance of multimodal and multiparametric imaging of the small intestine will enable better diagnostic performance and subsequently improved patient management.

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Impact of mass screening for gluten-sensitive enteropathy in working population

Meritxell Mariné, Fernando Fernández-Bañares, Montserrat Alsina, Carme Farré, Montserrat Cortijo, Rebeca Santaolalla, Antonio Salas, Margarita Tomàs, Elias Abugattas, Carme Loras, Ingrid Ordás, Josep M Viver, Maria Esteve

Meritxell Mariné, Fernando Fernández-Bañares, Rebeca Santaolalla, Carme Loras, Ingrid Ordás, Josep M Viver, Maria Esteve, Department of Gastroenterology, University Hospital of Mútua de Terrassa, Research Foundation Mútua de Terrassa, University of Barcelona, 08221 Terrassa, Catalonia, Spain

Antonio Salas, Department of Pathology, University Hospital of Mútua de Terrassa, Research Foundation Mútua de Terrassa, University of Barcelona, 08221 Terrassa, Catalonia, Spain

Montserrat Alsina, Department of Immunology (Catlab), University Hospital of Mútua de Terrassa, Research Foundation Mútua de Terrassa, University of Barcelona, 08221 Terrassa, Catalonia, Spain

Carme Farré, Department of Biochemistry, Sant Joan de Déu Hospital, Esplugues, 08950 Catalonia, Spain

Montserrat Cortijo, Margarita Tomàs, Elias Abugattas, Occupational Health Department, Egarsat (Prevention Society), Sant Cugat del Vallès, 08173 Catalonia, Spain

Author contributions: Mariné M, Esteve M, Fernández-Bañares F, Salas A and Viver JM contributed to the study concept and design. Mariné M, Esteve M, Santaolalla R, Fernández-Bañares F, Alsina M, Farré C, Salas A, Tomàs M, Abugattas E, Ordás I, Loras C, Cortijo M and Viver JM contributed to the acquisition of data; Salas A performed the histopathological analysis; Methodology design and laboratory analysis was carried out by Mariné M, Esteve M, Fernández-Bañares F, Santaolalla R, Alsina M, Farré C; Mariné M, Esteve M, Fernández-Bañares F, Salas A, Alsina M performed the analysis and interpretation of data; Mariné M, Esteve M, Fernández-Bañares F, Viver JM wrote the manuscript; Mariné M, Esteve M, Fernández-Bañares F, Alsina M, Farré C, Cortijo M, Santaolalla R, Salas A, Tomàs M, Abugattas E, Loras C, Ordás I, Viver JM carried out a critical revision of the manuscript for important intellectual content; Esteve M, Mariné M, Fernández-Bañares F performed the statistical analysis; The study was supervised by Esteve M, Fernández-Bañares F, Mariné M.

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Correspondence to: Maria Esteve, MD, Department of Gastroenterology, University Hospital of Mútua de Terrassa, University of Barcelona, Plaça Dr Robert nº 5, 08221 Terrassa, Barcelona, Catalonia, Spain. mestevecomas@telefonica.net
Telephone: +34-93-7365050 Fax: +34-93-7365043

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of gluten sensitive enteropathy (GSE) detected by serology in a mass screening program; (2) sensitivity of antitransglutaminase (tTGA) and antiendomysium antibodies (EmA); and (3) adherence to gluten-free diet (GFD) and follow-up.

METHODS: One thousand, eight hundred and sixty-eight subjects recruited from an occupational health department underwent analysis for tTGA and EmA and, if positive, duodenal biopsy, DQ2/DQ8 genotyping, clinical feature recording, blood tests, and densitometry were performed. Since > 98% of individuals had tTGA < 2 U/mL, this value was established as the cut-off limit of normality and was considered positive when confirmed twice in the same sample. Adherence to a GFD and follow up were registered.

RESULTS: Twenty-six (1.39%) subjects had positive tTGA and/or EmA, and 21 underwent biopsy: six Marsh III (one IIIa, four IIIb, one IIIc), nine Marsh I and six Marsh 0 (frequency of GSE 1:125). The sensitivity of EmA for GSE was 46.6% (11.1% for Marsh I, 100% for Marsh III), while for tTGA, it was 93.3% (88.8% for Marsh I, 100% for Marsh III). All 15 patients with abnormal histology had clinical features related to GSE. Marsh I and III subjects had more abdominal pain than Marsh 0 ($P = 0.029$), and a similar trend was observed for distension and diarrhea. No differences in the percentage of osteopenia were found between Marsh I and III ($P = 0.608$). Adherence to follow-up was 69.2%. Of 15 GSE patients, 66.7% followed a GFD with 80% responding to it.

CONCLUSION: GSE in the general population is frequent and clinically relevant, irrespective of histological severity. tTGA is the marker of choice. Mass screening programs are useful in identifying patients who can benefit from GFD and follow-up.

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Key words: Antitransglutaminase and antiendomysium antibodies; Celiac disease; Lymphocytic enteritis; Mass screening

Peer reviewer: Jose Castellote, PhD, Hospital Universitari de Bellvitge. L'Hospitalet de Llobregat Barcelona. C/Feixa Llarga S/N, L'Hospitalet de Llobregat, 08907, Spain

Abstract

AIM: To assess: (1) frequency and clinical relevance

Mariné M, Fernández-Bañares F, Alsina M, Farré C, Cortijo M, Santaolalla R, Salas A, Tomàs M, Abugattas E, Loras C, Ordás I, Viver JM, Esteve M. Impact of mass screening for gluten-sensitive enteropathy in working population. *World J Gastroenterol* 2009; 15(11): 1331-1338 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1331.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1331>

INTRODUCTION

Screening for celiac disease (CD) in the general population is still a controversial issue. Recent papers have reviewed the benefits and drawbacks of CD diagnosis in this situation^[1]. CD is a highly prevalent disease (1:100 to 1:300) which fulfils some of the criteria favoring mass screening. That is, the availability of accurate and non-invasive diagnostic methods and an effective treatment (gluten-free diet: GFD) which restores health and prevents disease-associated complications^[2]. The major concerns regarding mass screening are the reported lack of GFD adherence in asymptomatic patients detected in screening programs^[3,4] and the limited available data on the cost-effectiveness of such an approach^[5]. In addition, the benefits of early diagnosis in patients with mild disease, in terms of preventing late complications, are poorly understood^[6]. In this sense, knowledge of the natural history of mild CD has been hampered by its imprecise definition in the medical literature, with frequent overlapping of the terms "mild enteropathy" (i.e. lymphocytic enteritis and partial atrophy) and "silent CD". In fact, it was recently demonstrated that these terms may not be considered synonymous since gluten sensitive enteropathy (GSE) with preserved villous architecture may be symptomatically similar to patients with atrophy^[7] showing a good response to a GFD^[8,9], and conversely, silent patients with atrophy do exist, and are at risk of subsequent severe complications^[10].

Occupational health departments provide care for the working population on environmental issues associated with work processes, but also help detect very frequent preventable diseases. In this setting, the aims of the present study were: (1) to assess the frequency and clinical relevance of GSE (both Marsh I and Marsh III) detected by serology in a mass screening program; (2) to compare the sensitivity of antitransglutaminase antibodies (tTGA) and antiendomysium antibodies (EmA) in detecting the whole spectrum of GSE (Marsh I to Marsh III); and (3) to assess the degree of adherence to a mass-screening program and the effectiveness of GFD.

MATERIALS AND METHODS

Subjects and study design

One thousand, eight hundred and sixty-eight individuals (1308 males, 560 females, 36.6 ± 0.5 years) were recruited from the Occupational Work Surveillance Department in Catalonia, Northeastern Spain, between January 2004 and December 2005. None of the 1868

subjects included had previously been diagnosed with CD. After obtaining written informed consent, blood sampling for serum EmA and tTGA assay was carried out and clinical features for gastrointestinal and systemic symptoms, and associated diseases (yes/no questions) were obtained. HLA DQ2/DQ8 genetic study and duodenal biopsy were offered to those subjects positive for either EmA or tTGA. Furthermore, as previously described, a self-administered questionnaire of symptom severity using a visual analogue scale (VAS) ranging from 0 to 100 was recorded in these subjects^[7,11]. The symptoms evaluated were diarrhea, abdominal pain, abdominal distension, flatulence and asthenia. Anemia and hypertransaminasemia, as well as abnormalities in bone mineral density (BMD), were also recorded. Subjects were considered to be symptomatic when they complained of at least one of the above-mentioned symptoms and/or had impaired blood test and/or densitometry. A symptom was considered to be present when it scored more than 20 points and was considered severe when it scored more than 50 points on the VAS.

Antibody detection

Serum IgA-EmA was determined by indirect immunofluorescence (IFI) assay in serum samples at 1/5 dilution, as previously described^[12]. Commercial sections of monkey distal oesophagus (BioMedical Diagnostics, France) were used as the IFI substrate.

IgA-class tTGA was analyzed in serum using a quantitative automated ELISA method by means of a commercially available detection kit (Varelisa Celikey™, Phadia AB, Freiburg, Germany) using recombinant human tTG as antigen^[13]. Since > 98% of individuals had tTGA < 2 U/mL, this value was established as the cut-off limit of normality and was considered positive when confirmed twice in the same sample (recommended cut-off by the manufacturer > 8 U/mL). Total serum IgA was measured using rate nephelometry (BN II, Siemens Healthcare Diagnostics SL, Marburg, Germany). In cases of IgA deficiency, IgG-class EmA was measured.

When one or both serological markers were positive, an upper endoscopy with duodenal biopsy was proposed.

Genetic markers

Standard techniques for DNA extraction, PCR amplification and product detection were used. To purify genomic DNA from whole blood, a commercial reagent Generation® Capture Column Kit (Gentra Systems, Minnesota, USA) was used. HLA-DQ2 (DQA1*0501 and DQB1*0201 alleles) and HLA-DQ8 (DQA1*0301 and DQB1*0302 alleles) genotyping was performed by PCR amplification using sequence specific primers (PCR-SSP)^[14] on a GeneAmp PCR 2400 System (Applied Biosystems, Foster City, CA, USA). PCR products were detected by electrophoresis on 2% agarose gel and were visualized under UV light. Analysis of HLA-DQ8 haplotype was performed only on those patients with negative DQ2.

Duodenal biopsy and diagnosis criteria for GSE

Four endoscopic biopsies from the 2nd-3rd portions of

the duodenum were processed by using hematoxylin/eosin staining and CD3 immunophenotyping, and were blindly evaluated by an expert gastrointestinal pathologist (A.S.). Histopathological findings were staged according to the Marsh criteria^[15], as revised by Rostami *et al.*^[16]: 'Infiltrative' lesions with intraepithelial lymphocytosis are defined as Marsh type I, 'infiltrative/hyperplastic' lesions are defined as Marsh II, and 'partial (A), subtotal (B) and total (C) villous atrophy' as Marsh III. A cut-off of 25 intraepithelial lymphocytes (IELs)/100 epithelial cells was established to diagnose lymphocytic enteritis (LE)^[17]. Other frequent causes of LE, such as parasites, NSAID ingestion, Crohn's disease and autoimmune diseases were ruled out^[18]. *Helicobacter pylori* (*H. pylori*) infection was investigated by means of the urease test and histopathological assessment using hematoxylin/eosin staining of the gastric mucosa in all the cases.

The diagnosis of GSE was considered when some degree of histological abnormality was found and a good response to a GFD was demonstrated (see below) at least after one year of follow-up, according to AGA criteria^[19].

Measurement of BMD

BMD was assessed in all patients showing some degree of histological abnormality (Marsh I to III), both at baseline and after GFD. T and Z-scores were measured in the lumbar spine and left femoral neck using a dual-energy X-ray absorptiometry (DXA) (Lunar DPX-aph, Madison, WI, USA). According to World Health Organization criteria, osteopenia is defined as a value of BMD between 1 SD and 2.5 SD below the average for young adults (T score -1 to -2.5), and osteoporosis is defined as a value of BMD more than 2.5 SD below the average value for young adults (T score < -2.5)^[20].

Patient follow-up and response to GFD

GFD was recommended to all patients with villous atrophy and to all symptomatic LE patients, and adherence was recorded. To ensure the correct intake of a strict GFD, patients were referred to the Catalan Celiac Society ('Celiacs de Catalunya', Barcelona). Iron and/or calcium plus vitamin D supplements were prescribed when deficiencies or bone density impairment were detected. Clinical, histological, analytical and serological assessments were carried out in all patients who adhered to a GFD at least 1 year after starting the diet. In addition, a second densitometry assessment was carried out when the basal assessment was impaired. For the remaining individuals with positive tTGA at baseline, who were on a gluten-containing diet, clinical, analytical and serological assessments were requested, as a minimum. A patient was considered to have achieved a complete clinical response when all symptoms disappeared (VAS < 20 points) and when normalization of analytical and bone densitometry abnormalities occurred. A partial clinical response was defined as more than a 30-point reduction in the VAS score and/or a significant improvement but not normalization of analytical and bone densitometry abnormalities. A complete histological response was defined as a decrease from Marsh III to Marsh I or Marsh

0, and in Marsh I cases, a normalization in the IEL count or a reduction of at least 50% from the basal biopsy. An improvement in the degree of atrophy (i.e. from Marsh IIIc to Marsh IIIa) or a reduction in the IEL count from 25% to 50% of the basal biopsy in Marsh I cases, was considered a partial histological response.

In patients who did not accept a biopsy after GFD, negative serology was considered a criterion of at least partial response. Patients with LE and those with the lowest tTGA positive values were particularly encouraged to undergo histological retesting during follow-up.

Statistical analysis

Categorical parameters were expressed as proportions, whereas continuous variables were expressed as both mean and standard error of the mean (SEM). Since intestinal biopsy was performed when EmA and/or tTGA were positive, the ratio of sensitivities of the two serological tests was calculated by an estimation of test sensitivity when disease confirmation was limited to positive results. Differences in sensitivities were assessed using a modified McNemar test as previously described^[21]. Statistical comparisons for qualitative variables were made by an extension of Fisher's exact test for 2 × 3 contingency tables (Freeman-Halton test)^[22]. One-way ANOVA and paired Student *t* test were used to compare quantitative variables. *P* < 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS for Windows Statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

Frequency of GSE and histological severity related to positive serology and genetic markers

Figure 1 represents a flow diagram of the evaluated patients. Twenty-six of the 1868 individuals (1.39%) had positive markers for CD (18 males, eight females, mean age 37.7 ± 11.0 years). Of the 26 patients with positive markers, seven were positive for both EmA and tTGA, one was positive only for EmA, and the remaining 18 were positive only for tTGA. Twenty-one of these 26 individuals (80.7%) underwent an intestinal biopsy, which disclosed the following histological findings: six Marsh III (one IIIa, four IIIb, one IIIc), nine Marsh I and six Marsh 0. Three Marsh I cases had *H. pylori* infection but the IEL count remained unchanged after 6 mo of eradication therapy. Thus, 0.80% of subjects initially tested had a biopsy proven lesion of the GSE spectrum (1:125) and 0.32% had villous atrophy (1:312). Values of tTGA related to the degree of mucosal damage are shown in Figure 2. All Marsh III patients were positive for both EmA and tTGA, and all Marsh I and 2 Marsh III patients had tTGA values higher than 2 U/mL but lower than the cut-off recommended by the manufacturer (8 U/mL).

The sensitivity of EmA for GSE diagnosis was 46.6% (11.1% for Marsh I and 100% for Marsh III), whereas the sensitivity of tTGA was 93.3% (88.8% for Marsh I and 100% for Marsh III) (*P* = 0.04). The sensitivity ratio demonstrated a two-fold sensitivity

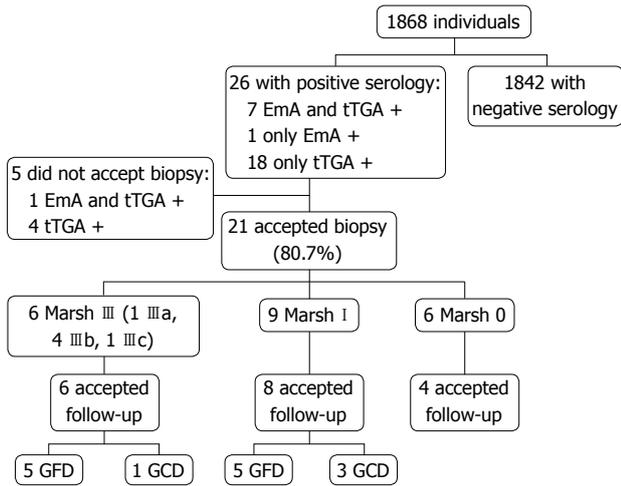


Figure 1 Flow diagram of recruited subjects. GCD: Gluten-containing diet.

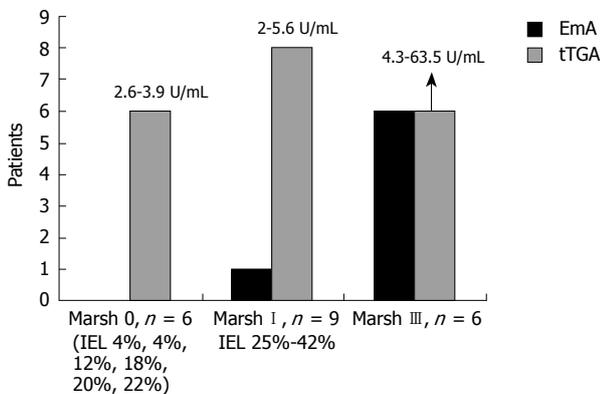


Figure 2 Values of tTGA related to the degree of histological damage.

for tTGA compared with EmA to diagnose the whole spectrum of GSE (from Marsh I to III).

Thirteen of the 21 biopsied subjects (62%) were DQ2 +, 1 (4.7%) was DQ8 +, six had one allele of the DQ2 + (28.6%) (DQB1*0201 in five subjects and DQA1*0501 in one) and only one Marsh III (4.7%) was negative for both alleles of DQ2 and DQ8. A detailed description of the genetic markers related to the degree of histological damage is shown in Figure 3.

In five subjects with positive markers a biopsy was not performed, four because they did not accept the procedure and one patient could not be located [one was positive for both EmA (1/20) and tTGA (3.94 U/mL) and four were positive only for tTGA (2.21 to 5.10 U/mL)].

Clinical characteristics of GSE related to the severity of intestinal damage

No significant differences were found in the percentage of autoimmune diseases ($P = 0.415$), thyroid diseases ($P = 0.632$), type 1 diabetes mellitus ($P = 1$), previous anemia ($P = 0.765$), diarrhea ($P = 0.764$), abdominal pain ($P = 1$), flatulence ($P = 0.965$) or abdominal distension ($P = 0.621$) between individuals, in the general working population, or in those with positive and negative serological markers.

All 15 subjects with abnormal histological findings had clinical features related to the disease. In one

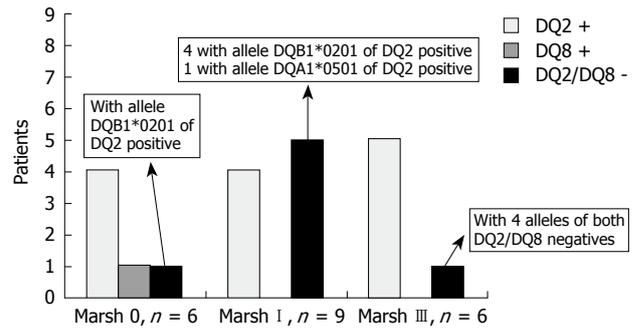


Figure 3 HLA DQ2/8 related to the degree of histological damage.

Table 1 Frequencies of clinical manifestations related to the degree of histological severity

	Normal mucosa (Marsh 0) n = 6 (%)	LE (Marsh I) n = 9 (%)	Atrophy (Marsh III) n = 6 (%)	P value
Flatulence	4 (66.7)	6 (66.7)	4 (66.7)	1
Distension	2 (33.4)	7 (77.8)	4 (66.7)	0.282
Abdominal pain	0 (0.0)	6 (66.7)	4 (66.7)	0.029
Diarrhea	1 (16.7)	4 (44.5)	4 (66.7)	0.300
Asthenia	2 (33.4)	2 (22.3)	4 (66.7)	0.213
Anemia	0 (0)	0 (0)	1 (16.7)	0.571
Hypertransaminasemia	1 (16.7)	1 (11.9)	2 (33.3)	0.783

Statistical comparisons were made by an extension of Fisher's exact test (Freeman-Halton test).

Marsh III and two Marsh I patients, the only clinical feature was osteopenia (20%). The frequencies of clinical manifestations related to the degree of histological severity are described in Table 1. Subjects with Marsh I and Marsh III lesions had significantly more abdominal pain (66.7%) than those with normal mucosa (0%; $P = 0.029$). A similar but non-significant trend was observed for distension and diarrhea, whereas asthenia was more often found in Marsh III patients. No differences were found for hypertransaminasemia and only one Marsh III patient had anemia. A progressive increase in the severity of most symptoms from Marsh 0 to Marsh III was observed when symptoms were assessed by means of a VAS (Table 2), reaching statistically significant differences for distension ($P = 0.035$) and asthenia ($P = 0.031$).

Moreover, severe abdominal pain (VAS > 50) was more frequent in Marsh I (33.4%) than in Marsh 0 (0%) and in Marsh III (16.7%) ($P = 0.006$), while distension and asthenia were more frequent in Marsh III (66.7%) than in Marsh 0 (0%) and Marsh I (11.2%) ($P = 0.001$ for both symptoms).

BMD was only assessed in those patients with abnormal biopsy. No significant differences in the percentage of osteopenia were found between Marsh I (55.6%) and III (33.4%) ($P = 0.608$). There were no patients with osteoporosis.

Follow-up after GFD

Eighteen of the 26 subjects with positive serology at

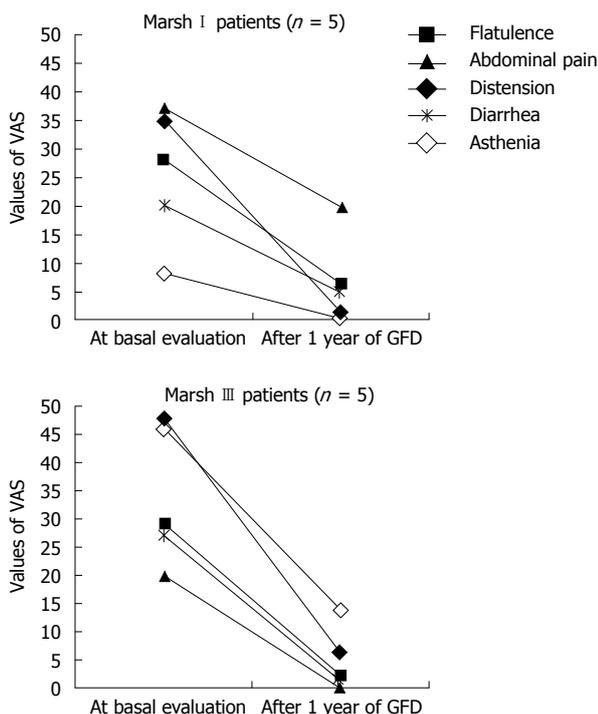


Figure 4 Evolution of the mean values of the VAS after GFD.

baseline accepted follow-up, disclosing a 69.2% adherence to the mass-screening program, with a mean follow-up of 28 months (range, 20 to 33). Of the 15 patients with histopathological lesions compatible with GSE, 10 followed a GFD (66.7%, five Marsh I and five Marsh III). Table 3 shows a detailed description of the patients who adhered to a GFD. Overall, nine of 10 patients (90%) (five Marsh III and four Marsh I) had a complete histological and/or serological response to a GFD. A dramatic clinical improvement was observed in both Marsh I and Marsh III patients; response was complete for two of the 10 patients (one Marsh III and one Marsh I) and partial for five (three Marsh III and two Marsh I). The main reason for Marsh I patients' adherence to GFD was the presence of osteopenia (four of five Marsh I patients). In contrast, osteopenia was only diagnosed in one Marsh I patient of the four who did not follow a GFD. No differences were found for either the number or the severity of symptoms between patients who followed a GFD and those who did not. At the end of follow-up, those patients who followed a GFD showed an improvement in the mean value of the VAS for all symptoms, and this was statistically significant for distension ($P = 0.014$), flatulence ($P = 0.028$) and abdominal pain ($P = 0.007$) (Table 4). In Figure 4, evolution of the mean values of the VAS are shown separately for Marsh I and Marsh III.

Anemia and hypertransaminasemia reverted after GFD in those patients who had these conditions at basal evaluation. BMD normalized in two of the six patients with abnormal values at baseline. In the remaining cases, a trend for T score improvement in both the femoral neck (initial -0.74 ± 0.22 , final -0.69 ± 0.18 ; $P = 0.144$) and lumbar spine (initial -1.27 ± 0.30 , final -1.09 ± 0.30 ; $P = 0.068$) was observed.

Table 2 Relationship between the values of VAS and degree of histological damage

	Normal mucosa (Marsh 0) n = 6	LE (Marsh I) n = 9	Atrophy (Marsh III) n = 6	P value
Flatulence	36.6 ± 12.0 (0-60)	28.8 ± 8.2 (0-60)	36.6 ± 14.7 (0-80)	0.839
Distension	6.67 ± 4.2 (0-20)	28.8 ± 6.7 (0-60)	50.0 ± 16.9 (0-100)	0.035
Abdominal pain	0.0 ± 0.0 (0-20)	31.1 ± 10.0 (0-80)	26.6 ± 9.8 (0-60)	0.06
Diarrhea	8.33 ± 8.3 (0-50)	17.7 ± 9.1 (0-80)	30.0 ± 12.3 (0-80)	0.384
Asthenia	10.0 ± 6.8 (0-40)	11.1 ± 7.5 (0-60)	50.0 ± 16.9 (0-100)	0.031

Statistical comparisons were made by one-way ANOVA. Results are expressed as mean (SE) and range.

Eight additional patients with initial positive serology, who decided to follow a gluten-containing diet (one Marsh III, three Marsh I and four Marsh 0), accepted a clinical, serological and/or histological follow-up (Table 5). A progression from Marsh 0 to Marsh I was observed in one case and from Marsh I to Marsh III in another. This progression was accompanied by worsening of symptoms and an increase in tTGA values in the Marsh III patient. In the remaining cases with the exception of one, tTGA values diminished below 2 U/mL in the follow-up, although clinical symptoms remained unchanged.

DISCUSSION

The frequency of biopsy proven CD with atrophy found in this study (1:312) is within the range previously described in our geographical area^[23]. All Marsh III cases in our study had positive tTGA and EmA, confirming that both serological tests have a similar high sensitivity for diagnosing CD with villous atrophy^[24]. However, it is well known that the sensitivity of serology sharply decreases in mild forms of GSE. Although not universally accepted, the recognition of Marsh I patients is important since a significant proportion of these patients have severe symptoms^[7] and could benefit from a GFD. The observation that less than 2% of individuals in the general population have tTGA values higher than 2 U/mL prompted us to establish this value as the normal cut-off limit, instead of the 8 U/mL recommended by the manufacturer. This fact allowed us to identify two additional Marsh III and eight Marsh I patients by using tTGA alone, and it increased the sensitivity of this serological marker with respect to EmA at the expense of a greater number of diagnosed patients with mild enteropathy (88.8% versus 11.1% sensitivity in Marsh I for tTGA and EmA, respectively). With the combined use of EmA and tTGA the prevalence of biopsy proven GSE increased to 1:125.

A recently published study performed in Iran^[25], evaluating both Marsh I and Marsh III patients, found a similar number of GSE patients to that found in our study, with most Marsh I patients detected only

Table 3 Follow-up of patients who adhered to GFD

	At basal evaluation					After GFD				
	Clinical features	EmA	tTGA (U/mL)	Biopsy	IELs	Clinical features	EmA	tTGA (U/mL)	Biopsy	IELs
Case 1 ♀ 47 years	Flatulence, distension, abdominal pain, diarrhea, asthenia, anemia, osteopenia	> 1/160	74	Marsh IIIb	---	Asthenia	Neg.	3.3	Marsh I	35%
Case 2 ♀ 24 years	Flatulence	> 1/160	159	Marsh IIIb	---	No symptoms	Neg.	1.9	---	---
Case 3 ♂ 41 years	Flatulence, distension, abdominal pain, diarrhea, asthenia, hypertransaminasemia	> 1/160	63.4	Marsh IIIc	---	Asthenia, hypertransaminasemia	Neg.	2.0	Marsh I	28%
Case 4 ♀ 32 years	Osteopenia	1/80	8.5	Marsh IIIa	---	Osteopenia	Neg.	0	---	---
Case 5 ♀ 22 years	Distension, abdominal pain, diarrhea, asthenia, hypertransaminasemia, osteopenia	1/10	4.34	Marsh IIIb	---	Distension, osteopenia	Neg.	1.0	Marsh I	42%
Case 6 ♀ 42 years	Flatulence, distension, osteopenia	Neg.	2.0	Marsh I	40%	Osteopenia	Neg.	2.4	Marsh 0	22%
Case 7 ♂ 41 years	Osteopenia	Neg.	3.34	Marsh I	26%	No symptoms	Neg.	1.24	Marsh I	26%
Case 8 ♀ 38 years	Flatulence, distension, abdominal pain	Neg.	4.04	Marsh I	42%	Flatulence	Neg.	2.0	Marsh 0	17%
Case 9 ♂ 37 years	Distension, abdominal pain, diarrhea, osteopenia	Neg.	3.20	Marsh I	26%	Abdominal pain, diarrhea, osteopenia	Neg.	2.4	Marsh I	28%
Case 10 ♂ 32 years	Flatulence, distension, abdominal pain, diarrhea, asthenia, osteopenia	Neg.	2.34	Marsh I	35%	Flatulence, distension, abdominal pain, diarrhea, asthenia, osteopenia	Neg.	1	Marsh 0	14%

Table 4 Evolution of symptoms after 1 year of follow-up for those patients who adhered to a GFD ($n = 10$; 5 Marsh I, 5 Marsh III)

	Basal (mean values of VAS)	After 1 year of GFD (mean values of VAS)	P value
Flatulence	28 ± 8.9	5 ± 3.4	0.028
Distension	42 ± 10.9	7 ± 4.7	0.014
Abdominal pain	28 ± 9.0	7 ± 5.1	0.007
Diarrhea	24 ± 10.2	4 ± 2.6	0.063
Asthenia	28 ± 12.3	12 ± 6.6	0.133

by positive tTGA and not with EmA. These results demonstrated, as did ours, the increased sensitivity of tTGA in detecting mild enteropathy. Unfortunately, the clinical characteristics of these patients and response to a GFD were not assessed in the Akbari *et al* study, raising some doubts about the reliability of the diagnosis of GSE in LE patients^[26]. In fact, other causes of LE should be ruled out before considering the possibility of GSE diagnosis^[18].

Interestingly, despite the limitations of small sample size, the clinical features of Marsh I patients identified in the general working population duplicate those previously published in a group of first degree relatives of CD patients, confirming that Marsh I patients may be symptomatic similar to patients with atrophy^[7]. Again, distension, abdominal pain and asthenia were the symptoms most consistently associated with GSE irrespective of the severity of the intestinal lesions, whereas non-significant differences were found for diarrhea and flatulence. In addition, as previously described, similar percentages of osteopenia were found in Marsh I and Marsh III patients, suggesting the

existence of a similar degree of calcium and/or vitamin D-impaired absorption. This study also demonstrated that the Marsh I patients detected in this mass-screening program, with low positive levels of tTGA, were true GSE patients with a gluten-dependent lesion and with a similar response to a GFD as those with Marsh III.

It is noticeable that tTGA values ranging from 2.6 to 3.9 U/mL were detected in six Marsh 0 individuals who could be considered false positives. However, a frequency higher than expected of DQ2/DQ8 positivity in these individuals, as well as the progression from Marsh 0 to Marsh I in one case, suggests that these individuals might have latent CD and therefore merit follow-up.

The genetic characteristics of the 21 individuals with available duodenal histology merit an additional comment. Sixty-two per cent and 4.7% were DQ2 and DQ8 positive, respectively, and 28.6% (five Marsh I and one Marsh 0) had only one allele of the DQ2 (DQB*0201 in five cases and DQA1*0501 in one more case). Thus, the percentage of DQ2 positivity in the present study was lower than that described for CD patients, among whom more than 90% express both DQ2 alleles^[27]. However, it has been reported that in the majority of DQ2-negative CD patients (approximately 5%), one of the DQ2 alleles is present, generally DQB*0201 and rarely DQA1*0501^[28]. Consistent with this, the DQ2 negative patients in the present study, most of them Marsh I, expressed only one allele of the DQ2, predominantly DQB*0201. It may be speculated that the presence of only the β chain or α chain of the DQ2 heterodimer, encoded by DQB*0201 or DQA1*0501, respectively, could impede the progression from mild enteropathy to atrophy in these subjects. Taking together all these data, individuals with tTGA values ≥ 2 U/mL

Table 5 Patients who accepted follow-up but did not adhere to GFD

	At basal evaluation					Follow-up				
	Clinical features	EmA	tTGA (U/mL)	Biopsy	IELs	Clinical features	EmA	tTGA (U/mL)	Biopsy	IELs
Case 1 ♂ 23 years	Flatulence, distension, abdominal pain, diarrhea, asthenia	1/40	6.76	Marsh III b	---	Flatulence, distension, abdominal pain, diarrhea, asthenia	Neg.	1.70	Not accepted	---
Case 2 ♂ 63 years	Osteopenia	Neg.	4.07	Marsh I	29%	Osteopenia	Neg.	2.2	Marsh I	25%
Case 3 ♂ 24 years	Flatulence, distension, abdominal pain, diarrhea, asthenia	Neg.	3.47	Marsh I	25%	Flatulence, distension, diarrhea, asthenia	Neg.	1.0	Not accepted	---
Case 4 ♀ 66 years	Flatulence, distension, abdominal pain	Neg.	5.60	Marsh I	35%	Diarrhea, abdominal pain, distension, flatulence	Neg.	15	Marsh III a	---
Case 5 ♂ 45 years	Asthenia	Neg.	2.68	Marsh 0	5%	Asthenia	Neg.	2.0	Marsh I	28%
Case 6 ♂ 47 years	Flatulence	Neg.	3.95	Marsh 0	22%	Flatulence	Neg.	1.0	Marsh 0	5%
Case 7 ♂ 36 years	Distension	Neg.	2.78	Marsh 0	20%	Distension, hypertransaminasemia	Neg.	1.2	Not accepted	---
Case 8 ♂ 36 years	Flatulence	Neg.	2.80	Marsh 0	18%	Flatulence	Neg.	1.0	Not accepted	---

detected in this study have, with a very high probability, some form of the GSE spectrum of conditions (from Marsh 0 to Marsh III).

The present study also shows that GSE patients in the general population may not be identified by clinical features, since a similar percentage of related CD symptoms was found in individuals with positive and negative markers. This fact explains why CD remains underdiagnosed in a high proportion of affected subjects and is an additional argument for mass-screening using other approaches, such as serology, irrespective of clinical symptoms. Unfortunately, serology had limitations due to its low sensitivity in detecting individuals with mild GSE. In addition, fluctuations from time to time in tTGA values may allow the identification of CD patients at some time points but not in others. In fact, most of the tTGA values in patients on a gluten-containing diet were negative in the follow-up.

Almost 70% of subjects with positive serology adhered to the follow-up program, which included GFD compliance or simple clinical, histological and serological surveillance. The reported degree of GFD adherence has been shown to vary greatly in different studies ranging from less than 10% (4) to 90%^[29], and is probably highly dependent on the patient-doctor relationship and confidence. In addition, the GFD adherence in this and other studies of CD detected by screening is similar to or better than that reported for other diseases, such as hypercholesterolemia or coronary heart disease^[30], in which specific diets or changes in lifestyle are required to prevent life-threatening complications.

It has been argued that the lack of adherence to a GFD in patients identified in screening programs is due to an absence of symptoms in these cases. We have demonstrated in the present study that, when systematically assessed, signs or symptoms related to GSE may be identified in all cases, taking into account that osteopenia was the only clinical feature detected in 20% of patients. Thus, the low GFD adherence due to an absence of potential benefit perceived by the

patient should never be used as an argument against the performance of mass screening for CD in the general population.

In conclusion, GSE in the general population is frequent and is clinically relevant, irrespective of the severity of the histological lesion. Mass screening programs are useful for identifying these patients in order to initiate either a GFD or close follow-up monitoring.

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COMMENTS

Background

Screening for celiac disease (CD) in the general population is still a controversial issue. CD is a highly prevalent disease (1:100 to 1:300) that fulfils most of the criteria favoring mass screening. The benefits of early diagnosis in patients with mild disease, in terms of preventing late complications, are poorly understood.

Research frontiers

In this study, the authors demonstrate that mass screening programs allow detection of CD cases with the whole gluten sensitive enteropathy (GSE) spectrum, which otherwise would have not been diagnosed, and that the adherence and response to a gluten-free diet (GFD) in these subjects was much better than that previously reported for other chronic diseases.

Innovations and breakthroughs

This study shows unpublished information on GSE detected in the general population. It was demonstrated that Marsh I subjects detected by antitransglutaminase (tTGA) in this setting are as symptomatic as Marsh III and that they also respond equally to a GFD, reinforcing the final diagnosis of GSE in mild enteropathy.

Applications

Understanding the evolution of GSE with mild enteropathy can help important decision-making, such as initiating a GFD in particular cases. Starting a GFD in symptomatic patients may prevent potential complications such as anemia and osteoporosis. The risk of lymphoma in mild GSE is at present unknown. A close follow-up of patients on a gluten-containing diet may help clarify this point.

Terminology

GSE is characterized by a permanent intolerance to ingested gluten in susceptible individuals and leads to immunologically mediated inflammation of the small

intestine mucosa. Common histopathology findings range from mild enteropathy, which consists of an increase in intraepithelial lymphocytes (> 25 IEL/100 IELs) (Marsh I lesion), to 'classic' celiac disease with crypt hyperplasia and partial (Marsh IIIa), subtotal (Marsh IIIb) or total (Marsh IIIc) atrophy.

Peer review

In this interesting retrospective study the power of screening tests for gluten sensitive enteropathy are critically evaluated in a cohort of 1868 patients. The authors conclude from their data that evaluation of tTGA is more essential in diagnosis of gluten sensitive enteropathy than EmA and histology. In order to select patients for gluten free diet and follow-up mass screening programs are proposed as useful. Statistical analyses are well. The manuscript is well-written and illustrated. The topic is of high interest for a readership interested in gastrointestinal diseases.

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Antioxidant activity of chito-oligosaccharides on pancreatic islet cells in streptozotocin-induced diabetes in rats

Wen-Peng Yuan, Bing Liu, Chang-Heng Liu, Xiao-Jun Wang, Mian-Song Zhang, Xiu-Mei Meng, Xue-Kui Xia

Wen-Peng Yuan, Chang-Heng Liu, Xiao-Jun Wang, Mian-Song Zhang, Xiu-Mei Meng, Xue-Kui Xia, Biology Institute of Shandong Academy of Sciences, Jinan 250014, Shandong Province, China

Bing Liu, Chinese Academy of Inspection and Quarantine, Beijing 100025, China

Author contributions: Yuan WP and Liu B designed the research; Yuan WP, Liu B, Liu CH and Wang XJ performed the research; Zhang MS, Meng XM and Xia XK contributed to the reagents, materials and analysis; Yuan WP and Liu B analyzed the data; Yuan WP and Liu B wrote the paper.

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Correspondence to: Bing Liu, Chinese Academy of Inspection and Quarantine, No. A3, Gaobeidian Beilu, Chaoyang District, Beijing 100025, China. icecream1221@126.com

Telephone: +86-10-85399005 Fax: +86-10-85399005

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Peer reviewer: Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico II University Medical School, VIA S. PANSINI, 5, Naples 80131, Italy

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Abstract

AIM: To investigate the antioxidant activity of chito-oligosaccharides (COSs) on pancreatic islet cells in diabetic rats induced by streptozotocin.

METHODS: The antioxidant effect of COSs on pancreatic islet cells was detected under optical microscopy and with colorimetric assay and gel electrophoresis. The activities of glutathione peroxidase and superoxide dismutase, total antioxidant capacity, and content of malondialdehyde in serum and tissue slices of pancreas were examined after 60 d to determine the effect of COSs in streptozotocin-induced diabetes in rats.

RESULTS: COSs can prohibit the apoptosis of pancreatic islet cells. All concentrations of COSs can improve the capability of total antioxidant capacity and activity of superoxide dismutase and decrease the content of malondialdehyde drastically. Morphological investigation in the pancreas showed that COSs have resulted in the reduction of islets, loss of pancreatic cells, and nuclear pyknosis of pancreatic cells.

CONCLUSION: COSs possess various biological activities and can be used in the treatment of diabetes mellitus.

INTRODUCTION

Chitosan's properties allow it to rapidly clot blood, and it has recently gained approval in the USA for use in bandages and other hemostatic agents^[1]. It has been further suggested that chitosan inhibits uptake of dietary lipids by increasing the thickness of the intestinal lumen boundary layer, a proposal again supported by numerous animal experiments that chito-oligosaccharides (COSs) possess various biological activities and can be used in diabetes treatment. Unlike high molecular weight chitosan, COSs, which are readily soluble in water due to their shorter chain and free amino groups in D-glucosamine units, and easily absorbed through the intestine, can quickly get into the blood flow and have versatile functional properties^[2-4].

Pancreatic beta-cell response to nutrient excess occurs by hypertrophy of existing β cells that increases insulin production and then formation of new β cells from progenitor cells. Failure of pancreatic β cells to adequately expand in settings of increased insulin demand ends up in hyperglycemia^[5]. Streptozotocin (STZ) selectively kills insulin-secreting β cells, and is widely used to generate animal models of diabetes. Previous studies have suggested that streptozotocin toxicity results from its N-nitrosourea moiety releasing nitric oxide and possessing DNA alkylating activity. More recently, it has also been proposed that streptozotocin induces apoptosis by inhibiting O-GlcNAcase, an enzyme that, together with O-GlcNAc transferase, is important for dynamic

intracellular protein O-glycosylation^[6].

In the present study, soluble COSs with low molecular weight were prepared by enzymatic hydrolysis of chitosan with chitosanase. The purpose of this study was to examine the antioxidant effect of chito-oligosaccharides on pancreatic islet cells and pancreatic islets in rats with STZ-induced diabetes.

MATERIALS AND METHODS

Materials

Chitosan (90% deacetylated, Mr: 500 000) was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd (Shangdong, China). Chito-oligosaccharides were prepared by enzymatic hydrolysis of chitosan with chitosanase. NIT-1 cell line was from Institute of Medicine, Ocean University of China. Male Wistar rats (200 ± 20 g) were from Laboratory Animal Centre, Institute of Medicine, Ocean University of China. STZ was from Sigma Chemical Co. Tissue-culture medium and reagents were from Gibco. All chemicals were purchased from Sigma Chemical unless otherwise stated.

Cell culture

NIT-1 cell line, a widely used β cell line for insulin secretion studies, was established from non-obese diabetic (NOD) mice transgenic for the SV40 T antigen under control of the insulin promoter, and cultured in DMEM containing 10% FBS and antibiotics (100 IU/mL of penicillin and 100 μg/mL of streptomycin). All cultures were kept at 37°C in 95% O₂ and 5% CO₂. The medium was changed every 2 d.

Effects of COSs on pancreatic β cells exposed to STZ

MTT assay: COS was dissolved in Dulbecco's modified Eagle's medium (DMEM) without FBS and then diluted the medium to 10, 100, 500, 1000 and 2000 mg/L degrades. The structure and function of cultured pancreatic β cells were observed under inverted phase contrast microscope. MTT assay was used to estimate the cell viability. Pancreatic β cells were digested by 0.25% trypsin until appearance of unicellular suspension. Then, 100-μL suspensions with the concentration of 5.0×10^4 cells/mL were transplanted into 96-well plates. Each well had eight parallels. After incubating for 24 h, the medium was changed with COSs at different concentrations. Cells were divided into normal control group; positive control group with NIT-1 cells treated with STZ; and COS protective group with NIT-1 cells treated with different concentrations of COS for 0.5 h, and then cultured with 2 mmol/L STZ for 12 h. Cells were incubated with 0.5 mg/mL of MTT in the last 4 h of the culture period (48 h). The medium was then decanted, 200 μL DMSO was added to the wells, and the absorbance was determined at 492 nm using an ELISA reader. The proliferation rate was calculated ($PR = A/A_0 \times 100\%$).

DNA gel electrophoresis: Groups were divided as

described above. After treatment, DNA was extracted from the cultured cells, washed in Tris-HCl for 3 times, and 0.5 mL STE extraction buffer (0.5 mol/L NaCl, 100 mmol/L Tris-HCl, 10 mmol/L EDTA, 1% SDS, 100 μg/mL protease K, pH = 8.0) was added, and the cells were re-suspended. The suspension was incubated in a water bath at 65°C for 30 min with occasional shaking, cooled to room temperature and incubated with chloroform/isoamylalcohol (24:1) for 10 min. After centrifugation at $5600 \times g$ for 10 min, the supernatant was mixed with dehydrated alcohol and incubated at -20°C for 2 h. Then, the mixture was centrifuged at $8600 \times g$ for 15 min. The DNA precipitation was washed with 70% ethanol twice and dissolved in sterilized TE (1 mol/L Tris-HCl, 0.1 mol/L EDTA, pH = 8.0). The DNA was examined in 1.2% agarose gel containing ethidium bromide (0.4 μg/mL) for about 1 h at 5 V/cm. The gel was photographed under ultraviolet light (254 nm) using GDS-760 image-analysis system (UVP).

Single cell gel electrophoresis (SCGE): After treatment, cells were washed in DMEM without serum, resuspended in PBS to a concentration of 2×10^5 cells/10 μL, mixed with 1% Low Melting Agarose (LMA), spread on a slide coated with a layer of 0.5% Normal Melting Agarose and covered with another layer of LMA. Slides were immersed in lysis solution at 4°C for 1 h. After lysis, the slides were placed in a horizontal gel electrophoresis tank filled with fresh alkaline electrophoresis buffer (300 mmol/L NaOH, 1mmol/L Na₂-EDTA, pH 13) and incubated in the solution for 20 min at 4°C. Electrophoresis (1 V/cm and 300 mA) was conducted at 4°C for 20 min, using a Bio-Rad 300 power supply. Once completed, the slides were washed 3 times with a neutralizing solution (0.4 mol/L Tris-HCl, pH = 7.5) and stained with ethidium bromide (5 μg/mL). The slides were examined at 200 × magnification under a fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. Twenty cells were selected randomly from each group as described above and analyzed by the Casp computerized image analysis system. To quantify the DNA damage, four different comet parameters were evaluated: tail length, tail DNA%, tail moment and olive tail moment.

Antioxidant ability of COS on STZ-induced diabetic rats

Male Wistar rats were rendered diabetic by intraperitoneal injection of STZ at 65 mg/kg body weight. The rats whose fasting blood glucose level was above 11.11 mmol/L were used for experiments after 7 d. Then, the rats were randomly divided into metformin treatment group, positive control group and diabetes mellitus (DM) and COS treatment groups. Test samples were given intragastrically with gavage needle for 60 d. COS treatment groups were given COSs orally at the concentrations of 250, 500 and 1500 mg/kg body weight daily. Normal control group and DM group received an equal volume of distilled water, about 10 mL/kg body weight daily. The metformin treatment group was

given metformin at a concentration of 200 mg/kg body weight. After 60 d, blood samples were collected by orbital sinus puncture after lightly anesthetizing the rats with ether. Serum was separated from the blood samples by centrifugation (5000 r/min, 10 min) and the activity of glutathione peroxidase (GPH-PX) and superoxide dismutase (SOD), the capability of total antioxidant capacity (T-AOC) and the content of malondialdehyde (MDA) were measured. Pancreas pathologic changes were observed under microscope after the small lumps (3 mm) were fixed in 4% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.2), embedded in paraffin, and stained with hematoxylin-eosin.

Statistical analysis

ANOVA was performed with Duncan's multiple range tests. SAS was used to compare the means (SAS Institute, Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant

RESULTS

COSs were prepared by enzymatic hydrolysis of chitosan. Components of the hydrolysis product were separated by Sephadex G-25 (Figure 1), and component 1 analyzed by TSK-GEL G3000PWXL had an average molecular weight of 1200 u, and a degree of deacetylation of 90% by the first derivative method of UV spectrometry.

The effect of COS on pancreatic islet cell viability was assessed by MTT assay (Figure 2). The degree of cell damage decreased with the increase of COS concentration (10-500 mg/L) and was significantly higher compared with the positive control group (Figure 3). The maximum protective effect on cell viability was achieved by 500 mg/L COS among the four samples (Figure 4).

Through DNA ladder (Figure 5) detection, we found that the injury degree of DNA fragmentation became more serious with the decrease in COS concentration (500-10 mg/L). As shown in Figure 5, the total DNA of the normal control group was a complete band and non-perceptible. An obvious DNA ladder could be seen in the positive control group treated with STZ. However, the DNA ladder disappeared with the increasing concentration of COS (10-500 mg/L). And the DNA ladder was more obviously decreased with 500 mg/L COS.

STZ can damage the DNA of the NIT-1 cells. The exposure to STZ produced significant DNA migration in the positive control group (Figure 6B). On the contrary, induction of DNA damage was rarely observed in the COS-treated group (500 mg/L) (Figure 6C). Similar differences in DNA damage between other COS groups (10-500 mg/L) and positive control group were observed after treatment with STZ. The concentration-dependent protective effect was found with COS at 10-500 mg/L, and the degree of DNA damage was decreased.

The results showed that exposure to STZ induced the breakage of DNA strands. COS can inhibit NIT-1

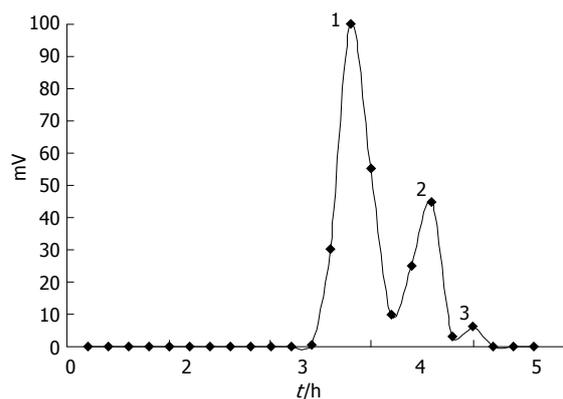


Figure 1 Chromatogram of COSs on Sephadex G-25 column.

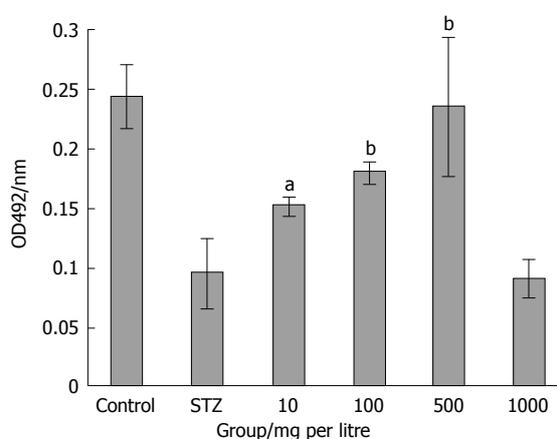


Figure 2 Protective effect of COS on the damage of NIT-1 cells induced by STZ. ^a $P < 0.05$, ^b $P < 0.01$ vs STZ group.

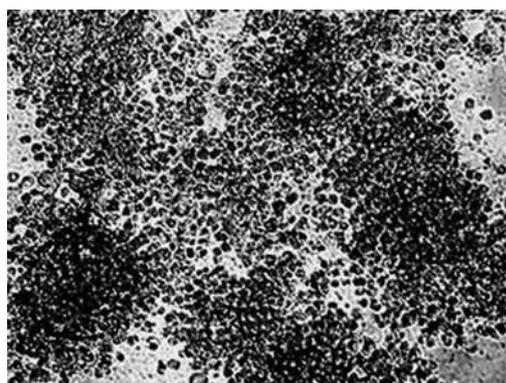


Figure 3 Morphological photograph of the positive control group ($\times 100$).

cell damage induced by STZ, and enhance the protective action of pancreatic β cells significantly (Table 1). All concentrations of COS could decrease the cells treated by STZ and had apparent dosage-dependent effects. The pretreatment of COS (500 mg/L) decreased the cells in tail length, tail DNA%, tail moment and olive tail moment significantly ($P < 0.01$).

COSs at all concentrations improved the capability of T-AOC and activity of SOD and decreased the content of MDA in serum drastically (Table 2). The capability of T-AOC was enhanced and the content of MDA decreased

Table 1 Effect of COS on changes of DNA damage in pancreatic β cells induced by STZ ($n = 9$, mean \pm SD)

Group	Concentration (mg/L)	Tail length (μ m)	Tail DNA (%)	Tail moment	Olive tail moment
Control		7.64 \pm 6.92 ^b	0.83 \pm 1.16 ^b	0.13 \pm 0.24 ^b	0.32 \pm 0.47 ^b
STZ		80.50 \pm 31.72	29.93 \pm 15.93	28.01 \pm 22.34	25.73 \pm 18.52
COS	10	49.90 \pm 33.83 ^a	28.41 \pm 23.35	20.69 \pm 21.67	17.10 \pm 14.73
	100	34.67 \pm 41.72 ^b	19.77 \pm 24.28	13.86 \pm 20.36	11.15 \pm 15.40 ^b
	500	23.00 \pm 20.79 ^b	6.56 \pm 7.57 ^b	2.90 \pm 4.12 ^b	3.64 \pm 4.54 ^b
	1000	51.00 \pm 15.44 ^a	20.14 \pm 10.51	11.48 \pm 9.42	13.06 \pm 6.61 ^a

^a $P < 0.05$, ^b $P < 0.01$ vs DM group.

Table 2 Effect of COS on changes of serum antioxidant in STZ-induced diabetes in rats ($n = 9$, mean \pm SD)

Group	Concentration (mg/kg)	T-AOC (U/mL)	GPH-PX (U/mL)	MDA (nmol/mL)	SOD (U/mL)
Control		12.10 \pm 2.58 ^b	468.61 \pm 4.35	6.21 \pm 0.53 ^b	182.51 \pm 15.73 ^b
DM		5.94 \pm 1.70	467.363 \pm 4.17	13.82 \pm 1.69	116.67 \pm 2.19
COS-H	1500	10.02 \pm 1.34 ^b	458.70 \pm 4.47	6.03 \pm 0.93 ^b	136.89 \pm 13.66
COS-M	500	7.49 \pm 1.03 ^a	461.82 \pm 10.20	6.88 \pm 1.08 ^b	141.65 \pm 11.13 ^a
COS-L	250	6.96 \pm 2.45 ^a	464.76 \pm 8.79	7.2 \pm 1.59 ^b	152.84 \pm 13.52 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs DM group.

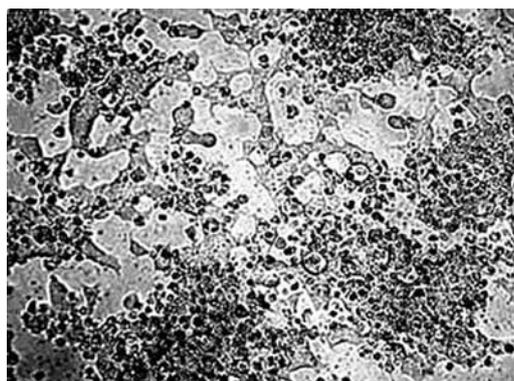


Figure 4 Morphological photograph of 500 mg/L COS protective group (\times 100).

with COS in a dosage-dependent manner.

Morphological investigation of pancreas in the DM group showed a reduction of islets, β cell loss, and nuclear pyknosis of β cells to various degrees after 60 d of STZ injection (Figure 7B). While COSs of all concentrations could protect the pancreas against STZ, the morphological observation indicates that the cells treated with medium dosage (500 mg/kg) were most protected (Figure 7C), showing similar morphological features to those of the normal control group (Figure 7A).

DISCUSSION

It has been observed that the radical scavenging properties of COSs are dependent on their DD and molecular weight. Based on the results obtained from studies carried out using electron spin trapping techniques, COSs with low molecular weight have been identified to have a higher potential to scavenge different radicals^[7]. In addition, highly deacetylated (90%) COS is more preferable to scavenge DPPH, hydroxyl, superoxide and carbon-centered radicals^[8].

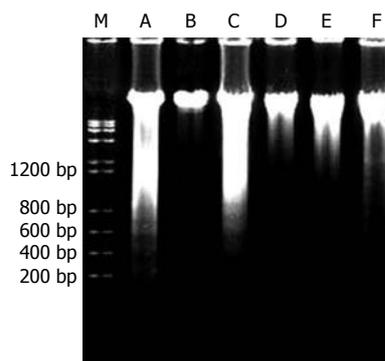


Figure 5 COS protective effect on NIT-1 cell genomic DNA presented by DNA Ladder method. M: 200 bp Marker; A: Positive control group; B: Normal control group; C: 1000 mg/L COS treatment group; D: 500 mg/L COS treatment group; E: 100 mg/L COS treatment group; F: 10 mg/L COS treatment group.

Recently, several approaches have been taken to prepare COSs, among which the enzymatic preparation methods have attracted great interest due to safety concerns. New improvements have been introduced to enzymatic production. Properties of COSs, such as DP, DA, charge distribution and nature of chemical modification to the molecule, strongly influence their observed biological activities. Therefore, molecular weight is considered as a principal characteristic of COSs that highly correlates with their biological activities. We have successfully obtained COSs with a DP of 7-9 by enzymatic hydrolysis and investigated their effects on pancreatic cell protection and their antioxidant activity *in vivo* and *in vitro*.

Many studies have suggested that the apoptotic events are often induced by the accumulation of reactive oxygen species (ROS)^[9]. The mechanisms of ROS damage to organisms mainly relate to the damage of DNA, especially the oxidative damage of mtDNA. The oxidative damage of mtDNA might cause some changes in the primary structure of DNA, such as base deletion,

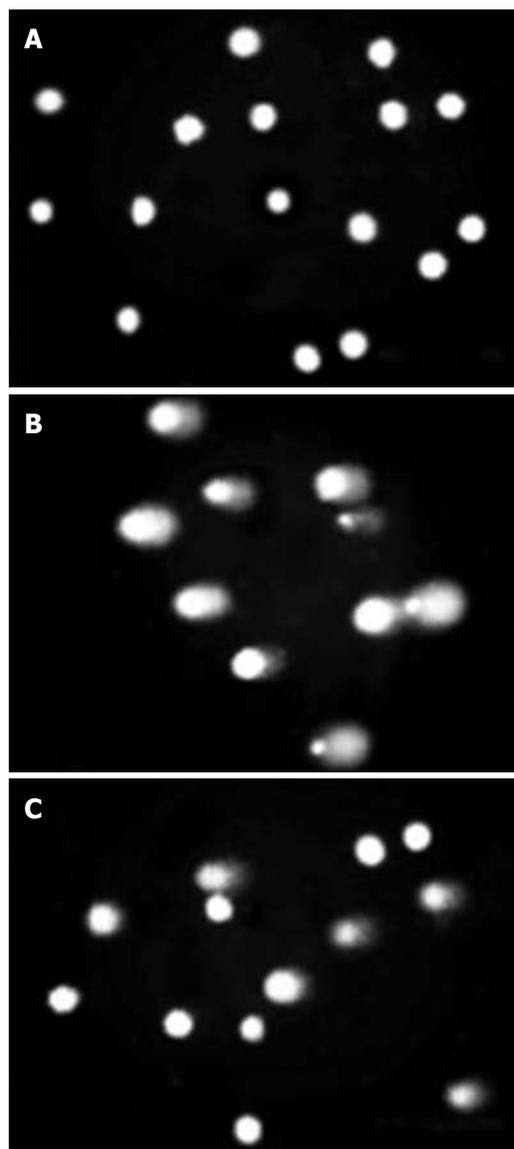


Figure 6 Photomicrograph of NIT-1 cells DNA migration in different experimental groups ($\times 200$). A: Normal control group; B: Positive control group; C: COS treatment group.

modification and insertion mutagenesis, among which base deletion is most universal. ROS can also induce base deletion, base modification, single and double-strand breakage, and interstrand DNA cross links in nuclear DNA. Most damage in DNA primary structure is investigated by DNA ladder and SCGE methods. A model of chemically induced islet cell death *in vivo* and *in vitro* is the STZ mediated islet cell destruction. STZ is an alkylating agent that causes DNA strand breakage in rat pancreatic islet cells. Recent studies strengthen the hypothesis that the toxic effects of STZ are in part mediated by NO, which is generated during its metabolism.

Our studies found obvious damage to DNA in the process of apoptosis induced by 2 mmol/L STZ. In the positive group treated with STZ only, the total DNA was degraded into approximately 180-200 bp and its multiples and a typical DNA ladder appeared on the agarose gel after electrophoresis. However, it could not

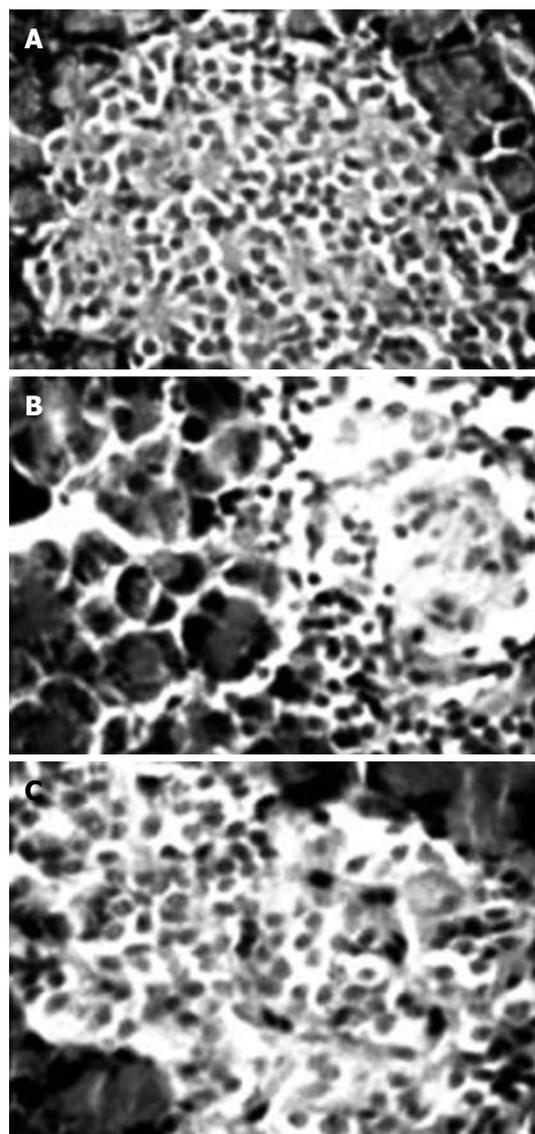


Figure 7 Photomicrograph of pancreas tissue slice in different experimental groups (HE $\times 400$). A: Normal control group; B: Positive control group; C: COS treatment group.

be detected in apoptosis induced by STZ after treatment with different concentrations of COS. SCGE assay is a powerful technique for the detection of DNA damage at alkali-sensitive sites in eukaryotic cells. Also known as the “comet” assay, it is a rapid, simple and sensitive method to quantify DNA damage in a small number of cells. During electrophoresis under alkaline conditions, cells with damaged DNA display increased migration of DNA from the nucleus towards the anode. Broken DNA migrates farther in the electric field, and the cell resembles a “comet” with a brightly fluorescent head and a tail region, which increases as damage increases. Our study showed that the long-time exposure to STZ was capable of causing a significant increase in DNA damage. Conversely, no induction of DNA damage was observed in the COS-treated group (500 mg/L). Similar differences in DNA damage between other COS groups and positive control group were observed after treatment with STZ (2 mmol/L). The results indicate that COSs have

prominent protective effects on the DNA damage in NIT-1 cells induced by STZ, and can prohibit the apoptosis of NIT-1 cells induced by STZ.

There is an effective defense mechanism of antioxidation in organisms, including SOD, CAT and GPX, which can scavenge ROS effectively. Cells are in the normal condition when the balance is maintained between oxidation and antioxidation. Once the balance is disturbed, the function will be affected, leading to apoptosis. Scavengers of free radicals are preventive antioxidants. The presence of radical scavenging compounds can break the oxidative sequence at different levels. Therefore, there has been a growing interest in the identification of natural antioxidants from many natural sources to overcome the radical-mediated deleterious effects in biological systems. Many biological compounds, including carbohydrates, peptides and some phenolic compounds, have been identified as potent radical scavengers. It has been described in previous studies that COSs can cause a rapid increase in the anti-oxidant enzymes activity to scavenge reactive oxygen. Even though the precise mechanism of radical scavenging activity of COSs is not clear, it is often attributed to the reaction between the amino and hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring and the unstable free radicals, to form stable macromolecule radicals. However, there are some discrepancies about hydroxyl radical scavenging activities of COSs and some of their derivatives. The latest studies have revealed that metal ion uptake ability of COSs has a great influence on their hydroxyl radical scavenging ability^[10]. According to their results, hydroxyl radical scavenging potency of COSs is partly due to chelating ability of transition Fe²⁺, molecular charge properties and proton donation *via* hydroxyl and amino groups. The uptake of metal ions by COSs can proceed through different mechanisms, including chelation *via* electron pairs of amino groups and ion exchange mechanisms of protonated amino groups^[11]. Therefore, it further strengthens the effect of DD on radical scavenging which is directly correlated with protonation of amine groups. However, there is not much information about the relationship between ion chelation and antioxidant properties of COSs to date.

Our study showed that COSs of all concentrations improved the capability of T-AOC and activity of SOD and drastically decreased the content of MDA. Morphological investigation of the pancreas showed that COSs have significant effects on the reduction of islets, pancreatic cell loss and nuclear pyknosis of pancreatic cells, and can protect the pancreas against STZ. The possible mechanism of COSs' protective function in the pancreas is that they have strong *in vivo* antioxidative properties, so that they can scavenge directly the reactive free radicals such as hydroxyl radical and superoxide anion, and inhibit DNA damage, to protect pancreas against oxidative damage induced by STZ.

The highly increasing prevalence of obesity and type 2 DM in the general population makes non-alcoholic fatty liver disease (NAFLD) the most common diagnosis

in clinical practice^[12]. Our study provided evidence for the protective effects of COSs in pancreatic β -cells against STZ *in vitro* and confirmed the usefulness of COSs in improving antioxidant ability and protecting the pancreas islet of STZ-induced diabetes in rats *in vivo*. These biological activities have considerable potential in DM and NAFLD.

ACKNOWLEDGMENTS

We thank Ms. Jin Liang for her help in revising this paper.

COMMENTS

Background

Chitooligosaccharide (COS) possesses various biological activities and has been shown to be particularly useful in diabetes treatment. Several methods have been recently used to prepare COS, and enzymatic preparation methods capture a great interest due to their safety and non-toxicity.

Research frontiers

Pancreatic β cells maintain the blood glucose concentration within a narrow range by modulating insulin secretion rate in response to the glucose levels in the blood. Proper insulin secretion requires the coordinated functioning of numerous β cells that form pancreatic islets. This coordination depends on a network of communication mechanisms whereby β cells interact with extracellular signals and adjacent cells *via* connexin channels. This study indicated that COSs improved the antioxidant ability and protection of the pancreas islet of streptozotocin (STZ)-induced diabetes in rats *in vivo*.

Innovations and breakthroughs

Soluble COSs with lower molecular weight were prepared by enzymatic hydrolysis of chitosan with chitosanase. This study indicated that COSs could improve the antioxidant ability and protect the pancreas islet against STZ-induced diabetes in rats *in vivo*.

Applications

This study indicated that COS could improve the antioxidant ability and protect the pancreas islets of STZ-induced diabetes in rats *in vivo*. These biological activities have considerable potential in diabetes mellitus treatment.

Terminology

COS is a polycationic copolymer consisting of β -1, 4-linked 2-acetamido-D-glucose and β -1, 4-linked 2-amino-D-glucose units. COS obtained by hydrolysis or degradation of chitosan, is not only water-soluble but also more effective than chitosan. NIT-1 cell line, a widely used β cell line in insulin secretion studies, is established from non-obese diabetic mice transgenic for the SV40 T antigen under the control of insulin promoter.

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Curcumin suppresses PPAR δ expression and related genes in HT-29 cells

Jin-Bo Wang, Li-Li Qi, Shui-Di Zheng, Heng-Zheng Wang, Tian-Xing Wu

Jin-Bo Wang, Tian-Xing Wu, Department of Chemistry, College of Science, Zhejiang University, Hanzhou 310027, Zhejiang Province, China

Jin-Bo Wang, Li-Li Qi, Shui-Di Zheng, Heng-Zheng Wang, Tian-Xing Wu, Ningbo Institute of Technology, Zhejiang University, Ningbo 315100, Zhejiang Province, China

Author contributions: Wang JB, Wu TX designed the study; Wang JB, Qi LL, Zheng SD and Wang HZ performed the research and analyzed the data; Wang JB wrote the paper.

Correspondence to: Tian-Xing Wu, Ningbo Institute of Technology, Zhejiang University, NO. 1 Xuefu Road, Ningbo 315100, Zhejiang Province, China. wangjbmail@yahoo.com.cn

Telephone: +86-574-88229516 Fax: +86-574-88229516

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Abstract

AIM: To investigate the effects of curcumin on the expression of peroxisome proliferator-activated receptor δ (PPAR δ) and related genes in HT-29 cells.

METHODS: HT-29 cells were treated with curcumin (0-80 μ mol/L) for 24 h. The effects of curcumin on the morphology of HT-29 cells were studied by Hoechst 33342 staining. The activity of caspase-3 was determined using DEVD-pNA as substrate. The levels of peroxisome PPAR δ , 14-3-3 ϵ and vascular endothelial growth factor (VEGF) in HT-29 cells were determined by Western blotting analysis and their mRNA expression was determined by real-time quantitative RT-PCR.

RESULTS: Treatment with 10-80 μ mol/L curcumin induced typical features of apoptosis and activated the caspase-3 in HT-29 cells. The expression of PPAR δ , 14-3-3 ϵ and VEGF was reduced and the activity of β -catenin/Tcf-4 signaling was inhibited by curcumin treatment.

CONCLUSION: Curcumin can induce apoptosis of HT-29 cells and down-regulate the expression of PPAR δ , 14-3-3 ϵ and VEGF in HT-29.

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Key words: Curcumin; 14-3-3 ϵ ; Peroxisome proliferator-activated receptor δ ; Vascular endothelial growth factor; HT-29 cells

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INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily that enable the cell to respond to extracellular stimuli through transcriptional regulation of gene expression^[1,2]. PPARs comprise three subtypes: PPAR α , PPAR δ and PPAR γ . Many of the functions of PPARs are associated with pathways of lipid transport and metabolism^[3-5]. Moreover, PPARs play important roles in cell replication, differentiation, tumorigenesis and apoptosis. For example, the expression of PPAR δ is elevated in human and rat colorectal cancer cells when compared with normal colon epithelial cells^[6,7]. PPAR δ has also been implicated in the growth of other human cancers, including hepatocellular carcinoma, cholangiocarcinoma, breast cancer and prostate cancer^[8,9].

14-3-3 proteins are anti-apoptotic and anti-inflammatory molecules in cells, which include at least 7 isoforms (β , γ , ϵ , η , ζ , σ , τ/θ)^[10]. 14-3-3 can bind phosphorylated Bad, sequester Bad in the cytosol, and inhibit cytochrome c release, caspase-3 activation, and the apoptosis of cells. PPAR δ can induce the expression of 14-3-3 ϵ protein. Elevated 14-3-3 ϵ augments Bad sequestration and prevents Bad-triggered apoptosis^[10]. C/EBP β protein is a mediator of PPAR δ -dependent 14-3-3 ϵ gene regulation in human endothelial cells. PPAR δ can regulate the expression of C/EBP β protein, which can bind to the C/EBP response element located at -160/-151 of the 14-3-3 ϵ gene^[11]. PPAR δ can directly bind to PPAR response elements located between -1426 and -1477 of the 14-3-3 ϵ promoter region, thereby activating 14-3-3 ϵ promoter activity and protein expression^[10]. PPAR δ can also regulate the expression of vascular endothelial growth factor (VEGF), which can promote colon tumor epithelial cell survival^[12,13].

Curcumin is an important polyphenol extracted from the rhizomes of *Curcuma longa* L. Several studies have shown that curcumin exerts antioxidant, anti-inflammatory, anti-carcinogenic and chemopreventive activities on many tumor cells^[14]. Curcumin can also down-regulate the activity of the β -catenin/Tcf signaling pathway^[15,16]. Curcumin affects the expression of the target genes of β -catenin/Tcf signaling pathway, such as c-Myc, cyclin D1 and c-Jun. PPAR δ has been identified as another β -catenin/Tcf-regulated gene^[6]. However, the effect of curcumin on the expression of PPAR δ remains unknown. In this study, we investigated the effects of curcumin on the expression of PPAR δ and related genes such as 14-3-3 ϵ and VEGF. The results showed that curcumin could inhibit the expression of PPAR δ and induce the down-regulation of the related genes, including 14-3-3 ϵ and VEGF.

MATERIALS AND METHODS

Reagents

RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin were purchased from GIBCO. Curcumin, sodium dodecylsulfate (SDS), phenylmethylsulfonylfluoride (PMSF), DNaseI and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-VEGF, anti-Tcf-4 and horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Epitomics. Anti-14-3-3 ϵ , anti-PPAR δ antibody and protein A/G plus-agarose were provided by Santa Cruz Biotechnology. Nitrocellulose membrane and the enhanced chemiluminescence (ECL) detection system were purchased from Amersham (USA). PrimeScript 1st strand cDNA Synthesis Kit and PCR Kit were from Takara, Japan. Caspase-3 assay kit and nuclear and cytoplasmic protein extraction kit were purchased from Beyotime Biotech, China. Other reagents used were of analytical grade and procured locally.

Cell culture and treatment

The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640, supplemented with 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Upon reaching 70%-80% confluence, the cells were exposed to 0-80 μ mol/L curcumin for 24 h.

Measurement of caspase-3 activities

Caspase-3 activities were measured as previously described^[17]. Briefly, cells were lysed in a buffer containing 5 mmol/L Tris (pH 8), 20 mmol/L EDTA, and 0.5% Triton-X 100. Reaction mixture contained 20 mmol/L HEPES (pH 7.0), 10% glycerol, 2 mmol/L dithiothreitol, 50 μ g protein per condition, and 200 μ mol/L DEVD-pNA as substrate. After incubation for 24 h at 37°C, the absorbance in each well was measured at 405 nm with a microplate ELISA reader.

Nuclear staining with Hoechst 33342

Chromatin condensation was detected by nuclear staining with Hoechst 33342^[18]. After treatment with 0-80 μ mol/L curcumin for 24 h, cells were harvested and washed with PBS three times. Then, the cells were stained with 1 μ L of Hoechst 33342 (5 mg/mL, Sigma) in 1 mL basal medium and incubated at room temperature in the dark for 15 min. Stained cells were imaged under a fluorescent microscope using 350 nm stimulation and 460 nm emission.

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was isolated using Trizol Isolation Reagent (Invitrogen, USA). RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density (OD) at 260 nm. One microgram total RNA was used for DNaseI treatment (Sigma) and subsequent cDNA synthesis. Reverse-transcription was performed with PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's instructions. Real-time qPCR was performed on the ABI 7500 Real Time PCR System using SYBR Premix Ex Taq II (Takara, Japan) for analyzing expression of genes. Table 1 shows the primers used for real-time quantitative RT-PCR. The amplification reactions were performed under the following PCR conditions: one cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 59°C for 15 s and 72°C for 30 s. mRNA fold changes in target genes relative to the endogenous GAPDH control were calculated as suggested by Schmittgen *et al.*^[19]. Each reaction was performed in triplicate.

Western blotting

Western blotting analysis was done as previously described with minor modifications to detect the expressions of PPAR δ , 14-3-3 ϵ , and VEGF protein^[20]. The total cellular protein and the nuclear protein were extracted according to the instructions of nuclear and cytoplasmic extraction reagents kit (Beyotime, Haimen, China). The lysates were used to estimate their protein content with BCA protein assay. Fifty micro-grams of protein from each sample was subjected to SDS-PAGE. After electrophoresis, proteins were electroblotted to a Hybond-C Extra nitrocellulose membrane (Amersham, USA). The membrane was blocked at room temperature with 5% non-fat dry milk in TBS containing 0.3% Tween (TBS-T). The membrane was washed three times with TBS-T and incubated overnight at 4°C with the primary antibody, anti-PPAR δ (1:500), anti-14-3-3 ϵ (1:2000), and anti-VEGF (1:1000), followed by 1 h incubation with a 1:5000 dilution of the appropriate horseradish-peroxidase-conjugated secondary antibody. After incubation, the membrane was washed with TBS-T for three times, the antigen-antibody complexes were visualized by enhanced chemiluminescence and exposure to X-ray film for 0.5 up to 30 min.

Table 1 Primers used for real-time quantitative RT-PCR

Apoptosis modulator	Forward primer (5'→3')	Reverse primer (5'→3')
PPAR δ	GCAGGCTCTAGAATTCATC	GTGCAGCCTTAGTACATGTC
VEGF	AGGAGGAGGGCAGAATCATCA	CTCGATTGGATGGCAGTAGCT
14-3-3 ϵ	GAGCGATACGACGAAATGGT	CCTTGGACTCGCCAGTGTAG
GAPDH	GGCAAATCCAACGGCACAGT	AGATGGTGATGGGCTTCCC

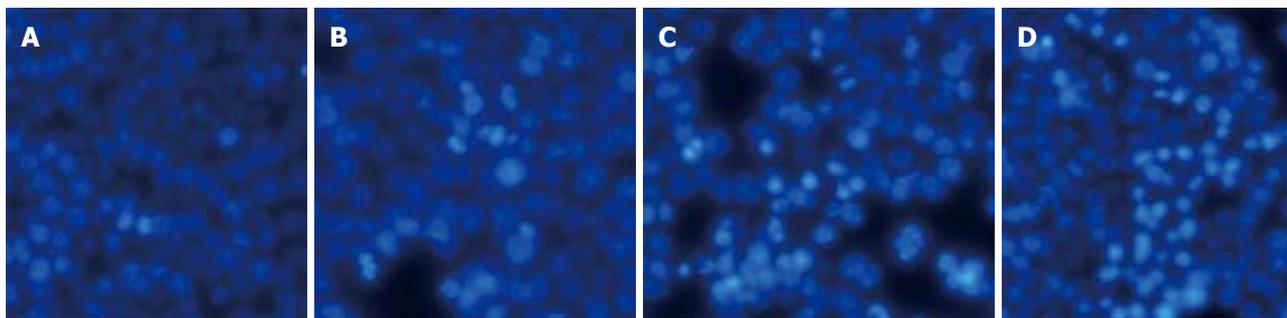


Figure 1 Induction of apoptosis in HT-29 cells by curcumin. Fluorescence images of HT-29 cells using Hoechst 33258 staining showed curcumin induced typical apoptotic morphological changes. A: Control; B: HT-29 cells incubated with 20 $\mu\text{mol/L}$ curcumin; C: HT-29 cells incubated with 40 $\mu\text{mol/L}$ curcumin; D: HT-29 cells incubated with 60 $\mu\text{mol/L}$ curcumin.

Immunoprecipitation

The immunoprecipitation was done as previously described with minor modifications^[16]. The nuclear lysates containing 500 μg protein were incubated with 5 μg primary antibody overnight at 4°C. Fifty microliters of protein A/G plus-agarose (Santa Cruz Biotechnology) was added and the complex was incubated at 4°C overnight. The beads were washed three times with high salt buffer (1 mol/L Tris-HCl, pH 7.4, 0.50 mol/L NaCl, and 1% Nonidet P-40) and twice with lysis buffer to eliminate non-specific binding. The immunoprecipitated complexes were released with 2 \times sample buffer for Western analysis.

Transfection and luciferase assay

Transient transfection was performed using Fugene 6 Transfection Reagent (Roche) in accordance with the manufacturer's instructions. Briefly, HT-29 cells were seeded at a density of 2×10^5 cells per well in six-well plates. After 24 h, cells were transfected with 0.5 μg luciferase reporter constructs (TOPflash or FOPflash, respectively) and 0.5 μg β -galactosidase gene. Three hours after transfection, the cells were treated with 0-80 $\mu\text{mol/L}$ curcumin and the incubation was continued for 24 h. Then, the cells were collected and resuspended in Luciferase Reporter Lysis Buffer (Promega, USA). The cell lysates were centrifuged and aliquots (70 μL) of the supernatant were assayed for the activity of luciferase and galactosidase. Reporter activity was normalized for variations in transfection efficiency using β -galactosidase as an internal control. Experiments were performed three times independently.

Statistical analysis

Results are presented as mean \pm SE. Comparisons

between multiple groups were performed using the one-way ANOVA followed by Dunnett's test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Induction of HT-29 cell apoptosis by curcumin

Hoechst 33342 staining assay was performed to observe the effects of curcumin on cell nuclear morphology. As shown in Figure 1, the control cells displayed intact nuclear structure, while nuclei with chromatin condensation and formation of apoptotic bodies were seen in cells incubated with curcumin in a dose-dependent fashion (Figure 1A-D). Caspase-3 activation is an important marker of apoptosis, so we determined the activities of caspase-3 in HT-29 treated with curcumin for 12 or 24 h. Caspase-3 activities were highly elevated by curcumin at the concentration of 20-80 $\mu\text{mol/L}$ (Figure 2).

Curcumin suppressed the expression of PPAR δ

PPAR δ plays important roles in the growth and proliferation of many cancer cells^[8]. PPAR δ has been identified as one of the down-streaming targets of the β -catenin/Tcf-4 pathway^[6]. We studied whether curcumin affected the expression of PPAR δ in HT-29 cells. HT-29 cells were incubated with curcumin at different concentrations. PPAR δ gene expression at the mRNA level was detected by quantitative RT-PCR. The results showed that 40-80 $\mu\text{mol/L}$ of curcumin could significantly reduce the level of PPAR δ in HT-29 cells (Figure 3A). Whole-cell extracts were prepared and analyzed by Western blotting. Curcumin reduced the level of PPAR δ protein in a dose-dependent manner (Figure 3B).

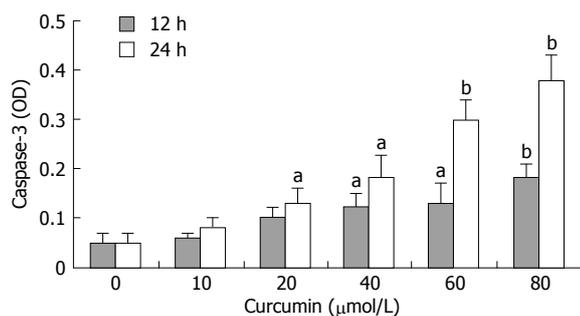


Figure 2 Caspase-3 activity was markedly increased by curcumin in a dose-dependent manner. Each bar denotes mean \pm SD ($n = 3$). (^a $P < 0.05$ vs control; ^b $P < 0.01$ vs control).

Curcumin decreased the expression of 14-3-3 ϵ and VEGF

14-3-3 ϵ plays important roles in protecting cells from apoptosis^[21]. It harbors three contiguous PPAR response elements (PPREs), which are the responsive promoter region of 14-3-3 ϵ activation. Deletion of PPREs abrogated PPAR δ -mediated 14-3-3 ϵ to PPAR δ up-regulation^[10]. On the basis of this study, we hypothesized that curcumin could inhibit the expression of 14-3-3 ϵ in HT-29 cells. The real-time quantitative PCR showed that treatment with 60 and 80 $\mu\text{mol/L}$ curcumin for 24 h could markedly reduce the 14-3-3 ϵ mRNA level in HT-29 cells (Figure 4A). The Western blotting showed that curcumin treatment also inhibited the 14-3-3 ϵ protein expression at the concentration of 20-80 $\mu\text{mol/L}$ (Figure 4C).

VEGF can stimulate endothelial cell proliferation and prevents apoptosis in the endothelial cells of newly formed vessels^[22]. VEGF has been identified as one of the potential targets of PPAR δ in colorectal cancer (CRC) cells^[13]. We hypothesized that curcumin can decrease the expression of VEGF. The real-time quantitative RT-PCR showed that 40-80 $\mu\text{mol/L}$ of curcumin could significantly down-regulate the expression of VEGF (Figure 4B). Consistent with the VEGF mRNA level, VEGF protein was also decreased by curcumin at the concentrations of 20-80 $\mu\text{mol/L}$ (Figure 4D).

Curcumin down-regulated β -catenin/Tcf signaling pathway and inhibited β -catenin associated with Tcf-4 in nuclei of HT-29 cells

The association of β -catenin with Tcf-4 is required for activation of β -catenin/Tcf signaling, so we determined the level of the β -catenin/Tcf-4 complex in the nucleus of HT-29 cells. We used anti-Tcf-4 antibody to co-immunoprecipitate the complex of Tcf-4 and β -catenin from nuclear extracts and determined the amount of β -catenin by immunoblotting. Curcumin markedly decreased the level of β -catenin/Tcf-4 complex (Figure 5A). However, the level of total β -catenin in the nucleus was not significantly affected by incubation of curcumin (Figure 5A). These results suggested that curcumin could inhibit β -catenin associated with Tcf-4 in the nucleus. Because PPAR δ is a target gene of β -catenin/Tcf-4, curcumin may decrease the expression of PPAR δ through

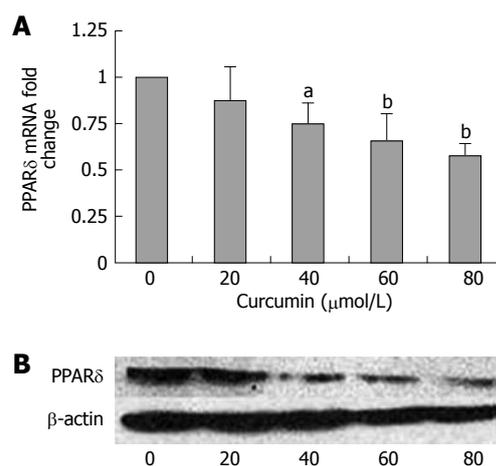


Figure 3 Curcumin inhibits the expression of PPAR δ in HT-29 cells. A: Real-time quantitative RT-PCR indicated that curcumin markedly reduced the level of PPAR δ mRNA. Values are % reduction in PPAR δ mRNA fold changes caused by curcumin compared with cells without curcumin treatment. Values are mean \pm SD from three samples per group. GAPDH was used as an internal control; B: The effects of curcumin on the level of PPAR δ protein were determined by Western blotting. β -actin was used as an internal marker. (^a $P < 0.05$ vs control; ^b $P < 0.01$ vs control).

blocking the β -catenin/Tcf-4 signaling pathway.

PPAR δ has been identified as a target of the β -catenin/Tcf signaling pathway^[6]. We hypothesized that curcumin can inhibit the expression *via* the β -catenin/Tcf signaling pathway. We transfected HT-29 cells with either TOPflash or FOPflash. The cells were incubated with 0-80 $\mu\text{mol/L}$ curcumin for 24 h and the luciferase activity was determined. As a result, curcumin could decrease the transcriptional activity of β -catenin/Tcf-4 signaling pathway in HT-29 cells (Figure 5B).

DISCUSSION

Curcumin can induce apoptosis of many cell lines. However, the mechanism is still unclear. In this study, we demonstrated that curcumin could induce the apoptosis of HT-29 cells and down-regulate the expression of PPAR δ , 14-3-3 ϵ and VEGF, which suggested that curcumin-induced apoptosis was attributed to the inhibition of the expression of PPAR δ , 14-3-3 ϵ or VEGF.

The PPARs are ligand-activated transcription factors that are members of the nuclear hormone receptor superfamily. PPARs form heterodimers with the retinoic X receptor and bind to DNA in correspondence to specific PPRE located in the promoter of target genes^[23]. The PPAR subfamily includes PPAR α , PPAR δ (or β) and PPAR γ , which share extensive structural homology^[23]. Studies have shown that PPAR α and PPAR γ play important roles in such physiological processes as fatty acid metabolism, glucose metabolism, immunity, and cellular differentiation^[24,25]. However, the physiological role of PPAR δ is less studied. Recently, PPAR δ has been found to be related to carcinogenesis^[26,27]. Curcumin has been proved to inhibit the growth and differentiation of many cancer cell lines^[28,29]. Administration of 30 mg/kg curcumin by intraperitoneal injection for 2 wk could

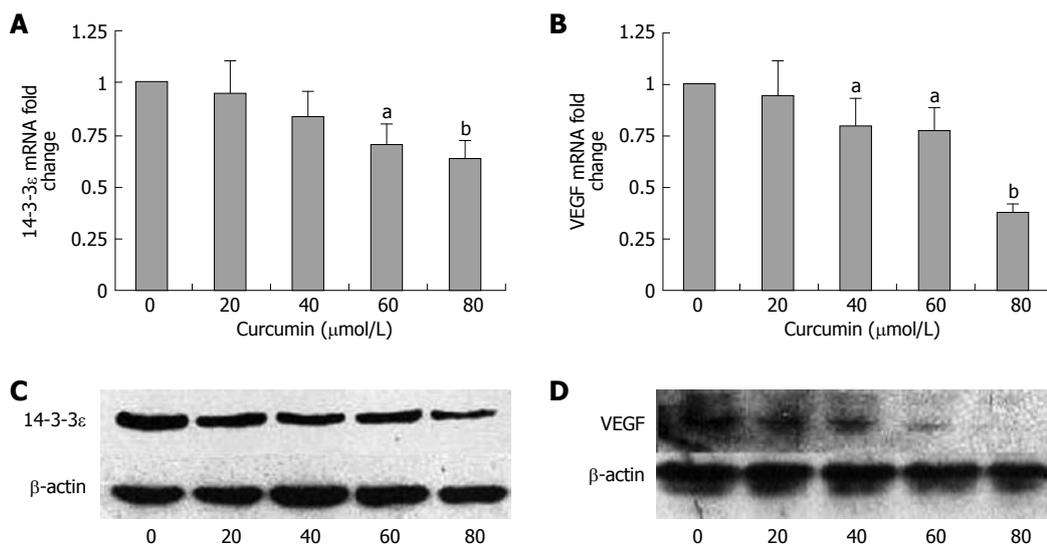


Figure 4 The expression of 14-3-3 ϵ and VEGF is down-regulated by curcumin. Real-time quantitative RT-PCR showed that curcumin decreased the mRNA levels of 14-3-3 ϵ (A) and VEGF (B). Values are means \pm SD from three samples per group. GAPDH was used as an internal control for quantitative RT-PCR. Western blotting was performed to determine the effects of curcumin on the expression of 14-3-3 ϵ (C) and VEGF (D). β -actin was used as internal marker for Western blotting. (^a P < 0.05 vs control; ^b P < 0.01 vs control).

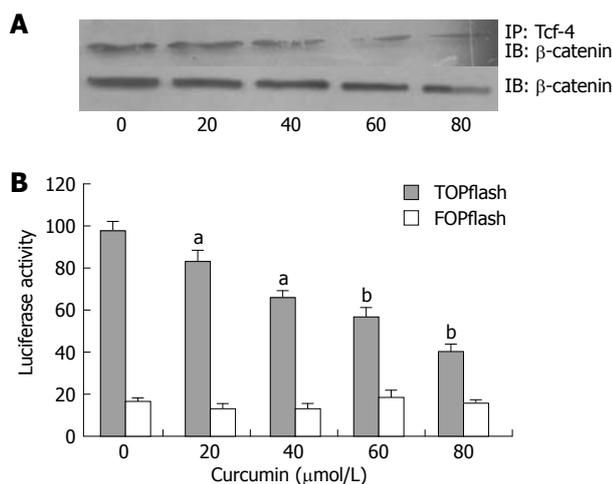


Figure 5 Curcumin inhibited β -catenin association with Tcf-4 and down-regulated the β -catenin/Tcf reporter activities in HT-29 cells. A: Immunoprecipitation and immunoblotting results showed that curcumin inhibited β -catenin associated with Tcf-4 but had no effects on the level of β -catenin in nucleus; B: Curcumin significantly reduced TOPflash luciferase activity but had no effect on the activity of FOPflash. Values represent means \pm SE of three independent experiments. (^a P < 0.05 vs control; ^b P < 0.01 vs control).

significantly improve the expression of PPAR γ in colonic tissues of rats^[30]. To the best of our knowledge, the effects of curcumin on PPAR δ have not been investigated. In this study, we found that curcumin could inhibit the expression of PPAR δ in HT-29 cells. We hypothesize that there are two possible reasons. First, He *et al*^[6] have identified PPAR δ as a β -catenin/Tcf-regulated gene. In this study, curcumin could inhibit the β -catenin associated with Tcf-4 and down-regulate the activity of the β -catenin/Tcf-4 signaling pathway, which is in agreement with the studies of Jaiswal *et al* and Park *et al*^[15,16]. These studies indicate that curcumin may reduce the expression of PPAR δ by blocking the β -catenin/Tcf-4 signaling pathway. Second, PPAR δ can also be up-regulated by oncogenic K-Ras^[31],

suggesting that curcumin may reduce the expression of PPAR δ by other signaling pathways. We will further study the mechanism that curcumin inhibits the expression of PPAR δ .

14-3-3 are cytosolic proteins serving as a scaffold to interact with a large number of proteins^[32]. They may protect cells from apoptosis through their binding and sequestering phosphorylated Bad in cytosol^[21]. Seven isoforms of 14-3-3 proteins have been identified in mammalian cells^[10]. It has been indicated that 14-3-3 ϵ is a target gene of PPAR δ , which can bind to the PPRE upstream of 14-3-3 ϵ promoter region^[10]. We found that curcumin decreased the expression of 14-3-3 ϵ in HT-29 cells. To the best of our knowledge, curcumin-induced down-regulation of 14-3-3 ϵ has not reported previously.

VEGF is also up-regulated by the activation of PPAR δ in colon carcinoma cells^[12]. Several studies have indicated that curcumin can down-regulate the expression of VEGF in different cell lines^[33,34]. In the present study, we found that curcumin could inhibit the expression of VEGF in HT-29 cells. VEGF plays important roles in tumor angiogenesis^[35]. Our results suggest curcumin may inhibit the angiogenesis of colorectal tumors.

In summary, our study shows that curcumin can suppress the expression of PPAR δ and the related genes such as 14-3-3 ϵ and VEGF in HT-29 cells. The reasons why curcumin inhibits the expression of PPAR δ and related protein are still unclear. Curcumin can down-regulate the activity of β -catenin/Tcf-4 signaling pathway, which suggests that curcumin may decrease the expression of PPAR δ by the inhibition of β -catenin/Tcf-4 signaling activity in HT-29 cells.

COMMENTS

Background

Colorectal cancer is one of the leading causes of death and is a major public health problem in Western countries. Peroxisome proliferator-activated

receptors (PPARs) play important roles in cell replication, differentiation, tumorigenesis, and apoptosis. The expression of PPAR δ is elevated in human and rat colorectal cancer cells when compared with normal colon epithelial cells.

Research frontiers

Curcumin can affect the expression of the target genes of β -catenin/Tcf signaling pathway, such as c-Myc, cyclin D1, c-Jun, *etc.* PPAR δ has been identified as another β -catenin/Tcf-regulated gene. However, the effect of curcumin on the expression of PPAR δ remains unknown. In this study, the authors demonstrate that curcumin could affect the expression of PPAR δ and related genes such as 14-3-3 ϵ and vascular endothelial growth factor (VEGF).

Innovations and breakthroughs

Recent studies have suggested the PPAR δ play important roles in colorectal carcinogenesis. It activates the expression of 14-3-3 ϵ , which can sequester the pro-apoptotic protein, Bad, to inhibit the apoptosis of cancer cells. It is the first report showing that the curcumin down-regulates the expression of PPAR δ and 14-3-3 ϵ .

Applications

PPAR δ is found as a new target of curcumin to induce the apoptosis of HT-29 cells. New curcumin derivatives can be developed to efficiently inhibit the growth and differentiation of colorectal cancer cells by down-regulating the expression of PPAR δ .

Terminology

Curcumin is an important polyphenol extracted from the rhizomes of *Curcuma longa* L. PPARs belong to the nuclear hormone receptor superfamily that enable the cell to respond to extracellular stimuli through transcriptional regulation of gene expression, including PPAR α , PPAR δ , and PPAR γ . 14-3-3 proteins are anti-apoptotic and anti-inflammatory molecules in cells, which include at least seven isoforms (β , γ , ϵ , η , ζ , σ , τ/θ). Elevated 14-3-3 ϵ augments Bad sequestration and prevents Bad-triggered apoptosis.

Peer review

Curcumin can induce apoptosis of many cell lines. However, the mechanism is still unclear. In this study, the authors demonstrated that curcumin could induce the apoptosis of HT-29 cells and down-regulate the expression of PPAR δ , 14-3-3 ϵ and VEGF. More importantly, the authors showed that curcumin could markedly lower the level of β -catenin/Tcf-4 complex without affecting the level of total β -catenin in nucleus after incubation of curcumin. These results suggested that curcumin could inhibit β -catenin associated with Tcf-4 in nucleus.

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Management of pancreaticobiliary disease using a new intra-ductal endoscope: The Texas experience

Douglas S Fishman, Paul R Tarnasky, Sandeep N Patel, Isaac Raijman

Douglas S Fishman, Texas Children's Hospital, Section of Pediatric Gastroenterology and Nutrition, Baylor College of Medicine, 6621 Fannin Street, Houston, TX 77030, United States
Paul R Tarnasky, Digestive Health Associates, Methodist Pavilion II 221 West Colorado Blvd. Dallas, TX 75208-2359, United States

Sandeep N Patel, Division of Gastroenterology and Nutrition, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive San Antonio, TX 78229-3900, United States

Isaac Raijman, Digestive Associates of Houston, 6620 Main Street. Houston, TX 77030, United States

Author contributions: Fishman DS wrote the manuscript, Tarnasky PR and Patel SN performed procedures and were involved in editing the manuscript; Raijman I designed the study, performed procedures and wrote the manuscript.

Correspondence to: Isaac Raijman, MD, Digestive Associates of Houston, 6620 Main Street. Houston, TX 77030, United States. iraijman@dahpa.com

Telephone: +1-713-7955260 Fax: +1-713-7955254

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Abstract

AIM: To evaluate a new single-operator mini-endoscope, Spyglass®, for its performance, feasibility and safety in the management of pancreaticobiliary disease.

METHODS: In a multicenter retrospective analysis of patients undergoing intraductal endoscopy, we evaluated 128 patients (71 men, mean age 57.6 years). Indications were therapeutic (TX) in 72 (56%) and diagnostic (DX) in 56 (44%).

RESULTS: Peroral endoscopy was performed in 121 and percutaneous in seven. TX indications included CBD stones in 41, PD stones in six, and biliary strictures in 25. DX indications included abnormal LFT's in 15, abnormal imaging in 38 and cholangiocarcinoma staging in three. Visualization of the stone(s) was considered good in 31, fair in six, and poor in four. Advancement of the electrohydraulic lithotripsy probe was not possible in three patients and proper targeting of the lesion was partial in four patients. A holmium laser was used successfully in three patients. Ductal clearance was achieved in 37 patients after one procedure and in four patients after two procedures.

Diagnosis of biliary strictures was modified in 20/29 and confirmed to be malignant in 10/23. Of the modified patients, no diagnosis was available in 17. Spyglass® demonstrated malignancy in 8/17 and non-malignancy in nine. Suspected pathology by imaging studies and abnormal LFT's was modified in 43/63 (66%). Staging of cholangiocarcinoma demonstrated multicentric cholangiocarcinoma in 2/3. There was no morbidity associated with the use of Spyglass®.

CONCLUSION: Spyglass Spyscope® is a first generation, single operator miniature endoscope that can evaluate and treat various biliary and pancreatic tract diseases.

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Key words: Intra-ductal endoscopy; Choledochoscopy; Cholangiopancreatography; Endoscopic retrograde cholangiopancreatography; Biliary disease; Sclerosing cholangitis; Cholangiocarcinoma; Lithotripsy; Pancreatic disease; Spyglass®

Peer reviewer: Taku Aoki, MD, Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

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INTRODUCTION

Intraductal endoscopy is an integral part of the evaluation and therapy of patients with biliary and pancreatic diseases. The use of cholangiopancreatography was first described in the mid-1970s. The initial "baby" or "daughter" scopes were passed through the operating channel of the "mother" duodenoscope. These early endoscopes were not widely used due to cost, fragility, difficult maneuverability, limited optical resolution, and were easily damaged at the level of the duodenoscope elevator. In addition, these endoscopes required two trained endoscopists to operate. Due to these challenges,

the field of intraductal endoscopy did advance for several years^[1,2]. Newer miniature endoscopes offer improved steering ability, durability, better optics and smaller size. These endoscopes have been increasingly used for diagnostic and therapeutic applications^[3-5]. Spyglass Spyscope® (Boston Scientific, Natick, MA), is a new single-operator endoscope that has been recently introduced into the endoscopic arena^[6]. It includes the Spyscope® which is 10 Fr in diameter, has a 4-way tip deflection and has a 1.2 mm working channel. It houses the Spyglass® optical fiber, an independent accessory channel and a separate channel for water irrigation.

We evaluated the Spyglass Spyscope® for its performance, feasibility and safety.

MATERIALS AND METHODS

In this multicenter (three tertiary institutions; Houston, Dallas and San Antonio, TX) retrospective analysis, we evaluated 128 patients (71 males, 57 women, mean age 57.6 years) with various pancreatobiliary disorders (Table 1). Of the procedures to which the patients had been subjected, 56 (44%) were diagnostic and 72 were therapeutic (56%). Diagnostic indications included abnormal serum liver tests in 15, abnormal imaging studies in 38, and staging of cholangiocarcinoma in three. Therapeutic indications included choledocholithiasis in 41, pancreatic stones in six and biliary strictures in 25. The majority of procedures were performed per-orally (121), with the remaining seven performed percutaneously. All procedures were performed under monitored anesthesia, had prophylactic intravenous antibiotics, and the majority were performed on an outpatient basis. Most patients had a previous endoscopic retrograde cholangiopancreatography (ERCP), and had a previous biliary sphincterotomy. All procedures were performed by an experienced biliary endoscopist.

Abnormal imaging studies included computed tomography, magnetic resonance and endoscopic ultrasound. Therapeutic intervention was performed with electrohydraulic lithotripsy (EHL) and a holmium laser. Biopsies were obtained with the Spybite® as well as with standard biopsy forceps.

Spyglass Spyscope® procedure was performed by attaching the Spyglass Spyscope® to the duodenoscope, at the junction of the head and the shaft. The Spyscope® was introduced over a guide wire through the accessory channel. The Spyglass® was advanced through the Spyscope® just to its tip. The Spyglass Spyscope® apparatus was then advanced through the duodenoscope and then into either the bile duct or the pancreatic duct. The guide wire was then removed to facilitate Spyscope® tip deflection.

RESULTS

Diagnostic purposes

The main indication was determination of biological behavior of bile duct strictures (Table 2). The diagnosis of biliary strictures was modified in 20 of 29 patients. All 29 patients had a preoperative diagnosis of

Table 1 Patient characteristics

Patient characteristics	Cases (n)
Patients	128
Male:female	71:57
Peroral usage	121
Percutaneous usage	7
Diagnostic indications	56 (44%)
Abnormal serum liver test	15
Abnormal imaging studies	38
Cholangiocarcinoma staging	3
Therapeutic indications	72 (56%)
Choledocholithiasis	41
Pancreaticolithiasis	6
Biliary strictures	25

Table 2 Diagnostic Spyglass® indications

Diagnostic Spyglass® indications	Cases (n)
Abnormal imaging and elevated liver panel	
Normal	9
Malignancy	9
Ductal ulcerations	7
Lithiasis	7
Extrinsic compression	7
Benign polypoid lesions	4
Total	43
Biliary strictures	
Diagnosis modified	20/29
Diagnosis confirmed malignant	10/23
Pre-operative diagnosis unknown	17
Post-operative diagnosis malignant	9
Post-operative diagnosis benign	8

malignant bile duct stricture (three had established cholangiocarcinoma considered resectable in two, one stricture by MRCP, 25 strictures by ERC). Spyscope® endoscopy demonstrated malignancy (visualization, cytology and biopsy) in 11 of 20 patients. In three patients, Spyscope® endoscopy demonstrated stone related strictures (stones not seen by standard cholangiography), extrinsic compression of the distal bile duct in two, and scar-like tissue that appeared benign in four patients [all with Primary sclerosing cholangitis (PSC)]. Visual components of malignancy included exophytic lesions, ulcerations, and raised lesions. Biopsy alone showed malignant features in 6/11 patients. Cytology showed malignant features in 4/20 patients. In patients with abnormal liver tests, abnormal Spyscope® endoscopy findings included stones in two, anastomotic ulceration in two, intrahepatic duct ulcer in one, intrahepatic adenoma in one, and normal in nine. Of patients with abnormal imaging studies, Spyscope® endoscopy demonstrated supra-papillary polypoid lesions in two, intraductal extension of ampullary cancer in one, stones in two, anastomotic surgical material in three, villous adenoma in three, cholangiocarcinoma in one, and normal in 26 (including no mucosal abnormalities in four patients with choledochal cyst type I) (Figure 1A and B). Of the patients with cholangiocarcinoma, two had resectable disease. Spyscope® endoscopy demonstrated intrahepatic disease in two and confirmed localized disease in one.

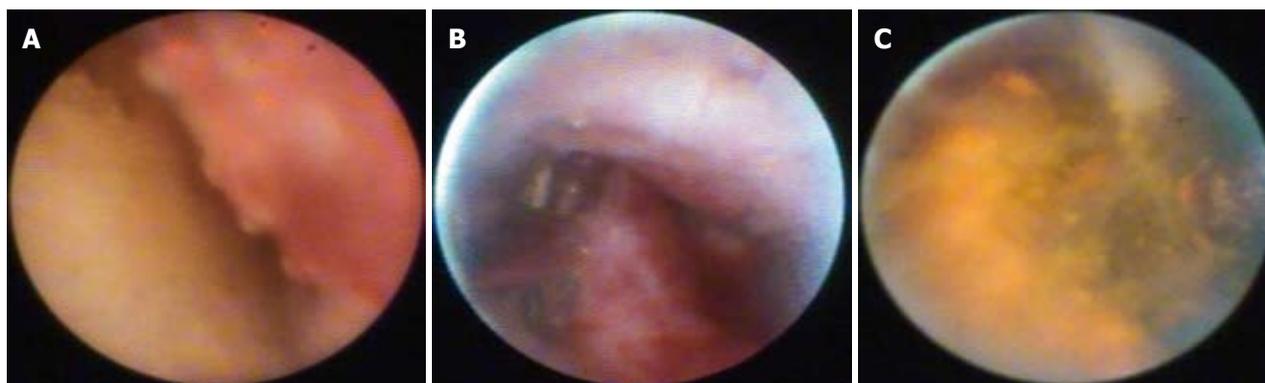


Figure 1 Spyglass® views of (A) bile duct adenoma, (B) cholangiocarcinoma, (C) bile duct stone with lithotripsy probe.

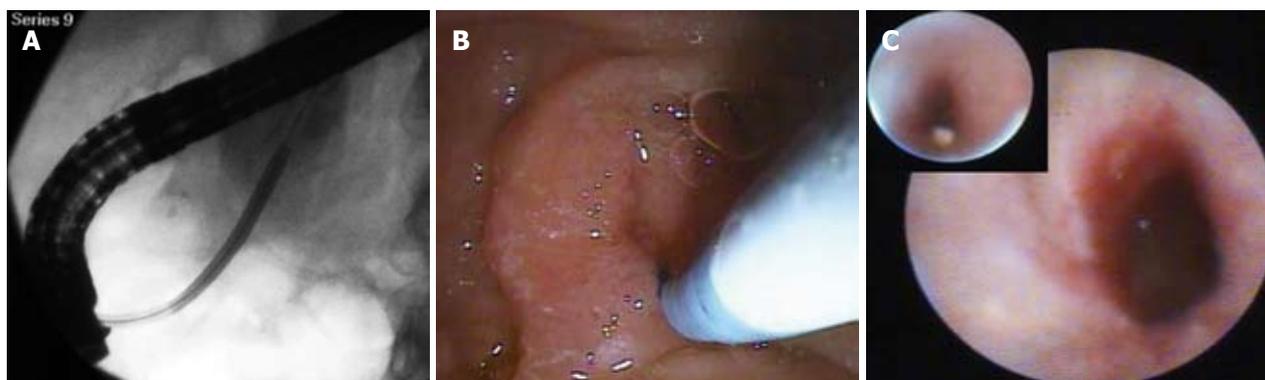


Figure 2 Pancreatic use of Spyglass® with (A) fluoroscopic view of cannulation, (B) duodenoscope view, (C) Spyscope® views of pancreatic stone and stricture.

Table 3 Spyglass® evaluation of choledocholithiasis

Spyglass® evaluation of choledocholithiasis	Cases (n)
Indications for choledochoscopy	
Large choledocholithiasis	26
Mirizzi syndrome	1
Intrahepatic lithiasis	10
Lithiasis with bile duct strictures (also with intrahepatic lithiasis)	11 (7)
Maneuverability	
Intrahepatic peroral advancement without difficulty	8/10
Percutaneous advancement	6/6
Holmium laser usage	3/3
Poor targeting of lesion with EHL	4/41
EHL not advanced	3/41
Overall visualization	
Good	31
Fair	6
Poor	4

Therapeutic purposes

Spyglass Spyscope® endoscopy was used in 41 patients for biliary stone disease (Table 3). In 35 patients, it was used per-orally, and percutaneously in six patients due to surgically modified anatomy. Indications for biliary therapy included large choledocholithiasis (26), Mirizzi syndrome (1), intrahepatic lithiasis (10), and lithiasis associated with bile duct strictures (11). Some of these indications pertain to the same patient (i.e. large stone and Mirizzi syndrome). EHL was used in 38 patients and a holmium laser in three

(Table 3). Lithotripsy was successful in 37/41 patients (Figure 1C). In five patients the EHL probe could not be advanced through the Spyscope® at the exit of the duodenoscope. In seven patients, the EHL probe could not be accommodated to fully target the stone. However, it produced enough fragmentation to remove the stone. Several factors were evaluated to assess feasibility, scope maneuverability, and technical success based on the ability to achieve ductal clearance of the Spyglass® system in the treatment of biliary disease. The initial assessment was based on the quality of visualization and related assessment of potential for intervention. Visualization (Table 3) was good in 31 (75.6%), fair in six (14.6%) and poor in four (12.9%). Maneuverability was superior if the guide wire was removed from the working channel. Percutaneous maneuverability was possible in 6/6 (100%) cases. The Spyscope® was advanced in the intrahepatics without difficulty in 8/10 (80%) patients. In 37/41 cases (87.1%) therapy for stones was completed by lithotripsy in one session (either by EHL or a holmium laser), and the remaining four patients required two sessions to achieve ductal clearance. A holmium laser was successfully used in three patients for stone destruction. Peroral use was easier than percutaneous. We noticed that the optical resolution of the Spyglass® deteriorated after ten uses. The best optical resolution was between uses one and seven.

Management and treatment for pancreatic stone disease

Six patients had pancreatoscopy for stones related

to chronic pancreatitis (Figure 2). All patients had a pancreatic sphincterotomy and a biliary sphincterotomy. All patients had a stricture distal to the stone and all the stones were localized to the head of the pancreas. The stone size varied between 5-14 mm. Two of the six stones were cast-like and impacted. In all patients, EHL was used *via* Spyglass Spyscope®. All patients underwent stricture dilatation before lithotripsy. Maneuvering the Spyglass Spyscope® in these patients was difficult due to its size (10 Fr). Stone clearance was successful in three of six patients, with the remainder treated with stent therapy.

Biliopancreatic disease overall results

Spyglass Spyscope® modified the preoperative diagnosis in 66% of the patients, prevented unnecessary surgery in two patients with cholangiocarcinoma, changed the diagnosis of malignant to benign disease in 45% of the patients with strictures, and provided successful therapy in 87% of the patients with stone disease. There was no morbidity or mortality associated with its use.

DISCUSSION

Since the introduction of intraductal endoscopy in the 1970s, several iterations have occurred to optimize the diagnosis and therapy for biliary and pancreatic disorders.

Reported uses include patient demographics (including young children and elderly) to diagnose and treat biliary and pancreatic stones or masses, strictures due to malignancy, primary sclerosing cholangitis and post-orthotopic liver transplantation.

The Spyglass® system is a single-operator three-channel, catheter based mini-endoscope. The system uses the Spyscope®, which has four-way maneuverability, accepts a 3.4-mm diameter catheter with a separate irrigation port, a channel for the 0.77 mm Spyglass® optical probe, and a 1.2-mm accessory channel. The accessory channel can be used to take biopsies with the Spybite and introduce EHL or holmium laser fibers.

Chen and Pleskow recently reported the initial experience with Spyglass Spyscope® (Boston Scientific, Natick, MA), a new single-operator endoscope. They evaluated 35 patients with the majority of cases (27) to evaluate indeterminate filling defects or strictures^[6]. PSC was the most common preoperative diagnosis present in 9/35 patients and 9/35 patients had common bile duct stones diagnosed by a pre- or peri-procedure, with EHL performed in five cases. The new endoscope had a 91% success rate for achieving either diagnostic or therapeutic endpoints, with a diagnosis by visual inspection yielding a 100% sensitivity and 77% specificity. When biopsies were performed, the overall sensitivity was 71% and 100% specificity for determining the behavior of a lesion. A low complication rate of 6% was reported with cholangitis seen in two patients, one requiring abscess drainage.

Chen reported an initial model system with Spyglass® that demonstrated improved targeting of lesions with improved steering ability compared to standard choledochoscopes^[7]. A follow-up evaluation with 35 patients described the first experience using the

Spyglass® in patients with strictures, filling defects, choledocholithiasis, and gallbladder stent placements. Our experience with intraductal endoscopy using Spyglass® is one of the largest series to date and represents description of both diagnostic and therapeutic modalities. The use of Spyglass® resulted in management changes in two-thirds of patients and no complications occurred.

The primary indication for diagnostic intraductal endoscopy in the biliary tree is to evaluate strictures seen on abnormal imaging study or prior ERCP. The majority of these patients are either post-OLT or at risk for cholangiocarcinoma (i.e. patients with PSC or history of choledochal cyst). Several modalities such as methylene blue^[8] and narrow band imaging^[9] have been used to optimize diagnosis in patients with various bile duct disorders^[6,7,10-12], including liver transplant^[5], PSC, and cholangiocarcinoma^[10,13-16]. Direct visualization and focused biopsy samples provide both endoscopic and pathological information to aid in diagnosis and treatment strategies. We described 29 patients who underwent Spyglass® to evaluate biliary strictures and the preoperative diagnosis was modified in 20 (68%) patients. All 29 patients had a preoperative diagnosis of malignant bile duct stricture, with 45% determined to be benign after Spyglass® evaluation. As with other intraductal endoscopes, bile duct stones were visualized in three Spyglass® cases, not seen on initial ERC.

Several reports detail the management of bile duct stones with standard choledochoscopy with EHL or laser therapies. Several authors have demonstrated the benefit of choledochoscopy in patients with filling defects or suspected choledocholithiasis by routine ERCP^[5,17-20]. Siddiqui and colleagues reported 18 patients with suspected stones, with 14/18 treated with EHL and the remaining four patients had either benign epitheloid tumors, large-cell lymphoma and cholangiocarcinoma^[5]. Arya *et al* described their experience of EHL in 111 patients^[17]. The authors' primary use for choledochoscopy and EHL were stones larger than 2 cm, or those with associated stone-related strictures, with successful stone fragmentation in 89 of 93 patients (96%) and overall stone clearance in 85/94 patients (90%). Treatment failures were due to targeting problems and hard stones. In their experience, 76% received one EHL session, 14% with two sessions, and 10% with three or more sessions. Farrell *et al* used a modified single operator choledochoscope system for treatment of stones, with 39 % of stones larger than 2 cm and 35% with stones above a stricture^[19]. More than half (54%) of patients in this cohort had multiple stones visualized and treated with choledochoscopy and EHL. They determined that the location of the stone, presence of multiple stones, presence of a stricture and the size of the stone were predictive factors for success and need for multiple EHL sessions.

Our series of 41 patients with bile duct stones using the Spyglass® system demonstrated similar results, with 87.1% of patients with clearance after one EHL session and the remaining patients with a second session. As with

previous reports, difficulties in stone removal were related to passage of the EHL probe through the Spyscope® and targeting of the lesion with the EHL probe. We found that removal of the guide wire facilitated visualization and manipulation of the EHL probe.

Several authors have described experience with peroral pancreatoscopy to evaluate pancreatic lesions (intraductal papillary mucinous neoplasms, strictures and treatment of stones)^[21-25]. We demonstrated the use of Spyglass® in six patients with adequate visualization of pancreatic stones, all with strictures distal to the stone. Half of the patients were successfully treated with EHL-directed therapy, with the remaining patients treated with stent-directed therapy. The outer diameter of the Spyscope® catheter and visibility limited better access in the presence of strictures.

CONCLUSION

Choledochoscopy has been available to endoscopists for several years. It is of high value in the management of difficult biliary stones, biliary strictures, and determining the presence or absence of ductal disease. The same has been applied to pancreatoscopy, however only recently since the advent of small caliber mini-scopes. Spyglass® is a new intraductal scope that differs fundamentally from other existing miniscopes in its structure, its four-way steering ability, its separate irrigation channel, its ease of use and requirement for only one operator. Due to its different structure, the Spyscope® component is disposable and the Spyglass® optical probe is reusable. The innovative design and ease in use will create an increase in choledochoscopy by more endoscopists. The applications are likely to remain for therapy of difficult stone disease. However, its use will increase for staging of various biliary cancers as well as in the management of biliary tract disease in liver transplant patients. Likewise, the use of Spyglass® will increase in patients with various pancreatic disorders, but not at the same pace as biliary disease. Our findings in redefining two-thirds of patients with malignant disease may influence both biliary and pancreatic oncologic management.

Future studies are planned to evaluate therapeutic intervention with the Spyglass® and related intraductal endoscopes. Additionally, we anticipate further improvements in optical quality, smaller catheter size, and maneuverability. Advanced imaging modalities such as narrow band imaging and chromoendoscopy offer further improvement in diagnostic capabilities, which can be assessed in the treatment of patients with biliary and pancreatic disease. With an increase in use, of critical importance is the recognition of disease (both endoscopically and histologically) and terminology. Thus, we believe that there will be an increase in the literature reporting the use of Spyglass® in biliary and pancreatic disorders.

COMMENTS

Background

Intraductal endoscopy has been used for more than 30 years to evaluate and

treat disorders of the biliary and pancreatic ducts. Cholangiopancreatography allows direct visualization and permits focused biopsies and lithotripsy. Use was limited for several years due to cost, ease of breakage, difficult maneuverability, and limited optical resolution.

Research frontiers

Spyglass Spyscope® (Boston Scientific, Natick, MA), is a new single-operator endoscope that can be used in the therapy of bilio-pancreatic disease. This intraductal endoscope is effective in the diagnosis of biliary strictures in patients with primary sclerosing cholangitis, orthotopic liver transplantation and cholangiocarcinoma. The Spyscope® can also be used in the management of biliary and pancreatic stones. The research hotspot is the significant improvement in cholangiopancreatography and the diversity of modalities that are demonstrated with the new endoscope.

Innovations and breakthroughs

The main advantages of the Spyglass Spyscope® are that it requires only one endoscopist, it has four-way steering ability, and a separate irrigation channel. This endoscope allows directed biopsies, can be used percutaneously, and permits the use of electrohydraulic lithotripsy or a holmium laser for the treatment of biliary and pancreatic stones.

Applications

This study demonstrates an effective new tool in the diagnosis and treatment of biliopancreatic disease using cholangiopancreatography.

Terminology

Cholangiopancreatography is the direct endoscopic evaluation of the bile or pancreatic ducts.

Peer review

The authors present a preliminary report of a new endoscope to directly evaluate the biliary and pancreatic ducts. Their promising results assessed the performance, feasibility and safety of the new endoscope. The new intraductal endoscope was useful for discriminating benign biliary strictures from malignancy.

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Ex-vivo evaluation of gene therapy vectors in human pancreatic (cancer) tissue slices

Michael A van Geer, Koert FD Kuhlmann, Conny T Bakker, Fibo JW ten Kate, Ronald PJ Oude Elferink, Piter J Bosma

Michael A van Geer, Conny T Bakker, Ronald PJ Oude Elferink, Piter J Bosma, Liver Center, Academic Medical Center of the University of Amsterdam, Meibergdreef 69/71, 1105 BK Amsterdam, The Netherlands

Koert FD Kuhlmann, Department of Surgery, Academic Medical Center of the University of Amsterdam, Meibergdreef 9, 1105BK Amsterdam, The Netherlands

Fibo JW ten Kate, Department of Pathology, Academic Medical Center of the University of Amsterdam, Meibergdreef 9, 1105 BK Amsterdam, The Netherlands

Author contributions: van Geer MA generated the Adenovirus preparations and performed the transduction experiments in the slices; Kuhlmann KFD provided the resection specimens and performed the immune histological stainings; Bakker CT produced the Lentivirus and part of the adenovirus preparations and performed the slicing of the resection specimens; ten Kate FJW scored the viability of the slices after *ex-vivo* culture; Oude Elferink RPJ designed the study and obtained the grant required for performing this study and corrected the paper; Bosma PJ designed the study, generated the AAV vector and wrote the paper.

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Correspondence to: Dr. Piter J Bosma, Liver Center, Academic Medical Center of the University of Amsterdam, Meibergdreef 69/71, 1105 BK Amsterdam,

The Netherlands. p.j.bosma@amc.uva.nl

Telephone: +31-20-5668850 Fax: +31-20-5669190

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Abstract

AIM: To culture human pancreatic tissue obtained from small resection specimens as a pre-clinical model for examining virus-host interactions.

METHODS: Human pancreatic tissue samples (malignant and normal) were obtained from surgical specimens and processed immediately to tissue slices. Tissue slices were cultured *ex vivo* for 1-6 d in an incubator using 95% O₂. Slices were subsequently analyzed for viability and morphology. In addition the slices were incubated with different viral vectors expressing the reporter genes *GFP* or *DsRed*. Expression of these reporter genes was measured at 72 h after infection.

RESULTS: With the Krumdieck tissue slicer, uniform slices could be generated from pancreatic tissue but only upon embedding the tissue in 3% low melting agarose. Immunohistological examination showed the presence of all pancreatic cell types. Pancreatic normal and cancer tissue slices could be cultured for up to 6 d, while retaining viability and a moderate to good morphology. Reporter gene expression indicated that the slices could be infected and transduced efficiently by adenoviral vectors and by adeno associated viral vectors, whereas transduction with lentiviral vectors was limited. For the adenoviral vector, the transduction seemed limited to the peripheral layers of the explants.

CONCLUSION: The presented system allows reproducible processing of minimal amounts of pancreatic tissue into slices uniform in size, suitable for pre-clinical evaluation of gene therapy vectors.

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Key words: Pancreas; Adenocarcinoma; Tissue slice technology; *Ex vivo*; Adenovirus

Peer reviewer: Julia B Greer, MD, MPH, Department of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, M2, Presbyterian University Hospital, 200 Lothrop Street, Pittsburgh, PA 15213, United States

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INTRODUCTION

Pancreatic cancer is a devastating disease. The mortality rate of this cancer almost equals its incidence rate. Genetic alterations in pancreatic cancer, as reviewed by Saif *et al*^[1], result in an aggressive cancer that is resistant to medical intervention. Surgical resection of pancreatic adenocarcinoma remains the cornerstone of treatment but is only available for a small minority of patients.

Even after surgical intervention prognosis remains poor, since median and 5-year survival after resection are 7.5 mo and 8%, respectively^[2]. A recent meta-analysis suggests that gemcitabine-based chemotherapy seems to improve overall survival of patients with advanced and metastatic disease to some extent^[3]. However, even with this treatment the prognosis continues to be poor. New therapies, such as viral-vector-mediated gene therapy, are needed to improve the survival of patients with pancreatic cancer.

Adenoviral vectors are used most frequently in gene therapy strategies aimed to treat solid cancers. Important advantages of this vector are its capability to infect and transduce dividing and non-dividing cells and that it can be propagated to high titers. Initially, non-replicating adenoviral vectors were used to introduce expression of a cytotoxic or a pro-drug activating gene into cancer cells. This so-called suicide gene therapy approach did show efficacy in pre-clinical cancer models. However, in the subsequent phase 1 clinical trials, no therapeutic effect was observed due to limited spread of the viral vector in the tumors. To improve the efficacy and the spread, adenoviral vectors were developed that were able to replicate in cancer cells but not in normal cells. These conditional replicating adenoviral vectors (CRADs) replicate specifically, in tumor cells and kill them as result of their lytic cycle. Upon replication a large amount of virus progeny will be generated in a tumor and infect other cancer cells. In pre-clinical models, these CRADs were more efficient than the non-replicating vectors. At least six different CRADs have subsequently been evaluated in clinical trials but only a minority of these studies suggested that these vectors could be effective^[4]. The first engineered oncolytic adenovirus that was implemented in a clinical setting was Onyx-015, which replicates specifically in p53-deficient tumor cells. Preclinical studies had shown that Onyx-015 efficiently kills human cancer cells in subcutaneous tumors in nude mice^[5,6]. Subsequent clinical studies showed that ONYX lacked efficacy as a single anti-tumor agent^[7]. Also, in phase I / II trials for pancreatic cancer, intra-tumoral injection of Onyx-015 lacked efficacy^[8,9]. Further studies showed that for these replicating adenoviral vectors, the transduction of cancer cells *in vivo* also appeared to be the limiting factor in treating patients.

Low expression of the coxsackie- and adenovirus receptor (CAR), *via* which adenovirus normally enters cells, was subsequently shown to cause the poor transduction of pancreatic cancer *in vivo*^[10]. To overcome this problem several strategies emerged to increase pancreatic cancer transduction by circumventing CAR-mediated entry^[11]. Insertion of the Arg-Gly-Asp (RGD) peptide motif in the fiber knob of adenovirus, for instance, enhances infectivity of pancreatic tumors in a mouse model^[12]. All such studies however rely on the use of human pancreatic cancer cells growing subcutaneously in a nude mouse model. The discrepancies in efficacy of novel treatments seen in these preclinical studies and in subsequent clinical trials demonstrates that this is not an ideal model. For instance, these mouse models do not

assess the cellular heterogeneity of solid tumors such as pancreatic cancers. In addition, they do not accumulate extracellular matrix, which occurs in human tumors. Finally, due to the lack of normal human pancreas cells, these models are not suitable for demonstrating increased tumor specificity of the retargeted adenoviral vector. Therefore new models are needed that more closely resemble the *in vivo* situation in patients. Tissue explants seem a good alternative in this respect. In these explants, the interaction between heterogeneous cell types comprising normal and tumor tissue is retained. In addition, these explants do contain extracellular matrix. Therefore, tissue explants seem a suitable model to study transduction efficiency in a setting more representative of the *in vivo* situation.

Several culture systems are available that allow the preservation of live pancreas tissue taken directly from patient specimens. Hoem *et al*^[13] cultured normal exocrine pancreatic cells, derived from pancreaticoduodenectomy specimens, as spheroids for at least 6 wk. A similar method was used for culturing pancreatic adenocarcinomas^[14]. Both groups manually dissected tumor specimens to small fragments or cubes. Manual dissection of fresh tissue, however, causes physical stress and generates fragments of variable size and shape. This will influence the transduction by viral vectors because it affects the viability of the tissue and surface/volume ratio. To generate tissue slices with a more consistent size and shape, we decided to use an automatic tissue slicer. These slicers are widely used to generate precision-cut organ slices for biotransformation research. Slices can be prepared from several organs including liver, lung, kidney, colon and intestine. They closely resemble the architecture of the original organ, which makes this technique a powerful instrument to perform toxicity and drug-metabolizing studies^[15]. Recently, the ultra-thin slices also proved to be a representative model for *ex vivo* evaluation of the transduction efficiency of adenoviral vectors^[16,17].

Precision-cut slicing of human pancreatic tissue was never reported. The aim of the present study was to evaluate the use of slices to test adenoviral-vector-mediated gene therapy for pancreatic cancer. We performed tissue slicing and subsequent culture of human pancreatic tissue slices and examined viability and morphology over time of normal and malignant pancreatic explants. Since we show that patient explants derived from minimal amounts of tissue are viable for up to 6 d with preservation of morphology, these slices appear to be a good model for studying viral-vector-mediated gene therapy and also seem suitable for pre-clinical testing of novel drugs to treat pancreatic disease.

MATERIALS AND METHODS

Materials

MD4000 Krumdieck tissue slicer was from Alabama Research & Development, Munford, Alabama; L-glutamine, penicillin, streptomycin, amphotericin were from Cambrex Bio Science, Walkersville, Maryland;

insulin, transferrin and selenium solution, MEM vitamins and MEM amino acids were from Invitrogen; the Innova 4300 incubator was from New Brunswick Scientific Co, Edison, New Jersey; the NOVostar fluorometer was from BMG Lab Technologies, Offenburg, Germany; WST-1 assay was from Roche, Almere, Netherlands; mouse anti-GFP JL-8 was from Clontech, Palo Alto, CA. The Powervision system was from ImmunoLogic, Duiven, The Netherlands; the biotinylated rabbit anti-mouse immunoglobulins and streptavidin–horseradish peroxidase conjugate were from DAKO Netherlands.

Methods

Virus generation and propagation: Plasmids encoding for E1-, E3-deleted adenovirus vectors were modified by using pAdHM15^[18]. pAdHM15 was digested with PI-Sce and Ceu- I to insert the reporter genes enhanced green fluorescent protein (GFP) and DsRed under control of the CMV promoter^[10]. Recombinant adenoviral vectors were generated by transfection of HEK 293 cells with PacI-linearized Ad-*GFP* and Ad-*dsRED*. Virus was purified and concentrated by performing two cesium chloride gradients according to standard protocols. Virus preparations were dialyzed two times against 1 L of PBS. After the second dialysis, glycerol was added to a final concentration of 10% (v/v) and virus preps were aliquoted, and stored at -80°C. The number of genomic copies was determined by quantitative real-time polymerase chain reaction by using the primers against hexon DNA^[19].

Lentivirus-CMV-*GFP* was constructed as described before, by transfection of 293T cells using a calcium phosphate method, concentrated by ultracentrifugation, and titrated on HeLa cells^[20]. AAV2-CMV-*GFP* was constructed and titers were determined as described previously^[21].

Primary tissue slices: Fresh human pancreatic specimens were obtained from patients undergoing a pancreaticoduodenectomy for pancreatic head tumors (pancreatic cancer slices) and carcinomas of the bile duct or ampulla of Vater (normal pancreas slices). Tumor specimens were embedded in 3% low melting point agarose/PBS before cutting in the MD4000 Krumdieck tissue slicer, to produce slices with a thickness of approximately 250 μm (estimated 2.5×10^5 cells), while submerged in oxygenated ice-cold Krebs. Slices were incubated in 1 mL DMEM including L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 $\mu\text{g/mL}$) and amphotericin (Fungizone 2.5 $\mu\text{g/mL}$) with or without insulin, transferrin and selenium (ITS), and 20 mmol/L HEPES pH 7.4. After 1 h, medium was replaced and slices were infected with 1×10^8 or 5×10^8 genomic copies Ad-*GFP* and/or Ad-*DsRED*, or with 1.0×10^6 transducing units of lentivirus-CMV-*GFP* or with 1.2×10^{10} genomic copies AAV-2-CMV-*GFP*. All experiments were performed in triplicate and were repeated at least three times.

Tissue culture plates were placed in an Innova 4300 incubator that was humidified and gassed with 95% O₂ and continuously shaken back and forth (90 times/min)

at 37°C. Virus was removed and medium was refreshed after 36 h. Slices were harvested at several time points and lysed overnight at 4°C in 60 μL RIPA buffer (25 mmol/L Tris HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). After one round of freeze/thawing, cellular debris was removed by centrifugation. Total fluorescence of GFP and dsRED in supernatant was measured with a NOVostar fluorometer. The filter settings were as follows: GFP excitation at 485 nm, GFP emission at 520 nm, DsRed excitation at 550 nm, DsRed emission at 580 nm. Several slices were fixed in 4% paraformaldehyde.

WST-1 and amylase secretion assay: Viability of tissue explants was determined with the WST-1 assay according to the protocol of Roche. Amylase activity in culture medium was determined using an enzyme-based colorimetric assay on a P800 Modular Roche Diagnostics apparatus.

Histology and immunohistology: Tissue slices were fixed in 4% paraformaldehyde, embedded in paraplast and 7- μm sections were made. Hematoxylin and eosin staining was performed according to standard protocols. Sections were assessed for viability and morphological characteristics. Paraffin-embedded sections were used for immunohistological detection of GFP as reported earlier^[21].

RESULTS

Slicing procedure and culture conditions

Several studies underline the relevance of tissue slice technology for experiments involving organ culture. Solid organs such as liver can be sliced directly with a tissue slicer. For human pancreas, this was not possible because it is too soft. We showed that this problem could be solved by embedding small (approximately 5 mm) pancreatic fragments in cylinders of 3% low melting agarose. The resulting core was solid enough for processing in a tissue slicer. Various slicing systems are available commercially.

Initially, we used the Brendel/Vitron tissue slicer. However, we found the pancreatic slices to vary significantly in viability, integrity and size. Therefore, these preparations contained a large percentage of tissue fragments not suitable for further experiments. Furthermore, operating this slicer requires both hands, which compromised the retention of aseptic conditions in the biosafety flow cabinet. Therefore, we switched to the fully automated Krumdieck tissue slicer. With this slicer, many more uniform slices with considerably less damage to the fragile pancreatic tissue were obtained.

The study of Wang *et al.*^[14] suggests that supplement-enriched culture medium extends tissue survival. We therefore added ITS, amino acids and vitamins to the culture medium. The oxygen tension varies between culture systems reported^[15]. We decided to gas the incubator with 95% O₂ to ascertain proper diffusion of oxygen to the inner cell layers.

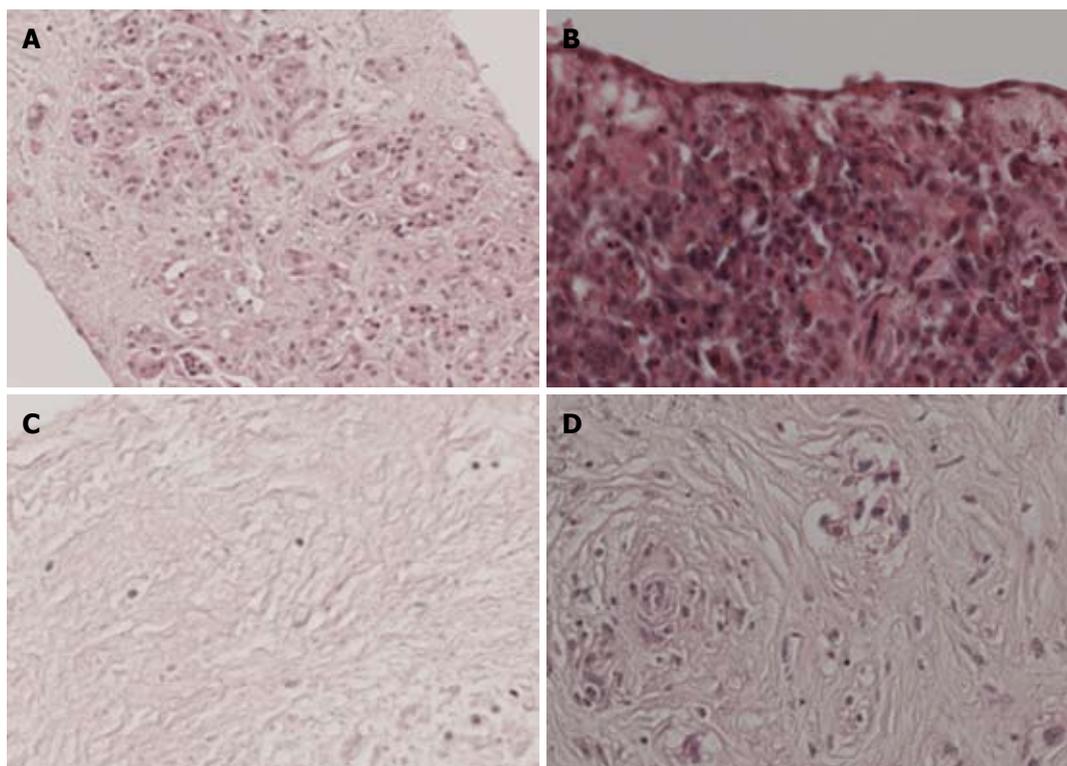


Figure 1 Histological staining of pancreatic tissue slices. Slices derived from normal pancreas and pancreatic cancer were cultured for 3 d. A, B: Normal pancreas of good viability; C: Normal pancreas of poor viability showing massive tissue slice necrosis; D: Poorly differentiated adenocarcinoma of good viability. Hematoxylin/eosin staining. Original magnification of all tissues (100 ×).

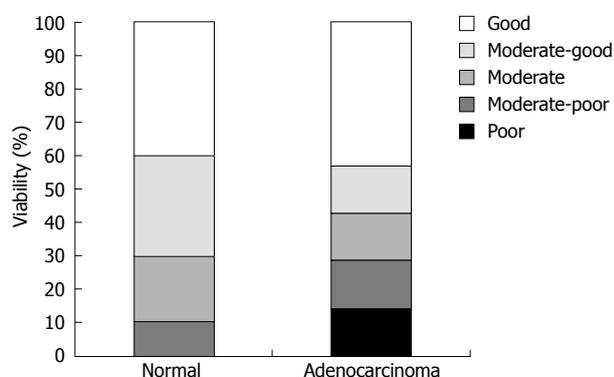


Figure 2 Viability of pancreatic (cancer) slices cultured for 3 d. All slices were cultured for 3 d and then stained with hematoxylin/eosin. The morphology of normal pancreas ($n = 10$) and pancreatic adenocarcinoma ($n = 6$) was studied microscopically and scored by an experienced pathologist.

Viability

To examine the quality over time of cultured pancreatic slices, we first assessed viability by histology. Parameters for viability included cell swelling, necrosis and nuclear pyknosis. We included slices from 10 normal pancreas specimens and six pancreatic adenocarcinomas for examination at 3 d after slicing. In normal pancreas, we were able to distinguish all cell types including exocrine cells, ductal cells, islets of Langerhans, and nerve cells. Figure 1A shows a normal pancreas tissue slice at day 3. We observed that a cell sheet consisting of squamous epithelial-like cells (Figure 1B) covered several tissue slices. Tissue slices of poor viability were characterized

by complete disappearance of viable cells (Figure 1C). Figure 1D shows poorly differentiated adenocarcinoma of good quality. Some pancreatic cancers were accompanied by stromal tissue consisting of fibroblasts and connective tissue. All 16 slices were scored for viability. For normal pancreas, 70% of the slices had moderate to good viability, while, for pancreatic cancer specimens, this was 57% (Figure 2). Not surprisingly, keeping the time between resection and start of tissue culture as short as possible improved the viability of the slices.

To examine the effect of medium formulation on the viability of the slices from normal human pancreas, we performed a WST-1 assay. Tissue slices were cultured either in normal culture medium or in medium supplemented with ITS, vitamins and amino acids. The WST-1 reaction was performed at day 0, 1, 3, 4 and 6. WST-1 measurements showed an increase in activity after 1 d of culture, which remained constant up to day 3 and then showed a gradual decline at day 4 and 6 (Figure 3A). Addition of ITS, vitamins and amino acids did not significantly improve viability of the tissue slices. After 6 d in culture, these slices were subsequently examined for morphology. Figure 3B and C shows a representative overview of normal pancreas of good viability, including Langerhans cells and nerve cells. We cultured two additional sets of normal pancreas slices for 6 d and performed histological staining. We noted moderate to good viability in one and moderate to poor quality, in the second. In slices with poor quality the deterioration of the tissue started in the peripheral cell layers. Even in these slices, moderate quality was maintained in

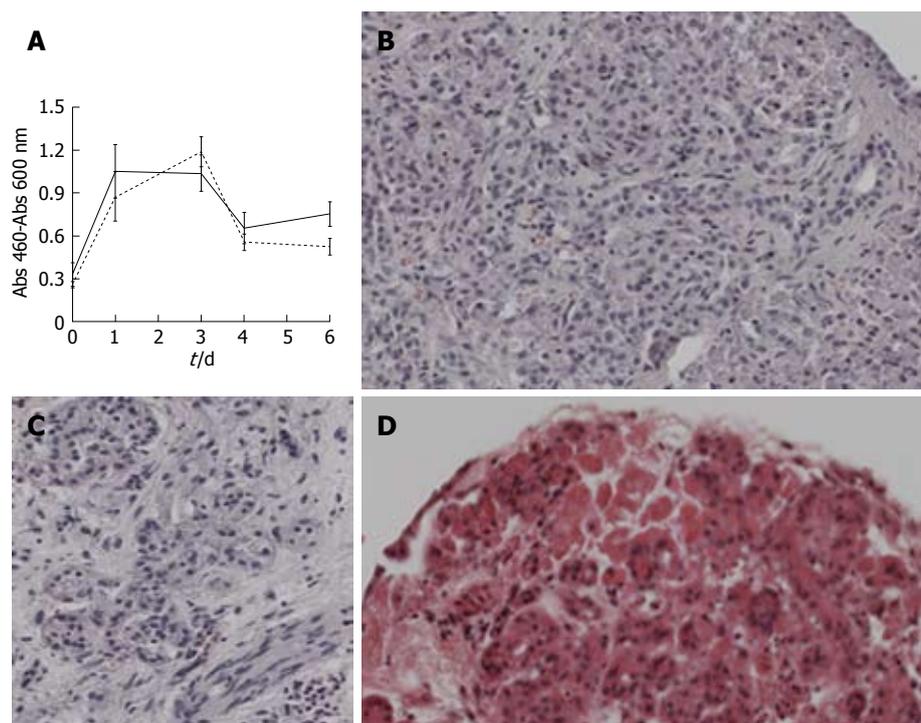


Figure 3 Viability of human pancreas tissue slices cultured *ex-vivo* for up to 6 d. A: Viability of explants cultured in 1 mL DMEM (black line) or in DMEM supplemented with ITS growth factors (dashed line) was determined at day 1, 3, 4 and 6 of culturing a WST assay. All data given as the means of at least five slices \pm SD; B: Normal pancreas with good viability at day 6; C: Normal pancreas of good viability at day 6 with Langerhans and nerve cells; D: Normal pancreas at day 6 with necrotic areas. Original magnification (100 \times).

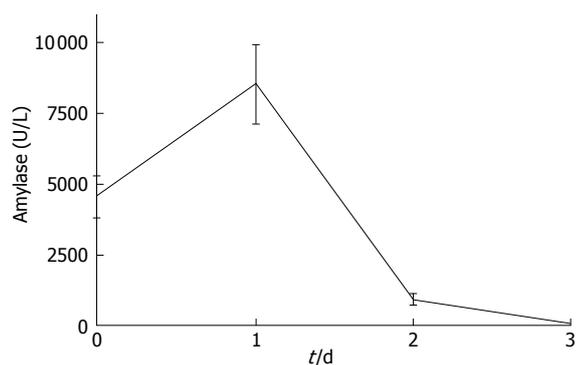


Figure 4 Amylase secretion by human pancreas tissue slices. Human pancreatic cancer tissues were cultured *ex-vivo* for up to 3 d. Amount of amylase present in medium was determined using a colorimetric enzyme based assay. Data are the means \pm SD ($n = 12$).

the center (Figure 3D). Based on these histological comparisons we concluded that pancreatic slices retain moderate to good morphology for at least 3 d in culture. The WST-1 conversion at day 6 and moderate to good morphology seen in several slices suggests that these cultured pancreatic tissue slices remain viable for even longer periods.

In addition to viability and morphology, we tested functionality of pancreas slices by determining amylase activity in the medium. Medium was analyzed during three consecutive days following explanting of tissue. As shown in Figure 4, amylase secretion is maximal at day 1 and declined below detection level at day 3. This secretion of amylase suggests that the slices are not just viable and retain the correct morphology, but that they are also functional at least up to day 2.

Transduction of slices with virus vectors

The possibility of culturing slices for up to 3 d renders

this system suitable for studying the transduction of pancreatic tissue by adenoviral vectors that require 48 to 72 h to reach maximal gene expression levels. We examined whether pancreatic tissue slices produce detectable levels of two different reporter proteins after transduction with adenoviral vectors. We chose to test the transduction in these slices with an adenovirus vector expressing GFP and with one expressing dsRed. Since both are Ad-5 vectors and will display their native tropism, we expect they should have overlapping transduction patterns. Figure 5A shows diffuse expression of GFP and DsRed upon transduction with these vectors, and as expected, the expression patterns of both reporter genes largely overlap. To quantify the expression levels of both reporter genes, the slices were lysed and used for fluorometric detection of GFP and ds-Red in the NOVOstar reader. Infection of normal pancreas and cancer tissues with 1.0×10^8 and 5.0×10^8 gc/slice, respectively, resulted in clearly detectable measurements of both reporter genes at 72 h after transduction (Figure 5B). Lower amounts of virus or measurements at earlier time points resulted in expression levels too low to measure accurately. As shown in Figure 5B, the expression of both reporter genes varies significantly between slices. These variations most likely reflect differences in viability, in size and in tissue composition between slices. Importantly, by dividing the remaining fluorescence values of GFP by DsRed we obtained an average GFP to DsRED ratio of 0.9. In addition to the overlapping expression pattern, this constant ratio between both reporter genes also indicates that one of these vectors can be used as internal control for slice quality. For instance, in case of co-transduction with a tropism modified vector, the GFP/DsRed ratio will reflect the improved transduction efficiency of the targeted vector in comparison to the wild type adenovirus.

The transduction of the slices by adenovirus is not

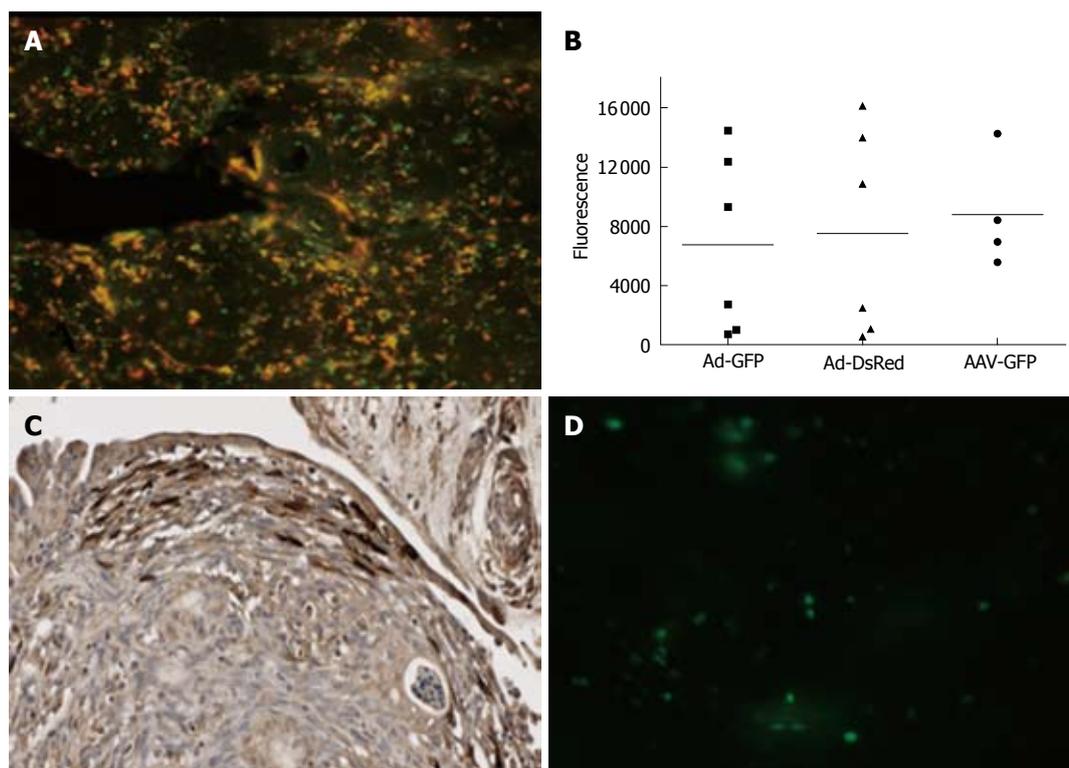


Figure 5 Transduction of pancreatic tissue slices with different viral vectors. A: Normal human pancreatic tissue slices are incubated with 1.0×10^8 Ad5.GFP and 1.0×10^8 Ad5.dsRed. After 48 h presence of GFP and ds.Red was detected using a fluorescent microscope; B: Normal pancreas slices were infected with 1.0×10^8 genomic copies per slice of Ad5.GFP and Ad5.dsRED, or 1.2×10^{10} viral genomes AAV2-GFP. At 48 h after infection slices were lysed, GFP and ds.Red were quantified using the Novostar; C: Pancreatic adenocarcinoma explants were transduced with Ad-GFP. At 72 h after virus addition expression of GFP was detected using an anti-GFP antibody and counterstained with hematoxylin; D: GFP expression in normal pancreas at 72 h after incubation with lentivirus-GFP.

homogeneous. As shown by the immunohistological staining in Figure 5C, transduction seemed limited to the peripheral layers of the explants. This non-homogeneous transduction pattern underscores the importance of co-transduction with an internal control when using this system to test the effect of retargeting of gene therapy vectors.

The possibility of culturing pancreatic slices for up to 3 d also seems long enough to study the transduction with other types of viral vectors such as lentiviral vectors and adeno associated virus (AAV). To explore this possibility we incubated normal pancreas with adeno-associated virus serotype 2-GFP (AAV2-GFP) or with lentivirus-GFP. Upon incubation with AAV-GFP, the fluorescence levels were comparable to those obtained with Ad-GFP (Figure 5B). In contrast, fluorescence was below detection limit when using lentivirus-GFP. As shown in Figure 5D, only a few cells were transduced by lentiviral vectors and expressed GFP. This poor transduction by lentiviral vectors may have been due to the vigorous shaking of the slices in combination with a low affinity of this vector for this tissue. Another possible cause is the fragility of lentiviral vectors. Production of proteases and/or lipases by these slices will affect lentiviral vector integrity and reduce transduction.

DISCUSSION

The limited intervention options for advanced and

metastasized pancreatic cancer require new treatment modalities such as gene therapy. The preclinical adenovirus-mediated gene therapy study models primarily rely on cultured cells and the xenograft nude mouse model. Both remain a poor representation of *in vivo* pancreatic cancer. Pancreatic cell lines acquire new genetic defects upon multiple passages *in vitro* and after subcutaneous injection *in vivo*^[22]. Moreover, xenograft tumors lack the natural interaction between tumor cells, stromal cells, normal cells and extracellular matrix. This is an important drawback since anatomical barriers such as fibrosis are a major hurdle for adenovirus spreading through the tumor^[23].

For these reasons, primary human tissue specimens seem more comparable to the *in vivo* situation and may provide a better prediction of virus-host interactions.

We studied the tissue slice system for culturing *ex vivo* pancreatic explants. Other groups have already reported slice culture of various organs from different species^[15-17,24]. Here, we demonstrated that this technique is also feasible for pancreatic tissue using the Krumdieck tissue slicer. Independently of the medium formulation, normal pancreas was viable for up to 6 d. Since the inner cell layers seemed more viable than the peripheral layers, oxygen and nutrient diffusion through the slices does not seem limiting. Microscopic examination of normal slices revealed an epithelial-like cell sheet surrounding the explants. This is in accordance with the findings of Hoem *et al*, who cultured *ex vivo*

pancreas for up to 6 wk^[13]. They hypothesized that these cells have a ductal or acinar cell origin, possibly resulting from transdifferentiation caused by prolonged culturing. Others showed that this so-called acinar-ductal transdifferentiation *in vitro* indeed depends on medium formulation^[25]. The loss of ductal and acinar cells from these explants may explain the decrease of amylase secretion by the slices over time.

We also showed that pancreatic adenocarcinoma explants can be cultured while retaining moderate to good viability and morphology. Resection specimens are small and the amount of tissue available for research purposes therefore is very limited. Manual dissection to fragments led to inhomogeneous samples that could not be used for further study. To optimize the yield of slices, we therefore used an automatic tissue slicer. The size of the resection specimens varied, and to be able to generate uniform slices, the specimens were included in low melting agarose. Upon stabilizing the tumor cubes in this manner, a significant number of slices could be obtained from limited amounts of tissue. This method does result in slices comparable in size and viability, with minimal loss of tissue. As such, this method seems superior to manual processing that resulted in slices with a high degree of heterogeneity and limited survival of explanted tissue^[14]. Furthermore, with this technique, several slices can be obtained from a small resection specimen. This enabled us to compare different viral vectors and experimental conditions in the resection specimens derived from each patient.

We were able to culture these slices for at least 3 d with good morphology. This period is sufficiently long to study the transduction efficiency since most viral vectors need between 48 and 72 h for optimal expression. We showed that adenovirus infects and transduces pancreatic slices efficiently. In addition, we showed that simultaneous transduction of slices with a control and experimental virus expressing different reporter genes provides an elegant procedure to determine adenoviral transduction efficacy while correcting for variability in tissue viability and composition. In this respect, this system seems highly suitable for determining the efficacy of adenoviral vectors that target pancreatic cancer cells. Transduction experiments with such infectivity-enhanced adenoviral vectors in slices from normal tissue and tumor tissue will indicate their targeting specificity. Others have already shown that slices that maintain viability for at least 3 d are suitable for replication of CRAds^[17,24]. Therefore, our tissue explant system seems suitable to test the transduction efficacy of CRAds, and more especially, to investigate the increased efficacy of infectivity-enhanced CRAds. However, an optimal comparison of actual oncolytic potency of CRAds requires several rounds of replication and therefore a prolonged culture time. For these investigations, patient-derived multicellular cancer spheroids seem more suitable^[26,27].

In addition to adenoviral transduction, these slices were efficiently transduced by AAV-2 but poorly by lentiviral vectors. These two vectors have been shown to be suitable for correction of inherited disorders in pre-

clinical models. Based on the transduction seen here, these slices can be used to investigate whether these vectors can be targeted; for instance, to the islet cells with the aim of providing correction for diabetes^[28].

In conclusion, we have developed a system that allows processing of minimal amounts of pancreatic tissue from resection specimens. The generated thin, homogenous and viable tissue slices retain their morphology for at least 3 d. Furthermore, with this method, a sufficient number of slices can be obtained from minimal amounts of tissue, enabling the comparison of several experimental conditions within a single tumor specimen.

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COMMENTS

Background

Pancreatic cancer is a devastating disease for which at present no therapy is available. Gene therapy was shown to be effective in preclinical animal models and was expected to improve the poor prognosis for patients suffering from pancreatic cancer. However, in patients, these methods lacked efficacy due to poor transduction of cancer cells. Therefore, other models more comparable to the situation in patients are essential for predicting the clinical efficiency of novel therapies such as gene therapy.

Research frontiers

To circumvent the low coxsackie- and adenovirus receptor expression in human cancers in patients, retargeting of adenovirus to receptors highly expressed on human cancers is an essential step to improve their clinical efficacy.

Innovations and breakthroughs

Available animal models for pancreatic cancer do not reliably predict the clinical efficacy of retargeted adenoviral vectors. In this study, we present a tissue slice model more representative of the situation in patients as a model to predict the clinical efficacy of retargeted adenoviral vectors.

Applications

This tissue slice model is suitable for determining the efficiency of viral vectors retargeted to pancreatic cancer. In addition, this system can be used for pre-clinical *ex-vivo* studies of novel drugs to treat pancreas cancer.

Peer review

This was a well-performed study in an area of interest to gastroenterologists and pancreatologists in particular.

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Investigation and prediction of enteral nutrition problems after percutaneous endoscopic gastrostomy

Shiro Yokohama, Masaru Aoshima, Yukiomi Nakade, Junya Shindo, Junichi Maruyama, Masashi Yoneda

Shiro Yokohama, Masaru Aoshima, Department of Gastroenterology, Asahikawa Rehabilitation Hospital, Asahikawa 078-8801, Japan

Junya Shindo, Department of Rehabilitation, Asahikawa Rehabilitation Hospital, Asahikawa 078-8801, Japan

Junichi Maruyama, Department of Internal Medicine, Asahikawa Rehabilitation Hospital, Asahikawa 078-8801, Japan

Yukiomi Nakade, Masashi Yoneda, Division of Gastroenterology, Department of Internal Medicine, Aichi Medical University School of Medicine, Aichi 480-1195, Japan

Author contributions: Yokohama S performed the majority of research; Yokohama S and Aoshima M designed research; Yokohama S, Shindo J and Maruyama J analyzed data; Yokohama S, Nakade Y and Yoneda M wrote the paper.

Correspondence to: Dr. Shiro Yokohama, Department of Gastroenterology, Asahikawa Rehabilitation Hospital, Midorigaoka Higashi 1-1-1-1, Asahikawa 078-8801, Japan. s44yokohama11@reha.or.jp

Telephone: +81-166-650101 Fax: +81-166-682459

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Abstract

AIM: To investigate and predict enteral nutrition problems after percutaneous endoscopic gastrostomy (PEG).

METHODS: We retrospectively analyzed data for 252 out of 285 patients who underwent PEG at our hospital from 1999 to 2008. Enteral nutrition problems after PEG were defined as: (1) patients who required ≥ 1 mo after surgery to switch to complete enteral nutrition, or who required additional parenteral alimentation continuously; or (2) patients who abandoned switching to enteral nutrition using the gastrostoma and employed other nutritional methods. We attempted to identify the predictors of problem cases by using a logistic regression analysis that examined the patients' backgrounds and the specific causes that led to their problems.

RESULTS: Mean age of the patients was 75 years, and in general, their body weight was low and their overall condition was markedly poor. Blood testing revealed that patients tended to be anemic and malnourished. A total of 44 patients (17.5%) were diagnosed as having enteral nutrition problems after PEG. Major causes of

the problems included pneumonia, acute enterocolitis (often *Clostridium difficile*-related), paralytic ileus and biliary tract infection. A multivariate analysis identified the following independent predictors for problem cases: (1) enteral nutrition before gastrectomy (a risk reduction factor); (2) presence of esophageal hiatal hernia; (3) past history of paralytic ileus; and (4) presence of chronic renal dysfunction.

CONCLUSION: Enteral nutrition problems after PEG occurred at a comparatively high rate. Patient background analysis elucidated four predictive factors for the problem cases.

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Key words: Percutaneous endoscopic gastrostomy; Enteral nutrition; Complication, Risk factor; Predictor

Peer reviewers: Nick P Thompson, MD, Department of Medicine, Freeman Hospital, Newcastle Upon Tyne, NE7 7DN, United Kingdom; Takayuki Yamamoto, MD, Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) was first introduced by Gauderer *et al* in 1980^[1]. Since that time, it has become one of the most useful and established enteral nutrition techniques performed at treatment centers. Compared to the use of a nasogastric tube, enteral nutrition using a PEG tube offers numerous advantages, including reduced laryngopharyngeal discomfort and a lower risk of aspiration lung disease^[2,3]. When performing PEG, the associated risks must always be kept in mind. While various devices have been developed^[4,5], the frequency of adverse events is higher as compared to other nutritional methods, since PEG is based on a surgical technique^[6-8]. Additionally, even

if PEG is successful, patients often encounter enteral nutrition problems after surgery. We investigated and analyzed the etiology of these problems in patients seen at our hospital.

MATERIALS AND METHODS

Patients and gastrostomy

Of the 285 patients who underwent PEG at our center from April 1999 to April 2008, we were able to statistically analyze the data for 252 subjects (157 males, 95 females). Our center admits many elderly patients who present poor general conditions in addition to having problems with ingesting food orally. PEG is primarily performed in the gastroenterology department after a request from a different department. After PEG is scheduled, upper gastrointestinal endoscopy is performed preoperatively in all cases, and abdominal computed tomography (CT) is carried out as needed in order to ascertain whether PEG can be done. If patients are taking anticoagulant or antiplatelet agents, a drug-free period is established, which depends on the type of drug being taken.

All patients in the present study underwent gastrostomy using the pull method^[1]. With the exception of one patient, no sutures were used to fix the abdominal and gastric walls at the gastrostomy site. In general, antibiotics were administered intravenously for 3 d following PEG. Two days after surgery, lukewarm water was injected, followed by injection of enteral nutrients starting 4 d after the surgery. A switch to enteral nutrition using the PEG tube was initiated 7-10 d after surgery. At our clinical center, we have been using PEG clinical paths since June 2003.

Data analysis

In the present study, patients were considered to have a problem with enteral nutrition after PEG if they met one of the following criteria: (1) patients who required ≥ 1 mo after surgery to switch to complete enteral nutrition, or who required additional parenteral alimentation continuously; or (2) patients who abandoned switching to enteral nutrition using the gastrostoma and employed other nutritional methods. The data on the patients' backgrounds and suspected reasons for their problems were collected and used for further analysis. To analyze the predictors among the problem cases, we chose 26 candidates that we believed could possibly have an influence on the postoperative enteral nutrition (Table 1). Binomial logistic regression analysis was performed using statistical software (SPSS Base 11.0j and SPSS Regression Models 9.0J; SPSS Japan Inc., Tokyo, Japan), with the presence or absence of enteral nutrition problems after PEG employed as the dependent variable. Since it was necessary to analyze numerous factors, univariate analysis was conducted to narrow down the candidates based on the significance probability ($P < 0.1$). Independent predictors were determined by conducting multivariate analysis based on a step-down procedure that used likelihood ratios.

Subsequently, after subjects were grouped in relation to each predictor, problem characteristics were investigated.

RESULTS

Patients' background factors

Table 2 shows the background factors for the 252 patients. Mean age of the patients was 75 years (range, 38-99 years), with men making up approximately 60% of the group. As to the general physical conditions, body weight was low and overall condition was markedly poor. Blood testing revealed that the patients tended to be anemic and malnourished prior to gastrostomy. There were inflammatory reactions in many patients. Cerebrovascular disorders accounted for about 70% of the underlying diseases. Even though the majority of the patients had central nervous system diseases, disuse syndrome and senile dementia were also noted. PEG was performed for enteral nutrition in all patients.

Cases of enteral nutrition problems after PEG

A total of 44 (17.5%) out of 252 patients exhibited enteral nutrition problems after PEG. There were 33 cases that met criterion (1), and three cases required continuous supportive parenteral alimentation. In 30 of these cases, the mean number of days required to switch to enteral nutrition was 69 ± 31 (mean \pm SD) d (range 32-145 d). Eleven cases met criterion (2).

Table 3 shows causes of the enteral nutrition problems for each of the criteria. Although various events were confirmed, pneumonia, paralytic ileus, acute enterocolitis and biliary tract infection were the most frequently seen in both criterion groups. In the pneumonia and acute enterocolitis patients, aspiration pneumonia and *Clostridium difficile*-associated enteric disease (CDED) accounted for the majority of the cases, respectively. For criterion (2), aggravation of chronic renal dysfunction and heart failure were noted.

Statistical analysis

In 252 patients, univariate analysis was performed for each of the 26 factors with the presence or absence of enteral nutrition problems after PEG used as the dependent variable (Table 1). Candidate predictors were narrowed down to the following: "enteral nutrition before gastrectomy"; "hemoglobin level the day before gastrostomy"; "albumin level the day before gastrectomy"; "presence of esophageal hiatal hernia"; "past history of paralytic ileus"; "past history of aspiration pneumonia"; and "presence of chronic renal dysfunction". A step-down procedure that employed likelihood ratios was used for the seven items subjected to multivariate analysis. The following four factors were identified as independent predictors for cases with enteral nutrition problems after PEG: (1) enteral nutrition before gastrectomy; (2) presence of esophageal hiatal hernia; (3) past history of paralytic ileus; and (4) presence of chronic renal dysfunction (Table 4). The sensitivity, specificity and overall accuracy using the prediction model were 30.0%, 97.0% and 85.8%,

Table 1 Potential factors and univariate analysis for each of the candidates

	Number of values	Significance probability	Odds ratio
Female sex	252	0.377	0.732
Age (yr)	245	0.377	1.007
Body mass index (weight ² /height)	209	0.454	0.957
Performance status (ECOG scale)	252	0.996	0.999
Enteral nutrition before gastrectomy ¹	252	0.000	0.272
Alimentation by peripheral infusion before gastrectomy	252	0.983	1.014
Fever ≤ 48 h before gastrostomy (≥ 37.5°C)	243	0.188	1.874
Blood examination the day before gastrectomy			
White blood cell count (/μL)	240	0.718	1.000
Hemoglobin (g/dL) ¹	240	0.010	0.754
Albumin (g/dL) ¹	240	0.092	0.483
C-reactive protein (mg/dL)	238	0.295	1.110
Fasting blood sugar (mg/dL)	240	0.150	1.006
Presence of esophageal hiatal hernia ¹	252	0.002	4.076
Presence of gastric ulcer or erosive gastritis	252	0.170	1.800
Past history of gastrectomy	252	0.315	2.428
Past history of CDED	252	0.258	1.774
Past history of paralytic ileus ¹	252	0.012	5.204
Past history of cholecystitis or cholangitis	252	0.367	1.489
Presence of arteriosclerotic disorder	252	0.835	0.898
Past history of aspiration pneumonia ¹	252	0.037	2.014
Presence of chronic renal dysfunction ¹	252	0.003	13.205
Past history of urinary tract infection	252	0.958	0.975
Presence of diabetes mellitus	252	0.611	0.805
Rehabilitation before gastrectomy	252	0.416	0.612
Use of clinical paths	252	0.843	1.078
Duration of procedure (min)	199	0.553	1.016

¹Items were used for the multivariate analysis.

Table 2 Patient background factors obtained on the day before PEG (mean ± SD)

	Patients with enteral nutrition problems after PEG	Patients without enteral nutrition problems after PEG
Number of patients	208	44
Sex (Male/female)	127/81	30/14
Age (yr)	75 ± 11 (range 38-99)	76 ± 9 (range 55-92)
Body mass index (weight ² /height)	19.3 ± 3.1	18.8 ± 4.3
Performance status (EGOC scale)	3.6 ± 0.6	3.6 ± 0.5
Blood examination		
White blood cell count (/μL)	6550 ± 2105	6421 ± 1954
Hemoglobin (g/dL)	11.9 ± 1.7	11.1 ± 1.6
Albumin (g/dL)	3.3 ± 0.4	3.1 ± 0.3
C-reactive protein (mg/dL)	1.12 ± 1.51	1.40 ± 1.66
Fasting blood sugar (mg/dL)	105 ± 36	114 ± 36

respectively. After the deletion of unselected factors, the sensitivity and overall accuracy were improved.

Investigation of each predictor

Table 5 summarizes the actual causes of the patients' problems for each predictor. Because enteral nutrition before gastrectomy is a risk reduction factor, we decided to investigate cases of parenteral alimentation before PEG. While pneumonia accounted for about 30% of the problems, paralytic ileus, acute enterocolitis and biliary tract infection were also noted. In the enteral nutrition group, blood albumin and hemoglobin levels prior to gastrectomy were significantly higher than those seen in parenteral alimentation group [mean ± SD; albumin level (g/dL): 3.3 ± 0.4 *versus* 3.1 ± 0.4, $P < 0.001$; hemoglobin level (g/dL): 11.9 ± 1.7 *versus* 11.1 ± 1.6, $P < 0.001$; unpaired *t*-test]. In cases with esophageal hiatal hernia,

pneumonia accounted for about 45% of the problems. In addition, the majority of these cases were caused by aspiration. On the other hand, in cases with a past history of paralytic ileus, the most frequent cause was a recurrence of ileus. Similarly, in cases with chronic renal dysfunction, an aggravation of chronic renal dysfunction accounted for about 30% of the cases.

DISCUSSION

Although predictors for postoperative enteral nutrition problems can be used to determine indications for PEG, there are no studies that have specifically examined these factors. In the current study, most patients were elderly and suffering from cerebrovascular disorders or dementia, and their general condition was markedly poor. Due to long-term recumbency and undernutrition,

Table 3 Causes of enteral nutrition problems after PEG

	No.	%
Cases that required \geq 1 mo after surgery to switch to complete enteral nutrition, or that required additional parenteral alimentation continuously		
Pneumonia (aspiration pneumonia)	13 (8)	25
Paralytic ileus	8	15
Acute enterocolitis (CDED)	7 (5)	13
Biliary tract infection	5	10
Peritonitis	3	6
Urinary tract infection	3	6
Hemorrhagic gastric ulcer	1	2
Diarrhea	1	2
Drug-induced liver injury	1	2
Bacterial endocarditis	1	2
Aggravation of ASO	1	2
Stenosis of upper respiratory tract	1	2
Aggravation of chronic renal dysfunction	1	2
Cerebral infarction	1	2
Infection to central venous catheter	1	2
Sepsis	1	2
Convulsive seizure	1	2
Progression of hyponatremia	1	2
Fever (unknown origin)	1	2
Total	52	100
Patients that abandoned switching to enteral nutrition using the gastrostoma and employed other nutritional methods		
Pneumonia (aspiration pneumonia)	6 (6)	33
Paralytic ileus	2	11
Acute enterocolitis (CDED)	2 (1)	11
Biliary tract infection	2	11
Aggravation of chronic heart failure	2	11
Aggravation of chronic renal failure	2	11
Bleeding from fistula	1	6
Fever (unknown origin)	1	6
Total	18	100

ASO: Arteriosclerosis obliterans; CV: Central vein.

these patients had various infections. The current report presents useful information for gastroenterologists who perform PEG in patients with similar backgrounds.

The enteral nutrition problems that occurred after PEG were defined according to previously described criteria. We excluded cases in which there was switching to enteral nutrition within 1 mo after PEG, as we believe that there are few disadvantages for such patients. We also excluded cases where the reason for the problem was unclear, even if these patients required longer than 1 mo to switch to enteral nutrition.

Our results demonstrated that enteral nutrition problems after PEG occurred at a comparatively high rate. Although various causes were confirmed, few cases were determined to be a direct complication of PEG. For both of the inclusion criteria, pneumonia occurred most frequently, although enterocolitis, paralytic ileus and biliary tract infection were also noted. Aspiration pneumonia accounted for the majority of the pneumonia cases. We also noted that cerebrovascular disorders accounted for approximately 70% of the underlying diseases. It is possible that dysphagia may promote aspiration in these patients. In and by itself, paralytic ileus can cause enteral nutrition problems. Furthermore, it may also promote or aggravate aspiration pneumonia,

as bowel paralysis induces vomiting or gastrointestinal reflux^[9]. In acute enterocolitis patients, CDED accounted for the majority of the cases. CDED is a drug (antibiotics)-induced enteric disease and we have previously reported that CDED can occur, with onset of the disease noted soon after the PEG procedure^[10]. In this study, we confirmed that the CDED that occurred after PEG was able to interrupt enteral nutrition over a long period of time. In almost all cases of biliary tract infection, stones or sludge were noted in the gallbladder. Other studies have reported that when patients are switched to enteral nutrition from parenteral alimentation, there is an increase in choleresis, along with a sudden contraction of the gallbladder^[11,12]. These events may promote obstruction and infection within the bile duct system.

We attempted to determine predictors for problem cases and our results indicated that only enteral nutrition before PEG was a risk reduction factor. Our analysis demonstrated there was a small but significant probability that preoperative enteral nutrition strongly inhibited enteral nutrition problems after PEG. As compared to parenteral alimentation, enteral nutrition offers the following advantages: (1) maintains a favorable and natural alimentation; (2) maintains gastrointestinal function; and (3) provides a check on the safety of enteral nutrition prior to the PEG procedure^[13-15]. Actually, we noted that aspiration pneumonia after vomiting or gastrointestinal disorder occurred in cases of preoperative parenteral alimentation. In addition, there was one patient after the PEG procedure who was afflicted by a central-venous-catheter-caused infection. Our results also showed that blood albumin and hemoglobin levels prior to gastrectomy were significantly higher in the enteral nutrition group. Therefore, if parenteral alimentation cases are scheduled for PEG, the procedure should be performed after switching to enteral nutrition.

Among the three risk factors examined, the presence of an esophageal hiatal hernia had the strongest association with the enteral nutrition problems that are found after PEG. In these cases, pneumonia accounted for about 45% of the causes, with the majority of the pneumonia cases occurring due to aspiration. Previous studies have shown that aspiration is a complication of esophageal hiatal hernia and gastroesophageal reflux disease^[16]. Recently, Kitamura *et al* reported that esophageal hiatal hernia was a risk factor for aspiration pneumonia after PEG^[17]. Our results indicate that preoperative upper gastrointestinal endoscopy is important for predicting enteral nutrition problems after PEG. After PEG, the posture of patients with esophageal hiatal hernia needs to be evaluated during nutrition. In addition, in such situations, it may also be necessary to consider using half-solid enteral nutrients^[18-20]. In patients with a past history of paralytic ileus, the recurrence of ileus accounted for about 40% of the causes. As chronic bowel dysfunction plays a role in the background of idiopathic paralytic ileus, its recurrence is not all that rare^[21]. In such cases, a rapid increase of enteral nutrient

Table 4 Predictors identified by multivariate analysis

	Regression coefficients (B)	Standard error	Significance probability	Odds ratio
Enteral nutrition before gastrectomy	-1.369	0.397	0.000	0.248
Presence of esophageal hiatal hernia	1.728	0.512	0.001	5.629
Past history of paralytic ileus	1.634	0.773	0.035	5.123
Presence of chronic renal dysfunction	2.011	0.954	0.035	7.470

Table 5 Causes of enteral nutrition problems after PEG with their respective predictors

	No.	%
Parenteral alimentation before gastrectomy		
Pneumonia (aspiration pneumonia)	14 (11)	33
Paralytic ileus	6	14
Acute enterocolitis (CDED)	5 (4)	12
Biliary tract infection	2	9
Aggravation of chronic renal dysfunction	2	5
Fever (unknown origin)	2	5
Peritonitis	1	2
Bleeding from fistula	1	2
Diarrhea	1	2
Drug-induced liver injury	1	2
Aggravation of chronic heart failure	1	2
Aggravation of ASO	1	2
Urinary tract infection	1	2
Sepsis	1	2
Infection to central venous catheter	1	2
Convulsive seizure	1	2
Total	43	100
Presence of esophageal hiatal hernia		
Pneumonia (aspiration pneumonia)	8 (5)	44
Acute enterocolitis (CDED)	3 (2)	17
Paralytic ileus	2	11
Peritonitis	1	6
Aggravation of chronic heart failure	1	6
Stenosis of upper respiratory tract	1	6
Aggravation of chronic renal dysfunction	1	6
Cerebral infarction	1	6
Total	18	100
Past history of paralytic ileus		
Paralytic ileus	3	38
Biliary tract infection	2	25
Pneumonia (aspiration pneumonia)	2 (2)	25
Peritonitis	1	13
Total	8	100
Presence of chronic renal dysfunction		
Aggravation of chronic renal dysfunction	3	33
Aggravation of chronic heart failure	2	22
Pneumonia (Aspiration pneumonia)	2 (1)	22
Diarrhea	1	11
Fever (unknown origin)	1	11
Total	9	100

after PEG may be responsible for the recurrent paralytic ileus. When there is coadministration of enterokines activators or gradual increases of enteral nutrients, this may prevent such recurrences^[22,23]. In cases with chronic renal dysfunction, an aggravation of renal dysfunction or heart failure accounted for about 60% of the causes. In most cases, there was an eventual discontinuation of the enteral nutrition after PEG. Therefore, in patients with poor renal function, the indication for PEG needs to be very carefully investigated. In addition, after performing PEG in such cases, it is necessary to finely control the infusions and medications by performing frequent blood

or X-ray tests.

Previous studies have reported that patients with diabetes or low body weight have a high frequency of complications^[24,25]. However, body mass index, fasting blood sugar levels and the presence of diabetes mellitus were not identified as predictors of enteral nutrition problems after PEG. In the present study, most patients were elderly, displayed a markedly poor general condition and tended to be underweight, malnourished and anemic. A bias in patient background factors may also have affected our analysis. Based on the mean preoperative blood glucose levels, it is also quite possible that a stricter control of the diabetes could have suppressed an increased number of adverse events. Although our univariate analysis indicated that blood albumin and hemoglobin levels obtained on the day before PEG could be regarded as strong candidates, both were excluded by multivariate analysis. In the enteral nutrition group, blood albumin and hemoglobin levels obtained the day before gastrectomy were significantly higher than those found in the parenteral alimentation group. Therefore, the relevance of these factors may have affected our current analysis.

CONCLUSION

Enteral nutrition problems after PEG occurred at a comparatively high rate. Analysis of patient background factors elucidated four predictors for these problem cases. Since characteristic causes exist for these respective predictors, it may be possible to analyze causal relationships and mechanisms of onset, thereby making it possible to devise several preventative methods.

COMMENTS

Background

Percutaneous endoscopic gastrostomy (PEG) has become one of the most useful and established enteral nutrition techniques. However, since PEG is based on a surgical technique and is mainly performed in elderly individuals with poor general conditions, the frequency of adverse events is higher compared to other methods of nutrition. Even if PEG is successful, patients often encounter enteral nutrition problems after surgery.

Research frontiers

Although knowledge of predictors of postoperative enteral nutrition problems may provide useful information, there are no studies that have specifically examined such predictors. The authors investigated the etiology of these problems, and tried to predict enteral nutrition problems after PEG.

Innovations and breakthroughs

The authors showed that enteral nutrition problems after PEG occurred at a comparatively high rate. Analysis of patient background factors elucidated the following four predictors for these problem cases: (1) enteral nutrition before gastrectomy; (2) presence of esophageal hiatal hernia; (3) past history of paralytic ileus; and (4) presence of chronic renal dysfunction.

Applications

Predictors for postoperative enteral nutrition problems can be used to determine indications for PEG. Since specific causes exist for these predictors, it may be possible to analyze causal relationships and mechanisms of onset, thereby making it possible to devise several preventive methods.

Peer review

This study reports a large number of patients with PEGs and identifies factors that seem to predict failure of enteral nutrition. This is likely to be of interest to readers and provides some novel data. In addition, the discussion gives some ideas about how to address individual patients with poor prognostic factors.

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Progression of diethylnitrosamine-induced hepatic carcinogenesis in carnitine-depleted rats

Salim S Al-Rejaie, Abdulaziz M Aleisa, Abdulaziz A Al-Yahya, Saleh A Bakheet, Abdulmalik Alsheikh, Amal G Fatani, Othman A Al-Shabanah, Mohamed M Sayed-Ahmed

Salim S Al-Rejaie, Abdulaziz M Aleisa, Abdulaziz A Al-Yahya, Saleh A Bakheet, Amal G Fatani, Othman A Al-Shabanah, Mohamed M Sayed-Ahmed, Department of Pharmacology, College of Pharmacy, King Saud University, PO Box 2457, Kingdom of Saudi Arabia

Abdulmalik Alsheikh, Department of Pathology, College of Medicine, King Saud University, PO Box 2457, Kingdom of Saudi Arabia

Author contributions: Al-Rejaie SS and Aleisa AM designed the study and were involved in editing the manuscript; Al-Yahya AA and Bakheet SA performed the majority of experiments; Alsheikh A performed the histopathology; Fatani AG provided vital reagents and analyzed the data, Al-Shabanah OA and Sayed-Ahmed MM provided financial support for the study and wrote the manuscript.

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Correspondence to: Dr. Mohamed M Sayed-Ahmed, Department of Pharmacology, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451,

Kingdom of Saudi Arabia. mmsayedahmed@hotmail.com

Telephone: +966-506065734 Fax: +966-1-14677200

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Abstract

AIM: To investigate whether carnitine deficiency is a risk factor during the development of diethylnitrosamine (DENA)-induced hepatic carcinogenesis.

METHODS: A total of 60 male Wistar albino rats were divided into six groups with 10 animals in each group. Rats in group 1 (control group) received a single intraperitoneal (i.p.) injection of normal saline. Animals in group 2 (carnitine-supplemented group) were given L-carnitine (200 mg/kg per day) in drinking water for 8 wk. Animals in group 3 (carnitine-depleted group) were given D-carnitine (200 mg/kg per day) and mildronate (200 mg/kg per day) in drinking water for 8 wk. Rats in group 4 (DENA group) were injected with a single dose of DENA (200 mg/kg, i.p.) and 2 wk later received a single dose of carbon tetrachloride (2 mL/kg) by gavage as 1:1 dilution in corn oil. Animals in group 5 (DENA-carnitine depleted group) received the same treatment as group 3 and group 4. Rats in group 6 (DENA-carnitine supplemented group) received the same treatment as group 2 and group 4.

RESULTS: Administration of DENA resulted in a significant increase in alanine transaminase (ALT), gamma-glutamyl transferase (G-GT), alkaline phosphatase (ALP), total bilirubin, thiobarbituric acid reactive substances (TBARS) and total nitrate/nitrite (NO_x) and a significant decrease in reduced glutathione (GSH), glutathione peroxidase (GSHPx), catalase (CAT) and total carnitine content in liver tissues. In the carnitine-depleted rat model, DENA induced a dramatic increase in serum ALT, G-GT, ALP and total bilirubin, as well as a progressive reduction in total carnitine content in liver tissues. Interestingly, L-carnitine supplementation resulted in a complete reversal of the increase in liver enzymes, TBARS and NO_x, and a decrease in total carnitine, GSH, GSHPx, and CAT induced by DENA, compared with the control values. Histopathological examination of liver tissues confirmed the biochemical data, where L-carnitine prevented DENA-induced hepatic carcinogenesis while D-carnitine-mildronate aggravated DENA-induced hepatic damage.

CONCLUSION: Data from this study suggest for the first time that: (1) carnitine deficiency is a risk factor and should be viewed as a mechanism in DENA-induced hepatic carcinogenesis; (2) oxidative stress plays an important role but is not the only cause of DENA-induced hepatic carcinogenesis; and (3) long-term L-carnitine supplementation prevents the development of DENA-induced liver cancer.

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Key words: Carnitine deficiency; D-carnitine; L-carnitine; Diethylnitrosamine; Hepatic carcinogenesis

Peer reviewer: Anna Linda Zignego, MD, PhD, Professor of Medicine, Department of Internal Medicine, Medical School, Center for the Study of Systemic Manifestations of Hepatitis Viruses "MaSVE", Center for Research, Transfer and High Education "DENOthe", University of Florence, Viale Morgagni 85, 50134 Florence, Italy

Al-Rejaie SS, Aleisa AM, Al-Yahya AA, Bakheet SA, Alsheikh A, Fatani AG, Al-Shabanah OA, Sayed-Ahmed MM. Progression of diethylnitrosamine-induced hepatic carcinogenesis in carnitine-depleted rats. *World J Gastroenterol* 2009; 15(11): 1373-1380 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1373.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1373>

INTRODUCTION

Liver cancer is one of the most common malignancies worldwide, especially in Asia and Africa^[1]. Hepatocellular carcinoma accounts for about 80%-90% of all liver cancer and is the fourth most common cause of cancer mortality^[2]. Major risk factors for liver cancer include hepatitis viral infection, food additives, alcohol, aflatoxins, environmental and industrial toxic chemicals, and air and water pollutants^[3,4]. Diethylnitrosamine (DENA) is a well known potent hepatocarcinogenic agent present in tobacco smoke, water, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics and pharmaceutical products^[5-7]. DENA is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models^[8]. Although there are many strategies for the treatment of liver cancer^[9-11], the therapeutic outcome of this cancer remains very poor. Therefore, prevention seems to be the best strategy for lowering the incidence of this disease. Recently, considerable research has been carried out in the search for natural or synthetic compounds as a means of chemically preventing liver cancer^[12-18]. In this regard, many compounds have been tested with proved efficacy against experimentally-induced hepatocarcinogenesis. These compounds include morin^[12], silymarin^[13], garlic^[14], star anise^[15], ganfujian granule^[1], apigenin^[16] and the crude extracts of *agaricus blazei*^[17].

L-carnitine is a naturally occurring compound that is primarily located in mitochondria and possesses potential protective effects against many mitochondrial toxic agents^[18-20]. It is derived from two sources: endogenous synthesis, in the liver and kidney, and from exogenous dietary sources such as red meat and dairy products^[19,21]. L-carnitine is an essential cofactor for the translocation of long-chain fatty acids from the cytoplasmic compartment into mitochondria, where beta-oxidation enzymes are located for ATP production^[22]. It has been reported that mitochondrial injury may exert a major influence on carcinogenesis^[23]. Consistent with this hypothesis, Chang *et al*^[24] have reported that mitochondrial dysfunction plays an essential role in hepatocarcinogenesis *via* production of reactive oxygen species (ROS), and that L-carnitine inhibits pre-neoplastic lesions and prevents hepatocarcinogenesis in Long Evans Cinnamon rats as a model of hepatocarcinogenesis. Furthermore, earlier studies have reported that carnitine effectively inhibits mitochondrial injury induced by ROS and mitochondria-dependent apoptosis^[25-27]. In addition, L-carnitine prevents the accumulation of free fatty acids and their toxic intermediates, thus preventing their harmful effects on mitochondrial and cell membranes^[20,26,28]. Despite the liver being the main organ responsible for endogenous synthesis of L-carnitine, we were unable to find any previously published studies investigating the role of long-term endogenous carnitine depletion and/or carnitine deficiency during induction of hepatic carcinogenesis. Therefore, this study was initiated with the following specific aims: (1) to determine whether or not endogenous

carnitine depletion and/or carnitine deficiency is a risk factor during the development of hepatic carcinogenesis and; (2) to gain insights into the possibility of mechanism-based protection by L-carnitine supplementation against DENA-induced hepatocarcinogenesis.

MATERIALS AND METHODS

Animals

Adult male Wistar albino rats, weighing 180-200 g, were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University (KSU), Riyadh, Kingdom of Saudi Arabia (KSA) and were housed in metabolic cages under controlled environmental conditions (25°C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless otherwise indicated. The protocol of this study was approved by the Research Ethics Committee of College of Pharmacy, KSU, Riyadh, KSA.

Materials

DENA and carbon tetrachloride (CCl₄) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DENA was dissolved in saline and injected in a single dose (200 mg/kg, i.p.) to initiate hepatic carcinogenesis, while CCl₄ was used in a single dose (2 mL/kg) by gavage as 1:1 dilution in corn oil to stimulate liver cell proliferation and regeneration according to previously published protocols^[29,30]. L-carnitine, D-carnitine and mildronate (trimethylhydrazinium propionate) were kindly supplied by Dr. Menotti Calvani, (Sigma-Tau Pharmaceuticals, Pomezia, Roma, Italy). All other chemicals used were of the highest analytical grade.

Carnitine-depleted rat model

Animal models of carnitine deficiency have been developed by many investigators^[31-33]. In the current study, carnitine deficiency was induced in rats by administration of D-carnitine (200 mg/kg per day) and mildronate (200 mg/kg per day) in drinking water. Depletion of L-carnitine by D-carnitine occurs *via* an exchange of the D- and L-isomers across the cell membrane where intracellular L-carnitine has been shown to exchange with extracellular D-carnitine^[31]. Moreover, mildronate inhibits carnitine biosynthesis by inhibition of the gamma-butyrobetaine hydroxylase enzyme^[32,33].

Experimental design

To achieve the ultimate goal of this study, a total of 60 adult male Wistar albino rats were divided into six groups with 10 animals in each group. Rats in group 1 (control group) received a single intraperitoneal (ip) injection of normal saline (2.5 mL/kg). Animals in group 2 (carnitine-supplemented group) were given L-carnitine (200 mg/kg per day) in drinking water for 8 wk. Animals in group 3 (carnitine-depleted group) were given D-carnitine (200 mg/kg per day) and mildronate (200 mg/kg per day) in drinking water for 8 wk. Two weeks after the experiment began, the animals in groups 1, 2 and 3

received a single dose of corn oil (0.5 mL/150 g body weight) by gavage. Rats in group 4 (DENA group) were injected with a single dose of DENA (200 mg/kg, i.p.) and 2 wk later received a single dose of CCl₄ (2 mL/kg) by gavage as 1:1 dilution in corn oil. Animals in group 5 (DENA-carnitine depleted group) received the same dose of DENA as group 4 and the same dose of D-carnitine-mildronate as group 3. Animals in group 6 (DENA-carnitine supplemented group) received the same dose of DENA as group 4 and the same dose of L-carnitine as group 2. At the end of the treatment protocol, animals were anesthetized with ether and blood samples were drawn from the orbital venous plexus. Serum was separated by centrifugation for 5 min at 1500 g and stored at -20°C until analysis. This was then used to determine ALT, G-GT and ALP activities and total bilirubin. All animals were sacrificed by decapitation and their livers were rapidly excised, weighed, washed with saline, blotted with a piece of filter paper and homogenized in normal saline or 6% perchloric acid, as indicated in the procedures of measurement of each parameter, to yield a 10% (w/v) tissue homogenate, using a Branson sonifier (250 VWR Scientific, Danbury, Conn., USA). Liver specimens from each group were removed for histopathological examination. The specimens were fixed in 10% neutral buffered formalin, sectioned at 3 µm and stained with hematoxylin and eosin (H & E) stain for light microscopic examination. To avoid any type of bias, the slides were coded and examined by a histopathologist who was blinded to the treatment groups.

Measurements of liver function

The activities of ALT, G-GT and ALP as well as total bilirubin in serum were determined using Randox commercially available kits (Randox Laboratories Ltd., Diamond Road, Cruclin, Co. Antrim, UK).

Determination of reduced glutathione and lipid peroxidation in liver tissues

The tissue levels of the acid soluble thiols, mainly GSH, were assayed spectrophotometrically at 412 nm, according to the method of Ellman^[34] using a Shimadzu (Tokyo, Japan) spectrophotometer. The content of GSH was expressed as µmol/g wet tissue. The degree of lipid peroxidation in liver tissues was determined by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant tissue from the homogenate^[35]. The homogenates were centrifuged at 1500 g and the supernatant was collected and used for the estimation of TBARS. The absorbance was measured spectrophotometrically at 532 nm and the concentrations were expressed as nmol TBARS/g wet tissue.

Determination of glutathione peroxidase and catalase activity in liver tissues

The activity of glutathione peroxidase (GSHPx) was determined according to the method of Lawrence and Burk^[36]. The changes in the absorbance at 340 nm were recorded at 1 min interval for 5 min and the results were expressed as µmol/min per g tissue. The catalase

(CAT) activity was determined spectrophotometrically by the method of Higgins *et al.*^[37] which is the assay of hydrogen peroxide (H₂O₂). The activity was expressed as µmol/min per g tissue using the molar absorbance of 43.6 for H₂O₂.

Determination of total nitrate/nitrite concentrations in liver tissues

Total nitrate/nitrite (NO_x), an index of nitric oxide (NO) production, was measured as the stable end product, nitrite, according to the method of Miranda *et al.*^[38]. The assay is based on the reduction of nitrate by vanadium trichloride combined with detection by the acidic Griess reaction. The diazotization of sulfanilic acid with nitrite at acidic pH and subsequent coupling with N-(10 naphthyl)-ethylenediamine produced an intensely colored product which was measured spectrophotometrically at 540 nm. The levels of NO_x were expressed as µmol/g wet tissue.

Determination of total carnitine levels in liver tissues

Liver homogenate was prepared in ice-cold 6% perchloric acid and centrifuged at 8000 g for 10 min. Part of the supernatant was used for the estimation of free carnitine, while the remainder was used for the determination of long-chain acyl carnitine after hydrolysis in 1 mol/L KOH at 65°C for 1 h according to Alhomida^[39]. Carnitine was determined using HPLC after pre-column derivatization with L-aminoanthracene as previously described by Longo *et al.*^[40]. The mobile phase was prepared by mixing 700 mL of 0.1 mol/L ammonium acetate, pH 3.5, with 300 mL of acetonitrile. Chromatographic separation was performed at a flow rate of 1.5 mL/min, using a Kromasil column (C18, 25 cm × 4.6 mm) from Saulentechnik Knayer, Berlin, Germany. The excitation and emission wavelengths of the spectrofluorimeter were 248 and 418 nm, respectively.

Statistical analysis

Differences between obtained values (mean ± SE, *n* = 10) were carried out by one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A *P* value of 0.05 or less was considered statistically significant.

RESULTS

Eight weeks after the experiment began, the administration of DENA resulted in a significant 16.6-fold, 316%, 152%, and 219% increase in serum ALT, G-GT, ALP and bilirubin, respectively, as compared to the control group. Long-term administration of either L-carnitine or D-carnitine alone for 8 wk showed a non-significant change. In carnitine-depleted rats, DENA resulted in a significant 85%, 56%, 49% and 29% increase in serum ALT, G-GT, ALP and bilirubin, respectively, as compared to DENA alone. Interestingly, administration of L-carnitine in combination with DENA resulted in a complete reversal of DENA-induced increases in serum ALT, G-GT, ALP and bilirubin, compared to the control values (Figure 1).

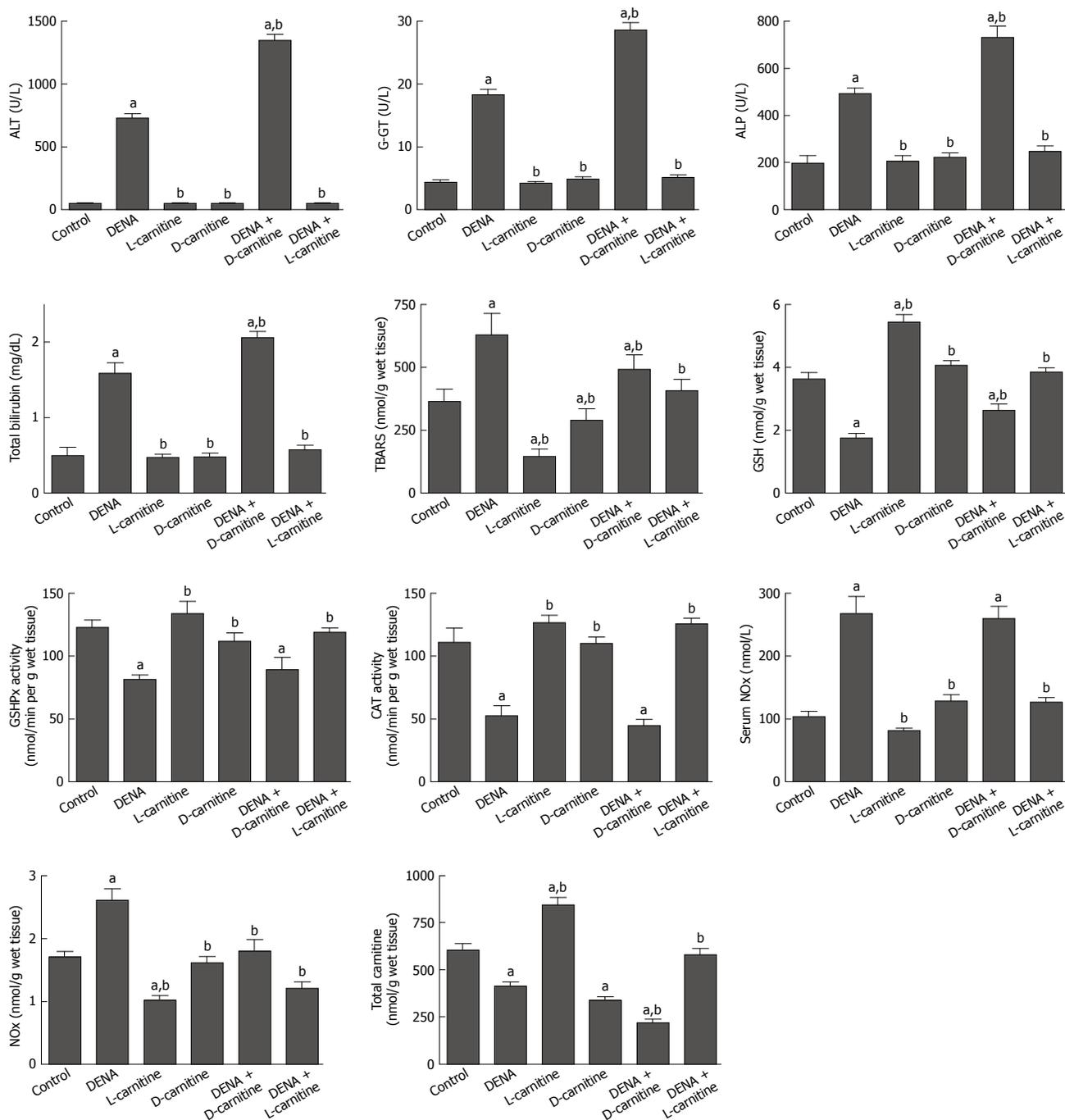


Figure 1 Effect of DENA on serum liver function indices, oxidative stress biomarkers, activities of antioxidant enzymes, NOx concentration and total carnitine levels in liver tissues from carnitine supplemented and depleted rats. Data are presented as mean \pm SE ($n = 10$). ^a $P < 0.05$ vs control; ^b $P < 0.05$ vs DENA, at $P < 0.05$ using ANOVA followed by Tukey-Kramer as a post ANOVA test.

The effects of DENA on the oxidative stress biomarkers, TBARS and GSH, in liver tissues from carnitine-supplemented and -depleted rats are shown in Figure 1. DENA resulted in a significant 73% increase in TBARS and a significant 52% decrease in GSH, as compared to the control group. Long-term administration of either L-carnitine or D-carnitine alone for 8 wk resulted in a significant decrease in TBARS and a significant increase in GSH as compared to either the control or the DENA groups. Moreover, L-carnitine resulted in a complete reversal of the DENA-induced decrease in GSH and increase in TBARS in liver tissues,

compared to the control values.

Administration of DENA resulted in a significant 34% and 52% decrease in GSHPx and CAT, respectively, as compared to the control group. Long-term administration of either L-carnitine or D-carnitine alone for 8 wk showed a non-significant change, as compared to the control group. In carnitine-supplemented rats, L-carnitine resulted in a complete reversal of the DENA-induced decrease in GSHPx and CAT in liver tissues compared to the control values (Figure 1).

Administration of DENA resulted in a significant 157% and 54% increase in the levels of NOx in serum

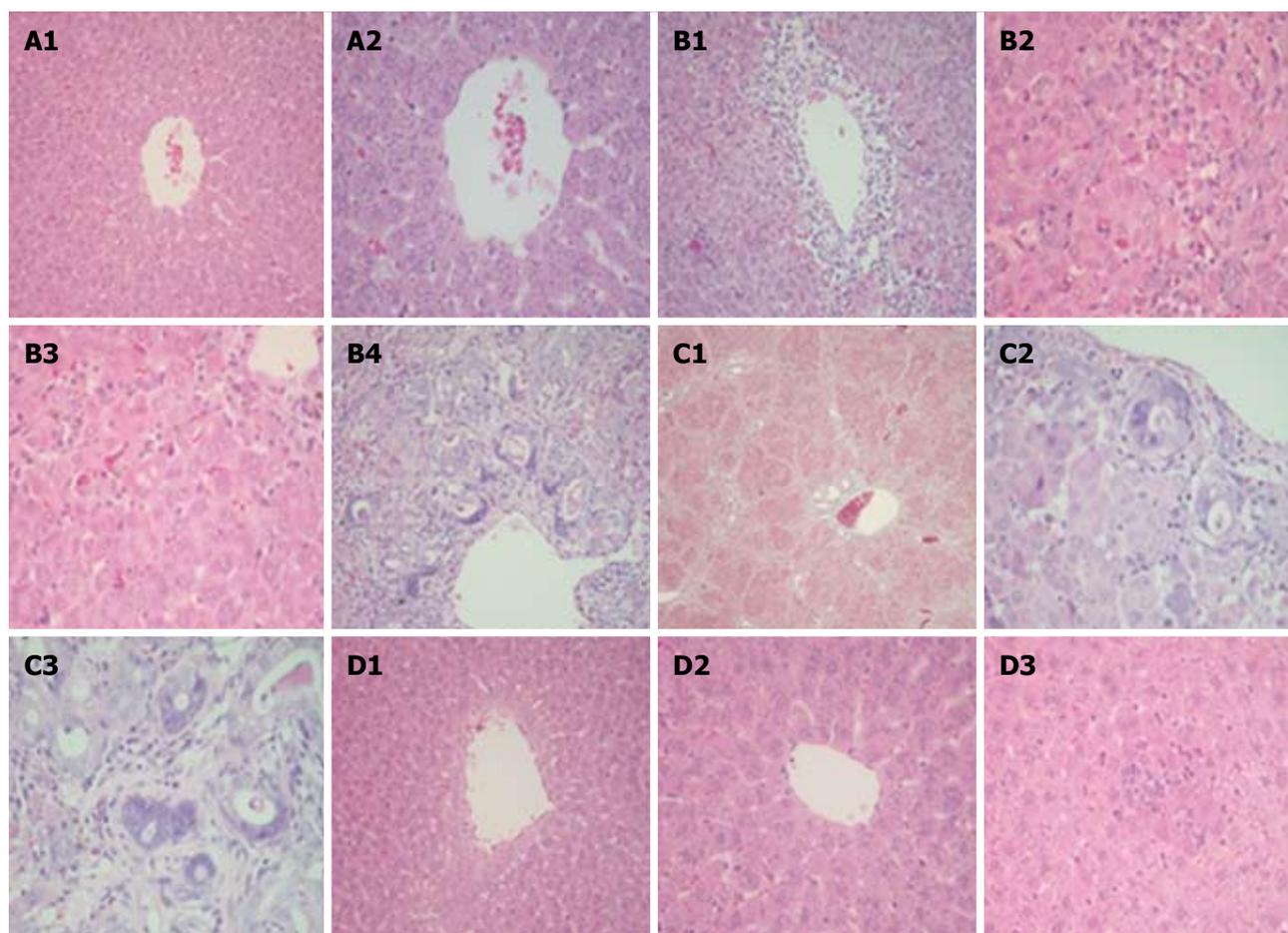


Figure 2 Photomicrographs of liver specimens stained with H&E. A: Liver from control rat showing normal liver histology with unremarkable central vein (A1, $\times 20$ and A2, $\times 40$); B: Liver from rat treated with DENA showing central vein surrounded by extensive necrosis and inflammatory infiltrate (B1, $\times 20$), considerable hepatocyte necrosis represented with arrows (B2 and B3, $\times 40$) and portal tract with bile duct proliferation and marked atypia (B4, $\times 20$); C: Liver from rat treated with DENA plus D-carnitine-mildronate showing diffuse bridging fibrosis and nodule formation (C1, $\times 10$) and bile ducts with marked reactive atypia showing nuclear enlargement, high nuclear/cytoplasmic ratio and prominent nucleoli (C2 and C3, $\times 40$); D: Liver from rat treated with DENA and L-carnitine showing normal liver (D1, $\times 20$) with unremarkable central vein (D2, $\times 40$) and hepatic lobule with a focus of inflammatory infiltrate but no necrosis (D3, $\times 40$).

and liver tissues, respectively, as compared to the control group. Long-term administration of either L-carnitine or D-carnitine-mildronate alone for 8 wk showed a non-significant change. In carnitine-supplemented rats, L-carnitine resulted in a complete reversal of the DENA-induced increase in NOx levels in serum and liver tissues compared to the control values (Figure 1).

Treatment with DENA resulted in a significant 32% decrease in total carnitine level in liver tissues, whereas long-term administration of D-carnitine-mildronate resulted in a significant 44% decrease in total carnitine level in liver tissues, as compared to the control group. Administration of L-carnitine resulted in a significant 40% increase in total carnitine level in liver tissues as compared to the control group. Administration of DENA to D-carnitine-mildronate-treated rats resulted in a significant 64%, 47% and 36% decrease in total carnitine content in liver tissues as compared to the control, DENA and D-carnitine-mildronate treatments, respectively. In carnitine-supplemented rats, L-carnitine resulted in a complete reversal of the DENA-induced decrease in total carnitine content in liver tissues compared to the control values (Figure 1).

Liver sections from control rats showed normal liver histology with unremarkable central veins (Figure 2A). Animals treated with DENA showed central veins surrounded by extensive necrosis and inflammatory infiltrate, clusters of hepatocyte necrosis and the portal tract with bile duct proliferation and marked atypia (Figure 2B). In carnitine-depleted rats, DENA-induced progressive histopathological and pre-neoplastic lesions that manifested as diffuse bridging fibrosis connecting central veins to portal tracts and nodule formation, bile ducts with marked reactive atypia showing nuclear enlargement, high nuclear/cytoplasmic ratio and prominent nucleoli (Figure 2C). Liver sections from rats treated with DENA and L-carnitine showed normal liver histology with unremarkable central veins, hepatic lobule with few scattered foci of inflammation, no evidence of necrosis, cirrhosis and no reactive atypia (Figure 2D).

DISCUSSION

Chemoprevention is defined as the use of naturally occurring and/or synthetic compounds in cancer therapy

in which the occurrence of cancer can be entirely prevented, slowed or reversed^[13]. Using Long Evans Cinnamon rats as a model of hepatocarcinogenesis, Chang *et al* was the first to report that L-carnitine inhibited pre-neoplastic lesions and prevented hepatocarcinogenesis^[24]. In our laboratory, earlier and more recent studies have demonstrated that carnitine deficiency is a risk factor and should be viewed as a mechanism in cisplatin-induced nephrotoxicity^[41,42], cardiomyopathy^[43] and hepatotoxicity^[44]. Although, the liver is the main organ responsible for endogenous synthesis of L-carnitine, we were unable to find any studies investigating the role of carnitine deficiency during DENA-induced hepatic carcinogenesis. Taken together, this prompted us to study whether or not long-term endogenous carnitine depletion is a risk factor during DENA-induced hepatic carcinogenesis.

The data presented here demonstrated that DENA increased serum indices of liver function including ALT, G-GT, ALP and total bilirubin (Figure 1) and caused severe histopathological lesions in liver tissues (Figure 2B). It is well known that the elevation of ALT and G-GT activities is repeatedly credited to hepatocellular damage^[45]. Also, the increase in ALP reflects a pathological alteration in biliary flow^[46]. G-GT is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain, and its liberation into serum indicates damage to the cell and thus injury to the liver^[12,46]. It is important to point out that serum G-GT activity is considered to be one of the best indicators of liver damage^[46]. In the current study, this observed increase in serum indices of liver function due to DENA could be a secondary event following DENA-induced lipid peroxidation of hepatocyte membranes, with a consequent increase in the leakage of ALT, G-GT, ALP and total bilirubin from liver tissues. An elevated level of serum indices of hepatocellular damage has been previously reported in many models of DENA-induced hepatocellular degeneration^[12,17,47,48]. Interestingly, long-term administration of L-carnitine prevented the increase in hepatic enzymes, suggesting that L-carnitine may have a potential protective effect against DENA-induced liver damage. This effect could be due to stabilization of hepatocyte membranes by L-carnitine with the consequent decrease in the leakage of liver enzymes. Indeed, the interaction of L-carnitine with sarcolemmal phospholipids and mitochondrial membranes has been previously reported^[49]. In carnitine-depleted rats, DENA produced a progressive increase in the activities of liver enzymes as well as massive degenerative changes and evidence of pre-neoplastic lesions in liver tissues (Figure 2C). Our results are consistent with the data presented by Hytiroglou *et al*^[50], which showed that precancerous lesions which may be detected in chronically diseased livers included clusters of hepatocytes with atypia and an increased proliferative rate.

Data from this study revealed that DENA significantly increased NOx and TBARS and decreased GSH, GSHPx and CAT in liver tissues, suggesting that reactive oxygen and nitrogen species induced by

DENA play an important role in DENA-induced hepatic carcinogenesis. Increased generation of ROS and decreased antioxidant enzymes in liver tissues has been reported in many models of DENA-induced hepatocellular carcinoma^[12-15]. It has been reported that ROS play a major role in tumor promotion through interaction with critical macromolecules including lipids, DNA, DNA repair systems, and other enzymes^[51]. Moreover, NOx is known to inhibit DNA repair proteins, thereby inhibiting the ability of the cell to repair damaged DNA^[52,53]. Data presented here demonstrated that long-term administration of both L-carnitine and D-carnitine completely reversed the increase in TBARS and NOx and the decrease in GSH, CAT and GSHPx induced by DENA in liver tissues. Our results are consistent with previous studies that have reported that the D- and L-forms of carnitine and its short-chain derivatives have similar non-enzymatic free-radical scavenging activity^[41,54,55]. Although, L-carnitine and D-carnitine have similar anti-lipid peroxidation activity, our study indicated that L-carnitine prevented the progression of DENA-induced hepatic carcinogenesis, while D-carnitine-Mildronate aggravated these lesions. Therefore, it is suggested that oxidative stress is not the only cause of DENA-induced hepatic carcinogenesis and that carnitine deficiency plays an important role.

The data presented here showed that DENA significantly decreased total carnitine in liver tissues. This effect could be a secondary event following inhibition of endogenous carnitine biosynthesis and/or decreased carnitine transport in DENA-induced hepatocellular damage. This hypothesis is consistent with data presented by Krahenbuhl *et al*^[56] which showed that biosynthesis of carnitine is decreased in rats with liver cirrhosis. It seems that our results are unique since there is no available data on the effect of DENA on hepatic carnitine content. The level of carnitine in hepatocytes is controlled by the specific carnitine transporter (OCTN-2) and endogenous synthesis^[18,19]. Decreased expression of OCTN-2 has been reported in the acute hepatitis phase in Long Evans Cinnamon rats as a model of hepatocarcinogenesis^[24]. Also, OCTN-2 located on hepatocyte membranes might be destroyed when exposed to ROS induced by DENA.

Although, D-carnitine-mildronate alone decreased hepatic carnitine content more than DENA, only DENA increased ALT, G-GT, ALP and total bilirubin. This argues against carnitine deficiency as a risk factor in DENA-induced hepatic carcinogenesis. A possible explanation for this is that D-carnitine, *via* its non-enzymatic antioxidant activity, causes stabilization of hepatic membranes and prevents the leakage of liver enzymes, whereas DENA, *via* the increasing generation of ROS, causes irreversible modification of membrane structures and functions with the consequent increase in liver enzymes. This marked decrease (64%) of carnitine level in liver tissue after combined treatment with DENA and D-carnitine-mildronate was parallel to the marked increase in ALT, G-GT, ALP and the massive histopathological lesions in liver tissues, which may point to carnitine deficiency as a possible risk factor during DENA-induced hepatic

carcinogenesis. Most probably, D-carnitine-mildronate *via* its depletion of L-carnitine, and DENA partly through generation of ROS and partly due to carnitine depletion produced such aggravated hepatocellular damage.

In this study, the chemopreventive effects achieved by long-term administration of L-carnitine against DENA-induced hepatic carcinogenesis is in good agreement with the data presented by Chang *et al.*^[24] which showed that L-carnitine inhibits pre-neoplastic lesions and prevents hepatocarcinogenesis in Long Evans Cinnamon rats as a model of hepatocarcinogenesis. Administration of L-carnitine will facilitate beta-oxidation, thereby minimizing the toxic effects of the free form of long-chain fatty acids and their intermediates in mitochondria. Consistent with this hypothesis is a report indicating that administration of L-carnitine decreased free fatty acids in serum and tissues, and prevented tissue injury in mice with juvenile visceral steatosis that lacked a carnitine transporter^[57,58]. In conclusion, data from this study suggest for the first time that: (1) carnitine deficiency is a risk factor and should be viewed as a mechanism in DENA-induced hepatic carcinogenesis; (2) oxidative stress plays an important role but is not the main cause of DENA-related hepatic carcinogenesis; and (3) long-term L-carnitine supplementation prevents the development of DENA-induced liver cancer. Therefore, carnitine supplementation alone or in combination with other natural chemopreventive compounds could be used to prevent, slow or reverse the occurrence of liver cancer.

COMMENTS

Background

Liver cancer is one of the most common malignancies worldwide, especially in Asia and Africa. In the last few years, considerable research has been carried out in the search for natural materials or foods as a means of chemically preventing liver cancer. L-carnitine is a naturally occurring compound which is primarily located in mitochondria and possesses potential protective effects against many mitochondrial toxic agents. It is derived from two sources: endogenous synthesis in the liver and from exogenous dietary sources such as red meat and dairy products. Despite the liver being the main organ responsible for endogenous synthesis of L-carnitine, the role of endogenous depletion and/or supplementation of L-carnitine during induction of hepatocarcinogenesis has not yet been studied.

Research frontiers

Absolute or relative carnitine deficits develop in chronic congestive heart failure, acute myocardial ischemia, diseases of peripheral blood vessels, and disturbances of lipid metabolism. However, the role of carnitine deficiency or supplementation during the development of hepatocarcinogenesis has not been addressed. In this study, the authors demonstrate that carnitine deficiency is a risk factor and should be viewed as a mechanism during the development of hepatic cancer.

Innovations and breakthroughs

Recent studies have highlighted the importance of L-carnitine in protecting against anticancer-drug-induced fatigue and multiple organ toxicity. This is the first study to report that carnitine deficiency is a risk factor and should be viewed as a mechanism during the induction of hepatic carcinogenesis, and that long-term L-carnitine supplementation prevents the development of liver cancer. Therefore, carnitine supplementation alone or in combination with other natural chemopreventive compounds could be used to prevent, slow or reverse the occurrence of liver cancer.

Applications

By understanding that carnitine deficiency provokes hepatocarcinogenesis, this study may represent a future strategy in preventing the occurrence of liver cancer by carnitine supplementation.

Peer review

This is a well written and well structured paper that provides new insights on the protective action of L-carnitine in the development of liver cancer. Interestingly, the scavenger action of L-carnitine against reactive oxygen species was not the only mechanism in exerting the protection.

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Correlation of p53 over-expression and alteration in *p53* gene detected by polymerase chain reaction-single strand conformation polymorphism in adenocarcinoma of gastric cancer patients from India

Sajjad Karim, Arif Ali

Sajjad Karim, Arif Ali, Department of Bio-technology, Jamia Millia Islamia, New Delhi 110025, India

Sajjad Karim, Center of Excellence in Genomic Medicine Research, King Abdulaziz University, PO Box 80216, Jeddah-21589, Kingdom of Saudi Arabia

Author contributions: Karim S designed research, performed all experimental work and wrote the paper; Ali A was involved in designing as well as supervising the research work.

Correspondence to: Arif Ali, Professor and Head, Department of Bio-technology, Jamia Millia Islamia, New Delhi 110025, India. aliarif@rediffmail.com

Telephone: +91-11-26988335 Fax: +91-11-26988335

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Abstract

AIM: To study the alterations in *p53* gene among Indian gastric cancer patients and to correlate them with the various clinicopathological parameters.

METHODS: A total of 103 gastric cancer patients were included in this study. The *p53* alterations were studied by both immunohistochemical method as well as polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis. We only studied four (exon 5, 6, 7, and 8) of the 11 *p53* exons. The alterations in *p53* were also correlated with respect to various clinicopathological parameters.

RESULTS: Among 103 cases, p53 over-expression and alteration were detected in 37 (35.92%) and 19 (18.44%) cases, respectively. Most of the *p53* alterations were found at exon 5 (31.54%), followed by exon 6 (26.31%), exon 7 (21.04%) and exon 8 (21.04%). A significant correlation of p53 over-expression was found with *p53* alteration ($P = 0.000$). Concordance between *p53* alteration (as detected by SSCP) and over-expression [as detected by immunohistochemistry (IHC)] was found in 75% cases. We found that IHC-positive/SSCP-negative cases accounted for 21% of cases and IHC-negative/SSCP-positive cases accounted for remaining 4% cases.

CONCLUSION: Our results show that *p53* gene

mutations are significantly correlated with p53 protein over-expression, with 75% concordance in over-expression and alteration in the *p53* gene, but 25% discordance also cautions against the assumption that p53 over-expression is always associated with a gene mutation. There may be other mechanisms responsible for stabilization and accumulation of p53 protein with no evidence of gene mutation that reflect an accumulation of a non-mutated protein, or a false negative SSCP result.

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Key words: Gastric cancer; *p53*; Single strand conformation polymorphism; Gene mutation; Immunohistochemistry

Peer reviewer: Minna Nyström, PhD, Department of Biological and Environmental Sciences, PO Box 56 (Viikinkaari 5 D), University of Helsinki, Helsinki FI-00014, Finland

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INTRODUCTION

Gastric cancer is a common disease worldwide^[1] and also one of the leading causes of cancer death (5th in male and 6th in female) in India^[2]. Its pattern and incidence vary widely between different parts of the world. Costa Rica and Japan have the first and second highest rate in the world with a death rate of 77.7 and 50.5 per 100 000, respectively^[1,3]. In the United States, 12 100 deaths from gastric cancer were expected during 2003 with a death rate of 6.8 per 100 000^[4]. The overall age-adjusted incidence (world population) of gastric cancer in Jordan is 5.8 per 100 000^[5]. The burden of new cancer cases has been estimated in the year 2002 based on the National Cancer Registry Programme in India. The number of

new digestive tract cancer cases was estimated to be approximately 145 000; out of which stomach cancer comprised 23 785 cases in men and 11 890 in women^[6]. Although aggressive surgical resection has improved the overall outcome of patients with this carcinoma, results of surgical resection for advanced cancer are still poor, and the search for predictors of disease survival and response to therapy is therefore mandatory^[7]. The molecular biology of gastric cancer has been widely studied in the developed world, particularly in Japan^[8-11]. However, only a few scattered reports from the developing world have been published^[12-16], and there are no similar published data reported from India. The molecular events leading to the development of gastric cancer are largely unknown, but there is now enough evidence to suggest that the functional inactivation of *p53* gene through allelic loss and point mutation plays an important part^[17,18].

Several genetic alterations have been shown to play a significant role in tumorigenesis. By and large, the most frequently observed molecular changes occur in the *p53* gene^[19]. The *p53* gene encodes a protein involved in control of the cell cycle and acts as a negative regulator in the cell's response to damaged DNA^[20]. Functional alteration of *p53* protein can occur through several mechanisms: point mutations, deletions, rearrangements in the *p53* gene, binding with viral proteins, binding with cellular proteins, and oligomerization^[21]. Wild-type *p53* protein has a very short half-life, whereas mutated *p53* is stable and can accumulate at high concentration in the nuclei of tumor cells. As a consequence, immunohistochemical staining with specific antibodies can be used to detect mutant *p53* protein. Detecting the precise alteration occurring at the genetic level is much more laborious and costly. The most widely used molecular approach is single strand conformation polymorphism (SSCP) analysis of DNA fragment amplified by polymerase chain reaction (PCR)^[22], with subsequent sequence analysis. The sensitivity of SSCP is influenced by the experimental conditions, and by the length of the amplified fragment under study.

The aim of present study was to determine *p53* status in Indian gastric cancer patients, who have undergone gastrectomy, and correlate it with various clinicopathological parameters. This study also correlated immunohistochemical staining of *p53* protein with SSCP analysis of the PCR-amplified DNA.

MATERIALS AND METHODS

Tumor specimens

From April 2002 to February 2007, cancerous and normal mucosa samples taken from 128 consecutive patients with suspected gastric cancer were enrolled for the study. Cancerous and normal mucosa samples collected from various hospitals after gastrectomy were aliquoted into two sterile tubes. The aliquots for histological and immunohistochemical studies were stored at 4°C in 10% buffered formalin. On the other hand, aliquots for PCR-SSCP studies were stored at -20°C in 1X PBS until DNA extraction. The tissue

specimen for SSCP analysis was taken from the center of the tumor mass. No patients had undergone previous chemotherapy or radiotherapy. All the clinicopathological data were collected prospectively and registered in the database. A detailed histopathological examination was performed to determine the depth of invasion on the gastric wall and the extent of metastases within regional lymph nodes. Among 128 cases, 25 were found to be histopathologically negative for gastric cancer and thus were excluded from the study. The remaining 103 cases were from West Bengal ($n = 60$), Jammu & Kashmir ($n = 30$) and New Delhi ($n = 13$). Patients included males ($n = 78$) and females ($n = 25$) with a mean age of 56 years (range 25-71 years). The clinical stage was determined by the tumor, node, metastasis (TNM) system^[23] and histological diagnosis was made according to the classification of Lauren^[24].

Immunohistochemical staining

For immunohistochemical analysis of *p53* protein, 4- μ m-thick tissue sections were cut and stained with anti-*p53* monoclonal antibody (DO7, Biogenex Laboratories, San Ramon, USA) by streptavidin-biotin immunoperoxidase technique (LSAB) using the standard avidin-biotin-complex (ABC) method, followed by antigen retrieval by microwave technique as previously described^[25,26]. Briefly, 4- μ m-thick sections were first mounted on glass slides, treated with methanol (with 4% H_2O_2) for 30 min, and incubated with anti-*p53* monoclonal antibody (1:100 dilution; DO1 and DO7, Biogenex Laboratories, San Ramon, USA) overnight at 4°C in a humid chamber. After washing thrice with PBS, slides were incubated with biotinylated anti-mouse IgG antibody (DAKO-LSAB kit, Peroxidase, M/s Dakopatts, Denmark) for 30 min at room temperature and developed using HRP-streptavidin and diaminobenzidine tetra hydrochloride (Sigma, St. Louis, USA). The slides were incubated at room temperature for 5-10 min under microscopic control till the optimum development of brown-colored product. Finally, sections were counterstained with Harris hematoxylin and mounted in DPX. The reaction was considered as positive when a strong coloration of the cell nucleus was evident. Sections with nuclear staining in less than 10% of the tumor cells were regarded as negative. Positive *p53* nuclear staining was categorized into three patterns as follows: (+), only a few scattered positively stained cells (10%-25% of all tumor cells); (++) , localized aggregation of positively stained cells (26%-70% of all tumor cells); and (+++) , diffuse aggregation of positively stained cells (more than 70% of all tumor cells). Over-expression of the *p53* protein was defined when a (++) or (+++) staining pattern was observed. Negative control sections were processed immunohistochemically without incubating with the primary antibody and positive control sections were from a breast cancer tissue known to express high level of *p53*.

Genomic DNA isolation and PCR amplification of the p53 exons

Genomic DNA was isolated from tissues of gastric

Table 1 Detail of the primers and cycling parameters

<i>p53</i> exons	Sequence (5'-3')	Cycling parameters ¹	PCR product (bp)
5	Sp5F: TGTTCACITGTGCCCTGACT Sp5R: CAGCCCTGTCGTCCTCCAG	45 s denaturation at 94°C, 30 s annealing at 54°C, 1 min extension at 72°C	266
6	Sp6F: GCCCTGATTCCCTCACTGAT Sp6R: TTAACCCCTCCTCCAGAGA	45 s denaturation at 94°C, 30 s annealing at 53°C, 1 min extension at 72°C	160
7	Sp7F: ACTGGCCTCATCTTGGGCT Sp7R: TGTCAGGGTGGCAAGTGGC	45 s denaturation at 94°C, 30 s annealing at 56°C, 1 min extension at 72°C	180
8	Sp8F: TAAATGGGACAGGTAGGACC Sp8R: TCCACCGCTTCTTGTCCTGC	45 s denaturation at 94°C, 30 s annealing at 54°C, 1 min extension at 72°C	230

¹Initial denaturation of 10 min at 94°C and final extension of 10 min at 72°C were used in all amplifications. Number of cycles in all amplifications was 35.



Figure 1 SSCP analysis of exons 5, 6, 7 and 8 of *p53* gene. Wild-type and one tumor sample containing mutation are shown for each exon. Only single-strand DNA bands are shown. Heterodimers and double-stranded bands have been omitted.

cancer patients by a modified proteinase K digestion and phenol/chloroform extraction technique. All four exons (5, 6, 7 and 8) were amplified separately and details of the primers and PCR cycling parameters used are mentioned in Table 1. The standard PCR reaction contained 0.5 µg genomic DNA, 200 µmol/L dNTPs, 1X PCR buffer, 1.5 mmol/L MgCl₂, 1 U of Taq DNA polymerase and 0.3 µmol/L of each primer. Each PCR reaction contained negative controls, where only water was added in place of genomic DNA. The PCR products were electrophoresed on a 20 g/L agarose gel to check the amplification. Samples with positive amplifications were processed for SSCP analysis.

SSCP analysis

Non-radioactive SSCP was performed as described previously^[27] with slight modification. A 2-µL volume of PCR product was denatured in 5 µL of formamide loading buffer by boiling for 10 min. Denatured samples were loaded onto 120 g/L acrylamide non-denaturation gel and electrophoresed at a constant 100 V for 4-6 h. Images of the ethidium-bromide-stained gels were captured using the BioRad Gel Documentation system. Samples with mobility shifts were verified by a second independent PCR-SSCP where silver staining was done for the same.

Statistical analysis

Descriptive statistics i.e. mean, standard deviation and frequency distribution were calculated for all the variables in the study. According to the different categories for the categorized variables, χ^2 test was used to estimate

the significance level of the association among them. Student’s *t*-test, Pearson correlation and analysis of variance were applied for the continuous variables according to the categories, wherever applicable. *P* < 0.05 was considered statistically significant. SPSS.60 software (SPSS Inc, Chicago, USA) was used for these calculations.

RESULTS

Detection of *p53* gene mutation by SSCP analysis

The *p53* exons 5, 6, 7 and 8 were successfully amplified from all 103 cases which gave expected PCR fragments of 266 bp, 160 bp, 180 bp, and 230 bp, respectively. After SSCP analysis, altered *p53* was identified by the presence of one or two extra bands migrating above or below the normal single-stranded products (Figure 1). Occasionally, mutant bands were also detected between the single and double-strand bands that may have been caused by formation of normal-mutant heterodimers. However, normal *p53* banding pattern was also observed among all cases. SSCP data showed that 17.47% (19/103) of the cases were harboring altered *p53* (Table 2). Out of these 19 cases, six showed alterations in exon 5, five in exon 6, four in exon 7 and four in exon 8. We further observed that the positivity for *p53* mutations was relatively higher in intestinal-type carcinoma (22.44%, 11/49) as compared to diffuse-type (15.66%, 7/45) adenocarcinoma^[28] (Table 3).

Detection of *p53* protein over-expression by immunohistochemistry (IHC)

We used DO-7 to detect *p53* over-expression by IHC

Table 2 Relationship between p53 protein over-expression detected by IHC and p53 gene mutation detected by SSCP analysis of 103 gastric carcinomas n (%)

IHC status	SSCP-negative	SSCP-positive	Total	P
IHC-negative	62 (60)	4 (4)	66 (64)	0.000
IHC over-expression	22 (21)	15 (15)	37 (36)	
Total	84 (81)	19 (19)	103 (100)	

Table 3 Correlation between clinicopathological characteristics and p53 genetic alteration in patients with gastric adenocarcinoma

Factors	p53 alteration		n (%)	P ¹
	Negative (%)	Positive (%)		
Age				
< 40 yr	12 (14.29)	1 (5.26)	13 (12.62)	0.533
40-65 yr	55 (65.48)	15 (78.95)	70 (67.96)	
> 65 yr	17 (20.23)	3 (15.79)	20 (19.42)	
Gender				0.407
Male	62 (73.81)	16 (84.21)	78 (75.73)	
Female	22 (26.19)	3 (15.79)	25 (24.27)	
Cell differentiation				0.462
Well	9 (10.72)	2 (10.53)	11 (10.68)	
Moderately	31 (36.90)	8 (42.11)	39 (37.86)	
Poorly	44 (52.38)	9 (47.37)	53 (51.46)	
Histology				0.522
Intestinal	35 (41.67)	10 (52.63)	45 (43.69)	
Diffused	41 (48.81)	8 (42.11)	49 (47.57)	
Undiff/mixed	8 (9.52)	1 (5.26)	9 (8.74)	
Site				0.606
Cardiac	19 (22.62)	2 (10.53)	21 (20.39)	
Fundus	14 (16.67)	2 (10.53)	16 (15.53)	
Body	18 (21.43)	5 (26.32)	23 (22.33)	
Antrum	33 (39.29)	10 (52.63)	43 (41.75)	
Stage				0.813
I	26 (30.95)	6 (31.58)	32 (31.07)	
II	32 (38.10)	6 (31.58)	38 (36.89)	
III	21 (25.00)	6 (31.58)	27 (26.21)	
IV	5 (5.95)	1 (5.26)	6 (5.83)	

¹ χ^2 test with Fisher's test were used to test the frequency distribution of p53 alteration. Undiff: Undifferentiated.

staining. Overall, 35.92% (37/103) of gastric carcinomas exhibited positive nuclear staining (Figure 2). The concordance of results between SSCP and IHC (i.e. both positive and negative) was 75% (Table 2). In addition, 21% of carcinomas (22/103) stained positively by IHC but failed to display any mutation by SSCP, while the remaining 4% (4/103) displayed mutant bands by SSCP but showed no staining by IHC.

DISCUSSION

The present study describes the status of p53 gene mutation and over-expression in Indian gastric cancer patients. The association between p53 abnormalities and pathological characteristics of tumor was also assessed. Mutations of the p53 gene are the most common genetic alteration, known to occur in a wide range of human cancers^[19]. A number of studies have reported the frequency of p53 alteration in gastric cancer, showing p53 mutation in 18%-58%^[18,29,30] and p53 protein over-

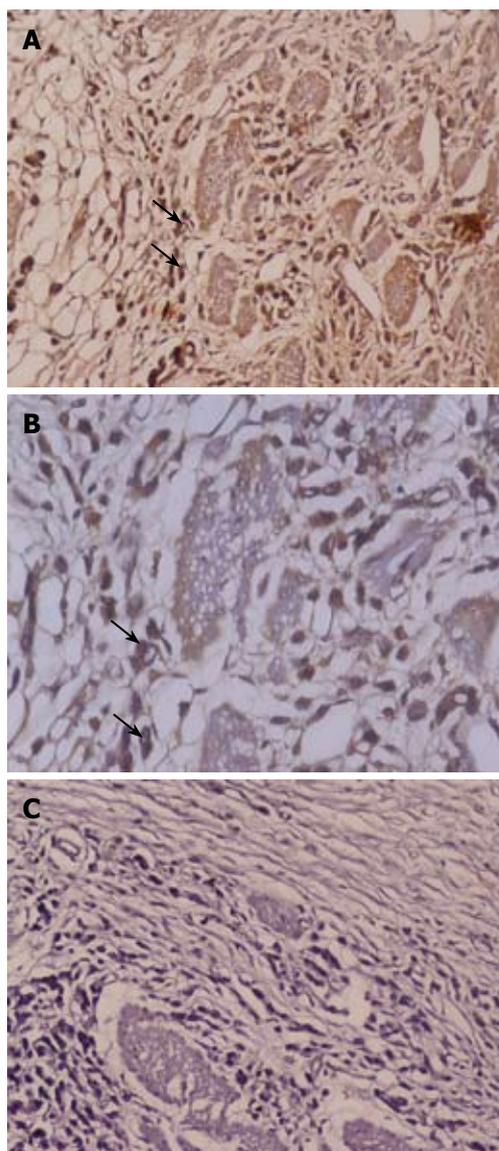


Figure 2 Immunohistochemical staining of p53 protein in diffused type-poorly differentiated gastric cancer. p53 protein over-expression is seen primarily on cell nucleus. A: Positive nuclear staining ($\times 200$); B: Positive nuclear staining ($\times 400$); C: Negative control ($\times 200$).

expression in 26%-65% of gastric adenocarcinoma^[31,32]. Under normal conditions, wild-type p53 protein is rapidly degraded but in carcinomas, the acquisition of a mutant genotype is thought to increase the half-life of the mutant protein, which leads to accumulation and detection by immunohistochemical techniques, an indirect method of screening tumors for mutation within the p53 gene. However, a number of discordant results have been reported^[33,34]. p53 over-expression can also reflect the accumulation of wild-type protein, which is stabilized *via* a mechanism other than mutation—for example, by binding with SV40 T antigen, which greatly increases the half-life of the protein^[35]. In the present study, we have attempted to address this issue by comparing p53 gene mutation and protein over-expression in a large number of gastric tumors. We analyzed here only exons 5 to 8; because most of the p53 mutations have previously been found to be clustered in

the highly conserved domains that roughly correspond to exons 5-8^[36]. Although approximately 90% of the *p53* point mutations have been reported from exons 5-8, the individual incidence rate of *p53* mutations in these exons is not known^[37,38]. Although our study is primarily focused on exons 5 to 8, mutations in other exons (13%-22%) could account for some negative SSCP cases^[39,40].

Several studies have addressed the relationship between p53 protein accumulation and *p53* alteration in different tumor types^[41-43]. The concordance rates between IHC staining and SSCP (both negative and positive) fall within a relatively wide range (50% to 80%), but this probably depends on differences both in methodology and tumor type. In the present study, for establishing a relationship between *p53* gene mutation and p53 protein accumulation, gastric carcinomas were analyzed for exons 5, 6, 7 and 8 of the *p53* gene. We found that p53 protein was over-expressed in 35.92% cases and *p53* gene was mutated in 17.47% of cases. A comparison of PCR-SSCP and immunohistochemical findings showed concordant (i.e. both positive and negative) results in 75% of the cases. In particular, 15 (78.94%) of the 19 cases showing a *p53* gene mutation were also positive for p53 protein accumulation on IHC, while 22 (26%) of the 84 cases without mutation were positive on IHC. In 26 (25%) cases, the immunohistochemical and SSCP results were discordant; negative IHC/positive SSCP in four and positive IHC/negative SSCP in 22. The presence of *p53* gene mutations was significantly correlated with p53 protein over-expression ($P = 0.000$).

Accumulation of p53 protein with no evidence of gene mutation may either reflect an accumulation of a non-mutated protein, or a false-negative SSCP result. The sensitivity of SSCP analysis is still a matter of debate; not only does it depend on the experimental conditions (i.e. temperature, ionic strength and presence or absence of glycerol), but also on the length of the amplified fragment being analyzed^[44]. Sheffield *et al.*^[45] reported that SSCP sensitivity dramatically decreased with increasing fragment size; the percentage of single base substitution detected by SSCP was 90% when the amplified fragment was 135 bp, 70% when it was 200 bp, and 67% as fragment size exceeded 300 bp^[46]. As the amplified fragment length we analyzed was 266 bp for exon 5, 160 bp for exon 6, 180 bp for exon 7 and 230 bp for exon 8, SSCP sensitivity for exon 5 and 8 might have been less than 70%. Although our study focused on exons 5 to 8, where the majority of the mutations are thought to be localized^[38], mutations in other exons (13%-22%) could account for some negative SSCP cases^[39,40]. A false-negative SSCP result may also occur when the tissue is not sufficiently represented in tissue from which the DNA is extracted. The search for *p53* gene mutation by molecular assays is important for defining the functional alterations that are derived from the different mutations. In this regard, it might be advisable to study all of the exons of the *p53* gene. We can justify the inconsistent cases of SSCP and IHC by

some possible explanations: first, there may be mutations in exons other than those we examined; second, binding of wild-type p53 protein to a cellular oncogene might have led to an elongated half-life; and third, the proportion of alleles containing *p53* mutations may be relatively low, and not shown up in the analysis^[47].

It was reported that wild-type p53 proteins could combine with viral oncoproteins or cellular oncoproteins to enhance their stability and prolong their half-life, leading to p53 protein accumulation in cells. In such cells, IHC staining was still positive even without *p53* gene mutations; furthermore, about 10% of *p53* gene mutations could take place outside of exons 5-8. Therefore, the positive rate of PCR-SSCP targeting only exons 5-8 was usually lower than that of IHC^[20,47-49].

In conclusion, PCR-SSCP is a simple and rapid method for the evaluation of *p53* gene mutations and its association with immunohistochemical analysis of the p53 protein expression might provide a more precise use of *p53* as a prognostic marker in gastric cancer.

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COMMENTS

Background

Gastric cancer is a common disease worldwide, including India. The molecular events leading to the development of gastric cancer are largely unknown, but various lines of evidence suggests that the functional inactivation of the *p53* gene plays an important role. The p53 protein is involved in control of the cell cycle. Wild-type p53 protein has a very short half-life, whereas mutated p53 is stable and can accumulate at high concentration in the nuclei of tumor cells, and is detected by immunohistochemical staining with specific antibodies. Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis was done to screen and detect the presence of any point mutation in *p53* gene.

Research frontiers

In general, the present study is a part of cancer research that is aimed at mutational analysis of the *p53* gene among gastric cancer patients. To the best of our knowledge, this is the first study where an attempt has been made to correlate the *p53* gene mutation with various clinicopathological features among Indian gastric cancer patients.

Innovations and breakthroughs

Authors have analyzed the status of p53 protein expression by immunohistochemistry (IHC) and *p53* gene mutation by PCR-SSCP among gastric cancer cases. This study also provides a significant correlation between immunohistochemical staining of p53 protein and SSCP analysis of the PCR amplified DNA.

Applications

PCR-SSCP in association with IHC of the p53 protein may provide a more precise use of *p53* as a prognostic marker for gastric cancer.

Terminology

SSCP is a technique used to screen and detect the mutational change at

the single nucleotide level. Additional bands of DNA is an indication of gene alteration. IHC is a technique used to detect the mutated p53 protein in the nucleus of cancer cells. Mutation in the p53 gene leads to increased half-life and accumulation of p53 protein in the nucleus.

Peer review

The paper describes p53 alterations among gastric cancer patients in India. The alterations were studied by IHC and PCR-SSCP analyses. A significant correlation of p53 over-expression and alteration in p53 gene detected by SSCP was found. It is an interesting study and this is also the first such study from an Indian population, including a large sample from a long research period.

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CASE REPORT

Anal metastasis from recurrent breast lobular carcinoma: A case report

Maria Puglisi, Emanuela Varaldo, Michela Assalino, Gianluca Ansaldo, Giancarlo Torre, Giacomo Borgonovo

Maria Puglisi, Emanuela Varaldo, Michela Assalino, Gianluca Ansaldo, Giancarlo Torre, Giacomo Borgonovo, University of Genova, Largo Benzi, 8. 16132 Genova, Italy

Author contributions: Varaldo E and Assalino M wrote the paper; Torre G, Ansaldo G and Puglisi M participated equally in the study; Borgonovo G revised and edited the manuscript.

Correspondence to: Giacomo Borgonovo, Dipartimento di Chirurgia, Università degli Studi di Genova. Largo Benzi, 8. 16132 Genova, Italy. borgonovo@unige.it

Telephone: +39-10-3537298 Fax: +39-10-3537286

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Abstract

We report a case of isolated gastrointestinal metastasis from breast lobular carcinoma, which mimicked primary anal cancer. In July 2000, an 88-year-old woman presented with infiltrating lobular cancer (pT1/G2/N2). The patient received postoperative radiotherapy and hormonal therapy. Four years later, she presented with an anal polypoid lesion. The mass was removed for biopsy. Immunohistochemical staining suggested a breast origin. Radiotherapy was chosen for this patient, which resulted in complete regression of the lesion. The patient died 3 years after the first manifestation of gastrointestinal metastasis. According to the current literature, we consider the immunohistochemistry features that are essential to support the suspicion of gastrointestinal breast metastasis, and since we consider the gastrointestinal involvement as a sign of systemic disease, the therapy should be less aggressive and systemic.

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Key words: Anal cancer; Breast cancer; Infiltrating lobular carcinoma; Estrogen receptors; Progesterone receptors

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INTRODUCTION

Over the last 10 years, the early diagnosis and development of new therapies have increased the survival time and disease-free survival of women with breast cancer, but long-term survivors are also at risk of developing distant metastases. Breast cancer is usually associated with lymphatic spread and blood metastases to the lung, bones and liver. In this paper, we report a case of isolated gastrointestinal metastasis from breast carcinoma, which mimicked primary anal cancer.

CASE REPORT

On July 2000, an 88-year-old woman presented with a right latero-cervical mass that was hard and increasing in volume, which extended up to the supraclavicular region. Physical examination showed a right breast tumor (1 cm in diameter) of the superior external quadrant, with some palpable homolateral axillary nodes. Abdominal ultrasonography and bone scanning did not reveal metastases, but thoracic computed tomography (CT) showed mediastinal lymph nodes < 1 cm in size. Carcinoembryonic antigen (CEA) and cancer antigen 15.3 (CA15.3) level was normal. The patient underwent right external superior quadrantectomy with right axillary and latero-cervical lymphadenectomy. The histological examination showed an infiltrating lobular carcinoma (ILC) with metastases in all 10 nodes removed (pT1/G2/N2). Immunohistochemical staining was positive for estrogen receptor (ER) protein (90%) and c-erbB2. The tumour cells showed a low mitotic index (15%).

The patient received postoperative radiotherapy and hormonal therapy (tamoxifen). Four years later, some new right axillary lymph node metastases were discovered at physical examination and therefore removed. The histological examination confirmed the diagnosis of lymphatic metastasis from ILC. Two months after lymphadenectomy, the patient presented with tenesmus and a painful anal polypoid lesion (Figure 1). The mass was removed for biopsy. The immunohistochemical staining was similar to primary breast tumor [95% ER and 10% progesterone receptor (PR)], which suggested a breast origin (Figure 2). Mib1 was 25% positive, while c-erbB2 was negative. No other site of disease was found by abdominal CT and bone scintigraphy. Radiotherapy was chosen for



Figure 1 The lesion as it appeared macroscopically at the time of diagnosis.

this patient, which led to complete regression of the lesion, but later an anal stenosis appeared without any changes in defecation. Treatment with anastrozole was then started. At follow-up, there was no evidence of progressive disease for 2 years, after which the level of CA15.3 began to increase, and some new right axillary lymph nodes metastases appeared. The patient underwent axillary lymphadenectomy and adjuvant hormonal therapy was continued.

Six years later, during follow-up, abdominal CT showed a suspicious increase in antral wall thickness, but endoscopic examination revealed only a peptic stenosis that was treated by pneumatic dilatation. Twelve months later, the patient underwent local radiotherapy for a relapse of anal lesions. Two months afterwards, during umbilical hernioplasty a diagnosis of peritoneal carcinosis was made. The patient died 3 years after the first manifestation of anal metastasis and 6 years after the diagnosis of breast cancer.

DISCUSSION

Gastrointestinal metastases of breast cancer are rare and usually associated with disseminated disease. The most frequent organ involved is the stomach^[1,2]. Survival after diagnosis of gastrointestinal metastases is poor, with few patients surviving beyond 2 years. The average survival from time of recurrence is 12-16 mo^[1,2].

Despite their rare clinical manifestations (only 0.07% of cases)^[3], some necroscopy studies have described an incidence rate of colon involvement that varies from 3% to 18%^[4,5]. The risk of a second primary tumor following breast cancer is about 12%^[6], and the incidence of metachronous primary colorectal cancer is estimated to be about 1%^[7,8]. In a review of the literature, we found 15 cases of solitary rectal metastasis from breast carcinoma, but only one case of anal localization has been reported^[9] and none from lobular breast carcinoma. Breast ILC is responsible for the majority of metastases in the GI tract, with a metastasis rate of 4.5% *vs* 0.2% from infiltrating ductal carcinoma^[10].

Clinical-histological features that lead us to suspect a diagnosis of gastrointestinal breast metastasis versus primary colon cancer are: (1) ER or PR protein and gross cystic disease fluid protein (GCDFFP-15) are strongly positive in metastatic breast carcinomas^[10-12];

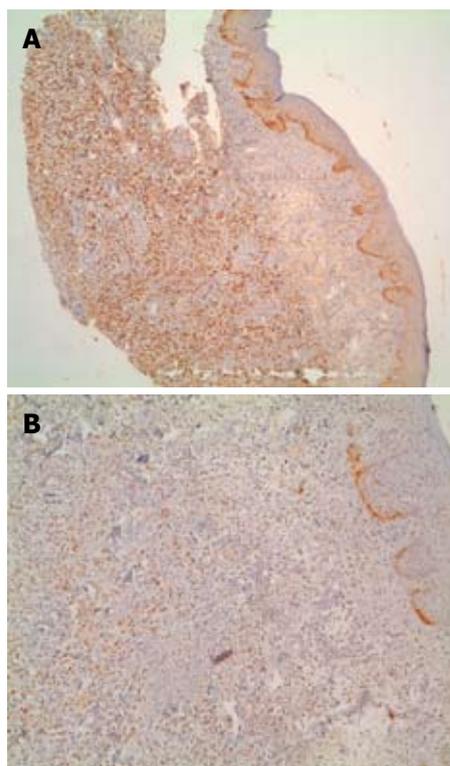


Figure 2 Positive immunohistochemical staining in anal lesion. A: ERs ($\times 4$); B: PRs ($\times 10$).

(2) absence of dysplasia in adjacent colonic epithelium suggests a metastatic growth; and (3) history of breast cancer. However, some authors have shown the presence of cells positive for ER or PR in colorectal cancer, but their concentration tends to be lower than in breast cancer^[13,14]. In our case, GCDFFP-15 was not tested for, but the immunohistochemical staining of anal biopsy was strongly positive for ERs (90%). The disease-free interval between primary breast cancer and gastrointestinal involvement varies from synchronous presentation to up to 30 years^[1].

If we consider that gastrointestinal involvement is a sign of systemic disease, systemic therapy should be initiated. In the literature, a common attitude towards isolated gastrointestinal lesions is to undertake local surgical treatment associated with hormonal or chemotherapy. Surgery is usually necessary for an exact diagnosis or for acute clinical manifestations. In our case, the diagnosis was made easily as the tumor was external to the anal canal, therefore, a more aggressive approach such as anorectal amputation (Miles' operation) was excluded.

CONCLUSION

It is very difficult to suspect anal metastasis from breast cancer by formulating the diagnosis by clinical, endoscopic and radiological features. Only a previous history of breast cancer and immunohistochemical analysis of anal biopsies compared with original breast carcinoma pathology allows a correct diagnosis. On the other hand, accurate identification of the disease

is essential since the treatment is different in the case of primary or secondary cancer. In the case of breast secondaries, several schedules of chemo- and hormonal therapy for ER/PR-positive tumors are of great benefit. Radiotherapy is strongly recommended in elderly patients rather than surgery, which should be limited to obtaining a diagnosis, or in cases with complications.

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Severe dystrophy in DiGeorge syndrome

Barnabás Rózsai, Ákos Kiss, Györgyi Csábi, Márta Czakó, Tamás Decsi

Barnabás Rózsai, Ákos Kiss, Györgyi Csábi, Tamás Decsi, Department of Pediatrics, University of Pécs, Pécs H-7623, Hungary

Márta Czakó, Department of Medical Genetics and Child Development, University of Pécs, Pécs H-7623, Hungary

Author contributions: Rózsai B as specialist in Pediatrics, Kiss Á as Pediatric resident and Csábi G as Pediatric neurologist participated in the diagnostic procedures and follow-up of the patient. Czakó M performed the genetic studies. Decsi T is the leader of our work-group and the chief of the Pediatric Internal Medicine Ward of our Department.

Correspondence to: Dr. Barnabás Rózsai, Department of Pediatrics, University of Pécs, József Attila u. 7., Pécs H-7623, Hungary. barnabas.rozsai@aok.pte.hu

Telephone: +36-72-535900 Fax: +36-72-535971

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Abstract

We present the case history of a 3-year-old girl who was examined because of severe dystrophy. In the background, cow's milk allergy was found, but her body weight was unchanged after eliminating milk from her diet. Other types of malabsorption were excluded. Based on nasal regurgitation and facial dysmorphisms, the possibility of DiGeorge syndrome was suspected and was confirmed by fluorescence *in situ* hybridization. The authors suggest a new feature associated with DiGeorge syndrome.

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Key words: DiGeorge syndrome; Dystrophy; Cow's milk allergy; Nasal regurgitation; Hypoparathyroidism

Peer reviewer: Dr. Phillip S Oates, Department of Physiology, School of Biomedical and Chemical Sciences, The University of Western Australia, Perth, WA, Australia

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INTRODUCTION

The main consequences of developmental abnormalities

of the third and fourth branchial pouches are congenital heart defect, hypoparathyroidism, hypoplasia or aplasia of the thymus, velopharyngeal insufficiency and facial dysmorphism; the clinical picture is named DiGeorge syndrome. Apart from these major symptoms, many others including low muscle tone, short stature, hypothyroidism, kidney problems, developmental delay, psychiatric disorders and learning difficulties are often present^[1]. In 40%-90% of children with DiGeorge syndrome feeding difficulties are also present^[2]; nasal regurgitation is one of the first manifestations of a feeding abnormality in this group^[2]. Eicher *et al*^[3] convincingly demonstrated, by videofluorography, that dysmotility through the pharyngoesophageal segment is the major cause of dysphagia in this syndrome. Recently, failure of upper esophageal sphincter relaxation was also demonstrated by a videomanometric technique in three children with velocardiofacial syndrome^[4]. Dysmotility of the upper gastrointestinal tract, velopharyngeal insufficiency and associated celiac disease^[5] may all contribute to developmental delay in DiGeorge syndrome.

Here we present a case of an almost 3-year-old girl with cow's milk allergy whose extreme dystrophy was not improved after eliminating milk from the diet, and in whom DiGeorge syndrome was later diagnosed as the background to the symptoms.

CASE REPORT

The girl was born after an uneventful pregnancy at the 38th week of gestation with a birthweight of 2950 g, and Apgar scores of 10-10 at 5 and 10 min. She was breast fed for 6 mo, her development was normal, and her body weight was 5500 g at the age of 6 mo. However, after finishing breast feeding and changing to follow-on formula, her body weight did not increase. In addition, episodes of infections with 38°C-38.5°C fever appeared, and several times, antibiotic treatments were initiated. Her mother reported that the infant sometimes had nasal regurgitation. No gross abdominal complaints were observed, and only moderate reflux was detected by ultrasonography. At 11 mo of age, routine laboratory tests and abdominal ultrasound were normal. An anti-endomysial antibody test, chloride sweat test, and stool cultures were negative. To exclude celiac-like lesions, a small bowel biopsy was performed, but normal bowel mucosa was found. With a background of dystrophy, cow's milk and soy-protein allergy was diagnosed by



Figure 1 Facial dysmorphisms in our patient.

specific immunoglobulin E (IgE) assay. The hypotonic musculature was explained as the result of dystrophy, and her creatine kinase level was only slightly elevated (276 U/L, normal range: < 200 U/L). Milk was removed from her diet and oral iron therapy was administered; however, her body weight did not increase. At 17 mo of age, reflux was no longer detectable, her appetite was normal, but still no weight gain was observed.

At 20 mo, the girl was admitted to our department for further examinations to resolve the cause of the severe dystrophy. Her body weight was 6000 g (< 3rd percentile) and her length was 76 cm (< 3rd percentile). In association with the signs of dystrophy, moderate psychomotoric retardation was seen and some minor anomalies were found during physical examination. These included ocular hypertelorism, micrognathia, flat nasal bridge, bulbous nasal tip, short philtrum, and slightly rotated ears (Figure 1). Laboratory tests including blood count, electrolytes, liver enzymes, pre-albumin, albumin, ammonia, iron and Ig levels, as well as the carbohydrate malabsorption test were normal. Serum amino acids showed a normal pattern. Otolaryngologic examination revealed dysfunctional soft palate movement. Echocardiography found a chorda tendinea in the left ventricle, which was thought to be a normal variant. Psychologic testing showed mental retardation (IQ: 61.6).

Based on facial dysmorphism, the nasal regurgitation seen earlier and the newly diagnosed velopharyngeal insufficiency, the possibility of DiGeorge syndrome was considered. Our suspicion was confirmed by fluorescence *in situ* hybridization analysis which showed microdeletion on the long arm of one of chromosome 22. We were unable to visualize the thymus either by X-ray examination, or by thoracic ultrasound. Flow cytometric analysis did not reveal immunodeficiency; the number of T lymphocytes was 3052/ μ L (normal range: 2100-6200/ μ L). A normal cytokine response occurred after simulating her lymphocytes with bacterial lipopolysaccharide. The ionized calcium, serum parathyroid hormone, thyroid stimulating hormone and vitamin D levels were in the normal range

[1.22 mmol/L (normal: 1.1-1.3 mmol/L), 1.9 pmol/L (normal: 1.6-6.9 pmol/L), 2.96 mU/L (normal: 0.27-4.2 mU/L), 155 nmol/L (normal: 47.7-170 nmol/L), respectively]. The daily calorie intake calculated from the dietary record of the mother was about 800-900 kcal, therefore hypercalorization was recommended (1200-1300 kcal/d). Following administration of this diet, her body weight began to increase, but it still remained below the 3rd percentile line.

The frequency of upper airway infections increased and *Candida* was cultured from the stools, therefore, long-term antifungal therapy was started. She complained of indefinite pain of the upper limbs. At 2 years of age she was hospitalized several times because of pneumonia. At this time the serum total calcium level was decreased (1.8 mmol/L; normal: 2.1-2.4 mmol/L), so calcium-gluconicum and cholecalciferol were given in doses of 3×500 mg/d and 400 IU/d, respectively. At the next examination, the total calcium level was found to be decreased further (1.54 mmol/L), the ionized calcium level was also low (0.68 mmol/L), and hyperphosphatemia (2.33 mmol/L; normal: 1.0-2.0 mmol/L) and hypoparathyroidism (1.58 pmol/L) were found. Cholecalciferol was changed to calcitriol (1×0.25 μ g/d). The total calcium level began to increase after a week of calcitriol administration (1.87 mmol/L). Calcium homeostasis normalized following a month of calcitriol therapy.

DISCUSSION

The diagnosis of DiGeorge syndrome in the present case was rendered difficult by the apparent explanation of the failure to thrive as serologically verified cow's milk allergy. Though our patient had neither atopic dermatitis nor recurrent episodes of obstructive bronchitis, which often accompany symptoms of cow's milk allergy^[6], cow's milk-protein-specific IgE levels indicated cow's milk allergy. However, diagnosing cow's milk allergy and eliminating milk from the diet did not result in the expected weight gain. So cow's milk allergy on its own did not explain the failure to thrive in our patient. Mention of nasal regurgitation provided important information, because this is a classic finding in infants with DiGeorge syndrome^[2]. Detailed physical examination revealed minor anomalies (Figure 1) that fit well into the suspected diagnosis. A cardiac defect was admittedly absent in our patient; however, in about 20% of patients with DiGeorge syndrome there is no heart disease^[1].

Dysmotility of the upper gastrointestinal tract is a well known complication in DiGeorge syndrome^[2-4], which together with growth hormone deficiency, hypothyroidism and velopharyngeal insufficiency may lead to developmental delay and dystrophy. Digilio *et al* reported that 8.3% of their patients with DiGeorge syndrome had body weight below the 3rd percentile and all of them were younger than 5 years of age. They also presented a 2-year-old girl who had high level of anti-endothelial antibodies and in whom celiac disease was

diagnosed after jejunal biopsy^[5]. As far as we know our case is the first in which an association between cow's milk allergy and DiGeorge syndrome is represented.

In summary, we recommend screening for DiGeorge syndrome in patients with classical diseases of the gastrointestinal tract if poor weight gain is also present. Nasal regurgitation during infancy is an important sign that suggests the possibility of DiGeorge syndrome.

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CASE REPORT

Resected case of eosinophilic cholangiopathy presenting with secondary sclerosing cholangitis

Fumihiko Miura, Takehide Asano, Hodaka Amano, Masahiro Yoshida, Naoyuki Toyota, Keita Wada, Kenichiro Kato, Tadahiro Takada, Junichi Fukushima, Fukuo Kondo, Hajime Takikawa

Fumihiko Miura, Takehide Asano, Hodaka Amano, Masahiro Yoshida, Naoyuki Toyota, Keita Wada, Kenichiro Kato, Tadahiro Takada, Department of Surgery, Teikyo University, School of Medicine, Tokyo, 173-8605, Japan

Junichi Fukushima, Fukuo Kondo, Department of Pathology, Teikyo University, School of Medicine, Tokyo, 173-8605, Japan

Hajime Takikawa, Department of Medicine, Teikyo University, School of Medicine, Tokyo, 173-8605, Japan

Author contributions: Miura F and Kondo F contributed equally to this work; Miura F, Asano T, Kondo F and Fukushima J designed the research; Miura F, Amano H, Toyota N, Wada K and Kato K performed the research; Takada T and Takikawa H analysed the data; and Miura F, Kondo F, and Yoshida M wrote the paper.

Correspondence to: Takehide Asano, MD, Department of Surgery, Teikyo University, School of Medicine, 2-11-1, Kaga, Itabashi-ku, Tokyo, 173-8605, Japan. asn@med.teikyo-u.ac.jp
Telephone: +81-3-39641228 Fax: +81-3-39622128

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with eosinophilic cholangitis might be due to fibrosis of the bile duct wall. Eosinophilic cholangiopathy might be confused as PSC with eosinophilia.

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Key words: Eosinophilic cholangiopathy; Primary sclerosing cholangitis; Secondary sclerosing cholangitis; Obstructive jaundice

Peer reviewer: Ravi S Chari, MD, Associate Professor, Division of Hepatobiliary Surgery and Liver Transplantation, Departments of Surgery and Cancer Biology, 1313 21st Avenue South, Suite 801 Oxford House, Vanderbilt University Medical Center, Nashville, TN 37232-4753, United States

Miura F, Asano T, Amano H, Yoshida M, Toyota N, Wada K, Kato K, Takada T, Fukushima J, Kondo F, Takikawa H. Resected case of eosinophilic cholangiopathy presenting with secondary sclerosing cholangitis. *World J Gastroenterol* 2009; 15(11): 1394-1397 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1394.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1394>

Abstract

Eosinophilic cholangiopathy is a rare condition characterized by eosinophilic infiltration of the biliary tract and causes sclerosing cholangitis. We report a patient with secondary sclerosing cholangitis with eosinophilic cholecystitis. A 46-year-old Japanese man was admitted to our hospital with jaundice. Computed tomography revealed dilatation of both the intrahepatic and extrahepatic bile ducts, diffuse thickening of the wall of the extrahepatic bile duct, and thickening of the gallbladder wall. Under the diagnosis of lower bile duct carcinoma, he underwent pylorus-preserving pancreatoduodenectomy and liver biopsy. On histopathological examination, conspicuous fibrosis was seen in the lower bile duct wall. In the gallbladder wall, marked eosinophilic infiltration was seen. Liver biopsy revealed mild portal fibrosis. He was diagnosed as definite eosinophilic cholecystitis with sclerosing cholangitis with unknown etiology. The possible etiology of sclerosing cholangitis was consequent fibrosis from previous eosinophilic infiltration in the bile duct. The clinicopathological findings of our case and a literature review indicated that eosinophilic cholangiopathy could cause a condition mimicking primary sclerosing cholangitis (PSC). Bile duct wall thickening in patients

INTRODUCTION

Benign disorders such as primary sclerosing cholangitis (PSC), IgG4-related autoimmune pancreatitis, eosinophilic cholangitis, intraductal stone disease, surgical or blunt abdominal trauma, intra-arterial chemotherapy, and portal biliopathy can present with obstructive jaundice. Eosinophilic cholangiopathy is also one of the causes of sclerosing cholangitis^[1-3]. The literature contains only about 40 case reports on eosinophilic cholangiopathy^[4-6], and therefore, little attention has been paid to this condition. The disease is characterized by a dense transmural eosinophilic infiltration of the biliary tract. It is highly responsive to oral steroid therapy^[4-6]. Surgery, including bile duct resection, is unnecessary if a diagnosis of eosinophilic cholangiopathy is made; therefore, a detailed histopathological examination of this disease has not yet been performed.

Sclerosing cholangitis from the above-mentioned etiology is sometimes difficult to differentiate from PSC. PSC patients in Japan have a higher incidence of eosinophilia, a less frequent association with

inflammatory bowel disease, and a relatively frequent association with chronic pancreatitis, compared with those in Western countries^[7,8]. Establishment of the concept of IgG4-related sclerosing cholangitis accompanying autoimmune pancreatitis has resulted in solutions for the different clinical characters of PSC between Western countries and Japan, except for the higher incidence of eosinophilia in Japan^[9,10].

We present a patient with sclerosing cholangitis and eosinophilic cholecystitis mimicking lower bile duct carcinoma, who underwent pancreatoduodenectomy and liver biopsy. To the best of our knowledge, this is the first reported case of eosinophilic cholangiopathy in which all layers of the bile duct wall and liver were histopathologically examined. This case was suggestive of an etiology of bile duct obstruction of eosinophilic cholangitis and PSC with eosinophilia.

CASE REPORT

A 46-year-old Japanese man with jaundice was admitted to our hospital. Ten months earlier, he had developed liver dysfunction with jaundice, which improved by conservative therapy. He had repeated episodes of urticaria from the age of 40 years. Physical examination disclosed no abnormalities except for scleral icterus and mild tenderness in the right upper quadrant of the abdomen. Liver function test results at admission were as follows: aspartate aminotransferase (AST), 37 U/L; alanine aminotransferase (ALT), 36 U/L; alkaline phosphatase (ALP), 695 U/L; γ -glutamyl transferase (GGT), 179 U/L; total bilirubin, 12.3 mg/mL. Total white blood cell count was 3900, with a differential cell count of 59% neutrophils (normal range, 40%-69%), 3% eosinophils (1%-8%), and 31% lymphocytes (21%-49%). Hepatitis virus screening was negative and no tumor markers were elevated. Computed tomography (CT) revealed dilatation of both the intrahepatic and extrahepatic bile ducts. Diffuse thickening of the extrahepatic bile duct wall and thickening of the gallbladder wall were identified on CT (Figure 1). Endoscopic retrograde cholangiography (ERC) showed an obstruction of the lower bile duct (Figure 2). Endoscopic naso-biliary drainage was performed. Upper gastrointestinal endoscopy showed no abnormal findings other than atrophic gastritis. Pylorus-preserving pancreatoduodenectomy and wedge liver biopsy were performed under the diagnosis of lower bile duct carcinoma. After the operation, minor leakage of pancreatojejunostomy developed, and conservative treatment was successful. The patient is alive without any symptoms 6 years and 6 mo after the operation at the time of writing.

In the resected specimen, the bile duct was slightly thickened and there was a narrow stenotic segment at the lower edge of the common bile duct. There was neither elevated lesion nor ulceration in the epithelium of the bile duct. The gallbladder was 11 × 6 cm with thickened wall, but had neither elevated lesion nor ulceration in the epithelium.



Figure 1 CT demonstrating dilatation of both the intrahepatic and extrahepatic bile ducts, diffuse thickening of the wall of the extrahepatic bile duct (arrow), and thickening of the gallbladder wall (arrowhead).



Figure 2 ERC showing an obstruction of the lower bile duct (arrow).

On histopathological examination, conspicuous fibrosis was seen diffusely in the bile duct wall (Figure 3A). Mild inflammatory cell infiltration, mainly composed of lymphocytes and plasma cells, was seen. Eosinophilic infiltration was very mild, and in some areas eosinophilic leukocytes were not detected (Figure 3B). Accordingly, this case was not diagnosed as definite eosinophilic cholangitis. However, careful observation revealed some areas with scattered eosinophilic leukocytes (Figure 3C). In the gallbladder wall, marked eosinophilic infiltration was seen (Figure 4), as well as scattered lymphocytes and plasma cell infiltration. Thickened fibromuscular layer and slight fibrosis in the subserosal layer were present. Hyperplastic change without atypia was identified in the gallbladder epithelium. There was no evidence of malignancy in either the bile duct or gallbladder. IgG4 immunostaining revealed hardly any positive plasma cells in the bile duct and gallbladder wall. Liver biopsy revealed mild portal fibrosis and concentric layers of fibrotic tissue surrounding the bile duct (Figure 5). This case was then definitively diagnosed as eosinophilic cholecystitis with sclerosing cholangitis of unknown etiology. However, focal infiltration of eosinophilic leukocytes in the bile duct wall suggested the possibility of eosinophilic cholangitis with very mild eosinophilic infiltration.

DISCUSSION

Sclerosing cholangitis is classified into two entities: PSC

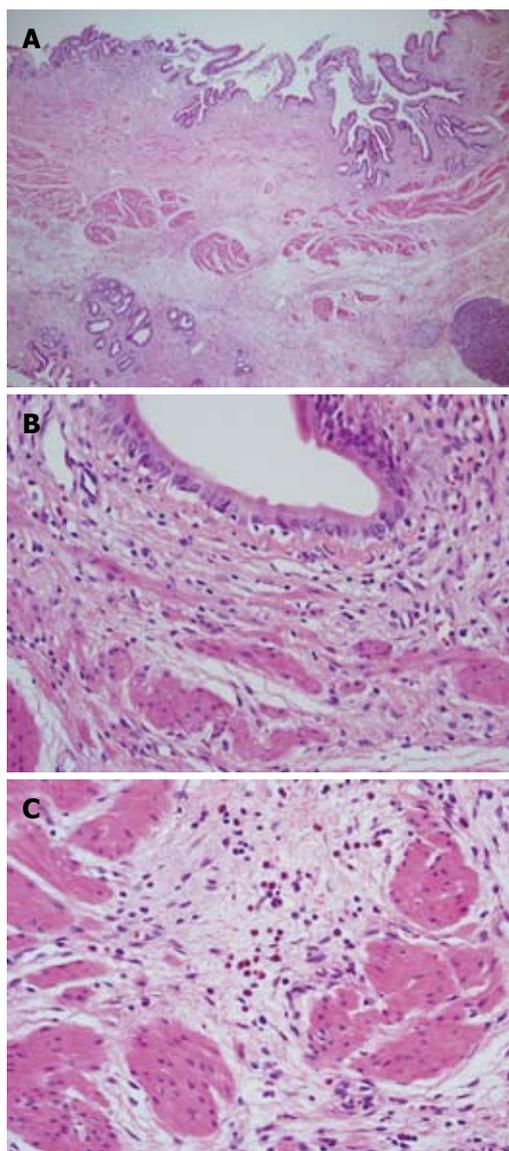


Figure 3 Histological examination of the bile duct wall. A: Conspicuous fibrosis was seen diffusely in the bile duct wall; B: Eosinophilic infiltration was very mild, and in some areas eosinophilic leukocytes were not seen; C: There were some areas with scattered eosinophilic leukocytes.

and secondary sclerosing cholangitis (SSC)^[2,11]. SCC is morphologically similar to PSC but it originates from a known pathological process. SSC includes IgG4-related autoimmune pancreatitis, eosinophilic cholangitis, intraductal stone disease, surgical or blunt abdominal trauma, intra-arterial chemotherapy, portal biliopathy, hepatic inflammatory pseudotumor, and AIDS-related cholangiopathy^[2,11].

In the present case, a certain diagnosis of eosinophilic cholecystitis or cholangiopathy could be made. Eosinophilic cholangiopathy is a rare benign cause of biliary obstruction^[1,3-6]. It is characterized by dense transmural eosinophilic infiltration of the biliary tract. When eosinophilic infiltration is localized in the bile duct, it is termed eosinophilic cholangitis. When it involves the gallbladder, it is called eosinophilic cholecystitis. Eosinophilic cholangiopathy is the term used to describe changes in either or both of them^[5,12].

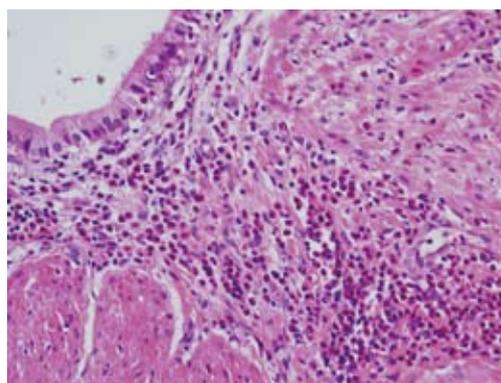


Figure 4 Histological examination showing marked eosinophilic infiltration in the gallbladder wall.

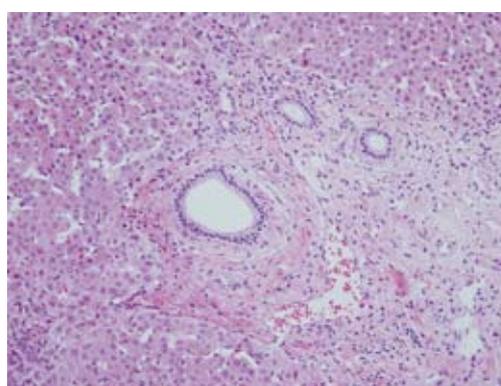


Figure 5 Liver biopsy showing mild portal fibrosis and concentric layers of fibrotic tissue surrounding the bile duct.

The cause of this disease is unknown. In more than half of the reported cases, eosinophilic infiltration is seen not only in the biliary tract but also in other organs, including the stomach, colon, pancreas, liver, and kidney^[4-6,13]. Eosinophilic cholangiopathy is thought to be part of a spectrum of diseases caused by eosinophilic infiltration of tissues and organs with or without peripheral eosinophilia. The recognition of peripheral eosinophilia (especially in the absence of leukocytosis) is important. However, peripheral eosinophilia was present in only about half of the reported cases^[6]. Some authors reported that patients with eosinophilic cholangitis had been treated successfully with oral corticosteroids^[4-6]. In fact, a few cases of eosinophilic cholangitis have been resolved within 3 wk, even without steroids^[1,3].

Bile duct wall thickening is a characteristic finding of eosinophilic cholangitis on imaging modalities^[4,6,14]. Thickening of the wall of the biliary tree usually, but not always, leads to biliary obstruction^[6]. In most cases, bile duct stricture is located diffusely from the hepatic hilum to the intrahepatic biliary tree, but there is a report describing lower bile duct stricture in a patient with eosinophilic cholangitis^[5]. The cause of thickening and obstruction of the biliary tract in patients with eosinophilic cholangitis is unclear because no resected case has been reported. Ours is believed to be the first reported case of eosinophilic cholangiopathy in which all layers of the biliary tract wall were histopathologically

examined. The most plausible cause of sclerosing cholangitis in our case was eosinophilic cholangiopathy, based on the fact that there was marked eosinophilic infiltration in the gallbladder wall and scattered eosinophilic infiltration in the bile duct wall. Close examination of all layers of the bile duct wall enabled us to find focal infiltration of eosinophilic leukocytes.

Experimental studies have demonstrated the close relationship between eosinophilic infiltration and fibrosis. It is considered that eosinophils play an important role in the fibrotic conditions of different etiopathology, including endomyocardial fibrosis, scleroderma and scleroderma like-conditions, idiopathic pulmonary and retroperitoneal fibrosis, asbestos-induced lung fibrosis, wound repair, and tissue remodeling^[15,16]. It has been demonstrated that transforming growth factor- β , a cytokine known for its ability to promote fibrosis, is produced by human eosinophils from patients with blood eosinophilia^[16,17]. Ten months before admission, our case had developed liver dysfunction with jaundice, which was diagnosed by observation only. This means that eosinophilic cholangitis might have existed 10 months earlier. This led to speculation that the etiology of our sclerosing cholangitis case was consequent fibrosis from previous eosinophilic infiltration in the bile duct wall. In this regard, bile duct thickening in patients with eosinophilic cholangitis might correspond to fibrosis.

Peripheral eosinophilia was observed in 27% of PSC in Japan, although reports of eosinophilia in PSC in other countries are rare^[8,18]. Based on the liver biopsy findings and cholangiography, the present case was not diagnosed as PSC. However, in some cases, eosinophilic cholangiopathy causes sclerosing cholangitis, which closely resembles PSC. In patients with eosinophilic cholangitis without peripheral eosinophilia, a differential diagnosis from PSC is difficult without bile duct biopsy. Eosinophilic cholangiopathy might also be confused with PSC with eosinophilia. There is a case report of PSC associated with increased peripheral eosinophilia and serum IgE^[19]. In that report, a 20-year-old Japanese man received oral corticosteroid therapy and improved in a few days. The possibility that he had eosinophilic cholangitis is high from the viewpoint of his clinical course, although a definite diagnosis could not be made because histopathological material was unavailable.

In summary, we have reported a case of SSC with eosinophilic cholecystitis. Close examination of the biliary tract of the resected specimen and a literature review suggested that bile duct wall thickening in patients with eosinophilic cholangitis may be due to fibrosis of the bile duct wall and that eosinophilic cholangiopathy might be confused with PSC with eosinophilia. Confirmation will depend on further investigations.

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CASE REPORT

Ileal angiomyolipoma manifested by small intestinal intussusception

Chang Ho Lee, Jong Hun Kim, Doo Hyun Yang, Yong Hwang, Myoung Jae Kang, Young Kon Kim, Min Ro Lee

Chang Ho Lee, Jong Hun Kim, Doo Hyun Yang, Yong Hwang, Min Ro Lee, Department of Surgery, Chonbuk National University Medical School, Jeonju 561-180, South Korea

Myoung Jae Kang, Department of Pathology, Chonbuk National University Medical School, Jeonju 561-180, South Korea

Young Kon Kim, Department of Radiology, Chonbuk National University Medical School, Jeonju 561-180, South Korea

Author contributions: Lee CH, Kim JH, Yang DH and Hwang Y collected data and performed the research; Kang MJ contributed to the work on pathology; Kim YK contributed to the work on radiology; Lee MR helped by supervising and approving the final manuscript.

Correspondence to: Min Ro Lee, MD, PhD, Department of Surgery, Chonbuk National University Medical School, San 2-20 Geumam-dong, Deokjin-gu, Jeonju, Jeonbuk 561-180, South Korea. gsmiro@chonbuk.ac.kr

Telephone: +82-63-2501570 Fax: +82-63-2716197

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Abstract

Angiomyolipomas (AMLs), a form of benign mesenchymal hamartoma, arise primarily in the kidneys of patients with or without tuberous sclerosis. Extra-renal AMLs are very rare and are most commonly found in the liver. AMLs of the small intestine are exceedingly rare. Here, a case of a 28-year-old man, who presented with ileal intussusception caused by ileal AML is reported. The clinicopathological and immunohistochemical findings of ileal AMLs are discussed and the literature on small intestinal AMLs is reviewed.

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Key words: Angiomyolipoma; Intussusception; Hamartoma; Ileum; Colectomy

Peer reviewer: Dr. Olivier Detry Department of Abdominal Surgery and Transplantation, University of Liège, CHU Sart Tilman B35, B-4000 Liège, Belgium

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INTRODUCTION

Angiomyolipomas (AMLs), benign mesenchymal tumors, are composed of blood vessels, smooth muscle cells, and mature fat cells. These mesenchymal hamartomas arise primarily in the kidney^[1] and extrarenal AMLs are very rare. AMLs of the small intestine are exceedingly rare and, to the best of our knowledge, only three cases have been reported in the literature^[2,3]. Herein, a case of ileal AML, manifested by small intestinal intussusception, is presented. This occurred in a 28-year-old man, and was confirmed by microscopic examination and immunohistochemical staining after right hemicolectomy.

CASE REPORT

A 28-year-old man presented to the emergency room complaining of progressive abdominal cramping pain, accompanied by nausea and vomiting for 6 h. He had no significant medical history except for having been admitted with pneumothorax 13 years previously. The pain was located over the right lower quadrant of the abdomen. He had no change in bowel habits, bloody stools, or tenesmus. Physical examination showed a palpable mass over the right lower quadrant with direct tenderness. Increased bowel sounds were noted during auscultation. Laboratory findings were normal; red blood cell count $5.02 \times 10^6/\text{mm}^3$, hemoglobin 15.7g/dL and hematocrit 44.1%, platelets $237 \times 10^3/\text{mm}^3$, and white blood cell count $7700/\text{mm}^3$. Serum analysis was as follows: total protein 8.6 g/dL, aspartate aminotransferase 19 U/L, alanine aminotransferase 13 U/L, blood urea nitrogen 13.5 mg/dL, and creatinine 0.7 mg/dL. An enhanced computed tomography (CT) scan of the abdomen showed ileocolic intussusception caused by an abnormal 3.0-cm soft tissue mass with increased thickness of the wall (Figure 1A). There was no evidence of any other soft tissue mass either in the liver or in the kidney. Colonoscopic examination could not be performed because of severe abdominal pain. The patient underwent surgery with the diagnosis of intussusception. During an exploratory laparotomy, the main polypoid mass was located approximately 60 cm from the ileocecal valve. An ileo-ileal intussusception, about 15 cm in length, was found. This mass protruded into the right colon, including the cecum. A right hemicolectomy was uneventfully performed.

Grossly, an approximately 3 cm × 3 cm × 2.5 cm sized polypoid mass was located in the ileum, 60 cm from the

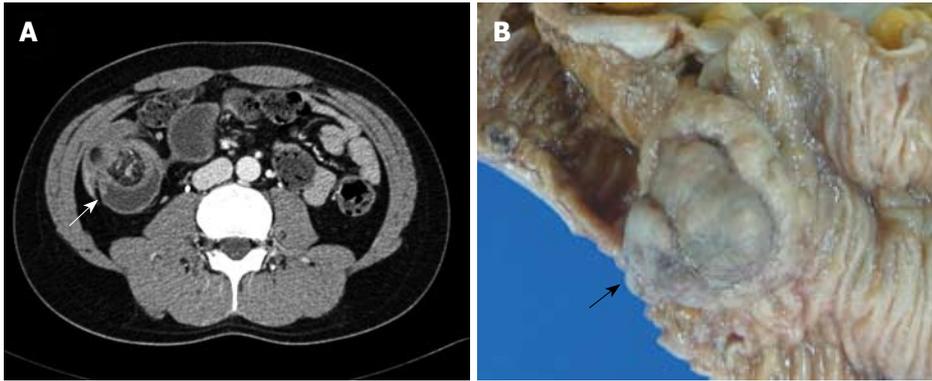


Figure 1 Enhanced abdominal CT and photograph of the pathological specimen. A: A soft tissue mass with fatty-tissue attenuation in the terminal ileum, and with intussusception is shown (arrow); B: Grossly, an approximate 3 cm × 3 cm × 2.5 cm sized polypoid mass was located in the ileum, 60 cm from the ileocecal valve (arrow).

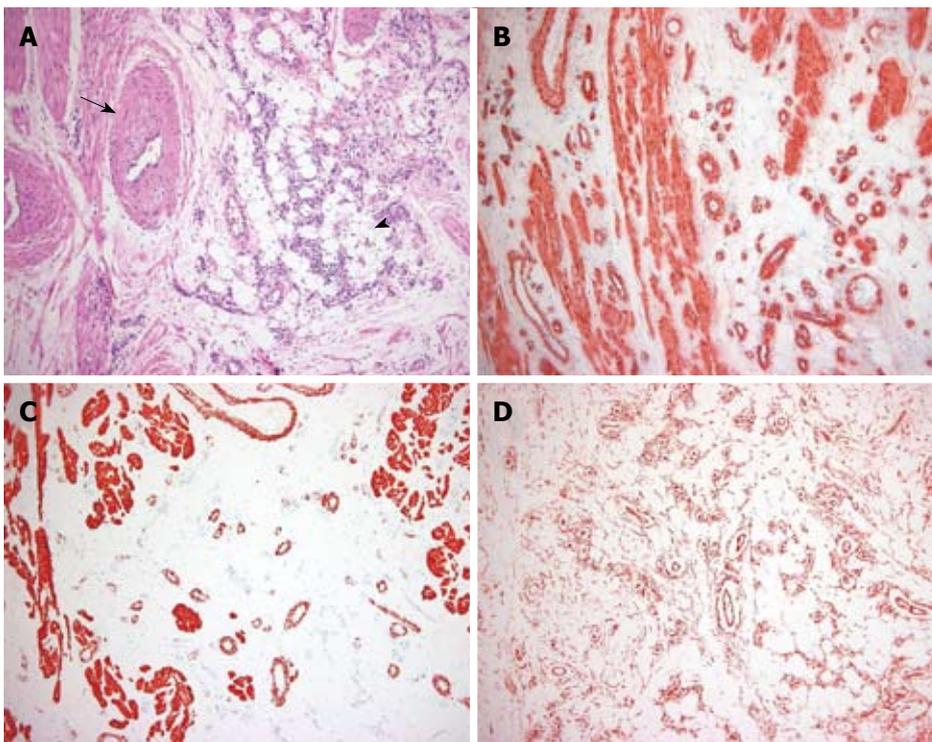


Figure 2 Photomicrograph of the histopathological specimen. A: The specimen was composed of three tissue components: mature adipose tissue (arrowhead) and smooth muscle surrounding thick-walled, medium-sized, vascular channels (arrow) (HE, × 40); B: Immunohistochemistry (IHC) staining showed that the proliferating smooth muscle cells were positive for α -SMA (IHC, × 40); C: Smooth muscle cells showed immunoreactivity for desmin (IHC, × 40); D: The scattered small blood vessels showed immunoreactivity for CD34 (IHC, × 40).

ileocecal valve, and many small polyps were located in the ileum about 25 cm from the ileocecal valve (Figure 1B). The cut surface of the main mass was grayish-yellow in color, with a lobulated appearance and exhibiting no hemorrhage or necrosis. Microscopically, it revealed a mixture of three components: mature adipose tissue, thick-walled vessels, and interspersed areas of spindle-shaped smooth muscle cells (Figure 2A). Immunohistochemically, the smooth muscle cell components of this lesion were consistently positive for α -smooth muscle actin (SMA: Clone 1A4; dilution 1:100; Dako; Figure 2B), desmin (Clone D33; dilution 1:200; Dako; Figure 2C), and vimentin (Clone V9; dilution 1:100; Novocastra). The vascular components were immunoreactive for CD34 (Clone QBEnd/10; dilution 1:50; Novocastra; Figure 2D), but tumor cells were negative for HMB-45 (Novocastra, 1:60) and C-kit (Clone 104D2; dilution, 1:200; Dako). The final pathological diagnosis was AML. The numerous small polyps were diagnosed as lymphoid polyps. The postoperative course was uncomplicated, and the patient was discharged 8 d later. He was seen in the outpatient department for follow-up, and had no tumor recurrence over the subsequent 6 mo.

DISCUSSION

AMLs are histologically benign tumors derived from mesenchymal tissue. The vast majority of AMLs arise in the kidney. In 45 to 80% of patients, they are associated with tuberous sclerosis, a multi-systemic disease with autosomal dominant inheritance, and the occasional association with the triad of epilepsy, mental retardation, and adenoma sebaceum^[1]. Extrarenal AMLs are very rare and have been reported in the liver^[4], nasal cavity^[5], vagina^[6], spermatic cord^[7], skin^[8], mediastinum^[9], and gastrointestinal (GI) tract, including the colon^[10-13] and small intestine^[2,3]. AMLs arising in the GI tract are extremely uncommon and usually present with melena, anemia, diarrhea, abdominal pain, and may even be clinically asymptomatic^[10-13]. Radiological diagnosis of extrarenal AMLs is difficult because of the rarity of the condition. CT is an effective means of imaging and identifying AMLs. Thin-section (3-5 mm) scanning is performed in an attempt to show fatty-tissue attenuation. Sonography can suggest the diagnosis, showing a well-defined hyperechoic lesion, but it is not diagnostic. On magnetic resonance imaging, lesions are bright on T1-

Table 1 Clinicopathological data and immunohistochemical staining results in small-intestinal AMLs

Case No.	1	2	3	4
Author	Han <i>et al</i>	Toye and Czarnecki	Lin <i>et al</i>	Present case
Age (yr)	60	60	48	28
Gender	Female	Female	Female	Male
Tuberous sclerosis	Absence	Absence	Absence	Absence
Symptoms	Periumbilical pain	Early satiety	Abdominal pain and bloody stool	Abdominal pain
Intussusception	Present	Absent	Present	Present
Location	Ileum	Duodenum	Ileum	Ileum
Size (cm)	4 × 4 × 3	3.6 × 3.6	4 × 4 × 2	3 × 3 × 2.5
Gross morphology	Polypoid	Polypoid	Pedunculated polypoid	Polypoid
IHC				
SMA	+	+	+	+
DES	+	NA	+	+
VIM	+	NA	+	+
CD34	NA	NA	+	+
HMB-45	-	-	+	-
CD117 (c-kit)	NA	NA	+	-

DES: Desmin; VIM: Vimentin; +: Positive; -: Negative; NA: Data not available; +*: Positive in few cells.

weighted images and dark on fat-suppressed images^[3]. Surgical excision is the treatment of choice, but inadequate resection may result in rapid local recurrence^[14,15]. Microscopically, most AMLs are composed of a variable mixture of mature fat, thick-walled poorly organized blood vessels, and smooth muscle (classic triphasic histology). Rarely, striking degrees of nuclear atypia may be seen in smooth muscle cells, raising the possibility of malignancy. Cells associated with thin-walled, branching vessels with a pattern similar to lymphangiomyoma is another variation of the smooth muscle component. The lipomatous component consists typically of mature adipose tissue but may contain vacuolated adipocytes, suggesting lipoblasts, thus mimicking a liposarcoma. The blood vessels are thick-walled and lack the normal elastic content of arteries. AMLs with a prominent vascular component may mimic a vascular malformation. Immunohistochemically, AMLs are characterized by coexpression of melanocytic markers (HMB45) and smooth muscle markers (SMA). However, previously reported cases of intestinal AML^[2,3,10-13] showed only focal or no immunoreactivity for HMB45, and the present case was also negative for HMB45. AMLs usually grow slowly. Malignant changes of AML are rare, and only sporadically reported cases have been documented in lesions arising in the kidney. However, to date, AMLs arising in the GI tract have never been reported to have malignant changes.

Only three cases of AML of the small intestine have been previously reported^[2,3]. All of the reported cases

of small intestinal AMLs are summarized in Table 1. The mean age at diagnosis was 49 years. In renal AMLs, the ratio of male to female patients was 1:9^[14]. All of the reported cases of small intestinal AMLs occurred in females, whereas the case presented here occurred in a male. None of the cases were associated with tuberous sclerosis. Three patients with the ileal lesion presented with intussusception, while the patient with the duodenal lesion did not. The tumor sizes ranged from 2 to 4 cm. Also, all four lesions appeared grossly to have a single polypoid pattern.

In conclusion, small intestinal AML is a very rare disease and preoperative diagnosis is difficult. When a patient has abdominal pain with associated ileal intussusception, AML should be considered in the differential diagnosis.

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Congenital absence of the splenic artery and splenic vein accompanied with a duodenal ulcer and deformity

Eun Kyung Shin, Won Moon, Seun Ja Park, Moo In Park, Kyu Jong Kim, Jee Suk Lee, Jin Hwan Kwon

Eun Kyung Shin, Won Moon, Seun Ja Park, Moo In Park, Kyu Jong Kim, Jee Suk Lee, Department of Gastroenterology, Kosin University College of Medicine, Busan, South Korea
Jin Hwan Kwon, Department of Radiology, Kosin University College of Medicine, Busan, South Korea

Author contributions: Shin EK and Moon W contributed equally to this work; Moon W carried out the design of the study; Shin EK wrote the paper; Lee JS was involved in the care of the patient; Kim KJ participated in acquisition of data; Park MI, Park SJ coordinated the study; Kwon JH performed the radiological reviews.

Correspondence to: Won Moon, MD, Department of Gastroenterology, Kosin University College of Medicine, 34 Amnam-dong, Seo-gu, Busan, South Korea 602-702, South Korea. moonone70@yahoo.co.kr

Telephone: +82-51-9905061 Fax: +82-51-9905055

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INTRODUCTION

Congenital absence of the splenic artery is a very rare condition. Only two cases have been reported to date^[1,2]. Both cases were associated with gastric bleeding and in both cases a normal splenic vein was present. To the best of our knowledge, congenital absence of the splenic artery accompanied with splenic vein absence has not been reported. We report a case of a patient with congenital absence of the splenic artery and congenital absence of the splenic vein, found during work-up of epigastric discomfort.

CASE REPORT

A 61-year-old woman was admitted to our hospital with postprandial epigastric discomfort of 15 d duration. The patient had no significant medical background and family history of disease. The patient had no history of alcohol intake or use of non-steroidal anti-inflammatory medications or tobacco. No abnormal findings were seen after a physical examination and laboratory testing. Upper gastrointestinal endoscopy showed a meandering, dilated, pulsatile vessel in the fundus (Figure 1), and severe stenosis with an active ulcer in the proximal part of the second portion of the duodenum, without active mucosal inflammation seen at other sites of the stomach and bulb. The endoscope (H-260; Olympus Optical, Tokyo, Japan) could not pass beyond the stenosis, and the duodenal second portion was observed by the use of a small-caliber upper endoscope (XP-260; Olympus Optical, Tokyo, Japan). There were no abnormalities in the other area of the second portion of the duodenum. *Helicobacter pylori* (*H. pylori*) infection was discovered using the rapid urease test. Double contrast gastrography showed acute angulation and an approximately 1-cm long focal stenosis located between the duodenal bulb and second portion of the duodenum (Figure 2).

Abstract

Congenital absence of the splenic artery is a very rare condition. To the best of our knowledge, congenital absence of the splenic artery accompanied with absence of the splenic vein has not been reported. We report a case of the absence of the splenic artery and vein in a 61-year-old woman who presented with postprandial epigastric discomfort. Upper gastrointestinal endoscopy showed a dilated, pulsatile vessel in the fundus and duodenal stenosis. An abdominal computed tomography (CT) scan revealed absence of the splenic vein with a tortuously engorged gastroepiploic vein. Three-dimensional CT demonstrated the tortuously dilated left gastric artery and the left gastroepiploic artery with non-visualization of the splenic artery. After administration of a proton pump inhibitor, abdominal symptoms resolved without any recurrence of symptoms during 6 mo of follow-up.

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Key words: Congenital; Anomaly; Splenic artery; Splenic vein; Duodenum; Ulcer; Deformity

Peer reviewer: Takafumi Ando, MD, PhD, Department of Gastroenterology, Nagoya University Graduate School of Medicine, Therapeutic Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan



Figure 1 Upper gastrointestinal endoscopy showing a meandering, dilated, pulsatile vessel in the fundus.

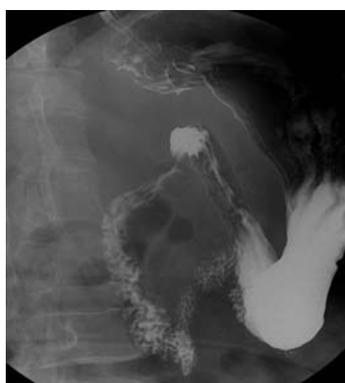


Figure 2 Double contrast view of the duodenum showing the acute angulation and stenosis between the duodenal bulb and second portion of the duodenum.



Figure 3 Abdominal CT findings. A: Oblique sagittal view demonstrates the absence of the splenic vein with confluence of the superior mesenteric vein (arrow) and the presence of the tortuously engorged gastroepiploic vein in the pancreas (asterisk), forming the main portal vein (arrowhead); B: Axial image of the abdomen shows the tortuously engorged gastroepiploic vein in the pancreas.

The use of abdominal computed tomography (CT) revealed the absence of the splenic vein as seen on a normal pancreatic and splenic image. The presence of a tortuously engorged, gastroepiploic vein located in the anterior portion of the pancreas was seen (Figure 3). Three-dimensional CT imaging demonstrated the presence of a tortuously dilated left gastric artery and left gastroepiploic artery with non-visualization of the splenic artery (Figure 4). It was concluded that the abnormally dilated vessel in the gastric fundus was the dilated left gastric artery. The patient was treated with 20 mg oral rabeprazole once a day. As the abdominal symptoms of the patient were gradually improving, the patient was discharged from the hospital and has been monitored with the use of regular check-ups. Three months later, follow-up endoscopy showed decreased activity of the duodenal ulcer, but the duodenal stenosis was sustained. The patient has remained asymptomatic for 10 mo.

DISCUSSION

A splenic artery that originates from the celiac trunk is the most common form according to the classic anatomical description of the splenic artery as described by Michels^[3]. Occasionally the splenic artery may arise from the aorta or the superior mesenteric artery, and a double splenic artery may rarely form^[4]. However, congenital absence of the splenic artery is an extremely rare occurrence.

The splenic vein follows a course located along the posterior aspect of the pancreas, and the splenic vein is



Figure 4 Three dimensional CT image demonstrates the tortuously dilated left gastric artery (arrow) and the left gastroepiploic artery (arrowhead) with non-visualization of the splenic artery.

encircled by pancreatic tissue. The hilum of the spleen receives both the short gastric and left gastroepiploic veins. The splenic vein may also receive the coronary and inferior mesenteric vein at the junction with the superior mesenteric vein to form the portal vein^[5,6]. When obstruction to normal flow occurs, venous blood returns to the portal vein by several collateral routes, including the short gastric vein and left gastroepiploic vein^[7-9].

Two cases of congenital absence of the splenic artery^[1,2] and only one case of congenital absence of the splenic vein^[10] have been reported to date. However, the present case is believed to be the first case of congenital absence of the splenic artery and congenital absence of the splenic vein together. In the two previous cases of

congenital absence of the splenic artery, gastrointestinal bleeding from a gastric mucosal lesion secondary to abnormally formed collateral blood flow was determined on arteriograms. Non-visualization of the splenic artery and filling of the intrasplenic branches *via* the collateral vessels was demonstrated. The two cases were treated with surgery due to repeated hematemesis^[1,2]. The previous case of congenital absence of the splenic vein showed gastric bleeding due to the presence of isolated gastric varices, which was diagnosed by the use of abdominal CT angiography. Marked torturous engorgement of the gastroepiploic vein was seen and the patient was treated with the use of conservative therapy^[10]. In the present case, there was a prominent vessel in the fundus without an overlying mucosal abnormality and hemorrhage. The differential diagnosis of the prominent fundal vessel included gastric varices resulting from cirrhosis, pancreatitis, cancer, a splenic vein thrombosis, splenic vein stenosis, a splenic artery aneurysm, surgery, coagulopathy^[11-13] and the presence of collateral arterial vessels resulting from congenital absence of the splenic artery or the presence of an occluded splenic artery^[14]. This patient had no evidence of cirrhosis, pancreatitis, a pancreatic neoplasm or coagulopathy, but the patient had a prominent, pulsatile vessel suggesting the presence of an artery.

In the present case, CT angiography demonstrated the presence of a dilated left gastric artery and left gastroepiploic artery without visualization of the splenic artery. The splenic vein is located at the posterior portion of the pancreas^[5,6], but in this case, abdominal CT images revealed absence of the splenic vein and the presence of a tortuously engorged, gastroepiploic vein at the anterior portion of the pancreas. Therefore, we diagnosed the case as a congenital absence of the splenic artery and congenital absence of the splenic vein. This patient complained of postprandial epigastric discomfort and the symptoms were caused by duodenal stenosis and acute angulation complicated by the presence of a chronic duodenal ulcer. The cause of the duodenal ulcer and deformity was not clear. This patient had no history of excessive alcohol intake, use of nonsteroidal anti-inflammatory drugs or tobacco use, and had a normal gastrin level, but the patient showed a positive result in a rapid urease test. Undoubtedly, *H pylori* infection is a major cause of duodenal ulcers^[15], but there is the possibility that a duodenal ulcer may have repeatedly developed due to other causes in this patient. We hypothesized that the duodenal ulcer resulted from sustained pressure and ischemia in the duodenal mucosa by the presence of abnormally dilated collateral

submucosal vessels secondary to absence of the splenic artery, and inadequate venous drainage through the collateral veins secondary to the absence of the splenic vein, which subsequently caused a duodenal deformity.

In conclusion, absence of the splenic artery and vein accompanied with a duodenal ulcer and deformity is a unique and very rare condition. When gastroduodenal mucosal lesions with prominent pulsatile gastric vessels in the fundus are present, although rare, the congenital absence of the splenic artery with or without the accompanying absence of the splenic vein should be considered for the differential diagnosis.

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Taku Aoki, MD

Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

Olivier Barbier, Professor

CHUQ-CHUL Research Center, Laval University, 2705 Laurier Boulevard, Québec (QUE) G1V 4G2, Canada

Jamie S Barkin, MD, Professor of Medicine, Chief

Sinai Medical Center Division of Gastroenterology, Mt. Sinai Medical Center, University of Miami, School of Medicine, 4300 Alton Road, Miami Beach, FL 33140, United States

Rosemary Joyce Burnett, MPH

Department of Epidemiology National School of Public Health, University of Limpopo, Medunsa Campus PO Box 173, MEDUNSA, Pretoria 0204, South Africa

Tatjana Crnogorac-Jurcevic, MD, PhD

Cancer Research UK, Molecular Oncology Unit, Barts and The London School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom

Dr. Paolo Del Poggio

Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

Inge I Depoortere, PhD

Centre for Gastroenterological Research, Gasthuisberg OandN, bus 701, Leuven 3000, Belgium

Eduardo R Marín-López, MD, AGAF, Professor of Medicine, Chief in Education and Research

Hospital Beneficencia Española de Puebla, GI and Liver Unit, 19 Norte # 1001, Col. Jesús García, 72090, Puebla, Pue, México

Robin Hughes

Institute of Liver Studies, King's College London School of Medicine, Bessemer Road, London, SE5 9PJ, United Kingdom

Dr. Pietro Invernizzi

Division of Internal Medicine, Department of Medicine, Surgery, Dentistry, San Paolo School of Medicine, University of Milan, Via Di Rudinfi 8, 20142 Milan, Italy

Dr. Aydin Karabacakoglu, Assistant Professor

Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Mitsuo Katano, MD, PhD, Professor

Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Shiu-Ming Kuo, MD

University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo 14214, United States

Walter E Longo, Professor

Department of Surgery, Yale University School of Medicine, 205 Cedar Street, New Haven 06510, United States

Wendy M Mars, PhD

Department of Pathology, University of Pittsburgh, S-411B South Biomedical Science Tower Pittsburgh, PA 15261, United States

Peter L Moses, MD, FACC, AGAF, Professor

University of Vermont College of Medicine Section of Gastroenterology & Hepatology, 111 Colchester Avenue, Smith 237B, MCHV, Burlington, VT 05401, United States

Christian D Stone, MD, MPH, Director

Inflammatory Bowel Disease Program, Assistant Professor of Medicine, Division of Gastroenterology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8124, Saint Louis, MO 63110, United States

Wing-Kin Syn, MD

Division of Gastroenterology, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham, NC27710, United States

Christer S von Holstein, Associate professor

Department of Surgery, Lund University Hospital, SE-221 85 Lund, Sweden

Zobair M Younossi, MD, MPH

Center for Liver Diseases at Inova Fairfax Hospital, 3289 Woodburn Road, Suite 375, Annandale, Virginia 22003-6800, United States

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February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

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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.apdwcgress.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

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John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
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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.

Instructions to authors

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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Za-zhi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID: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]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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INTRODUCTION

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Is transient elastography a useful tool for screening liver disease?

Paolo Del Poggio, Silvia Colombo

Paolo Del Poggio, Silvia Colombo, Hepatology Unit, Treviglio Hospital, Treviglio (Bg) 24047, Italy

Author contributions: Both authors wrote the manuscript.

Correspondence to: Paolo Del Poggio, Hepatology Unit, Treviglio Hospital, Treviglio (Bg) 24047,

Italy. pdpoggio@ospedale.treviglio.bg.it

Telephone: +39-363-424494 Fax: +39-363-424561

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L'Hospitalet de Llobregat Barcelona. C/ Feixa Llarga S/N, L' hospitalet de Llobregat Barcelona 08023, Spain

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Abstract

Transient elastography (TE) is a new non invasive tool for measuring liver stiffness, which is correlated to the histologic stage of liver fibrosis. Several studies in chronic liver disease (CLD) have determined a good accuracy of TE in predicting significant fibrosis and an optimal accuracy in predicting cirrhosis. Normal liver stiffness ranges between 3.3-7.8 KPa and using a cut off of 7.1 KPa, significant fibrosis and cirrhosis can be excluded with a very high negative predictive value (NPV). Positive predictive value (PPV) for the diagnosis of cirrhosis is lower using just a single scan but increases to 90% if high stiffness values are confirmed by a second independent scan. However the presence of fatty liver and metabolic syndrome slightly increases the readings and may reduce the accuracy of the test. It is uncertain if this increase is related to the presence of steatofibrosis or if it is caused by steatosis itself. TE can be used in screening patients attending the liver clinics to identify those with significant fibrosis or cirrhosis and may be particularly useful in discriminating HBV inactive carriers from chronic hepatitis B patients. TE, however, is not reliable in predicting the presence of esophageal varices in cirrhotics. Another potential indication for TE is the systematic screening of populations at high risk for CLD, such as intravenous drug users and alcoholics, but further studies are needed to determine its diagnostic accuracy in these settings.

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Key words: Transient elastography; Screening; Liver disease; Hepatitis B; Hepatitis C; Non alcoholic steatohepatitis; Cirrhosis

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INTRODUCTION

Transient elastography (TE) is a new non invasive tool for measuring liver stiffness, which is correlated to the histologic stage of liver fibrosis^[1]. The device (Fibroscan) generates an elastic wave by means of a vibrator applied to the thoracic wall at the level of the right liver lobe. The vibrator produces a shot and a low amplitude shear wave propagating through the liver parenchyma. The velocity of propagation is directly proportional to liver stiffness and is automatically calculated by the instrument. The range of measurements, expressed in kilopascals, varies from 2.5 to 74 KPa.

Many studies have been published on the use of TE in patients with already diagnosed chronic liver disease (CLD) but few have addressed the issue of its possible use as a first line examination in the liver clinic or in facilities where patients at risk of liver disease are attending.

HOW TO TAKE AND INTERPRET THE MEASUREMENTS

In order to obtain valid and reproducible measurements the probe should be placed at the center of the right liver lobe, two intercostal spaces below the upper liver margin and at the level of the anterior or middle axillary line. If measurements are taken below this point and too close to the lower liver edge both the percentage of valid shots and the median stiffness tend to decrease^[2]. Ultrasound assistance to locate the upper liver margin is usually unnecessary if the patient is reasonably thin because the liver can be recognized by percussion alone. The device incorporates an M-mode window enabling the operator to locate the liver parenchyma and avoid both ribs and lung. If the shot does not generate a readable wave the software classifies the measurement as unsuccessful. Liver stiffness

is defined as the median of 10 successful measurements and according to the manufacturer's recommendations at least 60% of the shots should be successful for each exam. The main reason for unsuccessful examination in the Western world is patients being overweight, while in the East intercostal spaces which are too narrow often hamper the appropriate contact of the probe. Overall failure rates in different studies range between 2.4% and 9.4%^[3-6]. The presence of diabetes and being a transplant recipient have also been identified as independent predictors of failure in a recent study of 215 patients with CLD^[7]. TE cannot be performed in ascitic patients because the interposed fluid blocks the progression of the shear wave. Other contraindications are pregnancy and the presence of a cardiac pacemaker because there are no safety studies on the use of TE in these conditions. TE is easy to perform, quick and reproducible although fatty liver and a low fibrosis stage may decrease reproducibility^[3]. TE can also be easily learnt and performed by nurses^[8] and the results are immediately available, thus saving physician's time and rendering this method particularly suitable for screening a large number of patients.

The validity of the results depends on one important parameter: the variability of measurements. This is reflected by the interquartile range (IQR), representing the range of values including 50% of patients above and below the median. According to the manufacturer's suggestion the IQR/median stiffness ratio should not exceed 30% of the median value, although it seems that 20% could assure the best concordance between liver biopsy and TE^[9]. There are no studies specifically dealing with the problem of excessive variability of readings and therefore the interpretation of results is derived more from personal experience and from the manufacturer's advice than from observational data. It is still unknown if variability is observed only in diseased or also in normal livers and how this variability affects the interpretation of the test. The cause may be an improper examination technique or it may be inherent to the liver disease itself e.g. in macronodular cirrhosis stiffness may change in different areas of the liver. When variable readings are obtained it is important to check if the probe is perpendicular to the thoracic wall, that the vibrator is not touching against a rib and if the elastographic wave is straight and narrow. If the wave that has been generated is broad, bifid or angulated the software may reconstruct the velocity curve in different parts of the wave and give variable readings. It is important in these cases to obtain a "good" elastogram. This can be obtained by placing the probe in the middle of the right lobe and avoiding contact with the rib as that may dampen the shot and distort the shear wave.

WHAT ARE THE NORMAL VALUES OF TRANSIENT ELASTOGRAPHY?

Paradoxically many studies have been published in CLD patients, but only three in apparently normal subjects (Table 1): the first as a full paper^[10] the second as a letter^[11] and the third as an abstract^[12]. In the first study,

Table 1 Liver stiffness in the normal population and factors influencing its measurement

	Corpechot C ^[11]	Roulot D ^[10]	Colombo S ^[12]
Number of subjects	71	429	327
Population	Healthy volunteers	Medical check-up	Blood donors
Mean stiffness (KPa)	4.8 (2.5-6.9) ¹	5.4 ± 1.5 ²	4.9 ± 1.7 ²
95th centile	-	8.6	7.8
Age	No effect	No effect	No effect
Gender	M > F	M > F	M = F
High BMI	Increased	Increased	Increased
Metabolic syndrome	-	Increased ³	-
Fatty liver	-	-	Increased ³

¹Range; ²Standard deviation; ³At multivariate analysis.

performed by Roulot, 429 apparently healthy subjects attending a free health check were studied by a single operator. Only values with an IQR/median stiffness of less than 30% were considered in the analysis, thus overcoming the problem of variability. Results could be obtained in 93.4% of the subjects, indicating that TE has a low failure rate in the general population. However, the percentage of failures rose to 25% in obese individuals (BMI > 30 kg/m²) and 88% in morbid obese individuals, confirming that TE is not a good method for screening overweight people. This is a significant drawback, because many obese subjects have fatty livers and need a rapid, non invasive method to rule out significant fibrosis. Using the 5th and 95th centiles normal values were set between 3.3-7.8 KPa in women and 3.8-8 KPa in men. In the main studies of TE in chronic liver disease^[13-18] the mean cut-off for significant fibrosis was established between 7 and 8 KPa, (Table 2), which is higher than the 95th centile of normal subjects. TE can thus reliably distinguish normal individuals from patients with significant fibrosis, although overlap exists with mild fibrosis. In addition none of the normal subjects studied by Roulot had values higher than 13-17 KPa, which is considered the cut off range for cirrhosis of all etiologies (Table 3).

Our group reproduced the same results in voluntary blood donors^[12]: in the absence of fatty liver we observed a mean normal liver stiffness of 4.6 KPa ± 1.52 SD. Using 6.9 as the optimal cut off for normal individuals and comparing it with the cut offs from the literature, we obtained a 96% NPV for ruling out significant fibrosis and a 100% NPV for ruling out cirrhosis. In conclusion, normal subjects can be reliably differentiated from CLD patients. TE could thus be proposed as a good screening tool to detect significant fibrosis and as an optimal tool for the detection of cirrhosis, irrespective of the etiology.

DO STEATOSIS AND TRANSAMINASE LEVELS AFFECT THE READINGS?

An important finding of Roulot's paper is that mean stiffness was found to be 1.3 KPa higher in subjects with metabolic syndrome than in those without.

Table 2 Diagnostic performance of TE in the diagnosis of significant fibrosis

	Oliveri ^[14]	Marcellin ^[13]	Castera ^[35]	Ziol ^[15]	Fraquelli ^[3]	Corpechot ^[18]	Kelleher ^[16]	Yoneda ^[17]
Patients	268	170	183	251	200	95	129	67
F2 or higher (%)	69	50	74	65	50	60	50	49
Etiology	HBV	HBV	HCV	HCV	80% HCV	PBC/PSC	Nafld	Nafld
Cut Off (KPa)	7.5	7.2	7.1	8.8	7.9	7.3	8.7	6.6
Sensitivity (%)	93	70	67	56	72	84	81	82
Specificity (%)	88	83	89	91	84	87	78	81
AUROC	0.96	0.81	0.83	0.79	0.86	0.92	0.86	0.87

Table 3 Diagnostic performance of TE in the diagnosis of cirrhosis

	Oliveri ^[14]	Marcellin ^[13]	Castera ^[35]	Ziol ^[15]	Fraquelli ^[3]	Ganne-Carrié ^[5]	Corpechot ^[11]	Foucher ^[33]	Nguyen ^[36]	Yoneda ^[17]
Patients	268	202	183	251	200	775	95	354	103	67
Cirrhotics (%)	24	8	25	19	26	15	16	13	32	7.50
Etiology	HBV	HBV	HCV	HCV	80% HCV	All	PBC/PSC	All	Alcohol	Nafld
Cut-off (KPa)	11.8	11	12.5	14.6	11.9	14.6	17.3	17.6	19.5	17
Sensitivity (%)	86	93	87	86	91	79	93	77	91	93
Specificity (%)	96	87	91	96	89	95	95	97	100	95
AUROC	0.97	0.93	0.95	0.97	0.91	0.95	0.96	0.96	0.92	0.99

Metabolic syndrome was also the main predictor of increased stiffness after adjustment for age, sex, BMI and liver enzymes. This finding suggests that the normal ranges for liver stiffness should be shifted upwards in overweight patients with metabolic syndrome. However no ultrasound examination was performed in this study and therefore it was unknown if the increased stiffness was dependent on metabolic syndrome itself or on fatty liver. To answer this question we investigated 327 healthy blood donors using TE and abdominal ultrasound performed on the same day by two operators with good concordant readings^[11]. Similarly to Roulot's study we had a very low failure rate (2.4%) confirming the good applicability of TE in population studies. At multiple regression analysis we found that the degree of steatosis, and not BMI, sex, age and liver enzymes, was related to liver stiffness. The central issue is whether steatosis itself increases liver stiffness or if it is caused by an underlying steatofibrosis. Data from the literature are inconclusive: in one study patients with chronic hepatitis C and the same fibrosis stage had increased liver stiffness if they had concomitant fatty liver^[19]. In addition, there was a close relationship between the augmentation of liver stiffness and the degree of steatosis. However studies in chronic hepatitis B have shown that patients with the same stage of fibrosis had lower^[20] or equal stiffness^[21] in the case of accompanying steatosis. It seems unlikely that steatosis might influence liver stiffness in discordant ways depending on the type of hepatitis and therefore further studies are needed to clarify this issue.

Another possible confounding factor is the effect of transaminase (ALT) level. It is well known that acute hepatitis may spuriously cause extreme and transient elevations of liver stiffness^[6,22,23], but also minor ALT elevations can alter TE readings and cause discordance with histological stage^[24]. If elevated ALT may overestimate fibrosis stage the opposite is also true: e.g.

elderly patients with normal or minimally elevated ALT may have their fibrosis stage underestimated. An algorithm has been proposed to correct for the underestimation of fibrosis in the elderly, but this algorithm has not yet been validated^[24]. In conclusion, abdominal ultrasound and ALT determination should always be used together with TE in population screening.

NEW TECHNIQUES: REAL TIME ELASTOGRAPHY

From the above considerations it would be attractive to use a new device incorporating liver stiffness measurements with conventional ultrasound. This task could be accomplished by real time ultrasonography. This technique is performed with conventional ultrasound probes and equipment such as Hitachi EUB-8500 and EUB-900 machines. The examined tissue is divided into up to 30000 finite elements and compression is applied with the probe itself to the skin overlying the liver. During compression the displacement of each element is measured and recorded: in hard tissue the amount of displacement is low, whereas in soft tissue the amount of displacement is high. The calculation of soft tissue elasticity distribution is performed in real time and the results are presented in a colour-coded scale with a conventional B-mode image in the background. Liver stiffness can thus be determined during a conventional routine upper abdominal ultrasonography. This new technique is rapid and cheaper than Fibroscan, but its accuracy should be tested against classic TE and liver biopsy. In one study comparing liver biopsy and real time ultrasonography the areas under the receiver operating curve (AUROC) were inferior to TE: 0.75 for equal or higher than F2 fibrosis, 0.73 for equal or higher than F3, 0.69 for F4 and APRI itself performed better than the new technique^[25]. Using heart beats instead of manual

compression for displacement^[26] may improve the accuracy and lead to better standardization. Clearly more studies are needed and at this time only TE has sufficient body of evidence to be proposed for screening studies.

WHEN TRANSIENT ELASTOGRAPHY CAN BE USED AS A FIRST LINE EXAMINATION

TE could theoretically be used to screen patients attending the liver clinic in order to identify: (a) patients with chronic hepatitis B and C with significant fibrosis, to establish an indication for antiviral therapy; (b) patients with non alcoholic fatty liver disease (NAFLD) or non alcoholic steatohepatitis with significant fibrosis in which aggressive dietary intervention or new therapies could be proposed; (c) patients with liver cirrhosis, in order to start sonographic surveillance for hepatocellular carcinoma; (d) patients with liver cirrhosis and significant portal hypertension, in order to start endoscopic surveillance of esophageal varices.

Outside the liver clinic TE could also be used to systematically screen populations at high risk for liver disease e.g. intravenous drug addicts or alcoholic patients attending rehabilitation programs.

In the clinical setting accuracy for the diagnosis of cirrhosis is higher than for significant fibrosis with a median AUROC of 0.95 *vs* 0.86 irrespective of the etiology of liver disease (Tables 2 and 3). The best accuracy is achieved in ruling out cirrhosis, with a NPV close to 100%^[1]. It should be stressed that despite similar AUROCs, cut offs for significant fibrosis and cirrhosis vary according to etiologies, being lower for hepatitis B, intermediate for hepatitis C and higher for NAFLD or alcoholic liver disease. In chronic hepatitis C and NAFLD there is a continuous spectrum of fibrosis irrespective of ALT levels and therefore it would be preferable to use ranges of values instead of cut-offs^[1]. On the contrary, in hepatitis B virus infection there is no spectrum of continuity between the inactive carrier state and the chronic hepatitis B patient^[27] and the use of a cut off value would be appropriate. TE can reliably differentiate the inactive carrier from chronic hepatitis B. In a recent paper^[14] the mean stiffness value of the liver of an inactive carrier was found to be 5 KPa \pm 1.8 SD, which is similar to normal controls and different from chronic hepatitis B patients with significant fibrosis. Therefore, if a patient suspected to be an inactive carrier has normal stiffness and elevated ALT, another cause for the increased ALT levels should be sought e.g. concomitant NAFLD. The capacity of correctly identifying chronic HBV carriers could be of immense value in regions with high prevalence of HBsAg, where it could be used together with ALT measurement as a quick and cheap screening test for a large proportion of the population.

If TE is a useful tool to diagnose significant fibrosis and cirrhosis in CLD patients and to define the inactive HBsAg carrier, it is not so for predicting portal hypertension and esophageal varices. In fact a

good correlation between stiffness and hepatic-vein portal gradient (HVPG) was found only up to HVPG values of 10-12 mmHg, whereas for higher values the correlation was suboptimal^[28]. This could be explained by the fact that TE measures the initial rise of portal pressure caused by the accumulation of a fibrillar matrix, but not the complex hemodynamic changes of late portal hypertension^[29]. Accordingly TE was not accurate in prediction of esophageal varices, with an AUROC ranging from 0.76 to 0.84 in various studies^[29-31]. Although sensitivity was good (71%-96%) , specificity and positive predictive values (PPV) were low (60%-80% and 48%-54%) and overall accuracy was inferior compared to simple tests like platelet count/spleen diameter ratio^[32]. Another problem arising from these studies is the wide range of proposed cut offs, varying from 13.9 to 21.3 KPa for all varices and from 19 to 30 KPa for F2 varices^[30,31]. The optimal cut offs therefore are still to be defined.

It would also be interesting to determine a cut off for liver stiffness associated with an increased risk for hepatocellular carcinoma, thus warranting enhanced surveillance for this type of patient. This issue was addressed in only one study^[33] in which 144 patients with cirrhosis or advanced fibrosis of varying etiologies were studied with TE and appropriate imaging. According to the authors, a cut of 53.7 KPa could identify cirrhotics harbouring hepatocellular carcinoma with good specificity (87%) and high NPV (90%). PPV and sensitivity were however too low (around 30%) to propose TE as a screening tool for determining the risk of hepatocellular carcinoma. Moreover the conclusions were drawn from only 19 liver cancer patients and clearly more studies are needed in larger cohorts of patients.

In different settings from liver clinics, TE has been studied in IVDU^[34] and alcoholics participating in a rehabilitation program (Melin P, personal communication). In the first study, conducted in Denmark, 434 IVDU from 6 methadone clinics were studied^[34]. Among the 394 subjects in which TE could be performed, 11 % had cirrhosis (> 12 KPa) and 16% significant fibrosis (8-12 KPa). Twenty-five patients with stiffness > 12 KPa had a repeated TE measurement at the time of liver biopsy. It is interesting to note that 6 patients had a stiffness < 12 KPa at the second scan and all of them had mild fibrosis at biopsy, while only 1 out of 19 patients confirmed at the second scan had mild fibrosis. The authors conclude that two consecutive and concordant scans are needed in order to establish a confident diagnosis of cirrhosis. In fact, PPV for the diagnosis of cirrhosis increased from 50% to 94% after 2 independent scans. The take home message of this study is that in population screening, it is advisable to confirm all elevated results with a second independent scan.

CONCLUSION

In conclusion, there are not enough data to recommend TE as a screening tool outside of liver clinics and

specific studies are needed on high risk populations. On the basis of existing evidence we can conclude that TE has a high NPV to exclude cirrhosis. PPV is low with a single scan but can increase to 90% if high stiffness values are confirmed by a second independent scan. Accuracy for diagnosing significant fibrosis is lower than for cirrhosis and different cut offs must be taken into account. However, TE is not useful for the prediction of esophageal varices, because PPV is low and cut-offs are still undefined.

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Natalia A Osna, MD, PhD, Series Editor

Isolated segmental, sectoral and right hepatic bile duct injuries

Radoje B Colovic

Radoje B Colovic, Institute of Digestive Diseases, First Surgical Clinic, Clinical Center of Serbia, 6 Koste Todorovica, 11000 Belgrade, Serbia

Author contributions: Colovic RB contributed all of this paper.
Correspondence to: Dr. Radoje B Colovic, Professor, Institute of Digestive Diseases, First Surgical Clinic, Clinical Center of Serbia, 6 Koste Todorovica, 11000 Belgrade, Serbia. marcolov@sbb.rs

Telephone: +381-11-2684044 Fax: +381-11-2684044
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Abstract

The treatment of isolated segmental, sectoral and right hepatic bile duct injuries is controversial. Nineteen patients were treated over a 26-year period. Group one was comprised of 4 patients in whom the injury was primarily repaired during the original surgery; 3 over a T-tube, 1 with a Roux-en-Y. These patients had an uneventful recovery. The second group consisted of 5 patients in whom the duct was ligated; 4 developed infection, 3 of which required drainage and biliary repair. Two patients had good long-term outcomes; the third developed a late anastomotic stricture requiring further surgery. The fourth patient developed a small bile leak and pain which resolved spontaneously. The fifth patient developed complications from which he died. The third group was comprised of 4 patients referred with biliary peritonitis; all underwent drainage and lavage, and developed biliary fistulae, 3 of which resolved spontaneously, 1 required Roux-en-Y repair, with favorable outcomes. The fourth group consisted of 6 patients with biliary fistulae. Two patients, both with an 8-wk history of a fistula, underwent Roux-en-Y repair. Two others also underwent a Roux-en-Y repair, as their fistulae showed no signs of closure. The remaining 2 patients had spontaneous closure of their biliary fistulae. A primary repair is a reasonable alternative to ligation of injured duct. Patients with ligated ducts may develop complications. Infected ducts require further surgery. Patients with biliary peritonitis must be treated with drainage and lavage. There is a 50% chance that a biliary fistula will close spontaneously. In cases where the biliary fistula does not close within 6 to 8 wk, a Roux-en-Y anastomosis should be considered.

INTRODUCTION

Dangerous anatomy, dangerous pathologies and dangerous surgery are all factors which lead to bile duct injuries in both open and laparoscopic cholecystectomy^[1]. Dangerous anatomical variants of the right liver bile ducts are known to occur in around 15%-20% of patients, and may be injured during cholecystectomy. These operative sectoral and segmental bile ducts injuries (SSBDI) can often pass by unnoticed and without serious symptoms, and thus the frequency is likely to be far higher. They can however also lead to serious complications such as cholangitis and liver abscesses or biliary fistula and peritonitis.

The treatment of SSBDI is controversial, and the different approaches often depend on the timing of the diagnosis and the types of complications. This study reviews our management of SSBDI.

ISOLATED SEGMENTAL, SECTORAL AND RIGHT HEPATIC BILE DUCT INJURIES

Dangerous anatomy, dangerous pathologies and dangerous surgery are all factors which lead to bile duct injuries in both open and laparoscopic cholecystectomy^[1]. There are three main dangerous anatomical variants. Firstly, the cystic duct may be near to the segmental (Figure 1A), or sectoral bile duct (Figure 1B) so that these ducts may be injured during dissection, transection or occlusion of the cystic duct. Secondly, the

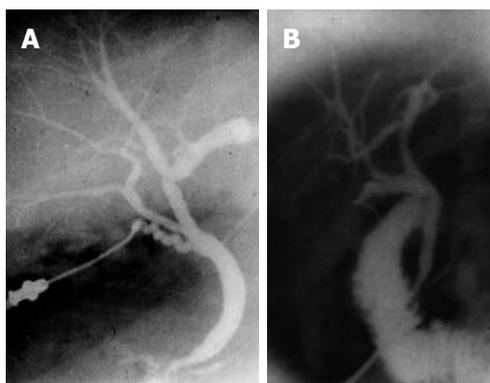


Figure 1 Cystic duct. A: near a segmental bile duct; B: very close to the sectoral bile duct.

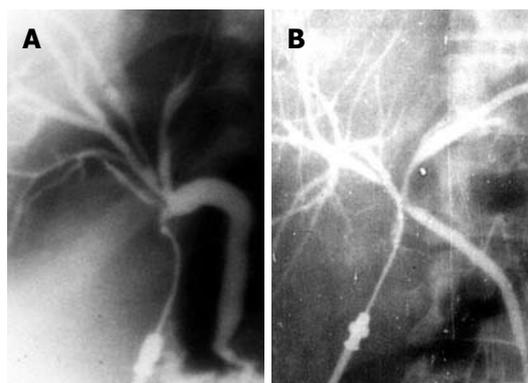


Figure 3 Cystic duct joins the convergence of the sectoral (A) and hepatic ducts (B).

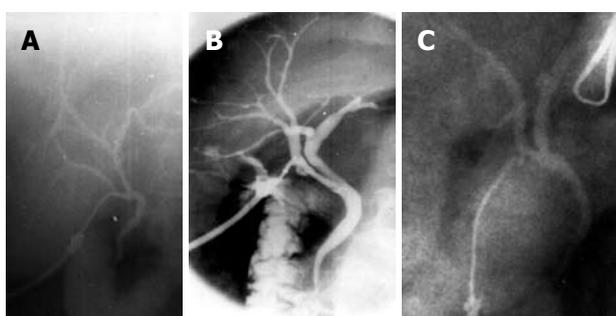


Figure 2 Cystic duct. A: Cystic duct joining the segmental; B: Sectoral hepatic ducts that could be misinterpreted as cystic ducts; C: Right hepatic ducts that could be misinterpreted as cystic ducts.

cystic duct may join one of these ducts, instead of the common bile duct (Figure 2A-C) so that one of them may be misinterpreted as a cystic duct and so transected, resected and occluded. Thirdly, the cystic duct may join the convergence of the sectoral or hepatic ducts (Figure 3A and B) so that the common bile duct may be misinterpreted as a cystic duct and so transected and resected.

Dangerous anatomical variants of the right liver bile ducts are known to occur in around 15%-20% of patients, and may be injured during cholecystectomy. These operative isolated segmental, sectoral and right hepatic bile duct injuries can pass by unnoticed and without serious symptoms, and thus the frequency is likely to be even greater. They can however also lead to serious complications such as cholangitis and liver abscesses or biliary fistula and peritonitis. The treatment of isolated segmental, sectoral and right hepatic bile duct injuries is controversial, and the different approaches often depend on the timing of the diagnosis and the types of complications.

We retrospectively reviewed the management of isolated segmental, sectoral and right hepatic bile duct injuries treated in our centre over a 26 year period (1982-2008). The 19 patients (14 women, with a mean age of 51 years) were divided into 4 groups in order to better analyze the different approaches (Table 1). The first group was comprised of 4 patients in whom the injury was recognized at the time of original surgery. Each patient

underwent a primary repair, 3 over a tiny T-tube inserted into the common bile duct and then passed through the anastomosis, and 1 patient had an anastomosis of the injured duct with a Roux-en-Y jejunal limb (Figure 4). All the 4 patients had an uneventful recovery and continue to remain well to this day.

The second group consisted of 5 patients in whom the injured duct was ligated. Four of the patients developed cholangitis, 2 of them then also developed liver abscesses requiring drainage and biliary repair. Two patients had good long-term outcomes, the third developed a late anastomotic stricture which was then successfully resolved by further surgery, while the fourth patient developed a small temporary bile leak and pain which resolved spontaneously and required no further treatment. The fifth patient developed serious complications (a liver abscess, an abdominal wound disruption, and pneumonia) from which he finally died.

The third group was comprised of 4 patients referred from other hospitals with neglected biliary peritonitis which had lasted for several weeks. All the 4 patients underwent laparotomy, drainage and lavage. All the 4 developed an external biliary fistula through the subhepatic drain, 3 of which resolved spontaneously, and 1 of which required Roux-en-Y repair. All 4 patients had good long-term outcomes.

The final group consisted of 6 patients with external biliary fistulae which had lasted for between 2 and 8 wk, also referred from other hospitals. Two patients, both with an 8 wk history of a fistula, underwent immediate Roux-en-Y repair. Two other patients also underwent a Roux-en-Y repair, as their fistulae showed no signs of spontaneous closure (Figure 5). The remaining 2 patients had spontaneous closure of their biliary fistulae and required no further treatment (Figure 6).

Thus 12 (63%) patients in this series underwent some form of repair, 3 over T-tube and 9 with Roux-en-Y anastomosis. Repair was not necessary in 6 (32%) patients. 5 of the 10 patients with biliary fistula formation required Roux-en-Y repair, while 5 had spontaneous resolution. One patient (5%) who had duct ligation died.

No single method of treatment for isolated

Table 1 Patient demographics including gender, age, intraoperative recognition of the bile duct injuries, immediate repair and the method utilised, complications, any treatment of the complications, delayed treatments, long-term outcomes and late complications

Patient number	Gender	Age	Injury recognised intra-op	Immediate intervention	Complication	Treatment for complication	Delayed treatment	Outcome	Late complication
1	F	60	Yes	Repair over a T tube	No	No	None	Excellent	None
2	M	54	Yes	Repair over a T tube	No	No	None	Excellent	None
3	F	45	Yes	Roux-en-Y	No	No	None	Excellent	None
4	M	76	Yes	Repair over a T tube	Wound infection	No	None	Good	Hernia
5	M	62	Yes	Ligature	Liver abscess peritonitis	Drainage	None	Died	None
6	F	65	Yes	Ligature	Cholangitis	Roux-en-Y	None	Excellent	None
7	F	45	No	Ligature	Pain, fever, liver abscess	Roux-en-Y	Roux-en-Y	Good	None
8	F	48	No	Ligature	Pain, fever	Roux-en-Y	None	Excellent	None
9	F	60	No	Ligature	Temp pain	No	None	Good	None
10	F	28	No	None	Biliary peritonitis	Lavage, drainage	None	Closure	None
11	F	24	No	None	Biliary peritonitis	Lavage, drainage	Roux-en-Y	Excellent	None
12	F	65	No	None	Biliary peritonitis	Lavage, drainage	None	Closure	Hernia
13	F	30	No	None	Biliary peritonitis	Lavage, drainage	None	Closure	Ileus
14	F	21	No	None	Ext. fistula	No	None	Closure	None
15	F	36	No	None	Ext. fistula	No	None	Closure	None
16	F	60	No	None	Ext. fistula	No	Roux-en-Y	Excellent	None
17	M	60	No	None	Ext. fistula	No	Roux-en-Y	Good	None
18	F	63	No	None	Ext. fistula	Roux-en-Y	None	Excellent	None
19	M	73	No	None	Ext. fistula	Roux-en-Y	None	Excellent	None

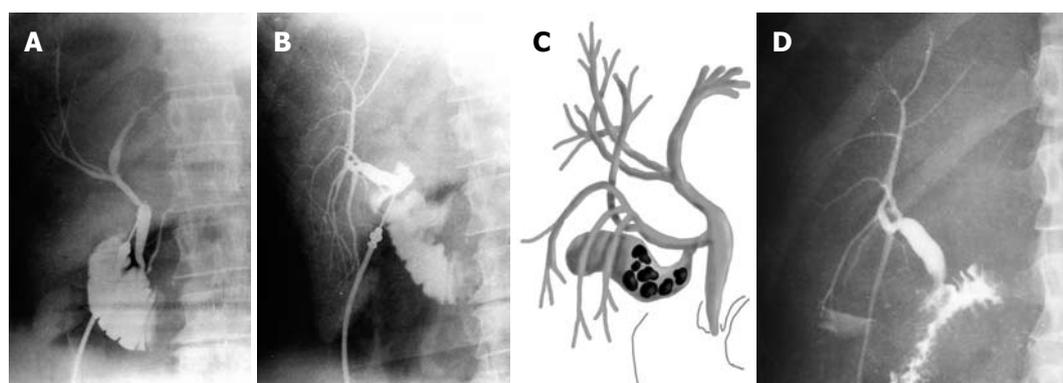


Figure 4 Cholangiography. A: An operative cholangiography with missing ducts within segments V and VIII; B: Cholangiography of the injured anterior sectoral duct; C: A schematic of the biliary anatomy before the injury took place (the anterior sectoral duct was misinterpreted as a cystic duct); D: Following hepatico-jejunostomy.

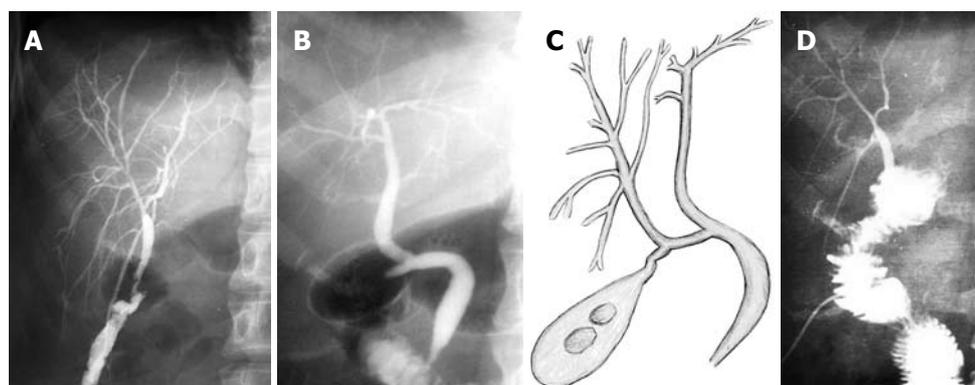


Figure 5 Demonstrates a case with an external biliary fistula that required Roux-en-Y repair. A: Fistulography showing the right hepatic duct; B: ERCP showing the rest of the biliary tree; C: A schematic of the biliary anatomy before the injury took place (the right hepatic duct was misinterpreted as a cystic duct); D: Following hepatico-jejunostomy.

segmental, sectoral and right hepatic bile duct injuries is universally accepted. Some authors believe that due to the uncertain outcomes and potentially harmful complications of reconstructive surgery, simple ligation

of injured ducts is the treatment of choice. This is felt to be the case irrespective of the size of the injured duct^[2], as unobstructed drainage of up to 50% of an otherwise normal liver, through either the right or left

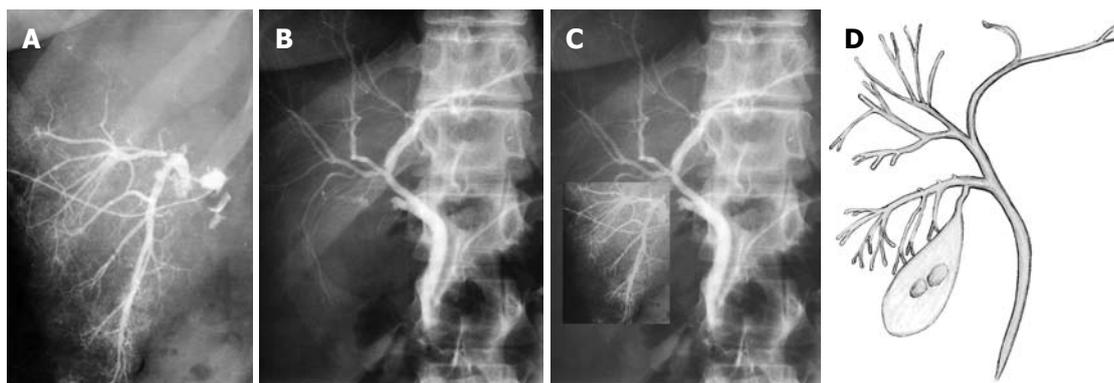


Figure 6 A biliary fistula that resolved spontaneously. A: Fistulography showing the ducts within segment V; B: ERCP showing the rest of the biliary tree; C: The entire biliary tree if the injury hadn't take place; D: A schematic of the biliary anatomy prior to injury.

unaffected ducts, is adequate to restore normal liver function; even with the obstructed lobe remaining in situ^[3,4]. The non-draining lobe of the liver would then undergo progressive asymptomatic atrophy, and not require further treatment; so long as it was completely obstructed and free from infection, and that there was normal biliary flow from the residual 50% of the liver^[3-5]. Although the incidence of “free communication” between the two main hepatic ductal systems above the hilum has been reported in up to 50% of patients^[6], there is no evidence that the bile from an obstructed ductal system drains through the other unobstructed ducts. Other authors believe that wherever possible isolated segmental, sectoral and right hepatic bile duct injuries should be immediately repaired if recognized, in order to restore normal anatomy and function^[7-9].

We favour a primary repair whenever an injury is recognized at original surgery. An end-to-end anastomosis over a tiny T-tube inserted in the common bile duct can be performed if the duct is of a reasonable size (at least 4 mm). There must be no loss of duct tissue, ductal tears or thermal injury, and the repair must be technically perfect using interrupted 5/0 slow-absorbable sutures. We believe that the end-to-end anastomosis is not indicated in injuries close to the common bile duct as any eventual stricture of the anastomosis may cause a stricture of the main bile duct. A Roux-en-Y hepaticojejunostomy can be performed in other situations and where the remaining duct is large enough to accommodate sutures.

Patients with isolated segmental, sectoral and right hepatic bile duct injury have also been successfully treated by nonsurgical methods such as endoscopic and/or percutaneous drainage and stenting^[10]. In some rare cases with high hepatic injuries segmental liver resection has even been performed in order to avoid long-term transhepatic stenting and its complications such as cholangitis and late stricture formation^[11].

Although surgeons have different approaches to the treatment of isolated segmental, sectoral and right hepatic bile duct injury recognized during or soon after the original surgery, there is no major disagreement in the treatment of cases with infection, biliary peritonitis and biliary fistula. Infection is treated stepwise, initially

with antibiotics and percutaneous biliary drainage where possible, and if required with a Roux-en-Y repair^[5] or even with hepatic resection of the infected parenchyma and ducts^[3]. Biliary peritonitis requires drainage and lavage, previously by laparotomy and now more commonly by laparoscopy, in order to prevent abdominal abscesses^[12]. Biliary fistulae are treated conservatively. If they fail to resolve spontaneously then a biliary repair with a Roux-en-Y may be necessary^[13].

Thus in conclusion, a primary repair, where possible, is a reasonable solution for isolated segmental, sectoral and right hepatic bile duct injury recognized during the original surgery; the larger the duct the greater need for primary repair. A patient with a ligated duct may develop an infection, and so should be closely followed up for several weeks. Infected ducts require further surgery and usually a Roux-en-Y repair. In the past, patients with biliary peritonitis were treated with open laparotomy, drainage and lavage of the abdominal cavity. Nowadays, lavage and drainage can be successfully carried out laparoscopically or even percutaneously. Biliary fistulae should be followed for several weeks as there is a 50% chance of spontaneous closure. In cases where the biliary fistula does not close within 6 to 8 wk, the patient should probably undergo a Roux-en-Y anastomosis without further delay.

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REVIEW

Individually administered or co-prescribed thiopurines and mesalamines for inflammatory bowel disease

Giovanni C Actis, Rinaldo Pellicano, Mario Rizzetto, Muhammad Ayoubi, Nicola Leone, Gianfranco Tappero, Paola Paziienza, Floriano Rosina

Giovanni C Actis, Muhammad Ayoubi, Nicola Leone, Gianfranco Tappero, Paola Paziienza, Floriano Rosina, Division of Gastro-Hepatology, Ospedale Gradenigo, Torino 10153, Italy

Rinaldo Pellicano, Mario Rizzetto, Division of Gastroenterology, Ospedale Molinette, Torino 10153, Italy

Author contributions: Actis GC identified the topics, and structured and drafted the text; Pellicano R chose and double-checked the references; Ayoubi M, Leone N and Tappero G were the clinicians in charge and supervised download of data; Paziienza P checked the accuracy of the basic science paragraphs; Rizzetto M and Rosina F were the chiefs of department, and read and approved the text.

Correspondence to: Giovanni C Actis, Division of Gastro-Hepatology, Ospedale Gradenigo, Corso Regina 10, Torino 10153, Italy. actis_g@libero.it

Telephone: +39-11-8151462

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has continued to inform indications and refine the prescriptions of mesalamines and thiopurines; these have not been restrained (they have been implemented in some cases) by the advent of the novel biological molecules with anti-cytokine activity.

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Peer reviewer: Emiko Mizoguchi, MD, PhD, Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States

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Abstract

Data from both basic research and clinical experience continue to suggest that mesalamines and thiopurines are effective and efficient for the maintenance of remission of inflammatory bowel diseases. Several decades following the formalization of their indications, attention on these two drugs has been fostered by recent achievements. Demonstration of the ability of mesalamine to activate a colonocyte differentiation factor has shed light on its chemopreventive effects on colorectal cancer; in addition to their anti-proliferative efficacy, thiopurines have been shown to be specific regulators of apoptosis. The two drugs are often co-administered in clinical practice. Recent advancements have shown that mesalamines exert a positive synergism in this context, insofar as they can inhibit side-methylation of thiopurines and hasten the function of the main immunosuppressive pathways. Considering that up to 40% of patients cannot tolerate thiopurines, such renovated targets have stimulated efforts to improve compliance by research on the toxicity mechanisms. The definition of genetic polymorphisms in the enzymes of thiopurine metabolism, and the uncovering of synergistic drug interactions, such as that with allopurinol, are just two of the results of such efforts. Interaction between basic research and clinical practice

INTRODUCTION

Maintenance of remission of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) is a crucial target for at least two reasons: unchecked bowel inflammation behaves as an independent factor in colon cancer development^[1], and the inflammatory pathways do mutate in relapsing inflammatory bouts, thus favoring development of drug unresponsiveness and activation of apoptosis-resistant non-professional immunocytes^[2]. Two classes of drugs form the traditional arsenal for IBD remission maintenance: mesalamines and thiopurines, and the remainder of this review emphasizes that they are far from being set aside. Mesalamines and thiopurines share a few facts in common: they have both been studied in the second half of the last century for indications that were eventually changed; they were studied by two female scientists, one of them was awarded the Nobel prize; they are both effective and cheap; and they both continue to remain under the limelight as novel pharmacological actions are being uncovered beyond those already known. Mesalamines and thiopurines are

often co-prescribed, and among other topics, we shall review the pros and cons of this association.

MESALAMINES

Brief history

The ancestor of mesalamine is a compound named salazopyrin (SASP) made by a sulfamidic moiety linked by an azo bond to 5-aminosalicylic acid (5-ASA). Such combined anti-bacterial and anti-inflammatory actions came to the attention of the Swedish rheumatologist Nanna Svartz, who believed that the joint lesions in her patients may have been caused by latent infections and inflammation. In the early 1940s, while giving SASP to her rheumatic patients, to take advantage of its double actions, she noticed an improvement in IBD in co-morbid subjects. Her observations were reported in 1942^[3] and were to be confirmed in a controlled fashion 20 years later^[4]. The abandonment of the infectious theory in rheumatoid arthritis and the acknowledgement that the significant allergic toxicity of SASP was mainly caused by its sulfa moiety led to the development of 5-ASAs compounds in the following decades, which are known as mesalazine in Europe and as mesalamine in the US.

Pharmacology, metabolism, and mechanism of action of 5-ASA

Upon ingestion, 5-ASA is partially oxidized in the stomach, absorbed in the proximal gut, and acetylated in the liver. Although it is not as efficiently absorbed in the terminal gut, the efficiency of the above described processes causes a significant portion of 5-ASA to be transferred to the bloodstream, thus posing the need for an efficient carrier to effect drug delivery to distal areas of disease. Diverse ways to address the need for the distal delivery of 5-ASA have been pursued in the last decades: use of a pH-dependent carrier that releases the active drug distal to the ileo-cecal valve, an ethyl-cellulose capsule to release 5-ASA evenly in the digestive lumen, or, the synthesis of dimers in which the azo bond is supposed to be broken by the colonic flora^[5]. *In vitro*, 5-ASA has been shown to share several pharmacological properties with non-steroidal anti-inflammatory compounds, including: inhibition of nuclear factor (NF)- κ B-dependent inflammatory pathways^[6]; limitation of the oxidative stress in epithelial cells^[7,8]; increase in the cellular heat-shock protein response^[9]; inhibition of leukotriene production^[10]; and modulation of prostaglandin metabolism^[11].

Indications

The indications for mesalamines do differ between UC and CD. At least one study^[12] has claimed that SASP is superior to placebo in the treatment of active left-sided CD. On the contrary, the results are mixed as to the indication for remission maintenance; a recent review^[13] has recommended that prescription of mesalamines for the maintenance of CD be avoided. Regarding the management of UC, there are different data. In one large study^[14], mesalamine doses ranging between 1 and

Table 1 Adverse effects of mesalamine and thiopurines in a cohort of IBD patients^[18]

Event	Number	Percentage (%)
Mesalamine (n = 44)		
Pulmonary dysfunction	3	6.8
Pancreatitis	1	2.2
Hemolytic anemia	1	2.2
Intolerance to local drug vehicle	1	2.2
Platelet reduction	1	2.2
Diarrhea	1	2.2
Total	8	17.8
Thiopurines (n = 57)		
Leukopenia	10	17.5
Hepatic damage	5	8.77
Infection	4	7.0
Pancreatitis	4	7.0
Idiosyncrasy	2	3.50
Nausea	1	1.75
Malignancy	1	1.75
Total	27	47

4 g/d have been shown to induce remission in 30% of patients, as compared with 12% remission achieved by placebo. A recent Cochrane^[15] analysis has shown that all of the FDA-approved formulations can offer a 30% therapeutic gain over placebo.

Toxicity

Although they are prescribed worldwide, SASP/mesalamines can exert occasionally complex toxicity that targets the skin, kidneys, pancreas, liver and cardiovascular system. As a result of the sulfa moiety, SASP can target the skin more often with manifestations ranging from rashes to major Stevens-Johnson lesions. In contrast, the phenacetin-like structure confers on mesalamine the ability to effect necrosis of the renal papilla, thus explaining the concern for clinically meaningful renal toxicity. In an English survey^[16], the frequency of 5-ASA-related interstitial nephritis was 11.1 cases per million prescriptions, with the figure being 7.5 for pancreatitis. The lung manifestations linked with mesalamine deserve particular attention^[17], being probably based on allergic mechanisms shown to occasionally cause a range of lung damage from eosinophilic pneumonitis to bronchiolitis obliterans. In our series^[18], an unexpected percentage of 6.8% patients out of 44 that received mesalamine showed respiratory distress or pleuro-pneumonitis (Table 1).

THIOPURINES

History

Belonging to the class of fraudulent nucleotides, the thiopurines are expected to exert a prevalent anti-proliferative and immunosuppressive effect by interfering with DNA replication and causing strand breakage. The thiopurines received a lot of attention in the 1950s and 60s from Gertrud Elion and George Hitchings, who aimed at exploiting their anti-proliferative actions for the treatment of pediatric malignancy. Some of the shrewd

perceptions of Elion, including the attempt to hasten the antineoplastic effects by adding the synergic drug allopurinol (see below), were already contained in her manuscripts of the 1960s^[19], and such work led to her award of the Nobel prize in 1988^[20]. In the following years, the oncological indications for thiopurines were challenged by the release of more potent antineoplastic drugs; However, an anecdotal claim at the beginning of the 1960s^[21] opened the era of their use for IBD, an era that it still far from being concluded.

Metabolism and mechanism of action

6-mercaptopurine (6-MP), derived in the liver after non-enzymatic, glutathione-dependent, elaboration of the pro-drug azathioprine (AZA) opens the thiopurine metabolic cascade, which leads to the final synthesis of the immunosuppressive 6-thioguanine metabolites (6-TGNs). While AZA is mostly prescribed in Europe, 6-MP is preferred in the US. At the initial pathway, two enzymatic systems compete for the common substrate 6-MP: thiopurine methyltransferase (TPMT) catalyzes the formation of the methylated non-immunosuppressive compounds of methyl-mercaptopurine (MMP); and hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) leads to the synthesis of 6-thio-inosine-monophosphate. This metabolite may either enter a phosphorylation loop regulated by a pyrophosphatase (ITPase), or it may undergo elaboration by a dehydrogenase to form 6-TGN, which finally exerts their specific DNA strand breakage effect by incorporation into the DNA replication pathways (Figure 1). A few typical features of the toxicity of thiopurines can be accounted for by at least three points of genetic/biochemical variability, as contained in the described metabolic pathway. Also, such points have recently become the target for finely tuned interventions that are aimed at modulating the patterns of the metabolic cascade, and at restraining the clinical meaningfulness of the relevant thiopurine toxicity^[22]. The three points include: the genetic polymorphism of TPMT; the possibility to inhibit the enzyme xanthine oxidase by allopurinol (Elion's initial idea); and the ITPase polymorphisms. The following paragraph on toxicity gives more insight into this matter.

Indications

The immunomodulatory action of thiopurines is characterized by a delayed onset that may take 3 mo. This feature, caused by some fine aspects of their immunomodulatory mechanisms as described below, has traditionally made the thiopurines specific remission maintenance drugs. A pivotal controlled study in 1980 has shown that 6-MP is superior to placebo for fistula closure and steroid sparing in CD^[23]. Drug withdrawal experiments have shown a significant trend to relapse, with > 50% of the withdrawal patients relapsing at the third year of follow-up^[24-26]. The efficacy of AZA in the maintenance of UC has been suggested by a similar withdrawal experiment conducted in 1992: out of 79 patients in remission on AZA and randomized to either continue or withdraw treatment, 36% of the former

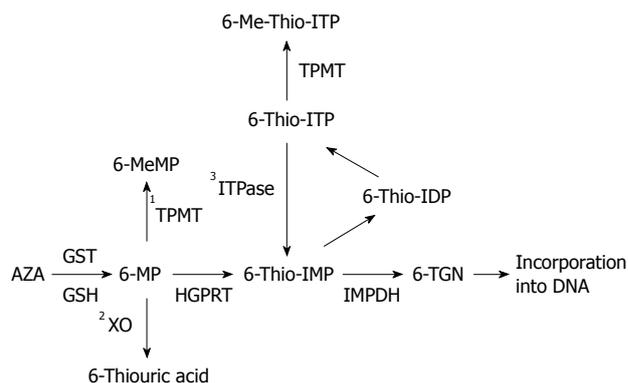


Figure 1 Thiopurine metabolism. For a caption to this figure please refer to metabolism and toxicity paragraphs and ref. 22. ¹TPMT is polymorphic and can be inhibited by mesalamine; ²Xanthine oxidase can be inhibited by allopurinol; ³ITPase is polymorphic.

relapsed during follow-up, but as many as 59% of the latter relapsed^[27]. Several multicenter and monocentric studies, including one from our group^[28], have confirmed the efficacy of thiopurines in the maintenance of remission and avoidance of colectomy for UC. In particular, we were able to show that AZA is effective in the maintenance of fastidious forms of UC that are initially responsive to cyclosporin^[29].

Toxicity

Owing to the high degree of genetic polymorphism that affects a few of the key enzymes in their metabolic pathway, thiopurines tend to exert varied toxicity. A recent relevant review^[30] includes the following: leukopenia 1.3%-12.6%; infection 0.3%-7.4%; liver damage 0%-4.2%; pancreatic damage 0%-4%; gastric intolerance 1.3%-6%; idiosyncratic reactions 0%-3.9%; and drug withdrawal 5.7%-22%. These figures have been upgraded in a recent review, which estimates that up to 40% of the subjects assigned to thiopurine treatment cannot benefit from these drugs because of the adverse effects^[31], and do not differ from our monocentric experience^[18] in which, of 57 IBD patients receiving thiopurines, 25 (43%) experienced adverse effects including leukopenia (17.5%), hepatic damage (8.7%), and infection (7.0%) (Table 1). Attempts at understanding and preventing leukopenia and hepatic and pancreatic events have brought about a significant increase in our basic knowledge. Examination of the thiopurine pathway suggests that leukopenia may chiefly be caused by dysfunction of the side methylator enzyme TPMT, which allows HGPRT to form excess levels of the bone-marrow-toxic 6-TGNs. Indeed, studies of TPMT activity have found it to be uneven, and the gene that encodes for TPMT has been shown to have a degree of polymorphism, as follows. Determination of erythrocyte TPMT activity has led to identification of three subsets of subjects: 90% show high activity; 10% show intermediate activity; and 0.3% are almost devoid of TPMT. The latter (1:300 in the Caucasian population) have a significant risk of developing fatal leukopenia if exposed to thiopurines. TPMT is encoded for by a highly polymorphic 27-kb long gene that is localized to chromosome 6p22.3. Seventeen

variant alleles have so far been described: three of these (TPMT*2, TPMT*3A, TPMT*3C) can cause the synthesis of an unstable protein, caused by an altered tertiary structure, and have been causally linked with 80%-95% of all cases of null TPMT activity. Such information in the last two decades has fostered the search for a screening test and has led to the standardization and release of commercial kits that are allegedly able to identify homozygous subjects at risk for developing fatal leukopenia. Such TPMT studies have provided the paradigm of the neonatal pharmacogenomic studies and have shown the extent of the clinical impact of these studies. If a hypoactive TPMT can cause leukopenia, recent studies^[32] have suggested that hyperactivity of the enzyme may cause accumulation of an excess of methylated by-products and liver damage that may affect up to 10% of the so-called hypermethylator subjects. This problem has been addressed^[31] by the co-prescription of allopurinol. By inhibiting xanthine oxidase and the production of the inactive thiouric acid, a 30% lower AZA dose can be administered, thus reducing the substrate for the hyperactive TPMT. Idiosyncratic pancreatitis has been claimed to respond to this strategy. In addition, idiosyncratic response to thiopurine, as shown by fever and flu-like syndromes, has been shown to be avoided by allopurinol. In these cases, probably the accumulation of ill-defined methylated toxins in the area of a polymorphic ITPase is the culprit. Although promising, research in the area of ITPase polymorphisms is still in its infancy.

CO-PRESCRIBED MESALAMINES AND THIOPURINES

Frequency in clinical practice

Data from a recent survey conducted by us and other four Italian centers has revealed a frequency of co-prescription of 71%.

Evidence of synergism between the two drugs

This comes essentially from withdrawal data in clinical practice. In two independent reports, others and ourselves have shown that patients on AZA and mesalamine in remission from their UC may relapse severely and progress to eventual colectomy if mesalamine is withdrawn; a consistent fall in the blood concentration of 6-TGNs is found in these circumstances^[33,34]. In addition, the above cited review has shown that regular mesalamine therapy behaves as the only independent factor of continuous remission before AZA withdrawal.

Mechanisms underlying the synergy

As illustrated above (Figure 1), the purine metabolic pathway unfolds primarily following a process by which the pro-drug AZA is finally converted to the metabolites of 6-TGN. The immunosuppressive power of this biochemical machinery depends on the accumulation of the latter compounds, insofar as they are able to decrease the number of dividing lymphocytes by DNA strand breakage. The immunosuppressive steps of

the pathway may be influenced by diversion of the metabolites towards two side-streams at the beginning of the process: one, catalyzed by TPMT, leads to the production of MMP, and the other, catalyzed by xanthine oxidase, produces thiouric acid, both of which are devoid of immunosuppressive activity. Of the two, TPMT has resulted in the most protean system, being under the influence of both genetic polymorphism and drugs. 5-ASA compounds have shown strong affinity for TPMT^[35], with a significant inhibitory activity that results in increased feeding of metabolites towards the main axis, which results in a boost to the immunosuppressive power of the pathway. Mesalazine, sulphasalazine and olsalazine have all been shown to influence TPMT activity, with the effects emerging at both the biochemical and clinical levels, as detailed below.

Results from clinical reports

An increase of the effectiveness of AZA in relation to administration of mesalamine, and a concomitant increase in 6-TGN concentration, with attendant leukopenia, have been described in several studies^[36-39]. Two other studies, on the contrary, have failed to find an advantage from co-prescription, whereas increased toxicity that has hastened the need to discontinue AZA has been emphasized^[40,41]. The overall available evidence speaks in favor of co-prescribing AZA and 5-ASAs. Together with allopurinol, the mesalamines seem to offer an effective strategy to optimize AZA administration in hypermethylator patients. Whether this readily translates into improved clinical outcomes remains debatable. A recent systematic review^[42], although not providing a definitive answer, has concluded that co-administration of thiopurines and mesalamines can lead to a decreased risk for colorectal cancer in long-standing disease.

REAPPRAISAL OF THE INDICATIONS FOR MESALAMINES: CHEMOPREVENTION

Background

Both CD and UC are known as pre-cancerous lesions, with the risk for transformation becoming significant within 8 years of the diagnosis of UC, and attaining 7%-14% at 25 years^[43]. Two orders of evidence achieved in the last decades have focused attention on mesalamine as a chemopreventive agent against UC-dependent colorectal cancer. On one hand, a pivotal paper^[1] has shown that unchecked inflammation acts independently in the promotion of cancer through dysplasia; and on the other hand, modern technology has provided evidence that mesalamine can exert a specific anti-neoplastic action thanks to its ability to interfere with both prostaglandins and nuclear transcription factors for the pro-inflammatory cytokines. The next question was whether mesalamine can protect against colon cancer *in vivo*. A literature search has retrieved at least three retrospective studies of correlation offering relevant answers: a 3-mo course of SASP significantly reduced

the cancer risk in a population of 3000 patients with colitis; in two subsets of colitis patients, of whom, only one received therapeutic doses of 5-ASA, the eventual percentage of cancer development was 3% *vs* 31%, respectively; and in the final study of 102 patients, the drug-induced risk reduction in cancer was 75%-90%^[44].

Mechanisms

Peroxisome proliferator activated receptor gamma (PPAR- γ). PPAR- γ is a nuclear receptor that belongs to a family of at least 50 members that are involved with an array of biological functions. Once located to the nucleus and heterodimerized with retinoid X receptor alpha, PPAR- γ begins to regulate four gene classes. Such a gene complex is known to direct four major biological functions: metabolism, proliferation, signal transduction, and cell motility. PPAR- γ is maximally expressed in the gut, with a gradient increasing from the proximal to the distal bowel. A local negative gradient of expression has been shown in the colon, with the lowest expression levels found in the distal colon; microscopically, the expression is high among the proliferating cells of basal crypts and progressively fades away from bottom to top, to almost indistinguishable levels in cells that detach from the crypt apex and fall free in the lumen. This clearly depicts PPAR- γ as a potent differentiation factor that exerts its pivotal role in an environment where a dramatic proliferative drift completely renews the epithelium every 3 d.

These effects favor the candidacy of PPAR- γ to be identified among the effective antineoplastic agents in the colon. The insulin-sensitizing drugs, the glitazones, which bind PPAR- γ show anticancerous activity in animal models. Hemizygous knock-out animals for PPAR- γ show lesser resistance to carcinogenic treatments. At this point, the final crucial question is whether mesalamines can bind PPAR- γ , and two lines of evidence have contributed to the answer: (1) colitic animals that are heterozygous for PPAR- γ respond least to 5-ASA, which implies a role for PPAR- γ in the mediation of the antineoplastic effect of 5-ASA; and (2) 5-ASA can be accommodated into a loop in the structure of PPAR- γ through hydrogen bonding. Taken together, the above indicate that 5-ASA can exert a chemopreventive action against UC-related colorectal cancer, and this action is mediated through anti-inflammatory activity, and depends on its binding to a potent differentiation factor of colonocytes^[45,46].

REAPPRAISAL OF THE POTENTIAL OF THIOPURINES: IMMUNOMODULATION

IBD patient caregivers have long become familiar with one of the hallmarks of the action of thiopurines: a latency of effect that may last for 3 mo. Traditionally, this delay has been attributed to the time supposed to be required for the 6-TGN metabolites to saturate myeloid precursors and exert their anti-proliferative effects, a tenet that has recently been challenged on the basis of two lines of evidence^[47,48]: (1) the use of an intravenous load

of AZA has not significantly reduced the latency; and (2) the process of myeloid cell saturation has been shown to be completed within 2 wk. The results of further studies prompted by these doubts have contributed to uncover that, well beyond their known anti-proliferative capacity, the thiopurines may exert a more finely tuned immunoregulatory action that is largely independent from DNA strand breakage and immune cell death. Focus on this novel aspect of their action is maintained by two recent publications.

Already back in 2003^[49], the Neurath group had shown that thiopurines can induce T-cell apoptosis in controls as well as in IBD patients, by replacing GTP as a ligand for the Rac-1 receptor, thus hindering its main function of inducing NF- κ B. This process is CD-28-dependent and triggers a mitochondrial pathway to apoptosis. This study was the first to demonstrate that a product of the intermediate thiopurine metabolism (6-thioguanine triphosphate, as generated from the phosphorylation loop described above) cannot simply break the native DNA, but specifically triggers apoptosis directed towards the autoimmune clones at the root of IBD perpetuation. This provides a fine immunological indication for the use of thiopurines, and hints at a disease-modifying role that is still to be studied.

The Ben-Horin group in Israel has recently developed research on this apoptosis process further, and has concentrated on timing and mechanistic issues^[50]. Using a double *in vitro* and experimental approach in animals, they have gathered evidence that thiopurines effect a proliferative arrest of T lymphocytes, without any apoptotic effect being obvious until the fifth day post-stimulation. During this latency, these lingering T lymphocytes are still capable of adherence and mediating inflammation; thus, the immunological events lying behind the clinical latency of thiopurines have been uncovered. In their second set of experiments, this time conducted *in vivo*, they have shown that the animals must be exposed for > 1 mo to mercaptopurine before it restricts the memory pool to antigenic re-challenge. These data depict thiopurines as fine immunomodulators that require several weeks to express full-blown activity. Far from being speculative laboratory exercises, these approaches serve (1) to remind the clinician of the underlying reasons for the specific indication for thiopurines, as maintenance drugs; and (2) to remind doctors to reiterate to their patients the need for maximum compliance in order to maximally exploit the drug and benefit from the longest disease-free period.

CONCLUSION

Despite the time elapsed since their initial study, mesalamines and thiopurines continue to remain under investigation as research from basic immunology fosters novel clinical approaches, as shown by a few publications that have appeared even while preparing this review. Mesalamine has recently been proposed as the first candidate drug to be endowed with a disease-modifying role in UC^[51]. Another review^[52] has raised the question of

the timing of the introduction of AZA for IBD, and has proposed to use it earlier in a top-down strategy in order to best exploit its effects, chiefly the mucosal-healing potential. Finally, mesalamines and thiopurines, together with cyclosporin, are still considered unbeaten in terms of cost-effectiveness, when compared with the most recent drugs made by genetic engineering, and have been awarded the status of “backbone therapy” for IBD^[53].

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Acute pancreatitis: Etiology and common pathogenesis

Guo-Jun Wang, Chun-Fang Gao, Dong Wei, Cun Wang, Si-Qin Ding

Guo-Jun Wang, Chun-Fang Gao, Dong Wei, Cun Wang, Si-Qin Ding, Institute of Digestive Surgery and Department of General Surgery, 150 Hospital of Chinese PLA, Gaoxin District, Luoyang 471031, Henan Province, China

Author contributions: All the authors contributed equally to this review.

Correspondence to: Chun-Fang Gao, MD, PhD, Institute of Digestive Surgery and Department of General Surgery, 150 Hospital of Chinese PLA, Gaoxin District, Luoyang 471031, Henan Province, China. cchunfang@163.com

Telephone: +86-379-64169001 Fax: +86-379-64169112

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Peer reviewer: Jia-Yu Xu, Professor, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, China

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Abstract

Acute pancreatitis is an inflammatory disease of the pancreas. The etiology and pathogenesis of acute pancreatitis have been intensively investigated for centuries worldwide. Many causes of acute pancreatitis have been discovered, but the pathogenetic theories are controversial. The most common cause of acute pancreatitis is gallstone impacting the distal common bile-pancreatic duct. The majority of investigators accept that the main factors for acute biliary pancreatitis are pancreatic hyperstimulation and bile-pancreatic duct obstruction which increase pancreatic duct pressure and active trypsin reflux. Acute pancreatitis occurs when intracellular protective mechanisms to prevent trypsinogen activation or reduce trypsin activity are overwhelmed. However, little is known about the other acute pancreatitis. We hypothesize that acute biliary pancreatitis and other causes of acute pancreatitis possess a common pathogenesis. Pancreatic hyperstimulation and pancreatic duct obstruction increase pancreatic duct pressure, active trypsin reflux, and subsequent unregulated activation of trypsin within pancreatic acinar cells. Enzyme activation within the pancreas leads to auto-digestion of the gland and local inflammation. Once the hypothesis is confirmed, traditional therapeutic strategies against acute pancreatitis may be improved. Decompression of pancreatic duct pressure should be advocated in the treatment of acute pancreatitis which may greatly improve its outcome.

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Key words: Acute pancreatitis; Pathogenesis;

INTRODUCTION

Acute pancreatitis, an inflammatory disease of the pancreas, is mild and resolves itself without serious complications in 80% of patients, but it has complications and a substantial mortality in up to 20% of patients^[1]. Its etiology and pathogenesis have been intensively investigated for centuries worldwide^[2]. In 1856, Claude Bernard suggested that bile reflux into the common pancreatic duct could trigger acute pancreatitis^[3]. Several subsequent studies led to theories fuelling the debate until 1901^[4], when Eugene Opie proposed that gallstone migration into the common bile duct is the main cause of acute pancreatitis^[5]. Since then, many other causes of pancreatitis have been discovered^[6]. However, the pathogenesis of acute pancreatitis is still controversial to date. Several theories attempt to explain the pathogenesis of acute pancreatitis. In terms of disease pathogenesis, whether acute pancreatitis is really one entity or it comprises a group of distinct pathogenic entities remains unclear. From a pathogenic perspective, acute pancreatitis is an identity crisis^[7].

CAUSES OF ACUTE PANCREATITIS

There are many causes of acute pancreatitis, which can be easily identified in 75%-85% of patients. In developed countries, obstruction of the common bile duct by stones (38%) and alcohol abuse (36%) are the most frequent causes of acute pancreatitis^[3,8]. Gallstone-induced pancreatitis is caused by duct obstruction by gallstone migration. Obstruction is localized in the bile duct and pancreatic duct, or both. Duct obstruction promotes pancreatitis by increasing duct pressure and subsequent unregulated activation of digestive

enzymes^[9]. Alcohol abuse is the second most frequent cause of acute pancreatitis, but the correlation between alcohol and pancreatitis is not completely understood^[10]. In experimental models, Gorelick showed that ethanol directly sensitizes acinar cells to cholecystokinin stimulation. As the development of pancreatitis might be affected by both genetic and environmental factors, failure to inhibit trypsin activity or to wash active trypsin into pancreatic ducts might promote alcoholic pancreatitis^[11]. In fact, the exact mechanism underlying alcoholic acute pancreatitis has not been extensively elucidated.

Pancreas divisum, a common congenital anatomical variant of the pancreatic duct in about 7% of autopsy series, results from the absence of fusion between the dorsal and ventral ductal systems. The possible consequence of pancreas divisum is a stenosed or inadequately patent minor papilla, preventing normal drainage of pancreatic secretions and leading to increased intraductal pressure. However, whether pancreas divisum is related to pancreatitis is highly controversial^[12]. Whether dysfunction of sphincter of Oddi can trigger acute pancreatitis by increasing intrapancreatic duct pressure is also controversial^[13]. Biliary sludge refers to a viscous bile suspension that contains cholesterol crystals and calcium bilirubinate granules embedded in strands of gallbladder mucus. Sludge is associated with bile stasis, long-lasting fast, distal bile duct obstruction, and total parenteral feeding. Most patients with biliary sludge are asymptomatic. Biliary sludge is commonly seen in patients with recurrent acute pancreatitis of unknown origin, and cholecystectomy might prevent the recurrence of pancreatic disease^[14].

Intraduct papillary mucinous tumor might be another cause of acute pancreatitis. Tumor or mucus produced by it obstructs the main pancreatic duct and its side branch, or both. Logically, the consequence is increased pancreatic duct pressure caused by pancreatic hyperstimulation and pancreatic duct obstruction. Thus, these tumors might trigger acute pancreatitis through the same mechanisms underlying acute biliary pancreatitis^[15].

Endoscopic retrograde cholangiopancreatography (ERCP) is a potential cause of acute pancreatitis. Asymptomatic hyperamylasaemia occurs in 35%-70% of patients after the procedure. ERCP has a higher risk of inducing acute pancreatitis when it is performed to treat Oddi sphincter dysfunction than to remove gallstones in the bile duct. Other risk factors for post-ERCP pancreatitis include young age, female sex, number of attempts to cannulate papilla, and poor emptying of pancreatic duct after opacification. Prevention of post-ERCP pancreatitis in high-risk patients might be achieved by placing a temporary pancreatic stent^[16].

Hypercalcaemia is another rare and inconsistent cause of acute pancreatitis. Because the incidence of pancreatitis is low in patients with chronic hypercalcaemia, additional factors are probably needed to induce pancreatitis^[17]. Drugs rarely induce acute pancreatitis. Cases of drug-induced pancreatitis have been reported^[18]. Many infectious agents are associated

with acute pancreatitis, but no microorganism has ever been identified within the pancreas. However, it was reported that acute pancreatitis is associated with viral or bacterial infections, and infestation with parasites^[19]. Although a few researchers speculated that unexplained recurrent acute pancreatitis might be associated with some known genetic mutations, no decisive and persuasive evidence supports the notion^[20].

In summary, many causes of acute pancreatitis have been discovered. The main causes are gallstone migration and alcohol abuse. Other causes are uncommon, situational, or controversial. Although there are many theories about the pathogenesis of acute pancreatitis, they are still controversial. These causes have not yet been completely elucidated.

MULTIPLE AND CONTROVERSIAL PATHOGENETIC THEORIES

For centuries, the pathogenesis of acute pancreatitis has been intensively investigated worldwide^[2]. Many theories have been proposed attempting to explain the pathogenetic mechanisms underlying acute pancreatitis^[21]. The important theories about the pathogenesis of acute pancreatitis include bile-pancreatic duct common pathway theory, pancreatic autodigestion theory, gallstone migration theory, enzyme activation theory, kinin and complement system activation theory, microcirculation disturbance theory, leukocyte excessive activation theory, pancreatic acinar cell apoptosis and necrosis theory, all of which are still controversial^[22]. They can only explain the pathogenesis of some specific pancreatitis cases, or specific aspects of pathogenetic process of some forms of acute pancreatitis. In fact, no ideal theories on the pathogenesis of acute pancreatitis are available at present.

Although 70%-80% of acute pancreatitis cases are due to alcohol abuse and gallstones, the exact mechanisms by which they initiate acute pancreatitis are unknown. In addition, because of its rapid course and the relative inaccessibility of pancreatic tissue for examination during pancreatitis, investigations of the mechanisms underlying these pathobiologic processes have been hampered. Considering these obstacles, investigators have turned to animal models of acute pancreatitis to reveal the molecular steps initiating these pathobiologic responses to identify potential targets for therapeutic intervention^[23-25]. Although the exact mechanisms underlying acute pancreatitis caused by alcohol and gallstones in humans have not been established, key steps in mediating the pathobiologic processes that define acute pancreatitis can be identified from animal models, and used to develop therapies that can be ultimately tested in human pancreatitis^[26].

Since early in the twentieth century, a good many of experimental studies based on animal models have been carried out^[27]. Many research results support that bile reflux and pancreatic auto-digestion by trypsin are central to the pathogenesis of gallstone pancreatitis^[28]. A century later, the following questions still remain to be

answered: whether it is rational to examine the possibility that gallstone pancreatitis develops without reflux of bile into the pancreatic duct, whether trypsinogen activation is an effect rather than the cause of pancreatitis, whether active trypsin is essential for the development of acute pancreatitis or whether it is merely a secondary factor that exacerbates pancreatitis. It has been shown that bile reflux is not a necessary factor for acute pancreatitis^[29]. In opossum, merely ligation of the pancreatic duct can cause necrotizing acute pancreatitis^[30], but in rats or in rabbits, this causes apoptosis and atrophy of pancreas^[31], suggesting that further study is needed to elucidate the pathogenesis of acute pancreatitis in order to explain the paradoxical experimental results with different animals.

Considering various causes of acute pancreatitis, the question of whether each cause of acute pancreatitis corresponds to specific pathogenesis or various causes of acute pancreatitis actually possess a common pathogenesis should be answered. We hypothesize that, irrespective of the etiology of acute pancreatitis, there is a common pathway that triggers various forms of acute pancreatitis.

COMMON PATHOGENESIS AND VARIOUS CAUSES OF ACUTE PANCREATITIS

The etiology and pathogenesis of acute pancreatitis have been intensively investigated^[2], but the pathogenetic theories are controversial. The predominant theories of acute biliary pancreatitis are common pathway theory and gallstone migration theory, which consent that the key factor for acute biliary pancreatitis is bile-pancreatic duct obstruction, which increases pancreatic duct pressure, bile reflux, trypsin activation and pancreatic auto-digestion^[32]. Acute pancreatitis occurs when intracellular protective mechanisms to prevent trypsinogen activation or reduce trypsin activity are overwhelmed^[33]. However, these theories are controversial.

Although pancreatic duct obstruction may play an important role in the pathogenesis of gallstone pancreatitis, it is not sufficient to cause the morphological changes of acute pancreatitis^[34], indicating that other events must occur if the changes induced by pancreatic duct obstruction lead to acute pancreatitis. Although acinar hyperstimulation has often been implicated in acute pancreatitis pathogenesis, there is no evidence that supports it^[35]. We hypothesize that pancreatic acinar hyperstimulation, in the presence of duct obstruction, triggers and exacerbate acute pancreatitis.

We speculate that the main preconditions that trigger acute biliary pancreatitis are pancreatic hyperstimulation and bile-pancreatic duct obstruction, which increase pancreatic duct pressure, active trypsin reflux, and unregulated activation of trypsin within pancreatic acinar cells. Enzyme activation within the pancreas leads to auto-digestion of the gland and local inflammation. However, little is known about the other causes of acute pancreatitis.

We hypothesize that there is a common pathogenic pathway that triggers various forms of acute pancreatitis: acute biliary pancreatitis and other forms of acute pancreatitis. In our hypothesis, there are various causes which may cause acute pancreatitis and lead to pancreatic duct obstruction and blockage of pancreatic juice outflow under certain circumstances. In the presence of exocrine pancreatic hyperstimulation, pancreatic duct pressure, active trypsin reflux, and unregulated activation of trypsin within pancreatic acinar cells would increase. When intracellular protective mechanisms to prevent trypsinogen activation or reduce trypsin activity are overwhelmed, acute pancreatitis occurs.

CONCLUSION

Acute pancreatitis has been intensively studied for centuries. Many causes of acute pancreatitis have been discovered, but its pathogenetic theories are multiple and controversial. The true nature of acute pancreatitis still remains to be elucidated. The causes of acute pancreatitis are various, and its mechanism is common. Once the hypothesis is confirmed, traditional therapeutic strategies against acute pancreatitis may be improved, and decompression of pancreatic duct pressure should be advocated in the treatment of acute pancreatitis which may greatly improve the outcome of acute pancreatitis^[36,37].

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Transforming growth factor- β 1 induces intestinal myofibroblast differentiation and modulates their migration

Julia Brenmoehl, Sandra Nicole Miller, Claudia Hofmann, Daniela Vogl, Werner Falk, Jürgen Schölmerich, Gerhard Rogler

Julia Brenmoehl, Sandra Nicole Miller, Claudia Hofmann, Daniela Vogl, Werner Falk, Jürgen Schölmerich, Gerhard Rogler, Department of Internal Medicine I, University of Regensburg, 93055 Regensburg, Germany

Julia Brenmoehl, Department of Internal Medicine II, University of Jena, 07747 Jena, Germany

Gerhard Rogler, Clinic of Gastroenterology and Hepatology, Department of Internal Medicine, University Hospital of Zurich, 8091 Zurich, Switzerland

Author contributions: Brenmoehl J performed the quantitative mRNA analyses (Taqman-PCR) and western blotting of fibronectin and the fibronectin isoforms, and wrote the manuscript; Miller SN performed the migration assays, western blotting and staining of α -smooth muscle actin, and wrote the manuscript; Hofmann C performed the western blotting of phosphorylated and total focal adhesion kinase and wrote the manuscript; Vogl D contributed to biopsies collection, data collection and isolation of colonic lamina propria fibroblasts; Falk W contributed to data interpretation and to writing the manuscript; Schölmerich J contributed to the overall interpretation of data and writing of the manuscript; Rogler G planned and co-ordinated the study and wrote the manuscript.

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Correspondence to: Julia Brenmoehl, PhD, Department of Internal Medicine II, University of Jena, Jena 07747, Germany. julia.brenmoehl@med.uni-jena.de

Telephone: +49-3641-9324227 Fax: +49-3641-9325827

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Abstract

AIM: To investigate the effects of transforming growth factor β 1 (TGF- β 1) on the differentiation of colonic lamina propria fibroblasts (CLPF) into myofibroblasts *in vitro*.

METHODS: Primary CLPF cultures were incubated with TGF- β 1 and analyzed for production of α -smooth muscle actin (α -SMA), fibronectin (FN) and FN isoforms. Migration assays were performed in a modified 48-well Boyden chamber. Levels of total and phosphorylated focal adhesion kinase (FAK) in CLPF were analyzed after induction of migration.

RESULTS: Incubation of CLPF with TGF- β 1 for 2 d

did not change α -SMA levels, while TGF- β 1 treatment for 6 d significantly increased α -SMA production. Short term incubation (6 h) with TGF- β 1 enhanced CLPF migration, while long term treatment (6 d) of CLPF with TGF- β 1 reduced migration to 15%-37% compared to untreated cells. FN and FN isoform mRNA expression were increased after short term incubation with TGF- β 1 (2 d) in contrast to long term incubation with TGF- β 1 for 6 d. After induction of migration, TGF- β 1-preincubated CLPF showed higher amounts of FN and its isoforms and lower levels of total and phosphorylated FAK than untreated cells.

CONCLUSION: Long term incubation of CLPF with TGF- β 1 induced differentiation into myofibroblasts with enhanced α -SMA, reduced migratory potential and FAK phosphorylation, and increased FN production. In contrast, short term contact (6 h) of fibroblasts with TGF- β 1 induced a dose-dependent increase of cell migration and FAK phosphorylation without induction of α -SMA production.

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Key words: Transforming growth factor β 1; Colonic fibroblasts; Myofibroblasts; Migration; Fibronectin

Peer reviewer: Zsuzsa Szondy, Professor, Department of Biochemistry and Molecular Biol, University of Debrecen, Debrecen H-4012, Hungary

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INTRODUCTION

Transforming growth factor- β (TGF- β) is involved in multiple fundamental biological processes such as cell proliferation^[1-3], cell migration^[4-8], cell differentiation^[9], extracellular matrix deposition^[6,7,10,11], and immune responses^[12]. TGF- β is secreted by a variety of cells

including platelets, monocytes, macrophages, and lymphocytes. It is thought to play a critical role in embryogenesis, host response to tumors, and the repair response that follows damage to tissues by immune and nonimmune reactions^[8].

TGF- β not only affects remodeling during normal wound healing after tissue injury, but also enhances fibrogenesis. Therefore, it is an important mediator in multiple fibrotic diseases, including pulmonary fibrosis, liver fibrosis, chronic pancreatitis^[12], and in stricture formation in Crohn's disease (CD)^[13]. Increased expression of TGF- β , especially of the isoform TGF- β 1, has been demonstrated in fibrotic tissues and regions of increased extracellular matrix deposition^[12]. TGF- β changes the balance between extracellular matrix synthesis and degradation, inducing an increase in the synthesis of matrix components and a parallel decrease in the matrix-proteolytic activity^[11,12].

TGF- β is synthesized as a pro-form which can be activated by multiple mechanisms. After activation of latent TGF- β and binding to its receptors, the activated receptors phosphorylate and assemble cytoplasmic Smad proteins. Subsequently, Smad complexes move to the nucleus as transcriptional regulators^[12,14].

TGF- β is also a potent chemoattractant for human dermal fibroblasts. Intact disulfide bonds and perhaps the dimeric structure of TGF- β are essential for its ability to stimulate migration of fibroblasts, since reduction of TGF- β results in a marked loss of its chemoattractant potency^[8].

Fibroblast migration plays an important role in tissue formation and wound healing. Following injury, tissue repair takes place involving inflammation, new tissue formation and scar constitution^[15]. Migration of fibroblasts into and through the extracellular matrix during the initial phase of wound healing appears to be a fundamental component of wound contraction. In recent studies, we found that colonic lamina propria fibroblasts (CLPF) conditioned media induce migration of primary human CLPF in the modified Boyden chamber^[16]. Furthermore, we demonstrated that fibronectin (FN) was mainly responsible for the autocrine induction of CLPF migration and was an essential requirement for the induction of CLPF migration, since different growth factors enhanced CLPF migration only in the presence of conditioned medium or recombinant FN^[16]. Reduced and enhanced migratory potential of CLPF correlated with decreased and increased amounts of FN or FN isoforms, respectively^[17]. FNs occur in up to 20 different isoforms as a result of alternative splicing of the primary transcript in the two homologous type III domains named ED-A and ED-B and the one non-homologous repeat termed III CS^[18-22]. The ED-A and ED-B segments are either entirely included or excluded. The type III repeat may be included, excluded, or partially included in FN^[23-25].

The alternatively spliced FN isoforms show distinct functional differences. The expression of FN containing ED-A and ED-B domains is significantly increased during physiological wound healing and pathological

tissue fibrosis^[26]. Changes in the migratory potential of CLPF from patients with CD were associated with changes in FN isoform level, while the expression of integrin α 5 β 1, the main FN receptor on the surface of CLPF, was unchanged^[17].

The differentiation or activation of fibroblasts into myofibroblasts is an important step in tissue repair. Transient appearance of myofibroblasts is a feature of normal wound healing, but the persistence of these activated cells is associated with excessive collagen deposition and fibrosis^[9]. Their prolonged presence and over-representation are hallmarks in the pathophysiology of tissue fibrosis^[27]. TGF- β 1 potently stimulates the production of α -smooth muscle actin (α -SMA) and stress fiber formation in fibroblasts and therefore their differentiation into myofibroblasts^[9].

Since the regulation of migration and differentiation of intestinal fibroblasts is an important mechanism during intestinal wound healing and fibrosis, the effect of TGF- β 1 on these processes and on FN and FN isoform production was investigated in this study.

MATERIALS AND METHODS

Patients

Primary CLPF cultures were obtained from endoscopic biopsies or surgical specimens taken from healthy areas of the mucosa of patients undergoing surveillance colonoscopy or surgery for colorectal carcinoma. The study was approved by the Ethics Committee of the University of Regensburg.

Isolation and culture of human colonic fibroblasts

Human CLPF were isolated and cultured as described earlier^[28]. Briefly, mucosa from surgical specimens was cut into 1 mm pieces while the biopsies were used directly for the isolation of CLPF. Epithelial cells were removed in Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+} (PAA, Cölbe, Germany) with 2 mmol/L EDTA (SIGMA, Deisenhofen, Germany). The remaining tissue was rinsed and then digested for 30 min at 37°C with 1 mg/mL collagenase 1 (SIGMA), 0.3 mg/mL DNase I (Boehringer, Mannheim, Germany) and 2 mg/mL hyaluronidase (SIGMA) in PBS (Gibco, Karlsruhe, Germany). The isolated cells were cultured in 25 cm² culture flasks (Costar, Bodenheim, Germany) with DMEM containing 10% FCS, penicillin (100 IE/mL), streptomycin (100 μ g/mL), ciprofloxacin (8 μ g/mL), gentamycin (50 μ g/mL) and amphotericin B (1 μ g/mL). Non-adherent cells were removed by subsequent changes of medium. The remaining cells were used between passage 3 and 8.

Incubation of CLPF with TGF- β 1

CLPF were incubated for two days or six days with 0, 0.1, 1, 10 and 50 ng/mL of TGF- β 1 (Biozol, Germany) in serum-free medium. The culture medium of 6 d treated cells was changed after 3 d and new TGF- β 1 was added for a further three days. Subsequently, cells were used for investigation of α -SMA production, cell

migration, quantitative mRNA analysis, FN, and FAK production.

Wounding assay: Induction of CLPF migration with platelet derived growth factor-AB

CLPF were incubated for 6 d with or without 10 ng/mL of TGF- β 1 (Biozol, Germany) in serum-free medium. The medium was changed after three days and new TGF- β 1 was added for a further three days. The cells cultured in 5 cm dishes were wounded with a comb, washed and incubated for a further 4 h with different concentrations of platelet derived growth factor (PDGF)-AB (R&D Systems, Minneapolis, MN, USA; 0, 5, 10, and 20 ng/mL). Cells were harvested and used for quantitative analysis of FN and FN splicing form mRNA and for Western blot analysis of FN and FN-splicing form protein as well as levels of total and phosphorylated FAK.

Immunocytochemical staining

For immunocytochemical staining of α -SMA, 20 000 cells were seeded onto LabTek[®] Chamber slides (Nunc, Wiesbaden, Germany) and fixed with ice-cold acetone. Immunocytochemical stainings were performed according to the manufacturer's APAAP protocol (Dako, Hamburg, Germany). The anti α -SMA antibody (1:25, clone 1A4, Dako) was used to investigate the differentiation of CLPF into myofibroblasts. IgG2a (MOPC-141, Sigma) was used as an isotype control.

Western blotting

Western blot analysis was performed as described earlier^[17,29]. Protein detection was carried out with a specific antibody to α -SMA (clone 1A4, Dako, Hamburg, Germany) and incubation with peroxidase-conjugated secondary antibody. Protein bands were visualized using a commercial chemoluminescence detection kit (ECL Plus; Amersham) according to the manufacturer's protocol. Loading was checked by Ponceau S staining.

For FN and FAK protein immunoblots specific antibodies to FN (1:1000; 35041A, Pharmingen), FN ED-A (1:400; clone IST-9, Abcam), FN ED-B (1:200; PhiloMab-2, Philogen), FAK (1:666; Chemicon), phosphoFAK (1:1000; Biosource) and β -actin (1:10000; Chemicon) were used. After washing of the membranes horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA) were added and incubated with chemiluminescent substrate (ECL Plus; Amersham Biosciences, Arlington Heights, IL) for 5 min before a film (Amersham Biosciences) was exposed.

After exposure, the antibody was stripped from the membrane with Re-Blot Plus-Strong (Chemicon, Hofheim, Germany) for 15 min and the blot was blocked again with 5% milk in 0.1% Tween 20-TBS before using it for the next antibody.

Migration assays

Migration assays were performed in a modified 48-well Boyden chamber as described earlier^[28]: A polycarbonate filter (8 μ m pore size, polyvinylpyrrolidone-free,

Gerbu Biotechnik, Gaiberg, Germany) divided the chamber into an upper and a lower compartment. Each test substance was placed in the wells of the lower compartment in replicates of three. Conditioned media with or without PDGF-AB, IGF-I (R&D Systems), EGF (Biosource, Germany) or TGF- β 1 (Biozol) were tested. DMEM high glucose medium with or without 1% bovine serum albumin (BSA, SIGMA) served as negative controls. A total of 20 000 CLPF/well in DMEM high glucose medium with 1% BSA were seeded into the wells of the upper compartment of the Boyden chamber. The Boyden chamber was incubated at 37°C in 10% CO₂ atmosphere for 6 h. The filter was removed from the chamber and the non-migrated cells on the upper side of the filter were scraped off with a rubber policeman. The migrated cells on the lower side of the filter were fixed and stained with Hemacolor staining kit (Merck, Darmstadt, Germany) and counted in 4 microscopic high power fields of view (hpf) at a 400-fold magnification. Unless otherwise indicated, each experiment was repeated at least three times ($n = 3$).

Conditioned media

The culture medium was removed from a confluent CLPF monolayer. The cells were washed twice with PBS and subsequently cultured with DMEM lacking FCS for 24 h. The conditioned medium was centrifuged to remove all cell debris and stored at -20°C for no more than 3 mo.

RNA isolation and cDNA synthesis

For the isolation of total RNA stimulated and unstimulated primary human CLPF cultures were washed twice with PBS. CLPF were scraped off, centrifuged for 5 min, and resuspended with lysis buffer from the RNeasy[®] kit (Qiagen, Hilden, Germany). Total RNA was prepared from these CLPF according to the manufacturer's protocol and stored at -80°C. The isolated RNA was reverse transcribed using the Promega Reverse Transcription System (Promega, Madison, WI, USA).

Quantitative mRNA analysis by real-time PCR

Amounts of FN, ED-A and ED-B mRNA were quantified by real-time PCR as previously described^[17].

Released reporter dye fluorescence during 40 cycles of amplification was monitored using Sequence Detector software (SDS version 2.0, PE Applied Biosystems). Reporter dye fluorescence versus PCR cycles was plotted. A threshold was set in the exponential phase of the fluorescence curves. The threshold cycle numbers (Ct) were calculated.

Ct values of GAPDH were subtracted from those of FN isoforms: $dCt = Ct(\text{FN isoforms}) - Ct(\text{GAPDH})$. The mean value of dCt values was calculated. The values of cDNA from stimulated CLPF were subtracted from those of untreated cDNA of the same CLPF: $ddCt = dCt(\text{stimulated}) - dCt(\text{unstimulated})$. The relative start

amount of cDNA was calculated in consideration of the exponential amplification: $x = 2^{-ddCt}$.

Statistical analysis

All data are given as mean \pm SE. The Student's *t*-test was used for analysis of parametric data, while the Mann-Whitney Rank Sum Test was used for evaluation of non-parametric data. Real time PCR data were pictured as boxplots. $P < 0.05$ were considered to be statistically significant.

RESULTS

Effect of TGF- β 1 on α -SMA production of CLPF

To investigate the effect of TGF- β 1 on α -SMA production of CLPF, CLPF were incubated with different concentrations of TGF- β 1 in serum-free medium. Subsequently, APAAP-staining of α -SMA or its isotype control IgG2 κ was performed. Since there was no remarkable change in α -SMA production after treatment for one day or two days with 0.1, 1, and 10 ng/mL TGF- β 1 (data not shown), we compared α -SMA production of CLPF incubated for two days (Figure 1A) or six days (Figure 1B) with 0, 10 and 50 ng/mL TGF- β 1. A time- and dose-dependent increase of α -SMA was observed: short-term treatment with 50 ng/mL TGF- β 1 resulted in enhanced α -SMA production, however, after long-term incubation α -SMA was detected in almost all cells.

In order to confirm these results, Western blot analysis was performed (Figure 2A). CLPF treated for two days or six days with 10 ng/mL or 50 ng/mL TGF- β 1 were lysed and levels of α -SMA were detected. In each lane 30 μ g/mL cell lysate protein were loaded and the loading was checked by Ponceau S staining (Figure 2B). Western blot data confirmed the induction of α -SMA production by long-term incubation of CLPF with TGF- β 1 in contrast to short-term incubation, where no α -SMA was detected.

Effect of TGF- β 1 on the migration of CLPF

Since TGF- β 1 potently stimulated the production of α -SMA in CLPF and as a consequence their differentiation into myofibroblasts, we investigated the effect of long term TGF- β 1 incubation on the migration of CLPF. Because 10 ng/mL TGF- β 1 induced similar α -SMA levels as 50 ng/mL TGF- β 1 (Figure 2), we treated CLPF for six days with 10 ng/mL TGF- β 1 and performed migration assays in the presence of PDGF-AB, IGF- I, EGF and TGF- β 1 diluted in 24 h CLPF-conditioned medium in a modified Boyden chamber (Figure 3). The Boyden chamber was incubated at 37°C in 10% CO₂ atmosphere for 6 h. We reported earlier that growth factors like PDGF-AB, IGF- I, EGF and TGF- β 1 diluted in conditioned media enhance CLPF migration^[28]. However, pre-incubation of CLPF with TGF- β 1 for six days significantly decreased CLPF migration. This decreased migratory potential of myofibroblast-like CLPF could not be re-enhanced by addition of growth factors (Figure 3).

Migration assays with different dilutions of PDGF-AB in conditioned medium showed that short-term incubation (6 h) in the modified Boyden chamber enhanced migration of TGF- β 1-untreated CLPF in a dose-dependent manner (Figure 3A). Addition of 5 ng/mL PDGF-AB to CLPF conditioned medium increased migration up to 60% (64 ± 5 cells/hpf) when compared to conditioned medium without additional PDGF-AB (control, 44 ± 4 cells/hpf). Addition of 20 ng/mL PDGF-AB to CLPF conditioned medium induced a more than two-fold migration rate (121 ± 19 cells/hpf) when compared to control. Pre-incubation of CLPF for six days with 10 ng/mL TGF- β 1 reduced migration to 37% (16 ± 2 cells/hpf) when compared to untreated CLPF (44 ± 4 cells/hpf) and could not be re-enhanced by PDGF-AB ($P < 0.0001$; Figure 3A).

Similar results were obtained in migration assays using different concentrations of IGF- I, EGF or TGF- β 1 in conditioned medium (Figure 3B-D). Addition of the growth factors to the conditioned medium enhanced the migration of TGF- β 1-untreated CLPF, although the effect was not as strong as with PDGF-AB. Again, pre-incubation of CLPF for six days with TGF- β 1 led to a reduced migratory potential of the cells and could not be re-enhanced by the growth factors IGF- I (Figure 3B: 100 ng/mL, $P < 0.005$; 200 ng/mL, $P < 0.05$; 300 ng/mL, $P < 0.0001$), EGF (Figure 3C: 10 ng/mL, $P < 0.05$) or TGF- β 1 (Figure 3D: 10 pg/mL, $P < 0.005$; 20 pg/mL, $P < 0.0001$; 30 pg/mL, $P < 0.05$).

Effect of TGF- β 1 on the production of FN and FN isoforms

It is known that TGF- β 1 promotes the insertion of the ED-A domain in FN^[30,31] and we have recently shown that changes in the migratory potential of CLPF were correlated with changes in FN isoform production^[17]. For this reason, the effect of TGF- β 1 on splicing of FN mRNA in CLPF was assessed. CLPF were incubated with different TGF- β 1 concentrations for two days or six days and the amounts of FN, FN ED-A-, and FN ED-B were determined. Therefore the cells were treated with 10 ng/mL and 50 ng/mL TGF- β 1 and untreated cells were used as controls. For the comparison of mRNA expression quantified by real time PCR untreated controls were set as 1. The results of 7 independent experiments were evaluated statistically (Figure 4).

After two days incubation with 10 ng/mL TGF- β 1 total FN mRNA expression was increased 20-fold and 25-fold with 50 ng/mL (both $P < 0.005$, paired *t*-test). When CLPF were incubated for six days, FN mRNA was increased 6-fold with 10 ng/mL TGF- β 1 and 10-fold with 50 ng/mL TGF- β 1 compared to control (Figure 4A).

mRNA amounts of FN ED-A and FN ED-B were increased 20-fold ($P < 0.05$) and 13-fold ($P < 0.005$), respectively, with 10 ng/mL TGF- β 1 after two days, while after six days 5-fold (FN ED-A, $P < 0.05$) or 3-fold (FN ED-B, $P < 0.05$) increased mRNA levels compared to controls were found (Figure 4B and C). Treatment with 50 ng/mL TGF- β 1 for 2 d caused a 23-fold ($P < 0.01$) increase of FN ED-A mRNA which

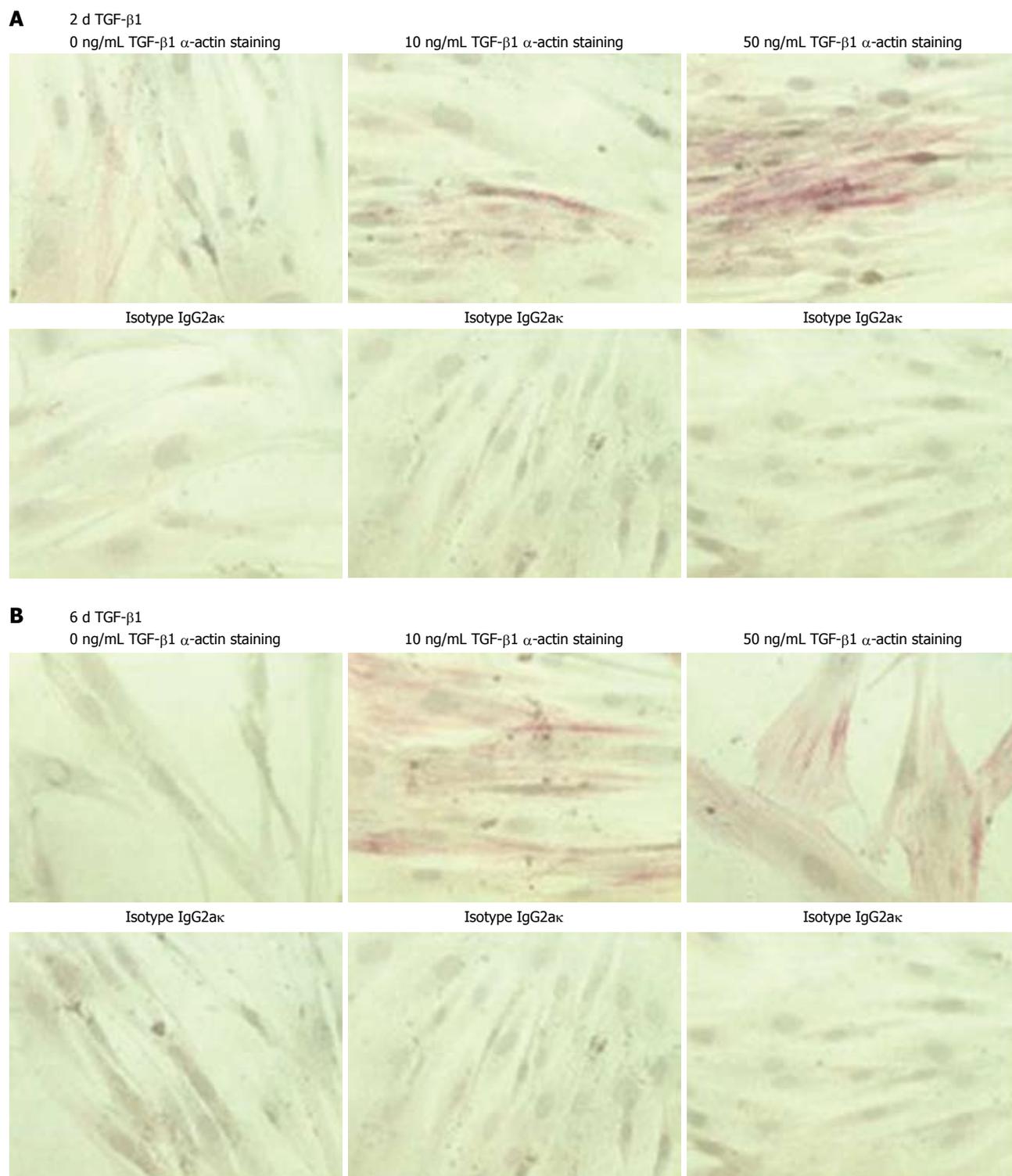


Figure 1 Effect of TGF- β 1 on α -SMA production in CLPF. CLPF were incubated for 2 d (A) or 6 d (B) with 0, 10 and 50 ng/mL TGF- β 1. Subsequently, APAAP-staining of α -SMA (α -actin) or its isotype control IgG2a was performed. Incubation of CLPF for 6 d with 10 ng/mL or 50 ng/mL TGF- β 1 showed the greatest effect on α -SMA, since almost all cells showed a red staining for α -SMA.

was reduced to 6-fold after a further four days. mRNA amounts of FN ED-B was 15-fold ($P < 0.01$) after two days and reduced to 4-fold ($P < 0.05$) after six days (Figure 4B and C).

Together these data indicate that TGF- β 1 causes a strong increase in mRNA expression of FN and migration inducing FN isoforms. However, this effect was not maintained but was clearly reduced after six

days. Values obtained after six days of incubation were not statistically different to unstimulated controls despite the presence of TGF- β 1. On average, FN mRNA amounts were 70% lower after pre-incubation with 10 ng/mL TGF- β 1 for six days than after pre-incubation for two days ($P < 0.05$), while FN ED-A and FN ED-B ($P < 0.05$) mRNA levels were reduced by 80%. Similar data were obtained for incubation with 50 ng/mL

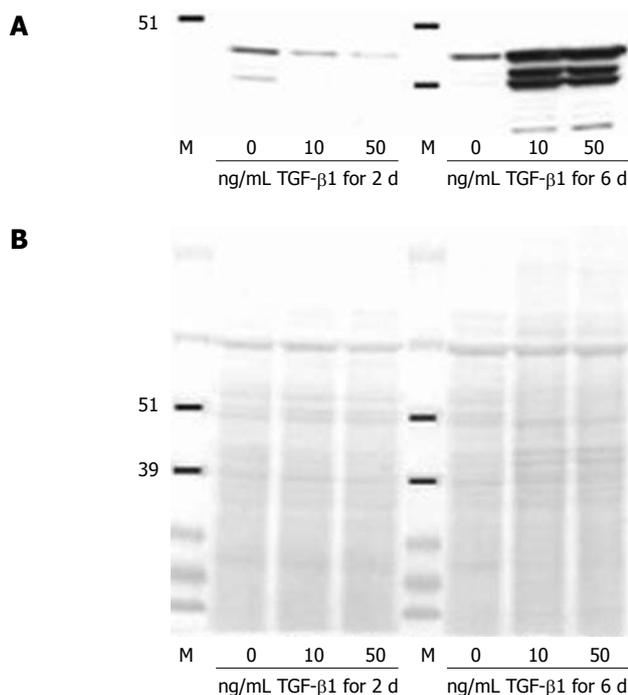


Figure 2 Effect of TGF- β 1 on α -SMA protein production. Control CLPF were incubated for 2 d or 6 d with 0, 10, and 50 ng/mL TGF- β 1 in serum-free medium. Subsequently the cells were washed and lysed. Amounts of α -SMA were analyzed by Western blotting (A). Equal loading was confirmed by Ponceau S staining (B). Incubation of CLPF for 6 d with 10 ng/mL or 50 ng/mL TGF- β 1 markedly enhanced α -SMA production.

TGF- β 1 for six days *versus* stimulation for two days (total FN 55% lower ($P < 0.05$) after six days compared to two days, FN ED-A and FN ED-B 75% lower ($P = 0.05$).

In addition, TGF- β 1-mediated induction of FN and FN splice variant production was confirmed by Western blot analyses. A time- and dose-dependent increase of total FN, FN ED-A and FN ED-B was detected after TGF- β 1 treatment (Figure 5). In contrast to the findings for mRNA expression after six days TGF treatment higher levels of FN, ED-A and ED-B protein compared to two days were observed. In the absence of TGF- β 1, levels of FN and FN isoforms were low and did not differ between two- and six- day cultures. A concentration of 0.1 ng/mL TGF- β 1 did not show a significant effect while 1 ng/mL TGF- β 1 induced an increase in FN and FN ED-A production after two days and six days compared to untreated cells. After two days FN ED-B protein was only found after incubation with TGF- β 1 concentrations of 10 ng/mL and 50 ng/mL, while this isoform could be detected after six days with 1, 10, and 50 ng/mL TGF- β 1. The most prominent effect on FN, FN ED-A, and FN ED-B production was observed after six days incubation with 50 ng/mL TGF- β 1.

Effects of TGF- β 1 and PDGF-AB on FN and FN isoforms in a wounding model

Because of the observed reduced migratory potential of CLPF incubated long-term with TGF- β 1 but increased protein levels of migration inducing factor FN and FN splice variants, we analyzed migrating cells in a wounding

assay in more detail. CLPF were pre-incubated with and without 10 ng/mL TGF- β 1 in serum free medium (six days). The CLPF monolayer was wounded with a comb, migration was induced by incubation with different concentrations of PDGF-AB (0, 5, 10, 20 ng/mL) in conditioned medium for 4 further hours and mRNA amounts of FN isoforms were assessed (Figure 6).

While CLPF without TGF- β 1 pre-treatment displayed a slight PDGF-dependent increase in the expression of FN, FN ED-A, and a slight decrease in FN ED-B mRNA in this wounding assay, TGF- β 1 pre-treated cells displayed a stronger enhancement of FN, FN ED-A, and FN ED-B mRNA-expression.

After stimulation with 5, 10, and 20 ng/mL PDGF-AB in conditioned medium FN mRNA-expression was 4.5- (Paired *t*-test, $P < 0.05$), 4- ($P < 0.01$), and 4.5-fold ($P < 0.05$) increased in TGF- β 1 pre-treated and 2-, 3.5-, and 1.5-fold enhanced in TGF- β 1 untreated CLPF as compared to the control, respectively (Figure 6A). PDGF-AB stimulation of pre-incubated CLPF led to 3- (5 ng/mL PDGF, $P < 0.005$), 2.5- (10 ng/mL PDGF, $P < 0.01$), and 4-fold increases (20 ng/mL PDGF, $P < 0.05$) in FN ED-A mRNA expression, whereas TGF- β 1 unstimulated cells showed a 2- ($P < 0.05$), 3.5-, and 1.5-fold enhancement, respectively (Figure 6B). PDGF caused a 3- (5 ng/mL, $P < 0.01$), 2.5- (10 ng/mL, $P < 0.01$), and 3-fold increase (20 ng/mL, $P < 0.01$) in FN ED-B mRNA in TGF- β 1-treated CLPF and a 1.5-, 2.5- (5 and 10 ng/mL, $P < 0.01$), and 1.5-fold (20 ng/mL) enhancement in TGF- β 1 untreated cells (Figure 6C).

In summary, pre-treatment of CLPF with TGF- β 1 (six days) and subsequent treatment with PDGF-AB (4 h) after wounding causes a much stronger induction of FN mRNA expression than without TGF- β 1 pre-treatment.

After TGF- β 1-incubation, wounding, and incubation without PDGF-AB mRNA levels of total FN, FN ED-A, and FN ED-B increased significantly by 4-fold when compared to TGF- β 1- and PDGF-AB-untreated controls ($P < 0.05$ for FN and FN ED-A; FN ED-B $P < 0.005$). With 5 ng/mL PDGF-AB mRNA amounts of FN splice forms doubled by TGF- β 1-stimulation [FN ED-A ($P < 0.05$)]. After TGF- β 1 pre-stimulation expression of FN and FN ED-B rose by 20% compared to untreated cells, while FN ED-A expression was even decreased by 30%. With 10 ng/mL TGF- β 1 and 20 ng/mL PDGF-AB, after cell wounding total FN expression showed a 3-fold ($P < 0.05$), FN ED-A expression a 2.5-fold ($P = 0.06$), and FN ED-B expression a 2-fold ($P < 0.005$) increase.

Western blot analyses of protein production confirmed these findings on mRNA expression (Figure 7). Protein amounts of FN, FN ED-A, and FN ED-B were strongly increased by TGF- β 1 incubation. In comparison to TGF- β 1 untreated controls the levels of FNs were enhanced after wounding and further enhanced by addition of PDGF-AB in conditioned medium. No significant differences were observed between protein amounts in TGF- β 1 pre- and not incubated CLPF by PDGF-treatment, respectively. FN

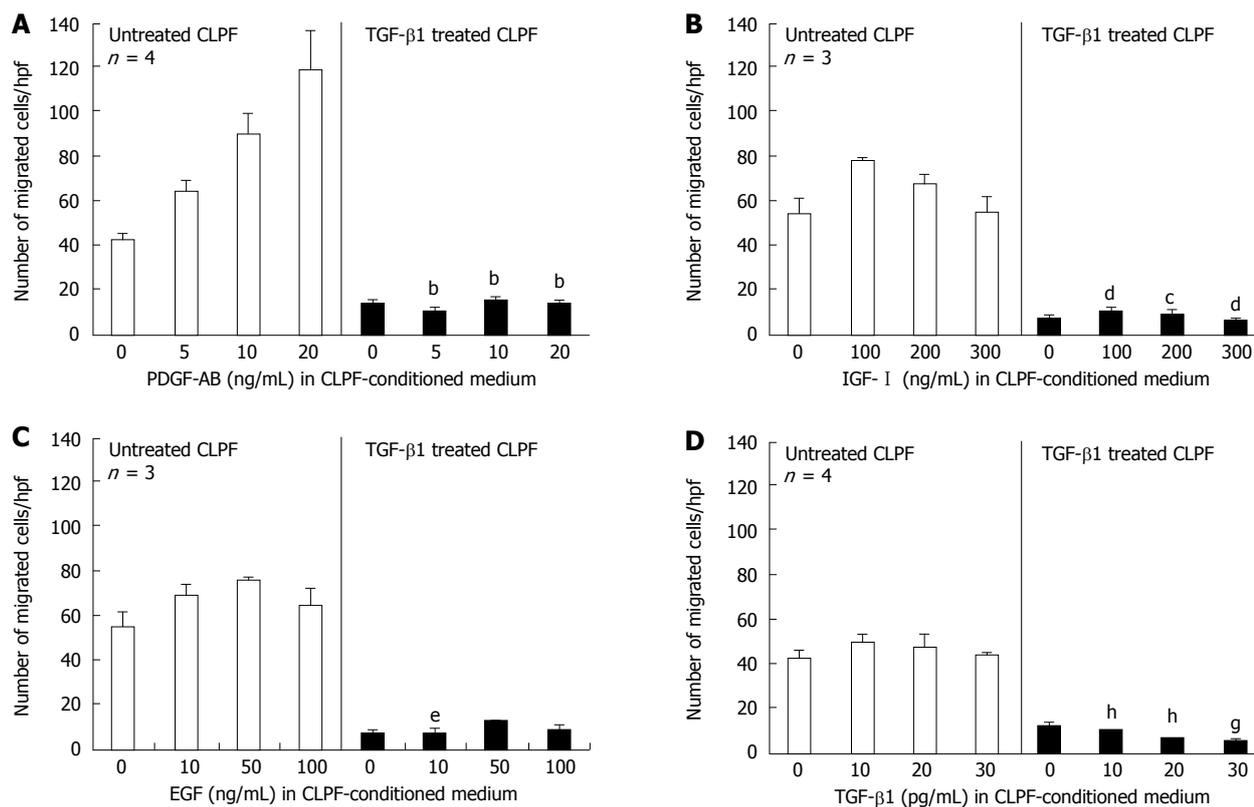


Figure 3 Effect of TGF- β 1 on growth factor-induced migration of CLPF. Control-CLPF were treated for 6 d with 10 ng/mL TGF- β 1 and subsequently migration assays in the presence of PDGF-AB, IGF- I , EGF and TGF- β 1 diluted in 24 h CLPF-conditioned medium were performed in the modified Boyden chamber. TGF β 1 treatment of CLPF significantly decreased the migratory potential, which could not be re-enhanced by cell migration-inducing growth factors. Statistics: The Mann-Whitney Rank Sum Test was used for the evaluation of the non-parametric data. A: ^b $P < 0.0001$ vs unstimulated CLPF; B: ^c $P < 0.05$, ^d $P < 0.005$ vs unstimulated CLPF; C: ^e $P < 0.05$ vs unstimulated CLPF; D: ^g $P < 0.05$, ^h $P < 0.01$ vs unstimulated CLPF.

ED-B protein levels of CLPF without pre-treatment were barely detectable.

Effects of TGF- β 1 on FAK phosphorylation in a wounding model

We have demonstrated in previous studies that enhanced cell migration of CLPF was associated with higher FAK phosphorylation and total FAK protein production^[29]. Therefore, we determined TGF- β 1-modulated FAK production and phosphorylation by Western blot (Figure 8) in CLPF that were induced to migrate by wounding and stimulation with PDGF-AB in conditioned medium. Pre-incubation of CLPF with 10 ng/mL TGF- β 1 for six days decreased FAK phosphorylation as compared to untreated controls. In addition, levels of FAK protein were reduced by TGF- β 1 stimulation. CLPF that were not pre-treated with TGF- β 1 contained enhanced levels of FAK with a constant higher FAK phosphorylation that was highest with an addition of 5 ng/mL PDGF-AB when compared to CLPF not treated with TGF- β 1.

DISCUSSION

In this study we demonstrate that TGF- β 1 potently stimulates the production of α -SMA in CLPF and therefore their differentiation into myofibroblasts as a result of long term contact. This differentiation into myofibroblasts is accompanied by a reduced migratory

potential, reduced FAK phosphorylation and increased synthesis of FN as a component of extracellular matrix (ECM).

Myofibroblast differentiation and activation by TGF- β 1 is a critical event in tissue repair and the pathogenesis of human fibrotic diseases. Myofibroblasts and TGF- β 1 are key elements for the generation of contractile force associated with wound contraction and pathological contractures as in the development of tissue fibrosis. Myofibroblasts are characterized by the presence of α -SMA-containing stress fibers, vinculin-containing fibronexus adhesion complexes, and FN fibrils containing the ED-A splice variant^[32]. TGF- β 1 promotes contraction of collagen gels by fibroblasts through their differentiation into myofibroblasts^[33]. In addition, TGF- β 1 induces a dose-dependent increase in the generation of contractile force and a concomitant increase in the production of α -SMA^[32].

In the current study much higher TGF- β 1 doses were required to induce α -SMA production than in the studies by Simmons *et al.*^[9] and Vaughan *et al.*^[32]. This may be a cell-type-specific effect. Simmons *et al.* used CCD-18Co fibroblasts isolated from the colon of a 2.5 mo old child, while in this study intestinal fibroblasts of adults have been used. Vaughan *et al.*^[32] used myofibroblasts obtained as explant cultures or by collagenase digestion of palmar aponeurosis from patients with Dupuytren's disease. Therefore, it may be

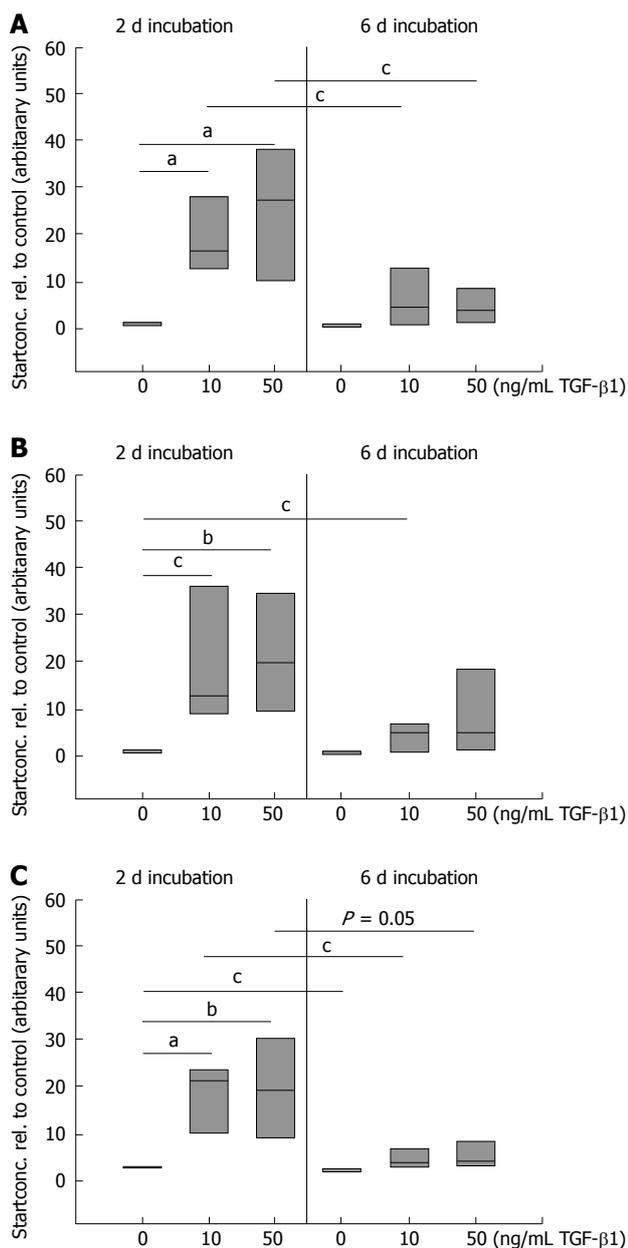


Figure 4 Quantitative mRNA analysis of FN (A) and the splicing forms FN ED-A (B) and FN ED-B (C) by real-time PCR in TGF stimulated CLPF. TGF-β1 stimulated and untreated control-CLPF (*n* = 7) were lysed and mRNA was isolated. The cDNA start concentration of the untreated control group was set as 1. Compared to the control cells a dose-dependent increase in mRNA expression of FN, FN ED-A, and FN ED-B was determined. TGF-β1-stimulation for 2 d caused a higher increase in FN mRNA expression than after 6 d. Paired *t*-test: ^a*P* < 0.005; ^b*P* < 0.01; ^c*P* < 0.05.

that fibroblasts obtained from palmar aponeurosis react more sensitively to TGF-β1 than CLPF.

Myofibroblast differentiation by TGF-β1 is dependent on cell adhesion and integrin signaling *via* FAK. TGF-β1 induces tyrosine phosphorylation of the autophosphorylation site Tyr-397 of FAK, an effect that is dependent on cell adhesion and is delayed relative to early Smad signaling. Pharmacologic inhibition of FAK or expression of kinase-deficient FAK, mutated by substituting Tyr-397 with Phe, inhibited TGF-β1-induced α-SMA production and stress fiber formation^[34]. In the current study we found that long term incubation

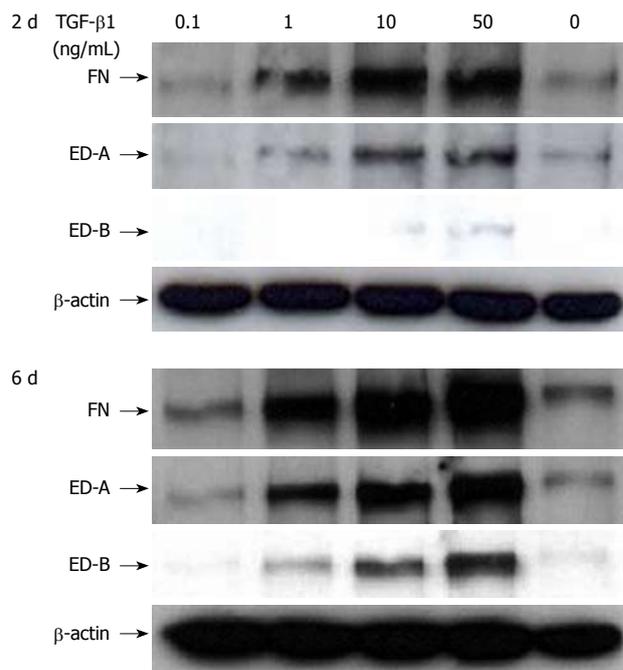


Figure 5 Effect of TGF-β1 on FN, FN ED-A, and FN ED-B protein in CLPF after 2 d and 6 d. Control-CLPF were incubated for 2 d or 6 d with 0, 0.1, 1, 10, and 50 ng/mL TGF-β1 in serum-free medium. Subsequently cells were washed and lysed. Amounts of FN were quantified by Western blotting. Loading was checked by β-actin production. Incubation of CLPF for 6 d with TGF-β1 markedly enhanced the levels of FN, FN ED-A, and FN ED-B.

with TGF-β1 reduced FAK phosphorylation and this was associated with a decreased migratory potential. TGF-β1 untreated CLPF displayed a PDGF-dependent increase of FAK-phosphorylation that correlated with an enhanced migration.

FAK is a nonreceptor protein tyrosine kinase involved not only in adhesion but also in cell migration. FAK-deficient fibroblasts exhibit defects in cell migration and elevated numbers of cell-substratum contact sites^[35]. The reduced migratory behavior of CD- and ulcerative colitis (UC)-CLPF is accompanied by a reduction of FAK and FAK phosphorylation^[29].

TGF-β1 production is increased in myofibroblasts at sites of fibrosis in experimental enterocolitis and CD^[9]. In CD, increased TGF-β1 expression is transmural whereas in UC, the increase is confined to the lamina propria and submucosa. The distribution of TGF-β1 coincides with the distribution of the inflammatory infiltrate as well as an increase in the collagen type III:I ratio in both CD and UC^[36]. Additionally, TGF-β1, -β2, -β3 and their receptors are increased in fibrotic CD mucosal tissue samples^[13]. Therefore, it may be assumed that a reduced migratory potential of inflammatory bowel disease (IBD)-CLPF^[29] is a result of enhanced α-SMA production and enhanced formation of focal contacts induced by increased levels of TGF-β1 in the tissue. However, we could not find higher α-SMA levels in *ex vivo* cultures of CD- and UC-CLPF compared to cultures isolated from control mucosa (data not shown). Therefore, a differentiation of IBD-CLPF into myofibroblasts was not the reason for the reduced migratory potential of these cells.

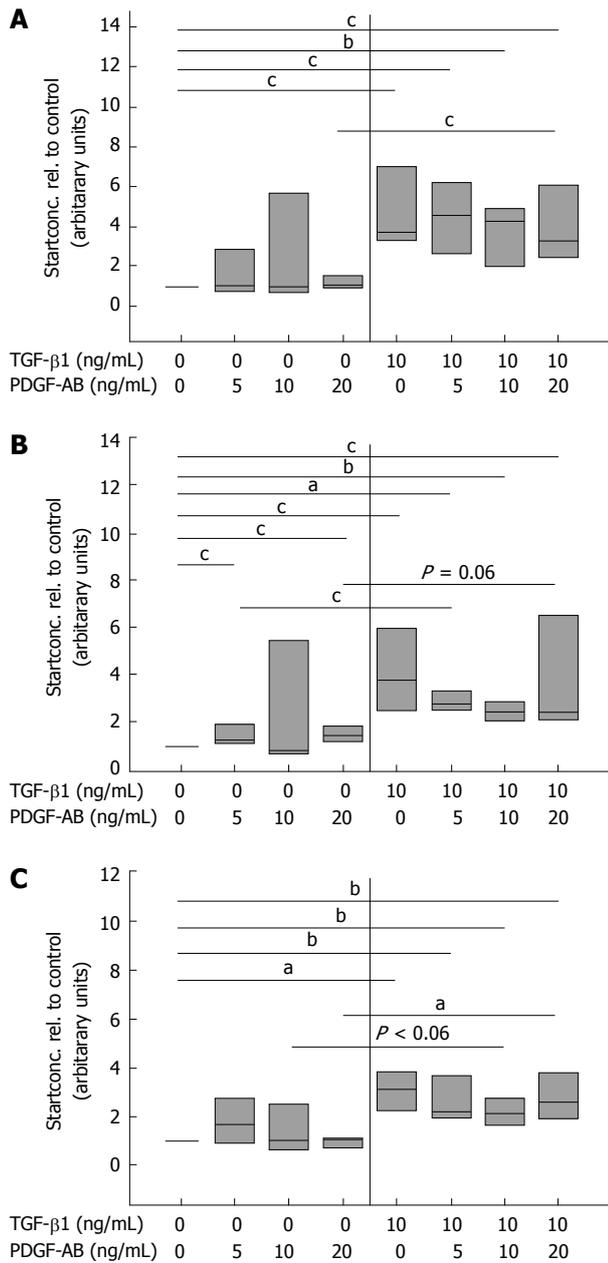


Figure 6 Quantitative mRNA analyses of FN (A) and the splicing forms FN ED-A (B), and FN ED-B (C) by real-time PCR in CLPF treated with TGF-β1. Control CLPF ($n = 7$) were pre-incubated with and without 10 ng/mL TGF-β1 for 6 d, the monolayer wounded with a comb, and incubated for further 4 h with increasing concentrations of PDGF-AB in conditioned medium. mRNA was isolated and cDNA transcribed. cDNA start concentration of the untreated control (0 ng/mL TGF-β1 and 0 ng/mL PDGF-AB) was set as 1. Paired t -test: ^a $P < 0.005$; ^b $P < 0.01$; ^c $P < 0.05$.

Contrasting migration-modulating effects of TGF-β are described in the literature. Ellis *et al*^[7] report a diverse pattern of motogenic response to the three TGF-β isoforms. Migration of subconfluent fibroblasts into 3D collagen gels was inhibited by all three TGF-β-isoforms, whereas migration of confluent cells was unaffected by TGF-β1 and TGF-β2, but stimulated by TGF-β3^[7]. Postlethwaite *et al*^[8] reported that TGF-β1 is a potent chemoattractant in the Boyden chamber for human dermal fibroblasts. Inhibitory effects of TGF-β1 on fibroblast migration into 3D collagen gels are in marked

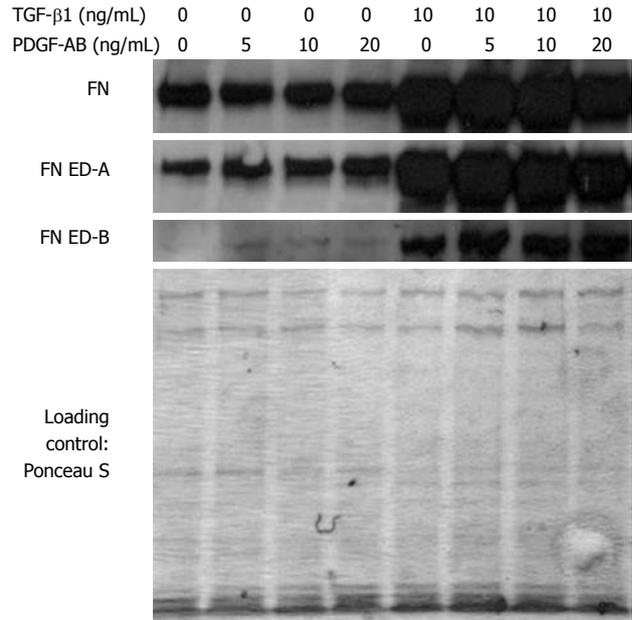


Figure 7 FN, FN ED-A, and FN ED-B protein levels of TGF-β1-pre-stimulated and unstimulated control CLPF in a wounding assay. Isolated proteins were analyzed by Western blotting. Protein levels of FN, FN ED-A, and FN ED-B were increased by TGF-β1-pre-treatment compared to untreated control. Loading was checked by Ponceau S staining.

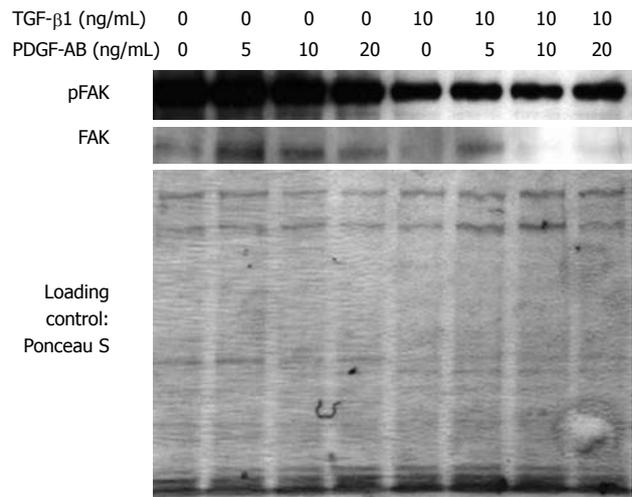


Figure 8 Analysis of FAK phosphorylation and FAK protein production of CLPF after TGF-β1 incubation in a wounding assay. Isolated proteins were analyzed by Western blotting. Phospho-FAK and FAK decreased after 6 d TGF-β1 pretreatment in comparison to untreated controls. Loading was checked by Ponceau S staining.

contrast to reports indicating that TGF-β1 stimulates the migration of human skin fibroblasts in the modified Boyden chamber^[6].

Previously we investigated the effect of TGF-β1 on CLPF migration in the modified Boyden chamber after a 6 h incubation period^[28] and found a significant migration-inducing effect of TGF-β1. Here we report that long term incubation for 6 d with TGF-β1 induces a marked reduction of migration in subsequent migration assays. This discrepancy obviously was determined by the time TGF-β1 could act on the target cells.

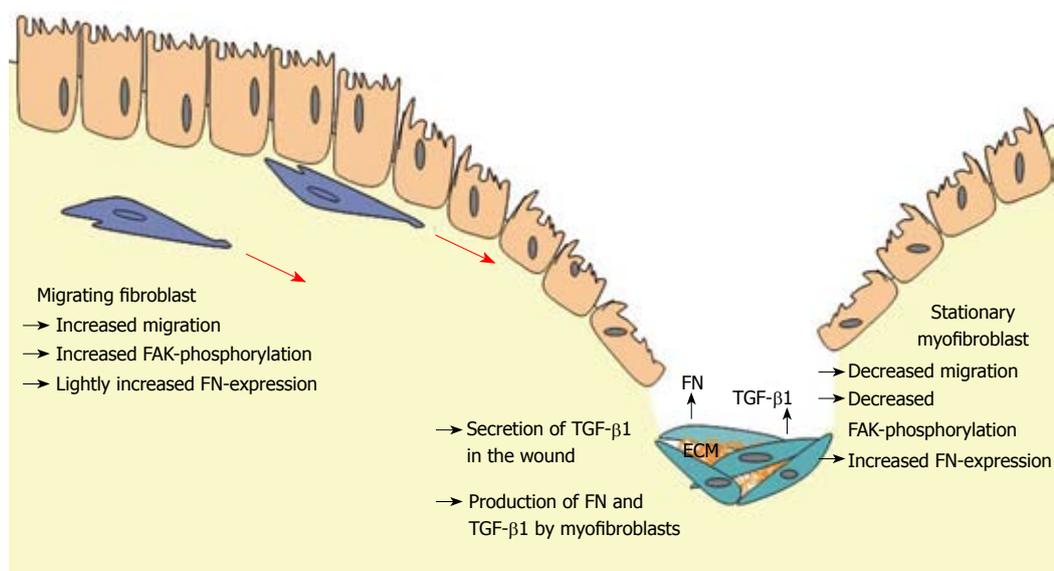


Figure 9 Schematic illustration of the role of TGF- β 1, FN, and FAK in the development of fibrosis in CD. After wounding TGF- β 1 is produced by different cell types and connective tissue cells. CLPF increase FAK phosphorylation and migrate into the wound along the TGF- β 1 gradient. FN that is also produced during migration induction and secreted at the site of injury may lead to an additional enhancement of the migratory gradient. Long term contact with TGF- β 1 within the wound allows fibroblasts to differentiate into myofibroblasts. These cells have a reduced migratory potential and support wound healing via wound contraction and production of extracellular matrix deposition like FN. Phosphorylation of FAK is reduced after longer contact with TGF- β 1 due to the differentiation into ECM-producing myofibroblasts.

Addition of 10 ng/mL and 50 ng/mL TGF- β 1 resulted in a dose-dependent increase of FN, FN ED-A, and FN ED-B expression. After two days incubation, expression of FN and the splice forms significantly increased. Enhanced mRNA expression was also observed after 6 d incubation with TGF- β 1 but was not as high as after 2 d. On the other hand, after six days higher protein levels of total FN and FN isoforms were observed in comparison to the unstimulated two day control. Therefore, the reduced migration of CLPF after six days incubation with TGF- β 1 is accompanied by enhanced protein levels of FN and FN isoforms. This increased production of FN and FN isoforms might lead to enhanced cell adhesion. Other groups also report that fibroblast attachment is significantly increased after TGF- β 1 treatment^[36]. Nevertheless, cell adhesion was not addressed in our investigations.

In CLPF treated for six days with TGF- β 1, after wounding and PDGF-AB incubation increased protein levels of FN, FN ED-A, and FN ED-B were found when compared to untreated controls. In these cells FAK protein and FAK phosphorylation were reduced. This reduced FAK phosphorylation correlated with the observed reduced migratory potential after long term incubation with TGF- β 1.

In conclusion, long term incubation of intestinal fibroblasts with TGF- β 1 induced differentiation into myofibroblasts with an enhanced α -SMA production and a reduced migratory potential that was accompanied by decreased FAK production and phosphorylation. In contrast, short term contact of CLPF with TGF- β 1 induced a dose-dependent increase of cell migration without induction of α -SMA. Therefore, the following scenario is conceivable: after wounding TGF- β 1 is produced by different cell types and connective tissue

cells, like CLPF, migrate towards the wound along the TGF- β 1 gradient. Long-term contact with TGF- β 1 in the wound allows the fibroblasts to differentiate into myofibroblasts. Myofibroblasts support wound healing *via* wound contraction and production of extracellular matrix deposition. In pathological situations the local increase in immigrated cell numbers with reduced dispersing potential and increased deposition of extracellular matrix may finally lead to tissue fibrosis. FN that is produced during the induction of migration and secreted at the site of injury may lead to an additional enhancement of the migratory gradient (Figure 9). The phosphorylation of FAK is reduced after longer contact with TGF- β 1 due to the differentiation into ECM-producing myofibroblasts.

However, this model remains speculative, since the modulating influence of other factors on fibroblast migration and the complex interactions of fibroblasts with immune cells or epithelial cells certainly play an important role in wound healing, too.

The role of TGF- β and the mechanism of mucosal wound healing or intestinal tissue fibrosis require further investigation to develop therapies for the modulation of the mucosal healing response in IBD.

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COMMENTS

Background

Migration of colonic lamina propria fibroblasts (CLPF) plays an important role during the progression of fibrosis and fistulae in Crohn's disease. Transforming growth factor- β (TGF- β) is involved in the regulation of cell migration, cell differentiation, extracellular matrix deposition, and immune responses. In recent studies, the authors found that CLPF conditioned media induced migration of primary human CLPF and that fibronectin (FN) was mainly responsible for the autocrine induction of CLPF migration. Since the regulation of migration and differentiation of intestinal fibroblasts is an important mechanism during intestinal wound healing and fibrosis, the effect of TGF- β 1 on these processes and on FN and FN isoform production was investigated in this study.

Research frontiers

A high number of patients with Crohn's disease have to undergo surgery because of fibrotic disease. The mechanism of stricture formation and the role of TGF- β 1 in this are not well understood.

Innovations and breakthroughs

This study adds to the understanding of the role of TGF- β 1 in intestinal wound healing and stricture formation. It shows that TGF- β 1 can both be beneficial and detrimental depending on the duration of secretion.

Terminology

Colon lamina propria fibroblasts (CLPF): CLPF are mesenchymal cells of connective tissue that are in an activated state and locally agile. After differentiation into myofibroblasts cells are in a less active state, concerned with maintenance. Transforming growth factor (TGF): TGF- β 1 is a cytokine that on the one hand stimulates migration of fibroblasts and on the other hand potentially stimulates the production of α -smooth muscle actin and stress fiber formation in fibroblasts and therefore their differentiation into myofibroblasts. Fibronectin (FN): FN is an extracellular matrix protein that is mainly responsible for the autocrine induction of CLPF migration and is essentially required for the induction of CLPF migration. Focal adhesion kinase (FAK): FAK is a nonreceptor protein tyrosine kinase involved not only in adhesion but also in cell migration. Enhanced migration of CLPF is associated with higher FAK phosphorylation and total FAK protein production. Myofibroblast differentiation by TGF- β 1 is dependent on cell adhesion and integrin signaling via FAK.

Peer review

The authors investigated the effects of transforming growth factor- β (TGF- β) on the differentiation of colonic lamina propria fibroblasts (CLPF) into myofibroblasts *in vitro*. They found that long term incubation of CLPF with TGF- β 1 induced differentiation into myofibroblasts with enhanced α -SMA, reduced migratory potential and FAK-phosphorylation, as well as increased FN production. In contrast, short term contact (6 h) of fibroblasts with TGF- β 1 induced a dose-dependent increase of cell migration and FAK-phosphorylation without induction of α -SMA production. The study is well designed, the methods they use are sound and their results are reliable.

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Down-regulation of extracellular signal-regulated kinase 1/2 activity in P-glycoprotein-mediated multidrug resistant hepatocellular carcinoma cells

Feng Yan, Xiao-Min Wang, Chao Pan, Quan-Ming Ma

Feng Yan, Department of Hepatobiliary Surgery, Postdoctoral Station, Zhongshan Hospital, Xiamen University, Xiamen 361004, Fujian Province, China

Xiao-Min Wang, Department of Hepatobiliary Surgery, Digestive Diseases Institute, Xiamen University, Xiamen 361004, Fujian Province, China

Chao Pan, Department of Pathology, Zhongshan Hospital, Xiamen University, Xiamen 361004, Fujian Province, China

Quan-Ming Ma, Department of Hepatobiliary Surgery, Zhongshan Hospital, Xiamen University, Xiamen 361004, Fujian Province, China

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Correspondence to: Xiao-Min Wang, MD, PhD, Professor of Medicine, Chief, Department of Hepatobiliary Surgery, Digestive Diseases Institute, Xiamen University, Xiamen 361004, Fujian Province, China. wxm@xmzsh.com

Telephone: +86-592-2292203 Fax: +86-592-2212328

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Abstract

AIM: To study the expression and phosphorylation of extracellular signal-regulated kinase (ERK) 1 and ERK2 in multidrug resistant (MDR) hepatocellular carcinoma (HCC) cells.

METHODS: MDR HCC cell lines, HepG2/adriamycin (ADM) and SMMC7721/ADM, were developed by exposing parental cells to stepwise increasing concentrations of ADM. MTT assay was used to determine drug sensitivity. Flow cytometry was employed to analyze cell cycle distribution and measure cell P-glycoprotein (P-gp) and multidrug resistant protein 1 (MRP1) expression levels. ERK1 and ERK2 mRNA expression levels were measured by quantitative real-time PCR (QRT-PCR). Expression and phosphorylation of ERK1 and ERK2 were analyzed by Western blot.

RESULTS: MTT assay showed that HepG2/ADM and

SMMC7721/ADM were resistant not only to ADM, but also to multiple anticancer drugs. The P-gp expression was over 10-fold higher in HepG2/ADM cells than in HepG2 cells ($8.92\% \pm 0.22\%$ vs $0.88\% \pm 0.05\%$, $P < 0.001$) and over 4-fold higher in SMMC7721/ADM cells than in SMMC7721 cells ($7.37\% \pm 0.26\%$ vs $1.74\% \pm 0.25\%$, $P < 0.001$). However, the MRP1 expression was not significantly higher in HepG2/ADM and SMMC7721/ADM cells than in parental cells. In addition, the percentage of MDR HepG2/ADM and SMMC7721/ADM cells was significantly decreased in the G0/G1 phase and increased in the S phase or G2/M phase. QRT-PCR analysis demonstrated that the ERK1 and ERK2 mRNA expression increased apparently in HepG2/ADM cells and decreased significantly in SMMC7721/ADM cells. Compared with the expression of parental cells, ERK1 and ERK2 protein expressions were markedly decreased in SMMC7721/ADM cells. However, ERK2 protein expression was markedly increased while ERK1 protein expression had no significant change in HepG2/ADM cells. Phosphorylation of ERK1 and ERK2 was markedly decreased in both HepG2/ADM and SMMC7721/ADM MDR cells.

CONCLUSION: ERK1 and ERK2 activities are down-regulated in P-gp-mediated MDR HCC cells. ERK1 or ERK2 might be a potential drug target for circumventing MDR HCC cells.

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Key words: Multidrug resistance; Extracellular signal-regulated MAP kinases; Hepatocellular carcinoma; P-glycoprotein; Multidrug resistance-associated protein

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third cause of cancer-related death^[1,2]. Drugs used in the treatment of HCC are cytotoxic with a high risk of side effects and none of them is specific for HCC^[3]. Moreover, HCC is a hypervascular solid cancer characterized by a high degree of drug resistance^[4]. Multidrug resistance (MDR) to chemotherapeutic agents plays a major role in the failure of cancer therapy^[5]. MDR phenotype, an intrinsic or acquired cross-resistance to a variety of structurally and functionally unrelated drugs, is almost constantly expressed in HCC and represents one of the major problems in cancer eradication by limiting the efficacy of chemotherapy^[6]. Resistance to therapy can result from decreased drug uptake, increased DNA repair or drug inactivation^[7].

Mitogen-activated protein kinase (MAPK) pathway is an attractive target or therapeutic intervention in cancer due to its integral role in the regulation of cancer cell proliferation, invasiveness, and survival. Pharmaceutical agents can inhibit various kinases and GTPases comprising the pathway^[8,9]. Extracellular signal-regulated kinase (ERK) 1/2 is a member of the MAPK family. ERK1 and ERK2 are isoforms of the "classical" MAPK^[10]. The activity of ERK1/2 has been implicated in the regulation of embryonic morphogenesis, cell proliferation, tumor transformation, and apoptosis^[11]. It has been recently found that P-gp expression in the MDR1-transduced human breast cancer cell lines MCF-7/MDR and MDA-MB-231/MDR is positively regulated by the ERK pathway and blockade of the MEK-ERK-RSK pathway can suppress cell surface P-gp expression by promoting its degradation^[12]. In addition, there are several lines of evidence that modulation of ERK activation may reverse MDR in prostatic, gastric and hematopoietic cancers^[13-16]. However, there is little evidence that ERK activity is related to MDR of HCC.

The aim of this research was to study the crucial kinases of ERK pathway, including expression and phosphorylation (activity) of ERK1 and ERK2 in MDR HCC cell lines, and to explore whether the relationship between MDR and ERK1/2 kinases involves specific molecular aspects of these cell lines. Once they are well characterized, the ERK pathway might be exploited for overcoming MDR of HCC.

MATERIALS AND METHODS

Cell culture

Human HCC cell lines, HepG2 and SMMC7721, were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences. HepG2 was cultured with DMEM (HyClone, Logan, UT, USA) and SMMC7721 was cultured with RPMI-1640 (HyClone, Logan, UT, USA). Both media were supplemented with 10% calf serum (HyClone, Logan, UT, USA) and maintained at 37°C in a humidified atmosphere containing 50 mL/L

CO₂ and 950 mL/L air. Multidrug resistant human HCC cell lines, HepG2/adriamycin (ADM) and SMMC7721/ADM, were developed by our group. To develop the HepG2/ADM and SMMC7721/ADM cells, ADM (Pharm-sh Pharmaceutical Co., Ltd., Shanghai, China) was added respectively to HepG2 and SMMC7721 cells at a stepwise increasing concentration from 0.01 to 0.2 mg/L. Resistant cells were selected by removing the non-resistant dead cells. Multidrug resistance was maintained by culturing the cells with 0.2 mg/L ADM and MDR cells were named HepG2/ADM and SMMC7721/ADM.

Measurement of cellular sensitivity to anticancer drugs

MTT (Sigma-Aldrich, St. Louis, MO, USA) assay was used to determine drug sensitivity. Sensitivity of cultured HepG2/ADM and SMMC7721/ADM cells to anticancer drugs, including ADM, fluorouracil, cisplatin, cyclophosphamide, mitomycin and vincristine (Pharm-sh Pharmaceutical Co., Ltd., Shanghai, China), was detected, respectively^[17]. IC₅₀ value was assessed by probit regression analysis using SPSS11.5 statistical software. Resistance index (RI) was calculated according to the formula: RI = IC₅₀ for MDR cells / IC₅₀ for parental cells.

Flow cytometric analysis of cell cycle distribution

Cultured HepG2/ADM and SMMC7721/ADM cells and their parental cells were collected respectively through trypsinization, washed with ice-cold PBS, centrifuged at 500 × g for 5 min at 4°C, washed twice with ice-cold PBS and fixed in 70% ethanol for 2 h at 4°C. Samples were rehydrated with PBS and the cells were incubated for 30 min at room temperature with a propidium iodide staining solution in PBS containing 0.2 mg/mL propidium iodide, 0.2 mg/mL DNase-free RNase A (Roche, Basel, Switzerland), and 0.1% Triton X-100. Using red propidium-DNA fluorescence, 4000 events were acquired with a Epics[®] XL Beckman Coulter FACS machine (Beckman Coulter Inc., Fullerton, CA, USA) for each sample and the percentage of cells in G₀/G₁, S and G₂/M phases of the cell cycle was calculated using the System II[™] software (Beckman Coulter Inc., Fullerton, CA, USA).

Flow cytometric analysis of cell p-gp and mrp1 expression level

Cultured MDR and parental cells were collected as above. Then, samples were immunostained with P-glycoprotein antibody (FITC) (Cat.ab66250, Abcam plc, Cambridge, UK) and MRP1 antibody (FITC) (Cat.No.557593, BD Biosciences Pharmingen, San Diego, CA, USA), respectively, according to the proper protocol. The cells were fixed and permeabilized with a BD Cytotfix/Cytoperm[™] solution (Cat.No.554722, BD Biosciences, San Jose, CA, USA) before they were immunostained with MRP1 antibody (FITC). Flow cytometry was carried out with a fluorescent-activated cell scan (FACS) using the System II[™] software. Fluorescence of the cells treated with fluorescent isotype control IgG (Cat.ab18455, Abcam plc) was evaluated in each experiment to measure the level of background fluorescence of negative cells.

Table 1 Determination of IC₅₀ and resistance index of different anticancer drugs (mean ± SD)

	IC ₅₀		Resistance index	IC ₅₀		Resistance index
	HepG2	HepG2/ADM		SMMC7721	SMMC7721/ADM	
Adriamycin (mg/L)	0.0063 ± 0.0022	0.135 ± 0.053	21.43	0.0139 ± 0.008	0.266 ± 0.036	19.14
Fluorouracil (μmol/L)	1.114 ± 0.271	25.34 ± 2.38	22.75	0.689 ± 0.082	48.5 ± 2.57	70.39
Cyclophosphamide (mg/L)	2.902 ± 0.369	32.68 ± 4.962	11.26	2.315 ± 0.279	60.08 ± 4.93	25.95
Cisplatin (mg/L)	0.0527 ± 0.013	1.084 ± 0.0749	20.57	0.0483 ± 0.011	1.637 ± 0.172	33.89
Mitomycin (mg/L)	0.061 ± 0.017	1.085 ± 0.246	17.79	0.032 ± 0.013	0.644 ± 0.168	20.13
Vincristine (mg/L)	0.0093 ± 0.0035	0.086 ± 0.0098	9.25	0.006 ± 0.004	0.247 ± 0.023	41.16

Mean fluorescence intensity (MFI) of positively stained cells was determined.

RNA extraction and quantitative real-time polymerase chain reactions (QRT-PCR)

ERK1 and ERK2 mRNA expression levels were measured by QRT-PCR. Total RNA was extracted using the TRIzol reagent (GIBCO BRL, Life Technologies Inc., Rockville, MD, USA) following the constructions of its manufacturer, and reverse transcribed to cDNA using the Gene Amp RNA PCR kit in a DNA thermal cycler (Bio-Rad). QRT-PCR was performed with SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) in an ABI Prism 7700 real time PCR machine (Applied Biosystems).

The synthesized cDNA served as a template in a (25 μL) reaction. A non-template control was included in all experiments. Primer sequences are as follows: ERK1 GenBank: NM_002746 forward, 5'-TCAACAC CACCTGCGACCTT-3', and reverse, 5'-GCGTAGCC ACATACTCCGTCA-3'; ERK2 GenBank: NM_002745 forward, 5'-GTTCCCAAATGCTGACTCCAA-3', and reverse, 5'-CTCGGGTCGTAATACTGCTCC-3'; β-actin GenBank: NM_001101 forward, 5'-TGACG TGGACATCCGCAAAG-3', and reverse, 5'-CTG-GAAGGTGGACAGCGAGG-3'.

QRT-PCR was performed at 94°C for 4 min, followed by 40 cycles at 94°C for 15 s, at 60°C for 25 s, and at 72°C for 25 s. Oligonucleotides and reagents for PCR assay were purchased from Qiagen GmbH, Hilden, Germany. Data were analyzed with the Sequence detector software (v1.9, Applied Biosystems). The mean Ct value for duplicate measurements was used to detect the expression of target gene with normalization to a housekeeping gene used as an internal control (β-actin) according to the $2^{-\Delta Ct}$ formula.

Western blot analysis

Protein was collected from cultured HepG2, SMMC7721, HepG2/ADM and SMMC7721/ADM cells and its concentration was measured (protein assay dye, Bio-Rad). Then, the protein was denatured in a LDS sample buffer for 5 min at 95°C, run on SDS-PAGE (NUPAGE, 4%-12% Bis-Tris, Invitrogen, Carlsbad, CA, USA) and blotted onto PVDF membranes (0.2 μm, Invitrogen). Membranes were blocked with 5% dry milk in TBS-T (TBS containing 0.05% Tween 20) for 1 h at room temperature and incubated overnight at 4°C with antibodies against

ERK1, ERK2, or phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Inc., Danvers, MA, USA). After incubation with the respective primary antibodies, the membranes were exposed to species-specific horseradish peroxidase-labeled secondary antibodies at room temperature, and developed using the ECL plus Western blotting reagent (GE Healthcare, Little Chalfont, UK) and Fuji Film LAS-1000 equipment (Fuji Film, Tokyo, Japan). Parallel membranes were incubated with 1:5000 rabbit monoclonal antibodies to GAPDH (Cell Signaling Technology, Inc.) and HRP-coupled rabbit anti-mouse secondary antibody. Primary and secondary antibody solutions were prepared in a PBS solution containing 2% bovine serum albumin and 0.1% Tween-20. After incubation with antibodies, the membranes were washed 3 times for 5 min in PBS containing 0.1% Tween-20. Calculation and statistics were performed using the ImageJ 1.37 software.

Statistical analysis

Statistical analysis was performed using Student's *t*-test to compare the two groups and ANOVA was used with Dunnett's post-test for multiple comparisons when the three groups or more were compared. *P* < 0.05 was considered statistically significant. The results were expressed as mean ± SE. Values were analyzed using the statistical package SPSS for Windows Ver.11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Determination of MDR

Each step of developing MDR HepG2/ADM and SMMC7721/ADM cells took 7-8 wk. MDR was maintained by culturing the cells with 0.2mg/L ADM. Cytotoxicity assay found that HepG2/ADM and SMMC7721/ADM were resistant not only to ADM but also to multiple anticancer drugs. Among them, fluorouracil (5-FU), cyclophosphamide (CTX), cisplatin (CDDP), mitomycin (MMC), and vincristine (VCR) were tested in our study. Their lethal dose (IC₅₀) was significantly higher for HepG2/ADM and SMMC7721/ADM cells than for non-resistant parental cells (Figure 1 and Table 1).

Cell cycle distribution

Cell cycle phase distribution was detected by flow cytometry to determine whether there is any difference in cell cycle kinetics between MDR HepG2/ADM and SMMC7721/

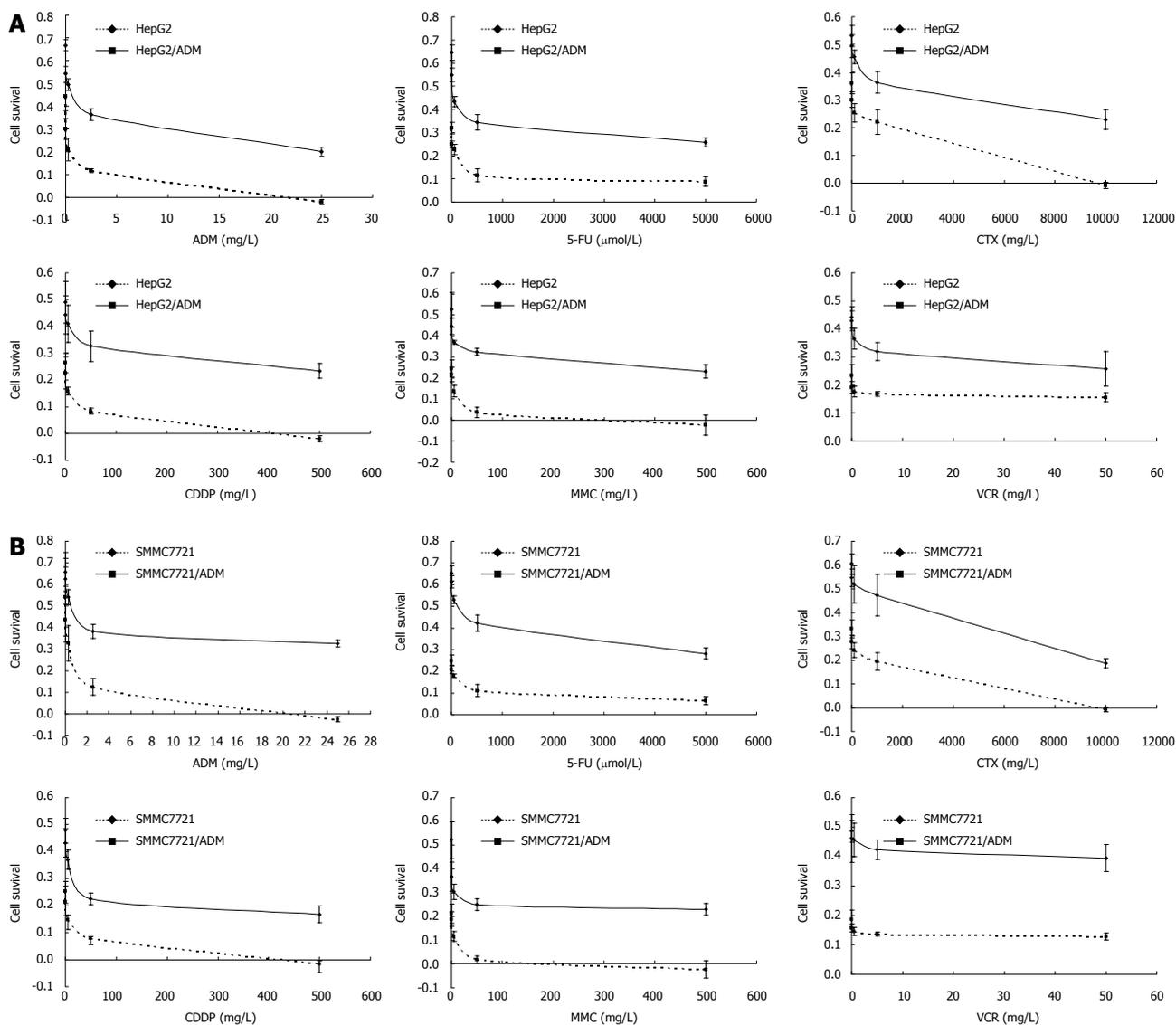


Figure 1 Measurement of cellular sensitivity to anticancer drugs and parental cells. Cytotoxicity assay for adriamycin, fluorouracil, cyclophosphamide, tested cisplatin, mitomycin, and vincristine was performed to evaluate the IC50 for HepG2 and HepG2/ADM cells (A), SMMC7721 and SMMC7721/ADM cells (B). Dose response curves were derived from five independent experiments using MTT assay. The data were shown as mean ± SE.

Table 2 Cell cycle distribution of parental and MDR HCC cells (mean ± SD)

Cells (n = 5)	G0/G1	S	G2/M
hepG2	65.08 ± 1.61	18.32 ± 1.37	16.58 ± 0.65
hepG2/ADM	62.44 ± 1.77 ^a	12.24 ± 1.21 ^b	25.36 ± 2.12 ^c
SMMC7721	71.12 ± 1.38	17.86 ± 1.91	11.02 ± 1.95
SMMC7721/ADM	67.8 ± 2.15 ^a	23.6 ± 0.93 ^b	8.62 ± 2.74 ^a

^aP < 0.05, ^bP < 0.01, ^cP < 0.001 vs control cells.

Table 3 P-gp and MRP1 expression in MDR and parental cells (mean ± SD)

Cells (n = 5)	P-gp (%)	MRP1 (%)
HepG2	0.88 ± 0.05	0.93 ± 0.15
HepG2/ADM	8.92 ± 0.22 ^a	0.9 ± 0.18
SMMC7721	1.74 ± 0.25	1.21 ± 0.35
SMMC7721/ADM	7.37 ± 0.26 ^a	0.79 ± 0.02

^aP < 0.001 vs parental cells.

ADM cells and their parental cells. The percentage of HepG2/ADM and SMMC7721/ADM cells was significantly decreased at the G0/G1 phase and increased at the S phase or G2/M phase (Figure 2 and Table 2).

P-gp and MRP1 expression

Protein expression of P-gp and MRP1 in HepG2/ADM, SMMC7721/ADM and their parental cells was evaluated. P-gp expression was over 10-fold higher in HepG2/

ADM cells than in HepG2 cells, and over 4-fold higher in SMMC7721/ADM cells than in SMMC7721 cells. However, the MRP1 expression was not significantly higher in HepG2/ADM and SMMC7721/ADM cells than in parental cells (Figure 3 and Table 3).

ERK1 and ERK2 mRNA expression

To examine the role of ERK signaling pathway in the development of MDR, ERK1 and ERK2 mRNA

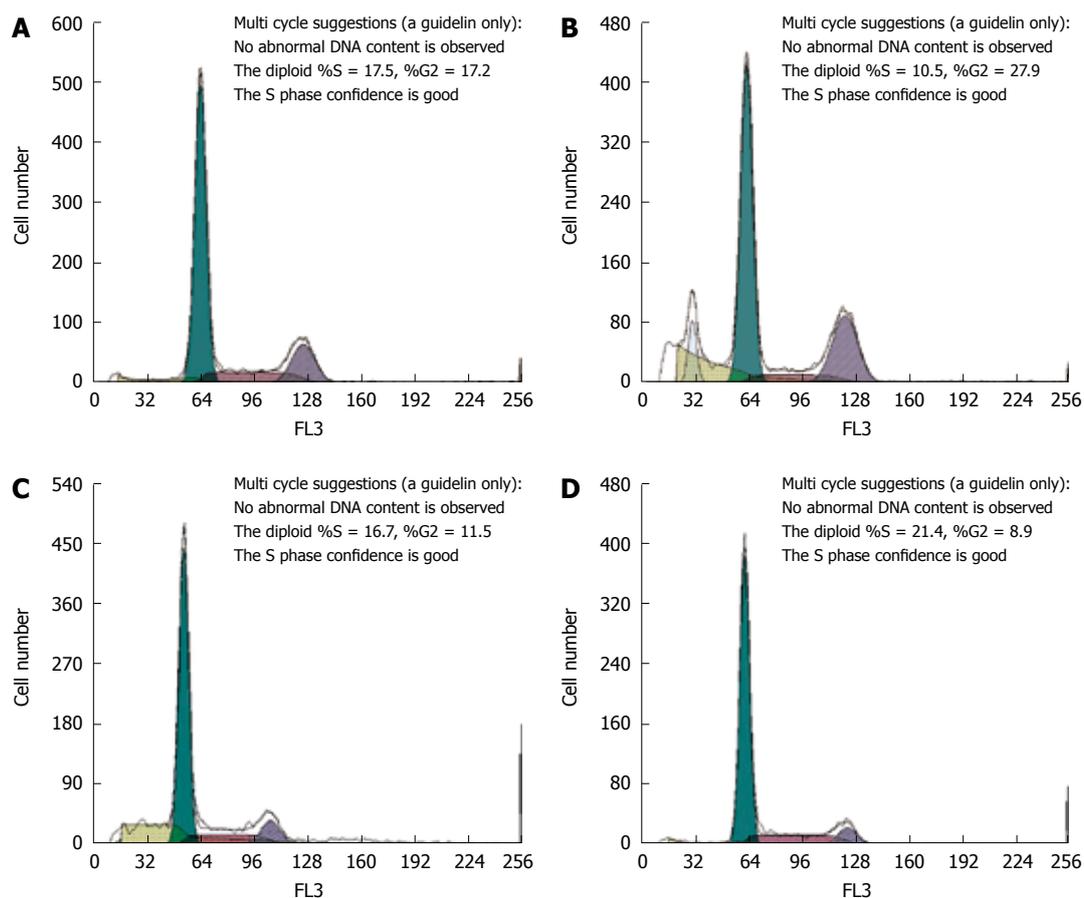


Figure 2 Cell cycle distributions in MDR and parental cells. Cell cycle distributions of HepG2 (A), HepG2/ADM (B), SMMC7721 (C) and SMMC7721/ADM (D) cells were analyzed by flow cytometry as described in Materials and Methods.

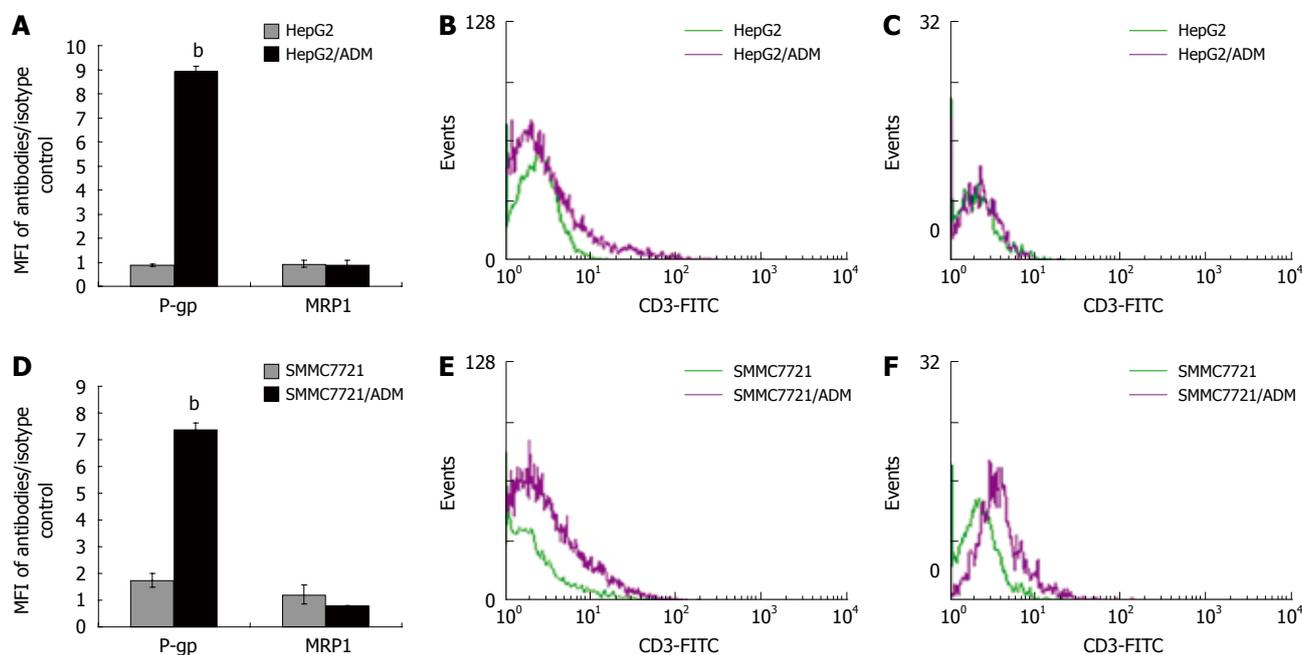


Figure 3 Expression of P-gp and MRP1 in MDR and parental cells. Histograms showing that P-gp expression was over 10-fold higher in HepG2/ADM cells than in HepG2 cells, and over 4-fold higher in SMMC7721/ADM cells than in SMMC7721 cells, but MRP1 expression had no significant difference (A, D). Corresponding flow histograms for P-gp (B, E) and MRP1 (C, F) are presented. The results are shown as mean \pm SE ($n = 5$). Statistical analyses comparing MDR cells with parental cells were performed using Student's *t*-test. ^b $P < 0.001$ vs SMMC7721 cells.

expression in parental and MDR cells was assessed, respectively (Figure 4). QRT-PCR analysis demonstrated that ERK1 and ERK2 mRNA expression was apparently higher in HepG2/ADM cells and significantly lower in SMMC7721/ADM cells.

Expression and phosphorylation of ERK1 and ERK2

The expression of ERK1, ERK2, p-ERK1 and p-ERK2 in HepG2/ADM, SMMC7721/ADM and their parental cells, was detected by Western blot analysis, respectively. The expression of ERK1 and ERK2 protein was

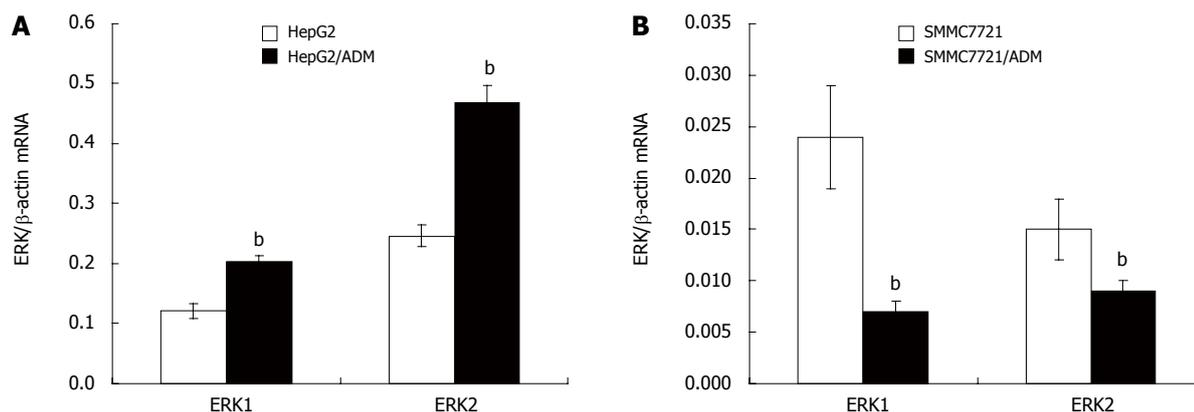


Figure 4 ERK1 and ERK2 mRNA expression in MDR and parental cells. ERK1 and ERK2 mRNA levels were measured by QRT-PCR. A: HepG2/ADM and HepG2 cells; B: SMMC7721/ADM and SMMC7721 cells. Results were normalized by β -actin mRNA expression and compared with the levels in parental cells ($n = 3$). The results are shown as mean \pm SE. Statistical analyses comparing MDR cells with parental cells were performed using Student's *t*-test. ^b $P < 0.01$ vs parental cells (data not shown).

markedly lower in SMMC7721/ADM cells than in their parental cells. However, the expression of ERK2 protein was significantly higher in SMMC7721/ADM cells than in their parental cells while the expression of ERK1 protein had no significant change in HepG2/ADM cells. The phosphorylation of ERK1 and ERK2 was markedly lower in HepG2/ADM and SMMC7721/ADM MDR cells than in their parental cells (Figure 5).

DISCUSSION

Multidrug resistant cancer cells may develop in patients upon prolonged treatment with anti-cancer drugs. MDR poses a great obstacle to chemotherapy for cancer because a higher dosage of drugs is needed to be administered to patients, which will cause severe adverse effects^[18]. Most strategies developed to reverse MDR phenotype involve use of resistance modulators, which have the common ability to reverse the phenotype by inhibiting MDR transporter function^[19,20]. A more efficient strategy to circumvent MDR is to down-regulate the expression of genes coding transporters. However, regulation of MDR-related gene expression is highly complex. For instance, such complexity is embodied in multiple transcription-regulatory elements in the 5' and 3' flanking sequences of the *mdr-1* gene, and numerous protein factors involved in transcription-regulatory processes in a cell type- and stimulus-dependent manner^[21]. The mechanism underlying the expression of MDR-related genes has not yet been fully understood. Thus, the molecular mechanism and signal-transduction pathways involved in regulation of MDR-related genes should be further studied in order to overcome MDR and improve chemotherapeutic efficacy.

The ERK signaling pathway mediates a number of cellular processes, including cell differentiation, growth, survival, and apoptosis. Several growth factors stimulate a protein kinase cascade that sequentially activates Raf, MEK, and ERK1/2. The role of ERK pathway in the generation of MDR has received more consideration than before, and some specific blocking agents of

the ERK pathway have been found. Recent studies demonstrated that modulation of ERK activation may be a new method to reverse MDR^[15,22]. However, whether ERK activation is positively or negatively correlated with MDR still remains controversial. Furthermore, the relationship between ERK activity and MDR of HCC is unknown. In this study, we found that ERK activity was down-regulated in MDR HCC Cells.

ADM is a chemotherapeutic agent principally used in treatment of solid tumors, including HCC^[23]. Although ADM can work through various mechanisms, it is still not immune to the MDR phenotype, and consequently, development of resistance to ADM has been well-documented in a broad range of cell lines^[24-26]. We established two MDR HCC cell lines, HepG2/ADM and SMMC7721/ADM. In this study, death of MDR cells with a lower drug resistance occurred at about 24-48 h after a higher concentration of ADM was added in the medium, and reached the peak at about 72 h. To recover the proliferation ability, the culture of remained adherent cells took at least 7-8 wk. The whole development of MDR HCC cell lines lasted ten months. The IC₅₀ of anticancer drugs was much higher for HepG2/ADM and SMMC7721/ADM cells than for their parental cells, suggesting that the acquired MDR of HepG2/ADM and SMMC7721/ADM is reliable.

To elucidate the mechanism involved in the development of acquired MDR of HepG2/ADM and SMMC7721/ADM cells, cell cycle distribution and expression of MDR-related proteins (P-gp and MRP1) were analyzed by FCM. The percentage of MDR HepG2/ADM and SMMC7721/ADM cells was significantly decreased in the G₀/G₁ phase and increased in the S phase or G₂/M phase than that of their parental cells, which probably contributes to the lower ability of cells to proliferate. Moreover, this kind of delayed cell cycle can result in cellular escape of cytotoxicity of cell cycle specific agents (e.g. vincristine, fluorouracil, *etc.*) and generate MDR^[27,28]. P-gp and MRP1 are members of the ATP-binding cassette transporter proteins. Over-expression of ATP-binding cassette transporter proteins

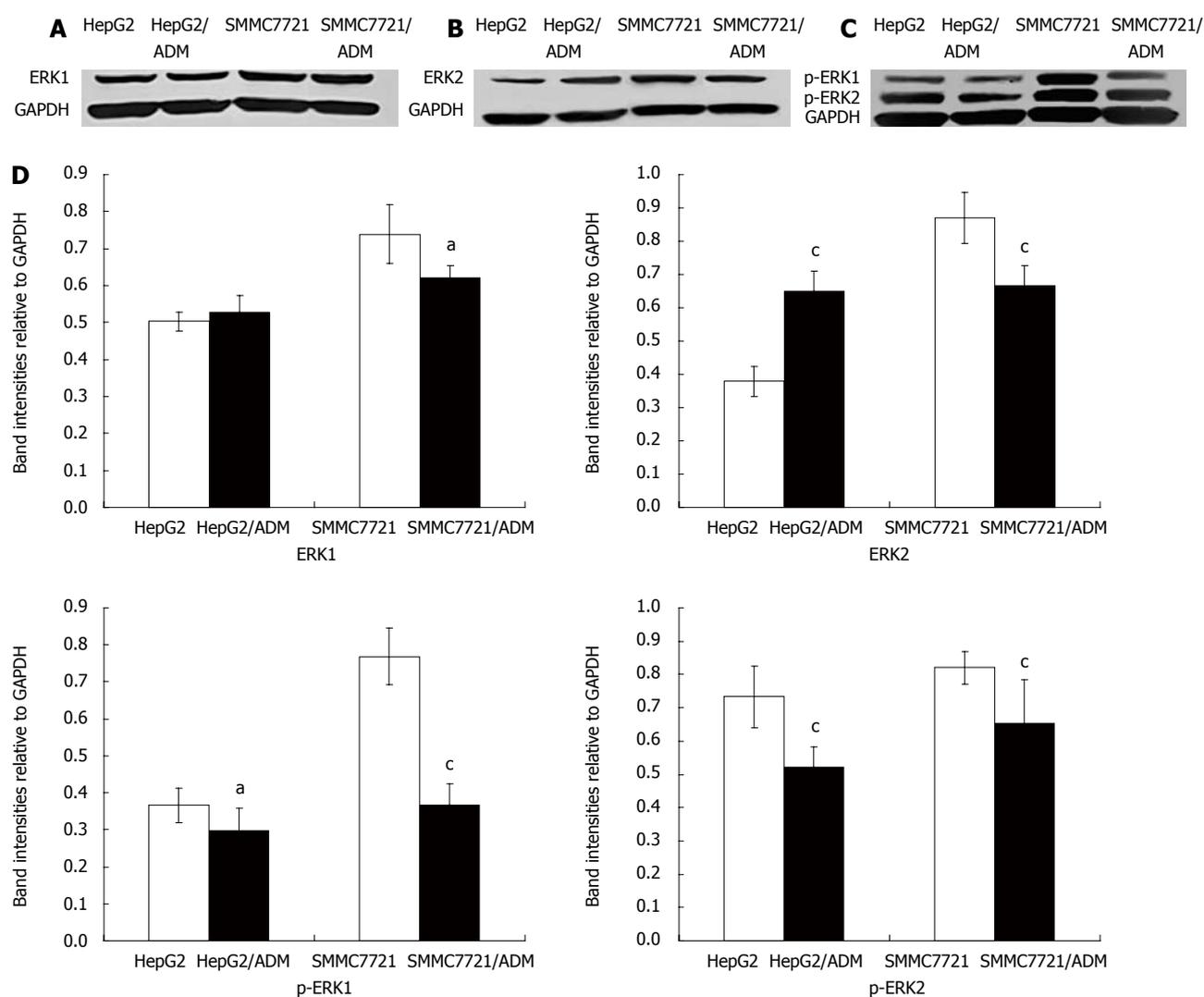


Figure 5 Expression and phosphorylation of ERK1 and ERK2 in MDR and parental cells. Western blot analysis of the ERK1 (A), ERK2 (B), p-ERK1 and p-ERK2 (C) expression in HepG2/ADM, SMMC7721/ADM as well as in HepG2 and SMMC7721 cells ($n = 3$) was performed. The expression of ERK1 and ERK2 was markedly lower in SMMC7721/ADM cells than in parental cells. However, the ERK2 expression was markedly increased and the ERK1 expression had no significant change in HepG2/ADM cells. The phosphorylation of ERK1 and ERK2 was lower in MDR cells than in parental cells (D). The results are shown as mean \pm SE. Statistical analyses comparing MDR cells with parental cells were performed using Student's *t*-test. ^a $P < 0.05$, ^c $P < 0.01$ vs parental cells (data not shown).

represents one of the major mechanisms that contribute to the MDR phenotype. Both P-gp and MRP1 function as a drug efflux pump that actively transports drugs from the inside to the outside of cells and causes a defect in the intracellular accumulation of drugs necessary for cancer cell killing. The results of our study show that P-gp was much higher in MDR HepG2/ADM and SMMC7721/ADM cells than in their parental cells, but the expression of MRP1 was low in both MDR and parental cells, indicating that MDR of HepG2/ADM and SMMC7721/ADM cells mainly attribute to the over-expression of P-gp but not MRP1. This phenomenon can partially be explained by the high expression of P-gp and low expression of MRP1 in liver tissue or HCC cell lines^[29].

ERK1 and ERK2, isozymes of ERK, are extensively expressed in cultured cell lines and mammalian tissues^[30]. To answer the question of whether ERK1 and ERK2 are involved in P-gp-mediated MDR in HCC cells, we detected the expression of ERK1 and ERK2 mRNA

in parental and MDR cells, and the expression and phosphorylation (activity) of ERK1 and ERK2 protein. The results showed that the expression of ERK1 and ERK2 mRNA was increased in HepG2/ADM cells and decreased in SMMC7721/ADM cells. However, ERK1 protein expression had no significant change in HepG2/ADM cells. The phosphorylation of ERK1 and ERK2 was markedly decreased in HepG2/ADM and SMMC7721/ADM MDR cells, suggesting that ERK1 and ERK2 activity is down-regulated in P-gp-mediated MDR HCC cells. However, the decreased activity was not in accordance with mRNA and protein expression in HepG2/ADM cells. The expression of ERK1 and ERK2 protein was diverse, which may contribute to the augments on whether ERK activation is positively or negatively correlated with MDR.

In summary, MDR of HepG2/ADM and SMMC7721/ADM cells mainly attribute to the over-expression of P-gp but not MRP1. ERK1 and ERK2

activity is down-regulated in P-gp-mediated MDR HCC cells, providing new insights into the complicated regulatory mechanism of MDR phenotype. ERK1 and ERK2 might be potential drug targets for circumventing MDR HCC cells. *In vivo* studies are warranted to examine whether ERK1 and ERK2 have a clinical potential in modulating the MDR phenotype during HCC chemotherapy.

COMMENTS

Background

The development of multidrug resistance (MDR) to chemotherapeutic agents plays a major role in the failure of cancer therapy, including hepatocellular carcinoma (HCC). Recent studies have shown that modulation of extracellular signal-regulated kinase (ERK) activation may reverse MDR in prostatic, gastric and hematopoietic cancers. However, there is little evidence that ERK activity is related with MDR of HCC cells.

Research frontiers

Multidrug resistant cancer cells may develop in patients upon prolonged treatment with anti-cancer drugs. Most strategies developed to reverse the MDR phenotype involve use of resistance modulators. A more efficient strategy to circumvent MDR is to down-regulate the expression of genes coding for transporters. Thus, to overcome MDR and improve chemotherapeutic efficacy, the molecular mechanism and signal-transduction pathway involved in the regulation of MDR-related genes should be further studied.

Innovations and breakthroughs

In this study, the MDR of HepG2/ADM and SMMC7721/ADM cells could attribute to the over-expression of P-gp but not MRP1, ERK1 and ERK2 activity was down-regulated in P-gp-mediated MDR HCC cells, thus providing new insights into the complicated regulatory mechanism of MDR phenotype.

Applications

ERK1 and ERK2 might be used as potential drugs targets for circumventing HCC MDR.

Terminology

Multidrug resistance: an intrinsic or acquired cross-resistance to a variety of structurally and functionally unrelated drugs, which is almost constantly expressed in cancer and represents one of the major problems in cancer eradication by limiting the efficacy of chemotherapy, and resistance to therapy can result from decreased drug uptake, increased DNA repair or drug inactivation.

Peer review

The authors examined the expression and phosphorylation of ERK1/2 in MDR HCC cell lines, and demonstrated that ERK1 and ERK2 activity was down-regulated in P-gp-mediated MDR HCC cells, indicating that ERK1 or ERK2 might be used as a potential drug target for circumventing MDR HCC cells.

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ORIGINAL ARTICLES

Melatonin ameliorates experimental hepatic fibrosis induced by carbon tetrachloride in rats

Ru-Tao Hong, Jian-Ming Xu, Qiao Mei

Ru-Tao Hong, Department of Geriatrics Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China

Ru-Tao Hong, Jian-Ming Xu, Qiao Mei, Department of Gastroenterology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China; The Key Laboratory of Digestive Diseases of Anhui Province, Hefei 230022, Anhui Province, China

Author contributions: Hong RT, Xu JM, and Mei Q designed the study; Hong RT performed the experiments, analyzed the data and wrote the manuscript.

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Correspondence to: Jian-Ming Xu, Professor, Department of Gastroenterology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China. xhkay@yahoo.cn

Telephone: +86-551-2922039 Fax: +86-551-3633742

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Abstract

AIM: To investigate the protective effects of melatonin on carbon tetrachloride (CCl₄)-induced hepatic fibrosis in experimental rats.

METHODS: All rats were randomly divided into normal control group, model control group treated with CCl₄ for 12 wk, CCl₄ + NAC group treated with CCl₄ + NAC (100 mg/kg, i.p.) for 12 wk, CCl₄ + MEL-1 group treated with CCl₄ + melatonin (2.5 mg/kg) for 12 wk, CCl₄ + MEL-2 group treated with CCl₄ + melatonin (5.0 mg/kg) for 12 wk, and CCl₄ + MEL-3 group treated with CCl₄ + melatonin (10 mg/kg). Rats in the treatment groups were injected subcutaneously with sterile CCl₄ (3 mL/kg, body weight) in a ratio of 2:3 with olive oil twice a week. Rats in normal control group received hypodermic injection of olive oil at the same dose and frequency as those in treatment groups. At the end of experiment, rats in each group were anesthetized and sacrificed. Hematoxylin and eosin (HE) staining and Van Gieson staining were used to examine changes in liver pathology. Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and protein concentration were

measured with routine laboratory methods using an autoanalyzer. Hydroxyproline (HYP) content in liver and malondialdehyde (MDA) and glutathione peroxidase (GPx) levels in liver homogenates were assayed by spectrophotometry. Serum hyaluronic acid (HA), laminin (LN), and procollagen III N-terminal peptide (P_{III}NP) were determined by radioimmunoassay.

RESULTS: Pathologic grading showed that the fibrogenesis was much less severe in CCl₄ + MEL3 group than in model control group ($\mu = 2.172$, $P < 0.05$), indicating that melatonin (10 mg/kg) can significantly ameliorate CCl₄-induced hepatic fibrotic changes. The serum levels of ALT and AST were markedly lower in CCl₄ + MEL treatment groups (5, 10 mg/kg) than in model control group (ALT: 286.23 ± 121.91 U/L vs 201.15 ± 101.16 U/L and 178.67 ± 103.14 U/L, $P = 0.028$, $P = 0.007$; AST: 431.00 ± 166.35 U/L vs 321.23 ± 162.48 U/L and 292.42 ± 126.23 U/L, $P = 0.043$, $P = 0.013$). Similarly, the serum laminin (LN) and hyaluronic acid (HA) levels and hydroxyproline (HYP) contents in liver were significantly lower in CCl₄ + MEL-3 group (10 mg/kg) than in model control group (LN: 45.89 ± 11.71 μ g/L vs 55.26 ± 12.30 μ g/L, $P = 0.012$; HA: 135.71 ± 76.03 μ g/L vs 201.10 ± 68.46 μ g/L, $P = 0.020$; HYP: 0.42 ± 0.08 mg/g tissue vs 0.51 ± 0.07 mg/g tissue, $P = 0.012$). Moreover, treatment with melatonin (5, 10 mg/kg) significantly reduced the MDA content and increased the GPx activity in liver homogenates compared with model control group (MDA: 7.89 ± 1.49 noml/mg prot vs 6.29 ± 1.42 noml/mg prot and 6.25 ± 2.27 noml/mg prot, respectively, $P = 0.015$, $P = 0.015$; GPx: 49.13 ± 8.72 U/mg prot vs 57.38 ± 7.65 U/mg prot and 61.39 ± 13.15 U/mg prot, respectively, $P = 0.035$, $P = 0.003$).

CONCLUSION: Melatonin can ameliorate CCl₄-induced hepatic fibrosis in rats. The protective effect of melatonin on hepatic fibrosis may be related to its antioxidant activities.

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Key words: Melatonin; Hepatic fibrosis; Oxidative stress; Hyaluronic acid; Laminin; Malondialdehyde; Glutathione peroxidase

Peer reviewers: Wendy M Mars, PhD, Department of Pathology, University of Pittsburgh, S-411B South Biomedical

Science Tower Pittsburgh, PA 15261, United States; Ana Cristina Simões e Silva, MD, PhD, Professor, Faculdade de Medicina UFMG, Departamento de Pediatria, sala 267, Avenida Professor Alfredo Balena, 190, Bairro Santa Efigênia, Belo Horizonte, Minas Gerais 30130-100, Brazil

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INTRODUCTION

Hepatic fibrosis, a common pathological process of chronic hepatic disease, can lead to irreversible cirrhosis, and involves multiple cellular and molecular events that ultimately result in accumulation of collagen and extra cellular matrix protein in space of Disse. If treated properly at fibrosis stage, cirrhosis can be prevented^[1]. However, no effective antifibrosis drugs are available at present. Several lines of evidence suggest that oxidative stress plays an important role in the etiopathogenesis of hepatic fibrosis^[2,3].

Melatonin (N-acetyl-5-methoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant, regulates circadian rhythms, sleep and immune system activity, behaves as a free radical scavenger^[4], eliminates oxygen free radicals and reactive intermediates^[5-9]. Both *in vitro* and *in vivo* experiments have shown that melatonin can protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes, such as safrrole, lipopolysaccharide (LPS), carbon tetrachloride (CCl₄), ischemia-reperfusion, amyloid-protein, and ionizing radiation^[10-12]. In addition, melatonin also has an indirect antioxidant effect by enhancing the levels of potential antioxidants such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione (GSH)^[10-12]. Recent studies showed that melatonin exerts its cytoprotective effects in various experimental models of acute liver injury and reduces fibroblast proliferation and collagen synthesis^[12,13], indicating that melatonin may have therapeutic effects on acute and chronic liver injury, through its antioxidant action.

The aim of our present study was to evaluate the possible antifibrotic effect of melatonin on a hepatic fibrosis model of rats. In addition, the antioxidant and anti-inflammatory properties of melatonin were investigated in rats with liver fibrosis.

MATERIALS AND METHODS

Drugs and materials

Crystalline melatonin was purchased from Sigma Chemical Company (St. Louis, MO, USA). The solvent used for melatonin was a mixture of ethanol (1%, v/v) and NaCl (0.9%). N-acetyl-L-cysteine (NAC) was purchased from Shanghai Sinopharm Chemical Reagent

Co. Ltd (Shanghai, China). Commercial kits used for determining malondialdehyde (MDA), glutathione peroxidase (GPx) and hydroxyproline (HYP) were obtained from Jiancheng Institute of Biotechnology (Nanjing, China). Commercial kits for radioimmunoassay of procollagen III N-terminal peptide (P_{III}NP), laminin (LN), and hyaluronic acid (HA) were obtained from Beijing North Institute of Biological Technology (Beijing, China). Other commercial chemicals used in experiments were of analytical grade.

Animal experiments and drug treatment

Male Sprague-Dawley rats, weighing 170-240 g at beginning of the study, purchased from Anli Experimental Animal Limited Company (Anhui, China), were kept at a constant temperature (22°C) in a 12-h light and dark cycle, with free access to food and water. All animals were treated humanely according to the National Guidelines for the Care of Animals in China. Rats were randomly divided into normal control group (*n* = 11), model control group (*n* = 20) treated with CCl₄ for 12 wk, CCl₄ + NAC group (*n* = 20) treated with CCl₄ + NAC (100 mg/kg, i.p.) for 12 wk, CCl₄ + MEL-1 group (*n* = 20) treated with CCl₄ + melatonin (2.5 mg/kg) for 12 wk, CCl₄ + MEL-2 group (*n* = 20) treated with CCl₄ + melatonin (5.0 mg/kg) for 12 wk, and CCl₄ + MEL-3 group (*n* = 20) treated with CCl₄ + melatonin (10 mg/kg) for 12 wk. Rats in treatment groups were injected subcutaneously with sterile CCl₄ (3 mL/kg of body weight) in a ratio of 2:3 with olive oil twice a week. Rats in normal control group received hypodermic injection of olive oil at the same dose and frequency as those in the treatment groups. At the beginning of CCl₄ injection, rats received intraperitoneal melatonin daily whereas rats that did not receive melatonin were given the same volume of vehicle (1% ethanol) at the same time point. After 12 wk, a laparotomy was performed and blood was drawn from the abdominal aorta under 3% pentobarbital sodium (1 mL/kg) anesthesia. The animals were then killed with their livers removed. Blood was collected into tubes and centrifuged. Serum was aspirated and stored at -80°C. Liver tissue was fixed in formalin for routine histological examination, or stored at -80°C until required.

Histopathological examination

Liver tissue samples, fixed in 40 g/L paraformaldehyde and embedded in paraffin, were cut into 5- μ m thick sections, which were stained with hematoxylin and eosin (HE) and Van Gieson (VG) according to the standard procedure. Van Gieson's method was used to detect collagen fibers. Hepatic fibrosis was divided into the following stages as previously described^[14]: stage 0: no fibrosis; stage 1: expansion of portal tracts without linkage; stage 2: portal expansion with portal to portal linkage; stage 3: expansive portal to portal and focal portal to central linkage; and stage 4: cirrhosis. Two pathologists with no knowledge of liver sources examined the stained sections independently.

Analysis of liver function

Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and protein concentration were measured with routine laboratory methods using an autoanalyzer (Hitachi Automatic Analyzer, Japan).

Measurement of MDA and GPx levels in liver homogenates

Liver samples were thawed, weighed and homogenized (1:9 w:v) in 0.9% saline. The homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C and supernatant was taken for assay of MDA and GPx with a commercial kit (Jiancheng Institute of Biotechnology, Nanjing, China) following its manufacturer's instructions. MDA was assayed by measuring the levels of thiobarbituric acid reactive substances (TBARS) at 532 nm and expressed as nmol/mg protein. GPx assay was based on its ability to inhibit oxidation of oxyamine by the xanthine-xanthine oxidase system. Total protein concentration in liver homogenates was determined using the Coomassie blue method with bovine serum albumin as a standard.

Detection of hydroxyproline content in liver

Total collagen content in fresh liver samples was determined by hydroxyproline assay. Hydroxyproline content was detected with a commercial hydroxyproline detection kit (Jiancheng Institute of Biotechnology, Nanjing, China) following its manufacturer's instructions.

Measurement of serum HA, LN, and PIII NP levels

Serum HA, LN and PIII NP levels were measured by radioimmunoassay with a commercial kit according to its manufacturer's instructions (Beijing North Institute of Biological Technology, Beijing, China).

Statistical analysis

Data were analyzed with SPSS software. Quantitative data were presented as mean \pm SD and analyzed by one way ANOVA analysis. Frequency data (pathologic grading of hepatic fibrosis) were analyzed by Ridit analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Pathological changes

Degeneration, necrosis, infiltration of inflammatory cells, and collagen deposition were found in liver tissues of model control group, CCl₄ + NAC group and 3 melatonin treatment groups (Figure 1C-F). Liver tissue samples from rats in normal control group showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A and B). Formation of fibrotic septa encompassing regenerated hepatocytes was observed in liver tissue samples from rats in model group (Figure 1D). A large number of inflammatory cells infiltrated intra- and interlobular regions (Figure 1C). Statistical analysis revealed significant differences in pathologic grading between CCl₄ + MEL3 group

Table 1 Pathologic grading of hepatic fibrosis in different groups

Group	Dose (mg/kg)	n	Pathologic grading of hepatic fibrosis					u value	
			0	I	II	III	IV		
Normal	-	11	11	0	0	0	0	0	5.5681 ^b
Model	-	13	0	0	1	6	6	-	-
NAC	100	12	0	2	5	3	2	1.8838	-
MEL	2.5	11	0	2	4	2	3	1.5568	-
	5	13	0	2	4	3	4	1.3662	-
	10	12	0	4	4	1	3	2.1720 ^a	-

u represents the Ridit value of the two groups, $P < 0.05$ indicates $u > 1.96$; $P < 0.01$ indicates $u > 2.58$; ^a $P < 0.05$, ^b $P < 0.01$ vs model group.

Table 2 Effect of melatonin on serum ALT, AST levels and A/G value in different groups (mean \pm SD)

Group	Dose (mg/kg)	n	ALT (U/L)	AST (U/L)	A/G
Normal	-	11	70.00 \pm 35.27	139.82 \pm 72.83	0.94 \pm 0.40
Model	-	13	286.23 \pm 121.91 ^b	431.00 \pm 166.35 ^b	0.74 \pm 0.09
NAC	100	12	194.42 \pm 90.83 ^{b,c}	293.33 \pm 94.60 ^{b,c}	0.78 \pm 0.11
MEL	2.5	11	211.09 \pm 97.03 ^b	357.09 \pm 153.26 ^b	0.68 \pm 0.15
	5	13	201.15 \pm 101.16 ^{b,c}	321.23 \pm 162.48 ^{b,c}	0.77 \pm 0.15
	10	12	178.67 \pm 103.14 ^{b,d}	292.42 \pm 126.23 ^{b,c}	0.73 \pm 0.07

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; A: Albumin; G: Globulin. ^b $P < 0.01$ vs normal control group; ^c $P < 0.05$ vs model control group; ^d $P < 0.01$ vs model control group.

and model control group ($P < 0.05$), indicating that fibrogenesis was much less severe in CCl₄ + MEL3 group than in model control group (Table 1, Figure 1C-F).

Detection of liver function

The serum ALT and AST levels were significantly higher in experimental and model groups than in normal control group ($P < 0.01$). The ALT and AST levels were significantly higher in model group than in CCl₄ + NAC and CCl₄ + MEL groups (5, 10 mg/kg) ($P < 0.05$, $P < 0.01$). Melatonin (5, 10 mg/kg) significantly decreased the elevated serum transaminase levels (Table 2), whereas no significant difference in the ratio of A/G was observed between model control group, CCl₄ + NAC and CCl₄ + MEL groups (Table 2).

MDA content and GPx activity in liver homogenates

The MDA level was significantly higher while GPx activity was significantly lower in liver homogenates of CCl₄ + NAC and CCl₄ + MEL groups than in those of normal control group ($P < 0.01$). The MDA level was significantly higher in model control group than in CCl₄ + NAC and CCl₄ + MEL groups (5, 10 mg/kg) ($P < 0.05$). Melatonin (5, 10 mg/kg) significantly blocked the elevated MDA level. GPx activity was significantly lower in the model control group than in CCl₄ + NAC and CCl₄ + MEL groups (5, 10 mg/kg) ($P < 0.05$, $P < 0.01$, Table 3).

HYP contents in liver tissue

Hepatic fibrosis was quantified by measuring hepatic

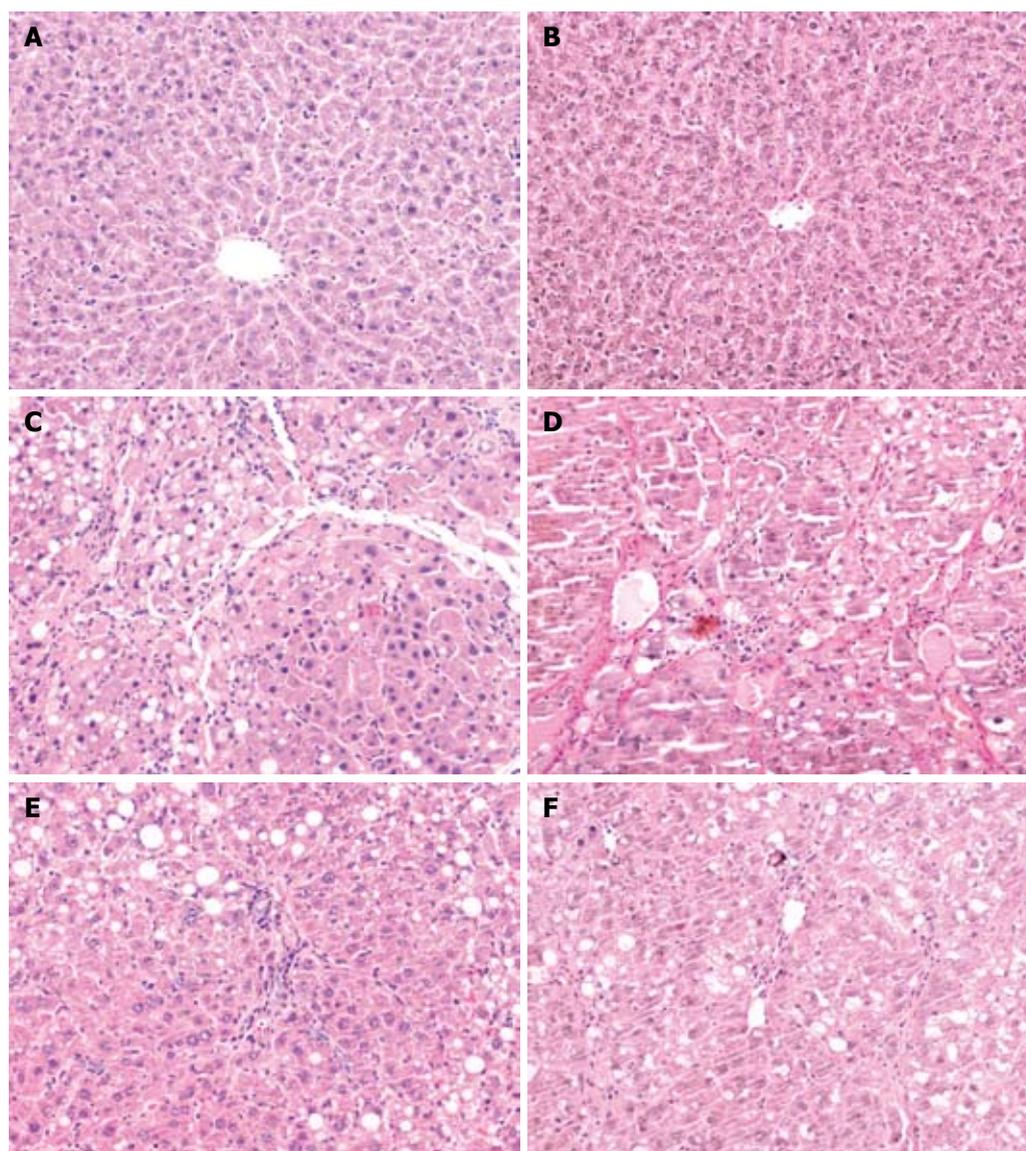


Figure 1 Pathological changes. Light microscopy of liver tissue sections showing normal liver lobular architecture with central veins in the normal control group (HE staining, × 200) (A), no collagen deposition in liver of normal control group (VG staining, × 200) (B), degenerated and necrotic liver cells associated with inflammatory cells in model group (HE staining, × 200) (C), formation of fibrotic septa in model group (VG staining, × 200) (D), and pathological change in liver of CCl₄ + melatonin (10 mg/kg) group was rather milder compared with the model group (HE staining, × 200; VG staining, × 200) (E, F).

Group	Dose (mg/kg)	n	MDA (noml/mg prot)	GPx (U/mg prot)
Normal	-	11	4.3 ± 1.87	80.68 ± 10.76
Model	-	13	7.89 ± 1.49 ^b	49.13 ± 8.72 ^b
NAC	100	12	6.29 ± 1.36 ^{b,c}	64.68 ± 8.22 ^{b,d}
MEL	2.5	11	6.84 ± 1.10 ^b	53.44 ± 9.35 ^b
	5	13	6.29 ± 1.42 ^{b,c}	57.38 ± 7.65 ^{b,c}
	10	12	6.25 ± 2.27 ^{b,c}	61.39 ± 13.15 ^{b,d}

MDA: Malondialdehyde; GPx: Glutathione peroxidase. ^b*P* < 0.01 *vs* normal control group; ^c*P* < 0.05 *vs* model control group; ^d*P* < 0.01 *vs* model control group.

hydroxyproline. The hydroxyproline content was significantly higher in model control, CCl₄ + NAC and CCl₄ + MEL groups than in normal control group (*P* < 0.01), and significantly higher in model group than

in CCl₄ + NAC and CCl₄ + MEL groups (10 mg/kg) (*P* < 0.05). Treatment with melatonin (10 mg/kg) or NAC reduced the hydroxyproline content in liver homogenates, and therefore prevented hepatic fibrosis induced by CCl₄ (Figure 2).

Measurement of serum HA, LN, and P_{III}NP levels

The serum LN and HA levels were significantly higher in model control, CCl₄ + NAC, and CCl₄ + MEL groups than in normal control group (*P* < 0.05, *P* < 0.01), and significantly decreased after treatment with melatonin (10 mg/kg) (*P* < 0.05). Treatment with NAC significantly reduced the serum HA level (*P* < 0.05). The serum P_{III}NP level was significantly higher in model control, CCl₄ + NAC and CCl₄ + MEL groups than in normal control group (*P* < 0.05). However, no significant difference was observed among the five groups (Table 4).

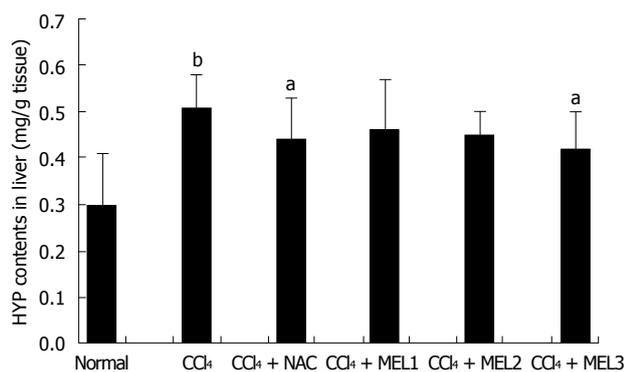


Figure 2 Effect of melatonin on hydroxyproline content in liver of rats with fibrosis fibrotic induced by CCl₄. ^a*P* < 0.05 vs model control group; ^b*P* < 0.01 vs normal control group.

Table 4 Serum HA, LN and PIII^{NP} levels in different groups (mean ± SD)

Group	Dose (mg/kg)	<i>n</i>	HA (μg/L)	LN (μg/L)	PIII ^{NP} (μg/L)
Normal	-	11	71.65 ± 27.64	37.65 ± 6.09	26.41 ± 7.28
Model	-	13	201.10 ± 68.46 ^b	55.26 ± 12.30 ^b	35.88 ± 5.92 ^b
NAC	100	12	131.31 ± 58.58 ^{ac}	48.28 ± 4.93 ^b	32.89 ± 3.90 ^b
MEL	2.5	11	174.41 ± 72.99 ^b	48.15 ± 6.40 ^b	34.21 ± 5.76 ^b
	5	13	153.54 ± 86.19 ^b	48.22 ± 9.51 ^b	33.12 ± 4.71 ^b
	10	12	135.71 ± 76.03 ^{ac}	45.89 ± 11.71 ^{ac}	31.99 ± 6.09 ^a

HA: Hyaluronic acid; LN: Laminin; PIII^{NP}: Procollagen III N-terminal peptide. ^a*P* < 0.05 vs normal control group; ^b*P* < 0.01 vs normal control group; ^c*P* < 0.05 vs model control group.

DISCUSSION

CCl₄ is widely used to induce hepatic fibrosis and cirrhosis in animal models. In this study, hepatic fibrosis was successfully induced by subcutaneous injection of sterile CCl₄ twice weekly for 12 wk. Through this hepatic fibrosis model, the effects of melatonin on hepatic fibrosis induced by CCl₄ in rats were examined.

N-acetylcysteine (NAC), a free radical scavenger, is a glutathione precursor which increases glutathione levels in hepatocytes^[15,16]. Increased glutathione levels limit the production of reactive oxygen species (ROS) which can cause hepatocellular injury. NAC can also inhibit the proliferation of hepatic stellate cells^[17]. Therefore, it was used as a positive control in this study.

It is well known that oxidative damage can induce hepatic fibrogenesis. ROS, such as H₂O₂, O₂⁻, and ·OH, are implicated in the development and pathological progress of hepatic fibrosis^[18,19]. Free radicals and biomolecular reaction products promote phagocytic and myofibroblastic activities. Lipid peroxidation accelerates collagen synthesis by stimulating stellate cells^[20]. It has been shown that melatonin is an effective antioxidant and a free radical scavenger. Due to its small size and high lipophilicity, melatonin can cross biological membranes easily and reach all compartments within the cell^[21], thus protecting DNA, proteins, and biological membrane lipids from the deleterious effects of free radicals^[22]. It has been found that melatonin has a higher antioxidant

efficiency than vitamin E and GSH, which are known as powerful antioxidants^[10]. The antioxidant properties of melatonin prevent acute liver injury induced by ischemia-reperfusion^[23], irradiation^[24], bile duct ligation^[25-27], and toxins^[18,28,29]. Several lines of evidence suggest that melatonin plays an important role in regulation of collagen levels and inhibition of collagen accumulation. Ostrowska *et al*^[30] showed that melatonin is negatively related with urine hydroxyproline levels in fasting rats. Cunnane *et al*^[31] demonstrated that primary biliary cirrhosis is related with melatonin deficiency in pinealectomized rats. Tahan *et al*^[12] reported that daily melatonin injection at pharmacological doses is effective against liver damage in a rat liver fibrosis model induced by a 14-d dimethylnitrosamine regimen. In the present study, liver injury was assessed with histological and biochemical parameters. The results suggest that melatonin could decrease the scores of hepatic fibrosis and serum ALT and AST levels in rats with hepatic injury caused by CCl₄. Melatonin at a dose of 10 mg/kg was as effective as 100 mg/kg NAC in reducing serum ALT and AST levels, indicating that melatonin can protect liver and alleviate the progression of hepatic fibrosis. However, further study is needed on the liver function protective effect of melatonin in cirrhotic patients.

HA and LN are known to be good serum markers of hepatic fibrogenesis^[32-34]. HYP in liver is an important index reflecting the degree of hepatic fibrosis and hepatic fibrosis can be quantified by measuring hepatic hydroxyproline^[33,35]. In the present study, treatment with melatonin (10 mg/kg) could significantly reduce HA and LN in serum and HYP in liver. The decreased of hepatic hydroxyproline and serum LN and HA levels indicate that melatonin can inhibit collagen deposition in liver.

Oxidative stress plays an important role in the formation of hepatic fibrosis *via* increasing stellate cell activation and collagen synthesis. MDA is the main product of lipid peroxidation and its concentration is generally presented as the total level of lipid peroxidation products^[36]. As an end product of lipid peroxidation, MDA can produce ozone, which reacts rapidly with cellular structures, generates hydrogen peroxide and other reactive oxygen species, leading to peroxidation and denaturation of membranes^[37]. It has been shown that MDA can activate stellate cells that produce collagen. The results of this study suggest that treatment with melatonin (5, 10 mg/kg) could significantly block increased MDA, suggesting that melatonin decreases lipid peroxidation and plays an anti-oxidative role in hepatic fibrosis induced by CCl₄ in rats.

Melatonin is not only a direct antioxidant but also an indirect antioxidant through enhancement of antioxidant enzyme activities in liver^[38]. It was reported that melatonin can reduce free radical damage by elevating GPx activation^[11,39]. Montilla *et al*^[25] reported that acute ligation of the bile duct is accompanied with decreased GSH levels both in plasma and in liver, as well as significantly reduced antioxidant enzyme activities. Treatment with melatonin is associated with a significant recovery of anti-oxidative enzymes such as

GPx^[25]. Tahan *et al*^[12] found that melatonin can restore GPx activity in a rat liver fibrosis model induced by a 14-d dimethylnitrosamine regimen. In this study, the GPx activity was significantly lower in model control group than in CCl₄ + NAC and CCl₄ + MEL groups (5, 10 mg/kg), indicating that melatonin can protect liver against CCl₄-induced hepatic fibrosis in rats, possibly through its direct and indirect antioxidant effects.

In conclusion, melatonin may have beneficial effects on hepatic fibrosis induced by CCl₄ in rats. The protective effect of melatonin on hepatic fibrosis may be related to its antioxidant activities.

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COMMENTS

Background

In China, the incidence of liver cirrhosis is still high. Liver cirrhosis results from fibrosis. If treated properly at fibrosis stage, cirrhosis can be prevented. However, no effective antifibrosis drugs are available at present. Several lines of evidences suggest that oxidative stress plays an important role in the etiopathogenesis of hepatic fibrosis. Melatonin can protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes. The possible fibrosuppressant effect of melatonin on hepatic fibrosis was evaluated in this study. In addition, the antioxidant and anti-inflammatory properties of melatonin were investigated in rats with fibrosis.

Research frontiers

Although the exact pathogenesis of hepatic fibrosis is still obscure, the role of free radicals and lipid peroxides in the development of hepatic fibrosis has attracted considerable attention. If treated properly at this stage, cirrhosis can be successfully prevented. However, it remains a problem to prevent hepatic fibrosis or to control its progression. Great efforts have been made to find safe and effective drugs. Recent experiments demonstrate that melatonin may have therapeutic effects on acute and chronic liver injury, possibly through its antioxidant activities.

Innovations and breakthroughs

Melatonin may have beneficial effects on hepatic fibrosis induced by carbon tetrachloride in rats. The protective effect of melatonin may be related to its antioxidant activities.

Applications

Melatonin can be used as an antifibrotic drug, protect liver cells against fibrosis and inhibits collagen fiber deposition in liver, thus providing a basis for further studies on its therapeutic effect on hepatic fibrosis.

Terminology

Melatonin (N-acetyl-5-methoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant. It regulates circadian rhythms, sleep and immune system activity, behaves as a free radical scavenger, and eliminates oxygen free radicals and reactive intermediates.

Peer review

This is a well-designed study describing the protective effect of melatonin on fibrosis induced by carbon tetrachloride. Methods are appropriate and results are consistent with the conclusion. The study is very interesting with a great amount of data, which corroborate the major conclusion.

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Risk of contrast-induced nephropathy in hospitalized patients with cirrhosis

Nilesh Lodhia, Michael Kader, Thalia Mayes, Parvez Mantry, Benedict Maliakkal

Nilesh Lodhia, Michael Kader, Thalia Mayes, Parvez Mantry, Benedict Maliakkal, Department of Medicine, Division of Gastroenterology and Hepatology, University of Rochester School of Medicine and Dentistry, Box 646 601, Elmwood Avenue, Rochester, NY 14642, United States

Author contributions: Maliakkal B and Mantry P designed the research; Kader M and Mayes T performed the research; Lodhia N, Kader M, Mayes T, Mantry P and Maliakkal B analyzed the data; Lodhia N and Maliakkal B wrote the paper.

Correspondence to: Benedict Maliakkal, MD, Department of Medicine, Division of Gastroenterology and Hepatology, University of Rochester School of Medicine and Dentistry, Box 646 601, Elmwood Avenue, Rochester, NY 14642,

United States. benedict_maliakkal@urmc.rochester.edu

Telephone: +1-585-2754711 Fax: +1-585-2717868

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cirrhotic patients, especially those with ascites, the risk of CIN is substantial.

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Peer reviewer: Mercedes Susan Mandell, MD, PhD, Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

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Abstract

AIM: To evaluate the incidence of contrast-induced nephropathy (CIN) in cirrhotic patients and to identify risk factors for the development of CIN.

METHODS: We performed a retrospective review of 216 consecutive patients with cirrhosis who underwent computed tomography (CT) with intravenous contrast at the University of Rochester between the years 2000-2005. We retrospectively examined factors associated with a high risk for CIN, defined as a decrease in creatinine clearance of 25% or greater within one week after receiving contrast.

RESULTS: Twenty-five percent of our patients developed CIN, and 74% of these patients had ascites seen on CT. Of the 75% of patients who did not develop CIN, only 46% had ascites. The presence of ascites was a significant risk factor for the development of CIN ($P = 0.0009$, OR 3.38, 95% CI 1.55-7.34) in multivariate analysis. Patient age, serum sodium, Model for End-stage Liver Disease score, diuretic use, and the presence of diabetes were not found to be significant risk factors for the development of CIN. Of the patients who developed CIN, 11% developed chronic renal insufficiency, defined as a creatinine clearance less than baseline for 6 wk.

CONCLUSION: Our results suggest that in hospitalized

INTRODUCTION

Renal failure is associated with a high morbidity and mortality in patients with cirrhosis^[1-4]. Cirrhotic patients may be particularly predisposed to renal failure because of intravascular volume depletion, hyperaldosteronism, and altered renal hemodynamics^[5]. Furthermore, aggressive use of diuretics, repeated large volume paracenteses, and gastrointestinal bleeding often contribute to renal insufficiency in these patients^[1,2].

Contrast-induced nephropathy (CIN) is a common cause of renal failure, and is associated with substantial morbidity and mortality^[6-9]. Multiple studies in the medical literature have estimated a risk of 2% in low-risk patients, rising to 50% in those with risk factors such as diabetes mellitus (DM), pre-existing renal disease, congestive heart failure, advanced age, anemia, and dehydration^[10-15].

Although cirrhosis has been suggested as a risk factor for CIN^[13-17], only two studies, to our knowledge, have specifically investigated this issue. A prospective study by Guevara *et al*^[18] did not find an increased susceptibility to CIN in cirrhotic patients. Although this was a well-conducted study, it was conducted under idealized circumstances. Our goal was to conduct a study that may be more representative of the cirrhotic patients we encounter on a frequent basis^[18]. The study by Najjar

et al^[19] was a retrospective study which also did not find an increased prevalence of CIN in cirrhotics. However, the authors did not precisely define CIN, making it difficult to interpret the results of the study^[15]. We believe that cirrhosis is a risk factor for the development of CIN, and we aimed to identify risk factors for the development of CIN. We thus performed a retrospective review of cirrhotics who received iodinated contrast during hospitalization for further analysis.

MATERIALS AND METHODS

After approval by the University of Rochester's Institutional Review Board, we reviewed the charts of 347 patients with cirrhosis who underwent computed tomography (CT) with intravenous contrast during hospitalization at our institution between the years 2000-2005. Inclusion criteria consisted of the presence of cirrhosis, inpatient hospitalization, and the use of iodinated contrast for CT. Patients with pre-existing sepsis, known congestive heart failure, gastrointestinal bleeding, spontaneous bacterial peritonitis (SBP), and chronic kidney disease (CKD), defined as baseline Cr > 18 mg/L, were excluded from our study. Patients who received peri-contrast intravenous sodium chloride, sodium bicarbonate, N-acetylcysteine, as well as those whose diuretic therapy was held during the day of contrast exposure were also excluded from our study.

Clinical data that were reviewed included the Model for End-stage Liver Disease (MELD) score, use of diuretics, serum sodium, a documented diagnosis of diabetes mellitus (DM) in the medical record, and the presence of ascites seen on CT. The use of potentially nephrotoxic medications, such as angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin receptor blockers (ARBs), nonsteroidal anti-inflammatory drugs (NSAIDs), and aspirin was documented. The age and race of each patient were also noted. Statistical analysis was performed by faculty of the Department of Biostatistics at the University of Rochester. Multivariate and univariate analyses were performed using logistic regression, and odds ratios were calculated.

All patients received 150 mL of iodinated contrast dye (iohexol, Omnipaque) intravenously per standard radiology protocol. Post-contrast creatinine was then reviewed and compared to baseline values. Serum creatinine on the day of contrast exposure was used to calculate the pre-contrast creatinine clearance (CrCl). Post-contrast CrCl was determined by the highest recorded creatinine value within one week after contrast exposure. The aim of this study was to evaluate the development of CIN, which we defined as a decrease in CrCl of 25% or greater, temporally associated with the use of contrast. CrCl was estimated using the Cockcroft-Gault equation. Patients who developed CIN were followed for 6 wk to assess the development of chronic renal insufficiency (CRI), defined as a CrCl less than baseline for 6 wk. The need for dialysis during this time period was also documented.

Table 1 Demographic data of the 216 patients included in the study

Factor	Patients	Percentage
Mean age	53.2	N/A
> 65 YO	34	16
< 65 YO	182	84
Male	128	59
Female	88	41
Caucasian	158	73
African American	37	17
Hispanic	10	4
Asian	3	1
Unknown	8	5

Table 2 Characteristics of patients who developed CIN (*n* = 53) vs those who did not develop CIN (*n* = 163)

Factor	Percent with CIN	Percent without CIN	P-value
Ascites	74	46	0.0009
MELD > 15	60	48	0.1774
Age > 65	9	18	0.2171
On diuretics	66	57	0.3200
Na ≥ 130	91	88	0.8449

Table 3 Relationship of ascites to incidence of CIN

Factor	Patients	Percentage
Ascites	114	53
CIN	39	34
No CIN	75	66
No ascites	102	47
CIN	14	14
No CIN	88	86

Ascites was a significant risk factor for the development of CIN (*P* = 0.0009, OR 3.38, CI 1.55-7.34).

RESULTS

A total of 216 patients met the inclusion criteria. 34 patients were greater than 65 years of age (16%). The mean age was 53.2 years. 158 patients were Caucasian, 37 were African American, 10 were Hispanic, and 3 were Asian (Table 1). The mean MELD score for all patients was 15.3.

A total of 53 (25%) patients developed CIN. Baseline characteristics of these patients included a mean MELD of 17, mean age of 51.9, and mean serum sodium of 134.7 mmol/L. In 36 of these patients (68%), renal insufficiency persisted for at least one week. A total of 39 (74%) of these patients had ascites (Table 2). Although 6 patients (11%) developed CRI, none of those patients required dialysis during our 6 wk review.

A total of 163 patients (75%) did not develop CIN. Their mean MELD score was 14.8, mean age was 53.6, and mean sodium was 135.8 mmol/L. Seventy-five of these patients (46%) had ascites seen on CT (Table 2).

The presence of ascites was a significant risk factor for the development of CIN (*P* = 0.0009, OR 3.38, 95% CI 1.55-7.34) in multivariate analyses (Table 3, Figure 1).

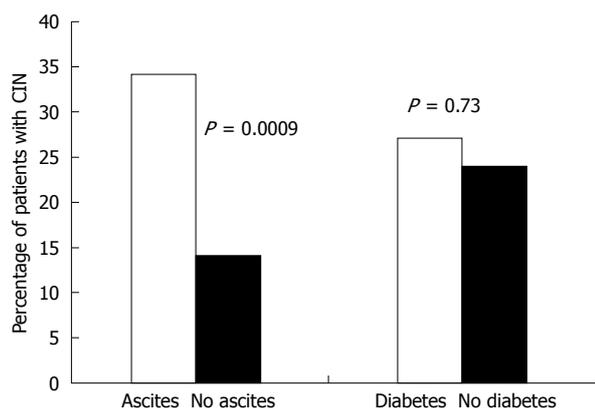


Figure 1 Percentage of patients experiencing CIN in the presence or absence of ascites ($P = 0.0009$) or diabetes ($P = 0.73$).

A total of 33 patients were on ACE inhibitors, ARBs, NSAIDs, or aspirin. Ascites remained a significant risk factor when these patients were excluded from this analysis ($P = 0.00006$, OR 3.98, 95% CI 1.83-8.69). Ascites was also a significant risk factor for the development of CRI, as 5/6 patients (83%) who developed CRI had ascites seen on CT scan. Age, serum sodium, and MELD score were not found to be significantly associated with a higher risk of CIN in multivariate analysis. Our analysis also did not show a significant association between the use of diuretics and an increased risk of CIN in patients with or without ascites (Figure 2).

In our study, the presence of DM was not a predisposing factor to CIN (Figure 1). A total of 66 diabetic patients were included in the analysis. The incidence of CIN was 18/66 (27%) in these patients, a nonsignificant difference compared to nondiabetics, where the incidence of CIN was 36/150 (24%). Among the total number of diabetic patients, three had evidence of mild kidney disease prior to the scan, defined as Cr > 15 mg/L. Only one (33%) of these patients developed CIN.

DISCUSSION

Intravenous contrast remains an important cause of acute renal failure in patients who receive CT scans. There is little data on whether the presence of cirrhosis serves as an important risk factor for the development of CIN.

We performed a large retrospective review at our institution of hospitalized cirrhotic patients who received intravenous contrast for CT imaging and found that there was a high rate of CIN. In multivariate analysis, the presence of ascites was a significant risk factor for the development of CIN, conferring over three times the risk compared to the absence of ascites. Factors such as MELD score, serum sodium, diuretic use, the presence of DM, and age failed to show a similar association.

The results of this study are dissimilar to those found in a prospective study by Guevara *et al.*^[18], who did not find an increased susceptibility to CIN in cirrhotic patients^[18]. However, this study was limited by a relatively small sample size and diuretic therapy was withheld for

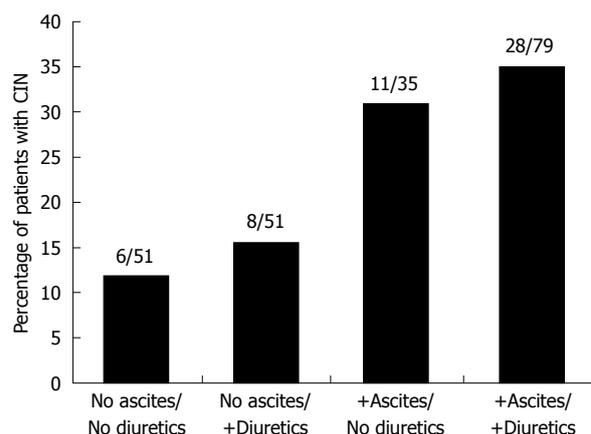


Figure 2 Percentage of patients experiencing CIN in relation to diuretic use and the presence of ascites.

at least 5 days prior to inclusion in the study. Our study included patients on diuretic therapy, and is therefore reflective of a broad range of cirrhotic patients.

A second retrospective study by Najjar *et al.*^[19] comparing 72 cirrhotic patients receiving intravenous contrast with 72 non-cirrhotic controls, revealed the development of CIN in 2 patients with cirrhosis (2.8%) and in 1 patient in the control group (1.4%), a non-significant difference. The authors of this study concluded that cirrhosis may not be a risk factor for CIN. However, the results of this study should be interpreted with caution because the study does not clearly define CIN^[19]. Without a precise definition of CIN, it is difficult to interpret the results of this study.

The exact mechanism by which iodinated contrast agents induce renal dysfunction is not entirely understood. The pathophysiology is complex, and a variety of factors act in concert to induce CIN. *In vitro* and animal studies suggest that damage secondary to iodinated contrast to the kidneys is likely mediated by a combination of toxic or obstructive injury to the renal tubules, ischemic injury by reactive oxygen species, and renal medullary hypoxia. The predominant factor is likely to be renal medullary hypoxia, in which adenosine, calcium, and endothelin bring about intrarenal vasoconstriction after contrast exposure^[17,20-25]. Tubular toxicity is also thought to play a role in CIN through both direct nephrotoxicity and tubular obstruction. The generation of reactive oxygen species can cause toxic, ischemic, and immune-mediated direct nephrotoxicity^[26-28]. Contrast dye increases urate excretion and leads to the deposition of Tamm-Horsfall proteins within the renal tubules, both of which can cause tubular obstruction^[29-31].

Factors such as age, serum sodium, MELD score, and diuretic use were not found to be associated with the development of CIN. It is surprising that there is no statistically significant relationship between diuretic use and CIN in our study. There are a fair number of studies suggesting that peri-contrast hydration can reduce the incidence of CIN^[32-36]. Furthermore, prophylactic forced diuresis with furosemide has been shown to

augment the risks of CIN^[32]. However, there are no data, to our knowledge, on the association between maintenance diuretic therapy and CIN. Although an increased frequency of CIN in the setting of diuretic use is biologically plausible, this relationship should be explored further before any conclusions can be made.

In our analysis, the presence of DM did not confer an increased risk of CIN. Although DM is considered to be a risk factor for CIN, the data on whether this relationship is present independent of underlying renal impairment are conflicting^[7,8,37]. Only three patients with DM in our analysis had evidence of mild kidney disease before receiving contrast, and one of these developed CIN. Because our study was retrospective, we were unable to assess our patients for the presence of microalbuminuria or overt proteinuria. Furthermore, our exclusion criteria of heart failure and CKD may have excluded many diabetic patients with significant vascular disease. Our sample may thus have consisted of a higher proportion of patients with uncomplicated diabetes and therefore at a lower risk of CIN.

In those patients that developed CIN, a large proportion (68%) had CIN that persisted for at least one week. 11% of these patients developed CRI as a possible result of contrast exposure. Although none of these patients required dialysis, even transient elevations in creatinine without progression to dialysis have been associated with prolonged hospital stay, adverse cardiac events, and increased mortality^[6-9].

There have been multiple studies performed investigating whether certain prophylactic regimens may reduce the risk of CIN. These have included agents such as N-acetylcysteine, diuretics, dopamine, hemofiltration, as well as hydration with sodium chloride or sodium bicarbonate^[38]. However, none of these have been performed in cirrhotic patients, and reviews of these trials have given discrepant results. Currently, only the use of low osmolality contrast medium at the lowest dose possible, in conjunction with saline hydration is recommended in a recent review article^[17].

The primary limitation of our study is the use of serum creatinine in determining the incidence of CIN. The assessment of renal function is notoriously difficult in patients with cirrhosis, and creatinine is likely a sub-optimal measure of renal function in these patients. Although we assessed CrCl rather than an absolute change in serum creatinine, studies suggest that creatinine-based formulas (e.g. Cockcroft-Gault, Modification of Diet in Renal Disease) can only provide a crude approximation of true glomerular filtration rate (GFR) in cirrhotics^[38-40]. However, direct measurement of kidney function (e.g. inulin clearance) is impractical in large cohorts, and cystatin C-based equations (e.g. Larsson and Hoek) are also unable to accurately assess GFR in patients with cirrhosis^[41]. Furthermore, the prognostic impact of serum creatinine in patients with cirrhosis is well-validated^[5,42-45], and its predictive value is reflected by its inclusion in the MELD score. Additionally, numerous studies have used either a 25%

rise in serum creatinine or a 25% decrease in estimated GFR to assess the development of CIN^[7-9,11,12,34,38]. While not ideal, we feel that using a 25% decrease in CrCl is an adequate means of detecting clinically significant CIN.

Another important limitation of our study is that we defined CIN based solely upon the peak CrCl within one week after receiving contrast. Although patients with heart failure, SBP, sepsis, and CKD were excluded from the study, there are other causes of increases in serum creatinine in cirrhotic patients exposed to intravenous contrast (e.g. hepatorenal syndrome, large volume paracenteses). Fluctuations in serum creatinine are common in the inpatient setting, and often no discernable cause is found for these variations^[46]. It is therefore possible that other factors may have contributed to alterations in CrCl in some of our patients. However, in hospitalized patients with no other apparent cause for a decline in renal function temporally associated with the administration of intravenous contrast, it is difficult to exclude CIN as a major contributing factor for this deterioration.

The retrospective nature of our study creates many limitations on our research, most notably the lack of a control group without intravenous contrast administration. The presence of a control group is particularly important when evaluating the incidence of CIN, as fluctuations in serum creatinine can have a multitude of causes. There are only two published studies, to our knowledge, that specifically compare the incidence of post-contrast renal dysfunction with the incidence of renal dysfunction in a control group of patients who did not receive intravenous contrast. Neither of these studies attributed a significant difference in the risk of renal failure to intravenous contrast, suggesting that the risk of CIN may be exaggerated. However, both of these studies were limited by a lack of randomization and a high threshold for the diagnosis of renal dysfunction (defined as a 50% increase in serum creatinine), and it is possible that differences in methodology may account for their findings^[47,48]. Nonetheless, future studies assessing the risk of CIN in cirrhotic patients would be strengthened by the inclusion of a parallel control group^[49,50].

It is also important to note that although diuretic use did not have an independent association with the development of CIN, there may be an association that our retrospective analysis was not able to elucidate. Patients with ascites are the most likely to receive high doses of diuretic therapy. It is difficult to sub-classify patients on the aggressiveness of their diuretic therapy, and we were thus unable to differentiate between patients on minimal doses of diuretics and those receiving aggressive diuresis. It is possible that high doses of diuretics may be a significant contributing factor to the development of CIN in patients with ascites. In patients with ascites receiving large doses of diuretic therapy, our retrospective study was unable to differentiate whether the development of CIN was from volume depletion from diuretic use or whether ascites was an independent predisposing factor.

In conclusion, our large retrospective study of hospitalized cirrhotic patients revealed a high incidence of CIN, especially in patients with ascites. CIN was associated with a significant percentage of patients progressing to CRI as a likely result of contrast exposure. These results suggest that in hospitalized cirrhotic patients, especially those with ascites, the risk of CIN is substantial. Alternative imaging strategies should be considered, and post-contrast renal function should be meticulously followed. Prospective studies evaluating the risk of contrast-induced nephropathy in cirrhotic patients, with and without the presence of ascites, should be performed for further investigation.

COMMENTS

Background

Contrast-Induced Nephropathy (CIN) is associated with substantial morbidity and mortality, especially in patients with cirrhosis. It is therefore important to determine whether cirrhosis is a risk factor for CIN, as well as to investigate which, if any, cirrhotic patients are particularly prone to CIN.

Research frontiers

The medical implications of CIN are substantial. As a result, there is a significant amount of research taking place on the identification of risk factors for CIN, as well as strategies to prevent its development. However, despite the vast amount of research on CIN, the data on the association between cirrhosis and CIN is scarce.

Applications

The study results suggest that in hospitalized cirrhotic patients, especially those with ascites, the risk of CIN is substantial.

Terminology

CIN: A decrease in CrCl of 25% or greater within one week after contrast exposure; Chronic Renal Insufficiency (CRI): a CrCl less than baseline for 6 wk; Chronic Kidney Disease (CKD): baseline Cr > 18 mg/L; Mild Kidney Disease: baseline Cr > 15 mg/L.

Peer review

Lodhia *et al* report the findings of a retrospective study that measured the risk of CIN in patients with cirrhosis. They found that the incidence of CIN was increased and that ascites was an independent risk factor for CIN. This is a very interesting and well written study.

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Interleukin-1 and TNF- α polymorphisms and *Helicobacter pylori* in a Brazilian Amazon population

Hivana Patricia Melo Barbosa, Luisa Caricio Martins, Sidney Emanuel Batista dos Santos, Samia Demachki, Mônica Baraúna Assumpção, Charliana Damasceno Aragão, Tereza Cristina de Oliveira Corvelo

Hivana Patricia Melo Barbosa, Charliana Damasceno Aragão, Tereza Cristina de Oliveira Corvelo, Laboratory of Immunogenetics, Federal University of Pará, 66075-110, Belém, Brazil

Luisa Caricio Martins, Nucleus of Tropical Medicine, Federal University of Pará, 66055-240, Belém, Brazil

Sidney Emanuel Batista dos Santos, Laboratory of Human and Medical Genetics, Federal University of Pará, 66075-110, Belém, Brazil

Samia Demachki, Department of Pathology, João de Barros Barreto University Hospital, Federal University of Pará, 66073-000, Belém, Brazil

Mônica Baraúna Assumpção, Department of Endoscopy, João de Barros Barreto University Hospital, Federal University of Pará, 66073-000, Belém, Brazil

Author contributions: Melo Barbosa HP, Martins LC, dos Santos SE and Corvelo TC designed and performed the research; Melo Barbosa HP and Martins LC performed the majority of experiments; Aragão CD took part in the experiments and collected the control samples; Assumpção MB performed endoscopy and provided clinical advice; Demachki S performed the histological evaluation and provided clinical advice; Melo Barbosa HP and Martins LC wrote the paper.

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Correspondence to: Hivana Patricia Melo Barbosa, Laboratory of Immunogenetics, Federal University of Pará, 66075-110, Belém, Brazil. hivana@ufpa.br

Telephone: +55-91-32696857 **Fax:** +55-91-32017568

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Abstract

AIM: To study the association between Interleukin-1 (IL-1) and tumor necrosis factor (TNF)- α polymorphisms, infection by *Helicobacter pylori* (*H. pylori*) and the development of gastrointestinal diseases.

METHODS: Genomic DNA was extracted from the peripheral blood of 177 patients with various gastrointestinal diseases and from 100 healthy volunteers. The polymorphisms in IL-1 β and TNF- α genes were analyzed using the polymerase chain reaction-restriction fragment length polymorphism method (PCR-

RFLP) and those from IL-1RN with PCR. The presence of infection due to *H. pylori* and the presence of the CagA toxin were detected by serology. The histopathological parameters in the gastric biopsies of the patients were according to the Sydney classification.

RESULTS: A comparison of the frequencies of the different polymorphisms studied among the patients and the control group demonstrated that the allele IL-1RN*2 was more frequent among patients with gastric ulcers and adenocarcinoma. Carriers of the allele IL-1RN*2 and those with reactive serology for anti-CagA IgG had a greater risk of developing peptic ulcer and gastric adenocarcinoma, as well as a higher degree of inflammation and neutrophilic activity in the gastric mucosa.

CONCLUSION: Our results indicate a positive association between IL-1RN gene polymorphism and infection by positive *H. pylori* CagA strains and the development of gastric ulcers and adenocarcinoma.

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Key words: *Helicobacter pylori*; Interleukin 1 β gene; Interleukin-1 receptor antagonist gene; TNF- α gene; Cag pathogenicity island

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is the major cause of chronic superficial gastritis in humans, and is a hypochloridria etiological factor in the pathogenesis of peptic ulcer disease (PUD) and some forms of stomach

cancer^[1]. However, most people harboring *H pylori* are asymptomatic, and only a few patients infected with this bacterium develop peptic ulcer or stomach cancer^[2]. The variability of clinical manifestations is associated with various factors, such as environmental, genetic susceptibility of the host and bacterial virulence^[1,2].

One noteworthy factor for bacterial virulence is the CitoCagA toxin, codified by the *cagA* gene, a marker for the Cag-PAI pathogenicity island^[3]. Infection by *H pylori* *cagA*-positive strains cause an intense inflammatory process, with dense neutrophilic infiltrate in the gastric mucosa^[2,3]. However, bacterial virulence factors alone are not sufficient for determining clinical evolution of the infection. While virulent strains are frequent in both patients with peptic ulcers and those with gastric carcinoma^[2,3], other factors in the host, mainly those that regulate immunological and inflammatory response may also contribute to a progression towards neoplasia^[4].

Genetic polymorphisms, particularly those that occur in the region promoting genes that codify inflammatory cytokines, have been associated with an increase in synthesis of those interleukins and have emerged as a hypochloridria determining factor for cancer susceptibility^[4,5].

Interleukin 1 beta (IL-1 β) is a hypochloridria initiator and amplifier of the immune response, and is also a potent inhibitor of stomach acid secretion^[5,6]. The antagonist of the IL-1 (IL-1RN) receptor is an anti-inflammatory cytokine that competes for the IL-1 receptors, modulating the effect of IL-1 β ^[7]. Studies carried out in Caucasian and Asian populations have demonstrated that polymorphisms in genes IL-1 β and IL-1RN are associated with an increased risk of hypochloridria and gastric carcinoma^[7-9]. Another important cytokine is TNF- α , the biallelic polymorphism in position -308 of the region promoting the gene codifying that cytokine, which has been associated with the development of gastric carcinoma in studies carried out in Caucasians^[10,11].

The objective of this study was to determine the frequency of polymorphisms in genes IL-1 β , IL-1RN and TNF- α in patients from the state of Pará, in the northern region of Brazil, with various gastrointestinal diseases and in a control group. The relationship of these polymorphisms to infection by virulent strains of *H pylori* (CagA+) and to the histopathological characteristics of gastric tissue were also determined.

MATERIALS AND METHODS

Patient and control samples

Peripheral blood and gastric fragment samples were collected from 177 consecutive patients from the state of Pará-Brazil who had various gastrointestinal disturbances. Gastric fragment samples were obtained by the endoscopy service of the João de Barros Barreto University Hospital. For the control samples, peripheral blood was collected from 100 patients who were without

clinical or metabolic diseases and who were asymptomatic for gastrointestinal disturbances, and were thus not submitted to endoscopic examinations.

All patients and controls were enrolled in the study between September 2003 and September 2004 and were from the same socioeconomic level, and had similar cultural habits. All were natives of Pará state with the same ethnic background, approximately 50% Portuguese, 40% Amerindian and 10% African^[12]. The study was approved by the Ethics Committee at João de Barros Barreto University Hospital.

Detection of *H pylori* infection

In both patients and controls, the presence of the specific *H pylori* and CagA IgG antibodies in serum samples was determined. To detect *H pylori* antibodies the commercial HIK anti-*H pylori* EIA kit was used (Monobind, USA), and the Helicobacter P-120 EIA commercial kit (VIVA Diagnostica, Germany) was used to detect anti-CagA antibodies. Kits were used according to the manufacturers' technical descriptions.

IL-1 β , IL-1RN and TNF- α genotyping

Genomic DNA was extracted from total blood using a leukocyte lysis solution (100 mmol/L Tris-HCl, 20 mmol/L EDTA, 200 mmol/L NaCl, 1% dodecylsodium sulfate, 0.2% β mercaptoethanol) and was purified using the phenol-chloroform method^[13].

Polymorphisms of the IL-1 β (-31, -511) and TNF- α (-308) genes were characterized using the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP). The volume for the PCR was 25 μ L, containing 0.5 mmol/L of each primer, 1 X PCR buffer, 1.5 mmol/L of MgCl₂, 0.2 mmol/L of each nitrogenated base, 1.25 U of *Taq* DNA polymerase, 50 ng of DNA and sterile water.

To determine the polymorphism of the IL-1 β gene in position -511, the PCR primers and conditions described by Wilkinson *et al*^[14] were used. The PCR products were digested with *Ava*I overnight at 37°C and separated by electrophoresis in 2% agarose gel stained with ethidium bromide. Those presenting two bands were called CC (114 and 190 bp), those with three bands, CT (114, 190 and 304 bp) and those with a single band, TT (304 bp).

Polymorphism of the IL-1 β gene in the -31 position was investigated using the primers described by El-Omar *et al*^[7]. The conditions of the PCR were as follows: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing and extension for 1 min, and final extension at 72°C for 10 min. Annealing temperatures were set at 58°C for primers. Negative and positive controls were used in all reactions. To discriminate alleles, the PCR products were digested with *Alu*I overnight at 37°C and then separated by electrophoresis in 2% agarose gel stained with ethidium bromide. Those presenting a single band (235 bp) were called CC; those with three bands (98, 137 and 235 bp)

were called CT and TT was used for those with two bands (98 and 137 bp).

For genotyping polymorphism -308 in the TNF- α gene, the PCR primers and conditions as described by Wilson *et al.*^[15] were used. The PCR products were digested with *NcoI* overnight at 37°C and separated by electrophoresis in 2% agarose gel stained with ethidium bromide.

The VNTR polymorphism at intron 2 of the IL-1RN gene was determined using the primers and conditions described by El-Omar *et al.*^[7]. The PCR products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide. They were named allele 1 = 442 bp (4 repeats), allele 2 = 270 bp (2 repeats), allele 3 = 528 bp (5 repeats), allele 4 = 356 bp (3 repeats), allele 5 = 614 bp (6 repeats).

Histological evaluation

Biopsy specimens from the lesion and the adjacent area in each patient were obtained. The specimens were fixed in 10% buffered formalin solution, embedded in paraffin, cut into sequential 0.4- μ m sections, and stained with hematoxylin and eosin (HE). The histopathological parameters were graded (0-3) using the criteria described in the updated Sydney classification system^[16] for analysis of chronic inflammation, polymorphonuclear activity, and intestinal metaplasia.

Statistical analysis

Hardy-Weinberg equilibrium and heterogeneity among groups were tested using the Guo and Thompson^[17] exact test. Maximum-likelihood haplotype frequencies were computed using an Expectation-Maximization (EM) algorithm^[18,19]. Linkage disequilibrium was tested using a likelihood-ratio test^[20]. All the aforementioned statistical procedures were carried out using Arlequin software^[21].

To compare the variables of sex, age, CagA status, *H pylori* and genotype frequencies between patients and controls, the G test was utilized. The risks of carriers with different alleles developing gastric ulcers and adenogastric carcinoma, as well as having alterations in the gastric mucosa were calculated using the Odds ratio. The data were analyzed with BioEstat version 4.0 software^[22]. Differences were considered statistically significant if *P* values were less than 0.05.

RESULTS

Clinical and demographic characteristics of patients and controls

One hundred and seventy-seven patients with various gastrointestinal pathologies were investigated; of these 80 (45%) had gastritis, 33 (19%) duodenal ulcer, 34 (19%) gastric ulcer and 30 (17%) had intestinal-type adenogastric carcinoma.

The epidemiological data of the two groups studied are described in Table 1. The patients had an average age of 45 years with ages ranging from 18 to 90 years. Subjects in the control group had an average age of

Table 1 Epidemiological characteristics of the control group and patients *n* (%)

Demographic data	Control <i>n</i> = 100	Gastritis <i>n</i> = 80	DU <i>n</i> = 33	GU <i>n</i> = 34	AC <i>n</i> = 30
Age (yr)					
> 50	31	26 (32)	11 (33)	12 (35)	21 (70)
< 50	69	54 (68)	22 (67)	22 (65)	9 (30)
<i>P</i> value		NS	NS	NS	0.00
Sex					
Male	57	39 (49)	19 (58)	21 (62)	19 (63)
Female	43	41 (51)	14 (42)	13 (38)	11 (37)
<i>P</i> value		NS	NS	NS	NS
IgG-anti <i>H pylori</i>					
Positive	61	74 (92)	30 (91)	33 (97)	29 (97)
Negative	39	6 (8)	3 (9)	1 (3)	1 (3)
<i>P</i> value		0.00	0.00	0.00	0.00
anti-CagA IgG (HP+)					
Positive	38	53 (66)	29 (97)	31 (94)	28 (97)
Negative	23	21 (34)	1 (3)	2 (6)	1 (3)
<i>P</i> value		NS	0.00	0.00	0.00

DU: Duodenal ulcer; GU: Gastric ulcer; AC: Adenogastric carcinoma; G Test: Control *versus* disease; NS: Not significant.

36 years, with ages ranging from 18 to 81 years. The patients with adenogastric carcinoma were older than those in the control group (Table 1).

The presence of IgG antibodies-*H pylori* and CagA specific was greater in patients with gastrointestinal diseases than in the control group (Table 1). A comparison of the presence of anti-CagA antibodies IgG in patients with various gastrointestinal diseases demonstrated that patients with gastric ulcer ($G = 6.330$, $P = 0.011$), duodenal ulcer ($G = 8.076$, $P = 0.004$) and adenogastric carcinoma ($G = 7.702$, $P = 0.005$) had greater seroprevalence of this antibody than patients with gastritis. However, when we compared the frequency observed in patients with gastric ulcer with that in patients with duodenal ulcer ($G = 0.007$, $P = 0.932$) and with stomach cancer ($G = 0.013$, $P = 0.908$) no differences were observed. A similar observation was made when we compared patients with duodenal ulcer and those with cancer ($G = 0.506$, $P = 0.476$).

IL-1 β , IL-1RN and TNF- α polymorphisms

The polymorphisms studied were in the Hardy-Weinberg equilibrium for both the control group (IL-1 β -31 $P = 0.811$; IL-1 β -511 $P = 0.902$; IL-1RN $P = 0.361$; TNF- α -308 $P = 0.363$) and for patients with gastrointestinal diseases (IL-1 β -31 $P = 0.321$; IL-1 β -511 $P = 0.791$; IL-1RN $P = 0.691$; TNF- α -308 $P = 0.552$). An imbalance in linkage among alleles IL-1 β -511T and IL-1 β -31C ($P > 10^{-5}$) was observed in both groups studied.

A comparison of the genotype frequencies of polymorphisms in genes IL-1 β analyzed in this study with those from other studies carried out in different countries, demonstrated that the frequency of the alleles for polymorphisms IL-1 β -31, IL-1 β -511 in our population did not differ statistically from that described for Caucasians^[7] and Asiatic populations^[23], and was

Table 2 Comparison of genotype frequencies for the polymorphisms of IL-1 β genes studied with results of other studies in different ethnic and population groups *n* (%)

Genotypes	Pará (Brazil)	Minas Gerais (Brazil)	Caucasians	Asians
IL-1 β -31				
TT	9	102 (35.8) ¹	7 (12) ²	7 (4) ⁴
TC	23	138 (48.4)	21 (36)	34 (20)
CC	68	45 (15.8)	30 (52)	128 (76)
<i>P</i> value	Reference	0.001	NS	NS
IL-1 β -511				
CC	31	108 (37.9) ¹	29 (50) ²	34 (20) ⁴
CT	46	137 (48.1)	21 (36)	97 (57)
TT	23	40 (14)	8 (14)	38 (23)
<i>P</i> value	Reference	NS	NS	NS
IL-1RN				
11	58	175 (61.4) ¹	37 (64) ²	163 (96) ⁴
12	36	92 (32.3)	14 (24)	4 (3)
22	6	18 (6.3)	17 (10)	2 (1)
<i>P</i> value	Reference	NS	NS	0.001
TNF- α -308				
GG	86	223 (78.3) ¹	152 (72) ³	274 (91.3) ⁵
GA	13	54 (18.9)	52 (25)	24 (8)
AA	1	8 (2.8)	6 (3)	2 (0.7)
<i>P</i> value	Reference	NS	0.025	NS

G Test: Our study versus other papers. ¹Queiroz *et al*^[25] 2004; ²El-Omar *et al*^[7] 2000; ³El-Omar *et al*^[24] 2003; ⁴Zeng *et al*^[23] 2003; ⁵Yea *et al*^[11] 2001. NS: Not significant.

in an intermediate position compared to that found in those populations. In contrast, the polymorphisms of the IL-1RN genes differed from those described in Asiatic populations^[11] and gene TNF- α -308 differed from descriptions in Caucasian populations^[24] (Table 2). When we compared our data with those obtained in Minas Gerais^[25], we observed a difference in the frequency of the IL-1 β -31 polymorphism, with the C allele being more frequent in our population than in the Minas Gerais population (Table 2).

In analyzing the distribution of the different genotypes among the patients and the control group we observed that the polymorphisms in genes IL-1 β -31, IL-1 β -511, TNF- α -308 did not differ. However, in relation to gene IL-1RN, we obtained a greater frequency of allele 2 carriers (IL-1RN*2) among patients with adenogastric carcinoma and gastric ulcer than in the control group (Table 3).

A combined risk analysis of the different polymorphisms studied demonstrated that there was no synergism between those polymorphisms and the development of gastric ulcers and adenocarcinoma. Individuals carrying only a polymorphism in gene IL-1RN*2 (OR = 3, *P* = 0.86) had a greater risk than individuals carrying polymorphisms in genes IL-31*C and IL-1RN*2 (OR = 1.2, *P* = 0.51) and the risk was similar to that for carriers of all the polymorphisms studied (OR = 3, *P* = 0.71).

In the association between the IL1-RN polymorphism and the presence of specific CagA antibodies, we found that carriers of allele IL-1RN*2 who were reactive for anti-CagA IgG had a greater risk of developing gastric

Table 3 Distribution of genotypes for IL-1 β (-511 and -31), IL-1RN and TNF- α in the control group and in patients with gastrointestinal pathologies *n* (%)

Genotypes	Control <i>n</i> = 100	Gastritis <i>n</i> = 80	DU <i>n</i> = 33	GU <i>n</i> = 34	AC <i>n</i> = 30
IL-1 β -31					
T/T	9	3 (4)	1 (3)	2 (6)	1 (4)
C/T	23	21 (26)	9 (27)	7 (20)	4 (13)
C/C	68	56 (70)	23 (70)	25 (74)	25 (83)
<i>P</i> value		NS	NS	NS	NS
IL-1 β -511					
C/C	31	26 (33)	9 (27)	10 (29)	6 (20)
C/T	46	34 (42)	13 (39)	14 (41)	11 (37)
T/T	23	20 (25)	11 (34)	10 (30)	13 (43)
<i>P</i> value		NS	NS	NS	NS
IL-1RN					
1/1	58	41 (51)	15 (46)	11 (32)	10 (33)
1/2	36	34 (43)	16 (49)	21 (62)	19 (63)
2/2	6	5 (6)	2 (6)	2 (6)	1 (4)
<i>P</i> value		NS	NS	0.00	0.00
TNF- α					
G/G	86	62 (77)	23 (70)	29 (85)	24 (80)
G/A	13	16 (20)	8 (24)	4 (12)	5 (16)
A/A	1	2 (3)	2 (6)	1 (3)	1 (4)
<i>P</i> value		NS	NS	NS	NS

DU: Duodenal ulcer; GU: Gastric ulcer; AC: Adenogastric carcinoma; G Test: Control versus disease; NS: Not significant.

ulcers and adenocarcinoma. This demonstrated an interaction between the presence of a virulent strain and allele IL-RN*2 in the development of these diseases (Table 4).

The relationship between IL-1RN genotypes and anti-CagA antibodies with histopathological data demonstrated that carriers of allele IL-1RN*2, who were seroreactive for CagA had high levels of inflammation and neutrophilic activity, with a heightened risk of developing intestinal metaplasia in the gastric mucosa (Table 5).

DISCUSSION

The state of Pará has a high prevalence and incidence of gastrointestinal diseases, principally adenogastric carcinoma. In addition, an increased prevalence of *H pylori* infection has been observed among patients with gastrointestinal diseases^[26,27], with a predominance of virulent strains (*vacA-s1b/m1/cagA*-positive) that are associated with the development of both peptic ulcers and adenocarcinoma^[28].

The patients with adenogastric carcinoma were older than those in the control group. These patients had been developing progressive gastric cancer lesions for a long time^[29].

Some studies have demonstrated that polymorphisms in genes IL-1 β , IL-1RN and TNF- α , together with *H pylori* infection are associated with an increased risk of developing stomach cancer^[7,10,11]; therefore, to better understand the factors related to the high prevalence of stomach cancer in our region we analyzed the frequency of the genotypes of polymorphisms in genes IL-1 β ,

Table 4 Combined risk of polymorphism in the IL1-RN gene and IgG CagA antibody for development of gastric ulcers and adenogastric carcinoma

IL1-RN	CagA	Control	GU	OR ^a (95% IC)	P	AC	OR ^b (95% IC)	P
1/1	(-)	15 (24%)	2 (6%)	Ref.	-	1 (4%)	Ref.	-
2* carrier	(-)	8 (13%)	2 (6%)	1.875 ^c	0.983	1 (4%)	1.875 ^c	0.735
1/1	(+)	21 (35%)	9 (27%)	3.214 (0.605-17.063)	0.289	8 (30%)	5.714 (0.644-50.648)	0.185
2* carrier	(+)	17 (28%)	20 (61%)	8.823 (1.762-44.181)	0.008	19 (62%)	16.764 (1.997-140.707)	0.004
Total		61	33			29		

GU: Gastric ulcer; AC: Adenogastric carcinoma. ^aControl x GU; ^bControl x AC; ^cThe confidence interval was not calculated, since, $n_1p_1q_1 < 5$ or $n_2p_2q_2 < 5$.

Table 5 Association of polymorphism for IL-RN gene and anti-CagA antibodies with histopathological findings from patients

IL1-RN	CagA	DI		OR (95% CI)	P	NA		OR (95% CI)	P	Metaplasia		OR (95% CI)	P
		1	2 and 3			1	2 and 3			+	-		
1/1	(-)	13	2	-	-	12	3	-	-	1	14	-	-
2* carrier	(-)	9	1	0.722 ^a	0.706	8	2	1 ^a	0.609	2	8	3.5 ^a	0.706
1/1	(+)	24	34	9.200 (1.90-44.606)	0.004	26	32	4.923 (1.254-19.314)	0.032	17	41	5.804 (0.706-47.693)	0.139
2* carrier	(+)	20	63	20.475 (4.253-98.556)	0.001	23	60	10.434 (2.695-40.388)	0.003	35	58	8.448 (1.064-67.065)	0.038

DI: Degree of inflammation; NA: Neutrophilic activity. Histopathological parameters: 1: Light; 2: Moderate; 3: Intense. ^aThe confidence interval was not calculated, since, $n_1p_1q_1 < 5$ or $n_2p_2q_2 < 5$.

IL-1RN and TNF- α and the presence of infection by CagA+ strains in patients with various gastrointestinal diseases and in a control group.

In this study, infection by virulent strains (CagA+) was greater in patients with peptic ulcers and adenocarcinoma than in patients with gastritis or in subjects in the control group. Similar results were found in a previous study carried out in Belém and other Brazilian states, where the presence of *H. pylori* CagA+ strains was associated with the development of peptic ulcers and adenocarcinoma^[28,29].

The frequency of polymorphisms in genes IL-1 β -31 and IL-1 β -511 in our study was similar to that described in Caucasian^[7,24] and Asiatic populations^[23,30], whereas the frequency of polymorphisms in genes IL-1RN and TNF- α -308 was significantly different from that reported in Asiatic^[23] and Caucasian^[7,24] populations, respectively. The genetic composition of the Brazilian population is made up of a genetic mix of various ethnic groups, including Portuguese, Africans and Amerindians^[12]. The differences and similarities between the allelic frequencies of the polymorphisms studied in our population with those of other ethnic groups are products of the genetic mix that has occurred in Brazil.

In comparing the frequencies of the polymorphisms studied in our population with those from another Brazilian study carried out in Belo Horizonte, Minas Gerais, located in the central-western region of the country, we observed differences in relation to IL-1 β -31 polymorphism. In Brazil, several states show differences in ethnic background, and the populations in the Amazon region are the ones which have an important indigenous genetic component^[12], greater than that described for the Minas Gerais population^[31],

which may be reflected in the gene frequency of those polymorphisms.

An analysis of the genotypes of the polymorphisms in the patients and the control group demonstrated that IL-1RN*2 carriers were more frequent among patients with gastric ulcers and adenocarcinoma. The IL-1Ra protein (codified by the IL-1RN gene) acts competitively to inhibit action by IL-1 β ^[7]. Carriers of IL-1RN*2 have higher levels of IL-1 β in the gastric mucosa than those with IL-1RN1/1^[32], and thus have a more severe and prolonged immune response, which may lead to hypochloridria due to destruction of the gastric glands and to action by IL-1 β that inhibits synthesis of chloridic acid by the parietal cells^[6,7]. El-Omar *et al.*^[7] have described an association between IL-1RN*2 and hypochloridria, and both gastric ulcer and stomach cancer reduce the synthesis of chloridic acid. Other studies have also described the association between the IL-1RN*2 polymorphisms and stomach cancer^[23,24,33]. In Brazil, Rocha *et al.*^[34] obtained similar results in relation to the association of allele IL-RN*2 and an increased risk of developing stomach cancer, as well as the absence of an association between the IL-1 β -31, TNF- α -308 polymorphisms and the risk of developing stomach cancer.

A combined analysis of the different polymorphisms demonstrated that there was no association between these polymorphisms and an increased risk of developing gastric ulcer or adenocarcinoma. In addition, we found an interaction between the presence of allele IL-RN*2 and infection by CagA+ strains. This finding is important for our region, which has a high incidence of stomach cancer, high prevalence of infection by CagA+

strains and a high frequency of the IL-1RN*2 allele. Reinforcing these data, our results have demonstrated that carriers of allele IL-1RN*2 infected by CagA+ strains had a greater risk of developing an intense inflammatory process in the gastric mucosa, which confirms a synergistic action between the polymorphism of gene IL-1RN and another type of infecting strain. Other studies have also observed that both infection by virulent strains and gene IL-1RN polymorphism are important risk factors for gastric carcinogenesis^[23,29,33]. In conclusion, our results suggest that bacterial virulence and genetic factors in the host act synergistically in the development of gastric ulcers and adenocarcinoma.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is associated with a broad spectrum of gastrointestinal disorders. However, most people harboring *H. pylori* are asymptomatic, and only a few patients infected with this bacterium develop peptic ulcer or stomach cancer. The variability of clinical manifestations is associated with various factors, such as environmental, genetic susceptibility of the host and bacterial virulence.

Research frontiers

This study indicated a possible association between IL-1RN gene polymorphism and infection by positive *H. pylori* CagA strains and the development of gastric ulcers and adenocarcinoma.

Innovations and breakthroughs

This study determined the frequency of polymorphisms in genes IL-1 β , IL-1RN and TNF- α in patients with various gastrointestinal diseases from the state of Pará, in the Brazilian Amazon, and in a control group. The relationship of these polymorphisms to infection by virulent strains of *H. pylori* and to the histopathological characteristics of the gastric tissue were determined.

Applications

This study may represent a future strategy for distinguishing patients with a risk of developing gastrointestinal diseases, such as gastric cancer.

Terminology

The CitoCagA toxin is a factor for bacterial virulence. The antagonist of the IL-1 (IL-1RN) receptor is an anti-inflammatory cytokine.

Peer review

In this study, confirmatory new data on gene polymorphism in a Brazilian population and message is clearly shown.

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BRIEF ARTICLES

Primary epithelial tumours of the appendix in a black population: A review of cases

Rondell Patrell Darrell Graham, Nadia Patricia Williams, Kamille Aisha West

Rondell Patrell Darrell Graham, Nadia Patricia Williams, Kamille Aisha West, Department of Pathology, University Hospital of the West Indies Mona, Kingston 7, Jamaica, West Indies

Author contributions: Graham RPD and West KA designed the research project, collected the data; Graham RPD, Williams NP and West KA analysed the data; West K prepared the first draft; Graham RPD and Williams NP edited the first draft, wrote the final paper.

Correspondence to: Rondell Patrell Darrell Graham, Department of Pathology, University Hospital of the West Indies Mona, Kingston 7, Jamaica,

West Indies. graham_ron@live.com

Telephone: +1-876-9271410 Fax: +1-876-9771811

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rare in our experience, and are represented principally by carcinoid tumours and adenomas. Carcinoid tumours occurred in younger patients but were slightly more common in men than women. Tumours were not suspected clinically and were diagnosed incidentally in specimens submitted for acute appendicitis supporting the need for histological evaluation in all resection specimens.

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Key words: Epithelial tumours; Appendiceal tumours; Carcinoid; Adenoma; Appendicitis

Peer reviewers: Dusan M Jovanovic, Professor, Institute of Oncology, Institutski Put 4, Sremska Kamenica 21204, Serbia; Jia-Yu Xu, Professor, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, 197 Rui Jin Er Road, Shanghai 200025, China

Abstract

AIM: To determine the prevalence, histologic types and clinical features of primary epithelial tumours of the vermiform appendix in a predominantly black population.

METHODS: All cases of primary tumours of the appendix identified by review of the histopathology records at the University of the West Indies between January 1987 and June 2007 were selected. Relevant pathologic and clinical data were extracted with supplementation from patient charts where available. Non-epithelial tumours were excluded. The total number of appendectomy specimens over the period was also ascertained.

RESULTS: Forty-two primary epithelial tumours were identified out of 6 824 appendectomies yielding a prevalence rate of approximately 0.62%. Well-differentiated neuroendocrine cell tumours (carcinoids, 47.6%) and benign non-endocrine cell tumours (adenomas, 45.2%) were most common with nearly equal frequency. The median age was 43 years, with no sex predilection. Carcinoid tumours occurred in younger patients (mean age 32 years), with a male-to-female ratio of 1.2:1. A clinical diagnosis of acute appendicitis was the most common reason for appendectomy (57.1%) and was histologically confirmed in 75% (18 of 24) of cases. In total, 16.7% of cases were diagnosed after incidental appendectomy.

CONCLUSION: Appendiceal epithelial tumours are

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INTRODUCTION

Primary appendiceal tumours are uncommon. They are often diagnosed incidentally after histopathological examination of the vermiform appendix submitted in the course of the management of another clinical diagnosis. This paper reviews the primary appendiceal tumours diagnosed at the University Hospital of the West Indies during the period from January 1987 to June 2007, and to our knowledge represents the first such analysis in a predominantly black population.

MATERIALS AND METHODS

The surgical pathology records of the Department of Pathology at the University of the West Indies were retrospectively reviewed over the time period January 1987-June 2007 and all cases of primary neoplasms of the appendix were selected. Non-epithelial tumours were excluded from the study population because of diagnostic controversy in the absence of immunohistochemical

Table 1 Demographic data and histologic types

Tumour type	Age range (yr)	Mean age (yr)	Median age (yr)
Carcinoid	18-70	32 ± 20.3	35.8
Adenoma	29-87	59 ± 15.2	58.6
Adenocarcinoma	36-74	55 ± 19.0	55

evaluation. The parameters examined included patient age, sex, the clinical history, the surgical procedure, the gross description of the specimen submitted and the histopathological diagnosis. Patient charts were retrieved where possible, for additional clinical information. The total number of appendectomies over the time period was also ascertained.

RESULTS

In the period under study, 6824 appendices were submitted for pathologic evaluation. Forty-two primary epithelial tumours of the appendix were identified, yielding a prevalence rate of approximately 0.62%.

The overall age range of patients was 18-87 years with a mean age of 45.9 ± 19.3 years and a median age of 43 years. The male-to-female ratio for all tumours was 1:1. Table 1 illustrates the demographic data with respect to each tumour type.

Of the 42 epithelial tumours identified, 20 (47.6%) were carcinoids, including 1 insular and 3 goblet cell carcinoids. Of the 19 (45.2%) adenomas, we identified 15 villous adenomas, the majority of which (11, 26.2%) were morphologically mucinous cystadenomas, as well as 1 tubular and 3 tubulovillous adenomas. All 3 cases of adenocarcinoma were of the mucinous type. Interestingly no cases of serrated adenomas were found in the records.

Only in 3 cases of carcinoid and 1 case of mucinous cystadenocarcinoma was the lesion identified grossly. Measurements were recorded for 2 out of 3 cases of carcinoid measuring 1.3 and 1 cm in maximum dimension.

In 26 of the cases the clinical diagnosis was acute appendicitis, appendiceal abscess in 3, appendix mass, caecal carcinoma, primary peritonitis and an appendiceal tumour in 1 case each. In a further 7 cases, the vermiform appendix was removed incidentally. No clinical diagnosis was submitted in 2 cases.

Of the 42 cases, only 7 patient charts were retrievable; review of the clinical notes revealed aggressive behaviour in one case of carcinoid tumour (goblet cell variant) with hepatic metastases, in a 50-year-old female, who presented with perforated appendicitis. The tumour was not identified grossly in this case.

In 4 of 42 cases there were synchronous colonic neoplasms giving a prevalence rate of 9.5%. Two of these 4, were cases of solitary synchronous caecal carcinoma accompanying a mucinous cystadenoma of the appendix in 1 case and an adenocarcinoma of the appendix in the other. In the other 2 cases, there was a single case of a caecal carcinoma and 2 tubulovillous and 1 villous colonic adenomas coexistent with an appendiceal tubulovillous adenoma in a right hemicolectomy specimen. The other case was that of a caecal carcinoma and 2 tubulovillous colonic adenomas coexistent with a mucinous cys-

adenoma of the appendix in a right hemicolectomy specimen. The carcinoma was diagnosed pre-operatively in 3 of the cases with no pre-operative diagnosis proffered in the other case. In none of these 4 cases was an appendiceal tumour suspected, nor was there evidence of any inherited syndromes in any of the cases.

No metachronous colorectal lesions were diagnosed up to June 2007. This search was limited by the retrieval of only 7 patient charts.

Pseudomyxoma peritonei was rare, with 1 such case diagnosed in a patient with a mucinous cystadenoma. Acute appendicitis was found to complicate a neoplasm in 54.8% of cases. Incidental appendectomy contributed to 16.7% of the neoplasms diagnosed.

DISCUSSION

Tumours of the appendix are uncommon. Our prevalence of 0.62% is comparable to the prevalence rate of 0.5%-0.9% found in other studies in New Zealand and the United Kingdom^[1-3]. No published reports are available for other predominantly black populations. The clinical picture was most frequently that of acute appendicitis (24 of 42) with histologic confirmation in 75% of these cases. Of note, incidental appendectomy provided 7 cases (16.7%). This highlights the utility of routine histopathologic examination of appendiceal specimens because the diagnosis is often made without antecedent clinical suspicion and these diagnoses can potentially alter patient management^[4].

Importantly, the index of suspicion of appendiceal tumours should be raised in cases clinically suggestive of acute appendicitis in the middle aged and elderly, given the median age of 43 at diagnosis of these tumours in our experience.

Carcinoid tumours show epithelial and neuroendocrine differentiation, and may arise in many sites, but most commonly in the gastrointestinal tract^[5]. These tumours frequently arise at the tip of the appendix^[6] and are reportedly the most common tumours of the appendix^[2,7]. Gaskin *et al*^[8] in a previous study of carcinoids of the gastrointestinal tract from our institution reported that the appendix was the most common location with a similar mean age to that documented in this study. In our study, there were almost equal numbers of carcinoids and adenomas, but patients with carcinoids were younger than those with adenomas and adenocarcinomas. There is a paucity of literature with regards to neuroendocrine tumours in black populations, however, in one study the incidence of carcinoid tumours in all sites was found to be highest in African-American males^[9]. Other studies, including those from the SEER data (1973-2001) report that appendiceal neuroendocrine tumours are more common in females^[8,10,11], in contrast to our data, which revealed a slight male predilection. This may be due in part to the small size of the study population.

While the overall behaviour of carcinoids is unpredictable, appendiceal carcinoids have an excellent prognosis^[5,11]. Importantly, where lesions are identified in the gross assessment of the specimen, they should be measured. Appendectomy is appropriate for lesions

< 1 cm but for lesions over 2 cm in diameter there is a significant increase in metastatic spread and thus right hemicolectomy is required in such cases^[3,6]. There remains controversy around what is the appropriate treatment for lesions between 1-2 cm. Authors have suggested that additional criteria be examined in such cases. These criteria include proliferation markers, mitotic activity, vascular and mesoappendiceal invasion^[6,12]. Unfortunately, there was one case of an unmeasured grossly visible lesion in our review. This potentially exposed the patient to not receiving further surgery which may have been necessary.

After diagnosis of an epithelial non-endocrine neoplasm of the appendix, the entire colorectum should be examined for synchronous lesions^[13,14]. In this series, appendiceal adenomas were associated with synchronous colonic tumours in 9.5% of cases, further underscoring the need for colorectal examination and surveillance post diagnosis of appendiceal adenoma. The relative unavailability of colonoscopy in our population over the period and the small sample size may be responsible for such a high rate when compared to Khan *et al*^[14]. The absence of cases of serrated adenomas may reflect past alternative classification of these lesions, or a lack of reporter awareness or it may be highlighting a lesser contribution by the serrated pathway to colorectal carcinoma in our population. This area requires further study. Based on the available records, there were no cases of colorectal carcinoma syndromes with appendiceal involvement diagnosed during the period. Pseudomyxoma peritonei, a term best avoided for diagnostic purposes, was extremely rare.

COMMENTS

Background

Primary appendiceal tumours are uncommon. They are often diagnosed incidentally after histopathological examination of the vermiform appendix submitted in the course of the management of another clinical diagnosis. No previous research has been done in a predominantly black population to determine the prevalence or histologic types of primary epithelial tumours of the appendix.

Research frontiers

Carcinoids (neuroendocrine tumours) are considered the most common primary appendiceal neoplasm and there is some evidence that African-American males have the highest incidence of carcinoids in all sites. However, several articles including the SEER database identified carcinoids more frequently in females. The roles of gender and ethnicity, if any, are unclear and require further investigation. The serrated pathway to colorectal carcinoma has been well described but the absence of such lesions in this study may reflect inappropriate classification or lack of reporter awareness. Further specific research into the serrated pathway to colorectal carcinoma in predominantly black populations is required.

Innovations and breakthroughs

This and other articles have validated the use of routine histologic examination of appendectomy specimens given the frequency of diagnosis of incidental tumours which potentially require specific management. Generally carcinoid tumours portend a favourable prognosis but in some cases these tumours behave aggressively. Several criteria including proliferative markers, mitotic activity, vascular and mesoappendiceal invasion are being examined to determine their utility in predicting the behaviour of carcinoid tumours. The gross documentation of tumour size is important in determining if further management is required. While carcinoids are generally thought to be the most common tumour of the appendix, in this study an almost equal number of

adenomas were diagnosed. The published description of the sessile serrated adenoma and its recognition as a precursor to adenocarcinoma of the colon and rectum opened a new paradigm of research into colorectal carcinoma. It also highlighted that these lesions may have been previously misdiagnosed as hyperplastic polyps.

Applications

Routine histopathological examination of appendectomy specimens is justified given the not infrequent incidental finding of appendiceal tumours. In cases of appendicitis in the elderly, the index of suspicion for epithelial tumours of the appendix should be raised. Moreover, once the diagnosis of an adenomatous lesion is made, colonoscopic examination of the entire large bowel is mandatory given the frequency of synchronous colorectal neoplasia in our population.

Terminology

The term carcinoid refers to tumours which show epithelial, endocrine and neural characteristics which have been identified by light microscopic, ultrastructural and immunohistochemical means. The term serrated pathway of colorectal carcinoma refers to those lesions which arise from precursors with a peculiar hyperplastic morphology associated with architectural dysplasia but no cytologic abnormalities. These tumours have specific mutations which further distinguish them from the adenomatous and the hereditary nonpolyposis colorectal cancer (HNPCC) pathway.

Peer review

This retrospective brief report is the first publication describing the prevalence and types of primary epithelial tumours of the appendix in a predominantly black population. It also describes the rate of synchronous neoplasia and highlights the need for routine histopathology. Thus, it is of reference value to the international scientific medical community.

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Carbon dioxide for gut distension during digestive endoscopy: Technique and practice survey

Filip Janssens, Jacques Deviere, Pierre Eisendrath, Jean-Marc Dumonceau

Filip Janssens, Jean-Marc Dumonceau, Division of Gastroenterology and Hepatology, Geneva University Hospitals, Micheli-du-Crest street, 24, 1205 Geneva, Switzerland
Jacques Deviere, Pierre Eisendrath, Medical-Surgical Department of Gastroenterology and Hepato-Pancreatology, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium

Author contributions: Janssens F and Dumonceau JM designed research; Deviere J, Eisendrath P and Dumonceau JM performed research; Janssens F and Dumonceau JM analyzed data; Janssens F, Dumonceau JM, Deviere J and Eisendrath P wrote the paper.

Correspondence to: Jean-Marc Dumonceau, MD, PhD, Division of Gastroenterology and Hepatology, Geneva University Hospital, Micheli-du-Crest street, 24, 1205 Geneva, Switzerland. jmdumonceau@hotmail.com

Telephone: +41-22-3729340 Fax: +41-22-3729366

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Abstract

AIM: To assess the adoption of Carbon dioxide (CO₂) insufflation by endoscopists from various European countries, and its determinants.

METHODS: A survey was distributed to 580 endoscopists attending a live course on digestive endoscopy.

RESULTS: The response rate was 24.5%. Fewer than half the respondents (66/142, 46.5%) were aware of the fact that room air can be replaced by CO₂ for gut distension during endoscopy, and 4.2% of respondents were actually using CO₂ as the insufflation agent. Endoscopists aware of the possibility of CO₂ insufflation mentioned technical difficulties in implementing the system and the absence of significant advantages of CO₂ in comparison with room air as barriers to adoption in daily practice (84% and 49% of answers, respectively; two answers were permitted for this item).

CONCLUSION: Based on this survey, adoption of CO₂ insufflation during endoscopy seems to remain relatively exceptional. A majority of endoscopists were not aware of this possibility, while others were not aware of recent technical developments that facilitate CO₂ implementation in an endoscopy suite.

INTRODUCTION

Use of Carbon dioxide (CO₂) as the insufflating gas during colonoscopy was proposed in 1974 to decrease the explosion hazard associated with polypectomy^[1]. As it appeared that there was less bloating and likely less pain after procedures using CO₂ for gut distension compared to air^[2], randomized controlled trials (RCTs) were performed to compare post-procedure pain when using CO₂ versus room air as the insufflation agent. The results of all of these RCTs were unambiguous, with significantly less pain reported after CO₂ colonoscopy^[3-7]. For other endoscopic procedures also, CO₂ was found to be superior to air: (1) for double balloon enteroscopy, small bowel intubation is deeper^[8]; (2) for endoscopic retrograde cholangio-pancreatography (ERCP), post-procedural pain is less^[9,10]; and (3) for complex colorectal procedures (endoscopic submucosal dissection), fewer sedative drugs are required^[11]. This is explained by the pathophysiology of gases: intestinal gases leave the body through alimentary orifices and exhaled air (gases can diffuse through the gut into splanchnic blood and subsequently pulmonary circulation). Experimental studies in live animals have shown that the clearance of gas from isolated bowel segments is much faster for CO₂ than nitrogen or oxygen (the two main components of air), and this by a factor of 160 and 12, respectively^[12]. The most important reason for this is the higher solubility of CO₂ compared to other gases in water. Other factors that influence the diffusion of gases through the intestinal barrier are less significant

(e.g. gas tension gradient between the intestinal lumen and blood) or identical for all digestive gases (e.g. surface and thickness of the exchange membrane, and tissue perfusion)^[13].

Despite the high level of evidence supporting the use of CO₂ for gut distension during colonoscopy and other endoscopic procedures, this gas does not seem to be used in many endoscopy practices. We here report a survey that was performed in a large group of endoscopists to assess the use of CO₂ insufflation in daily endoscopy practice, including reasons for possible non-adoption.

MATERIALS AND METHODS

Survey design and administration

A questionnaire was developed by the authors for the study. Content validity of the survey was determined based on input by experts in the field and a review of the relevant literature. The final, two-page, 26-item, survey contained two parts: the first one addressed respondents' demographic characteristics and knowledge about the use of CO₂ as room air replacement during gastrointestinal endoscopy; and the second part was divided in two sections directed to endoscopists who, either did ("practitioners"), or did not ("non-practitioners") use CO₂. Non-practitioners were asked for which reasons they did not use CO₂, while practitioners were asked about their actual use of CO₂.

The survey was performed during the 26th European Workshop on Gastroenterology and Endotherapy held in Brussels, Belgium, on 16-18 June 2008. Questionnaires were placed in cases distributed to course participants, and attendees were asked to deposit completed surveys in a dedicated box at the registration desk. Consent to participate in this study was inferred from voluntary completion of the survey. Efforts to increase response rates included two rehearsals by the course director (Deviere J), projection of a reminder slide during breaks, and collection of surveys by staff members who passed between rows of participants or were posted at the exits of the projection rooms. No gift or financial incentive was proposed to attendees.

Statistical analysis

Results are expressed as mean \pm SD or as a percentage. Each response was included in the analysis, regardless of the completeness of the survey. In cases when not all survey respondents answered to an individual question, the number of respondents (i.e. the denominator for percentage calculations) is indicated.

RESULTS

Study population

Surveys were distributed to 580 medical doctors attending the course, and 142 of them completed the study (response rate, 24.5%). All of them answered all the demographic questions (Table 1). The respondents

Table 1 Demographic characteristics of the 142 survey respondents (mean \pm SD)

Characteristics	n (%)
Male gender	109 (76.8)
Age (yr)	47.7 \pm 9.1
Years in practice	17.5 \pm 9.2
Country	
Belgium	25 (17.6)
Greece	18 (12.7)
Italy	18 (12.7)
France	16 (11.3)
Spain	10 (7.0)
Switzerland	9 (6.3)
Other	46 (32.4)
Main practice setting	
Private	36 (25.4)
Community Hospital	54 (38.0)
University Hospital	52 (36.6)
No. of colonoscopies performed/year in the center	
< 500	7 (4.9)
500-1000	40 (28.2)
1000-1500	33 (23.2)
> 1500	62 (43.7)
Proportion of colonoscopies performed with propofol/general anesthesia	
< 20%	74 (52.1)
20%-39%	4 (2.8)
40%-59%	0
60%-79%	15 (10.6)
80%-100%	49 (34.5)
Main patient pattern	
Outpatients	48 (33.8)
Inpatients	5 (3.5)
Mixed	89 (62.7)

had their endoscopy practice in 21 countries, but six of these (Belgium, Greece, Italy, France, Spain and Switzerland) made up two-thirds of the respondents. Main practices were roughly equally distributed between private practice, community hospitals and university hospitals. Sedation with propofol or general anesthesia was used for more than 50% of colonoscopies by about half the respondents.

Answers to the survey

Fewer than half of the respondents (66/142, 46.5%) were aware that room air could be replaced by CO₂ for gut distension during endoscopy. Thirty-eight respondents (26.8%) had previously seen ($n = 24$) or performed ($n = 14$) an endoscopy procedure using CO₂, with only six of them actually practicing this technique (adoption rate of the technique in the whole population, 4.2%). Fifty-eight (87.9%) of the 66 respondents who were aware of the technique also stated that all RCTs had shown that CO₂ insufflation decreased pain and gut distension compared to air insufflation. The proportions of survey respondents who correctly answered questions relating to various aspects of CO₂ use during endoscopy are shown in Figure 1.

One hundred and thirty endoscopists answered why they did not use CO₂: 73 (56.1%) of them were not aware of this possibility, and those who were aware most often cited "technical difficulties in implementing the

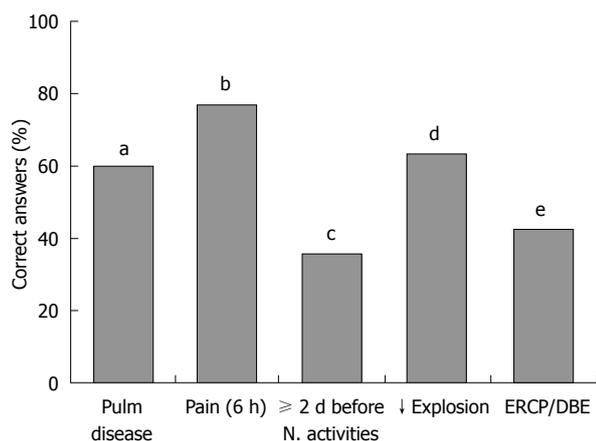


Figure 1 Percentages of correct answers (yes/no choice; correct answer was yes in all cases) to the following questions. ^aCO₂ insufflation is not advised in patients with severe pulmonary diseases; ^bAbout 20% of patients still have pain 6 h after colonoscopy using air insufflation; ^cAbout 20% of patients need ≥ 2 d before they are able to return to their normal activities after screening colonoscopy; ^dCompared to air, CO₂ colonoscopy decreases the risk of bowel explosion; ^eCompared to air, CO₂ insufflation is better for ERCP and double balloon enteroscopy DBE.

system” and “advantages not significant enough for the patient” [$n = 48$ (84%) and 28 (49%), respectively; two answers were permitted for this item]. Marginal answers included the risk of patient carbonarcosis ($n = 6$) and of CO₂ inhalation by the endoscopy personnel ($n = 4$).

Reasons that could motivate a change in their practice were stated by 127 endoscopists: a demonstration of the use of CO₂ [in a workshop ($n = 61$; 48.0%) or in their endoscopy unit ($n = 50$; 39.4%)], and proposal of a CO₂ insufflator as an option when buying a colonoscope ($n = 44$; 34.6%) were the most frequently cited answers. Other answers were less frequent [5% higher reimbursement for CO₂ compared to air colonoscopy ($n = 16$; 12.6%); virtual colonoscopy performed close to their practice using CO₂ insufflation ($n = 9$; 7.1%)]. Four endoscopists reported that they had attempted implementing CO₂, but that they had abandoned it because of costs.

Four endoscopists who were actually using CO₂ compared it with air colonoscopy. CO₂ was rated as similar to air in terms of ease of use, endoscopist comfort and patient comfort during colonoscopy, but better with respect to post-procedure patient comfort, and more expensive (all answers were identical, except for one endoscopist who rated CO₂ as better for patient comfort during colonoscopy).

DISCUSSION

CO₂ was used for gut distension during endoscopy by < 5% of survey respondents, even though all RCTs performed since the description of the technique 35 years ago have shown that pain is lower with CO₂ compared to air^[1,3-6,9,14]. Indeed, the adoption rate found in the present study was even lower than that reported 20 years ago in a survey of US hospitals in Illinois (13% for colonoscopy)^[15]. A majority of endoscopists

were not aware at all of the possible use of CO₂ during endoscopy, while the remainder ignored recent practical developments (they cited technical difficulties in implementing CO₂ as the main factor limiting its adoption, even though CO₂ insufflators have become more widely available). The other major reason cited for not adopting CO₂ was that advantages for the patients were not sufficiently significant. This likely relates to a lack of information among endoscopists about post-colonoscopy patient inconvenience (only one-third of them knew that 20% of patients need ≥ 2 d before being able to return to their normal activities after screening colonoscopy)^[16].

Endoscopists currently pay more attention to patients' comfort; for example, polyethylene glycol is being replaced by sodium phosphate for bowel preparation before colonoscopy^[17]. However, recent reports have shown that phosphate nephropathy may complicate bowel preparation using sodium phosphate, even after a single preparation^[18]. Another example is the use of propofol for sedation in replacement of benzodiazepines^[19]. CO₂ deals with the post-procedure phase of colonoscopy by reducing bloating and abdominal pain, the most frequent side effects of colonoscopy^[16]. However, it remains to be demonstrated if the advantages conferred by CO₂ are sufficiently significant to improve patient acceptance of endoscopic procedures and cost-effectiveness (by reducing loss from normal activities after endoscopy). These two criteria, namely patient acceptance and cost-effectiveness, are of paramount importance for colorectal cancer screening as computed tomography (CT) colonography has been shown to be superior to colonoscopy for both of them^[20,21]. Incidentally, one of the three CO₂ insufflators that are available for endoscopy was developed initially for gut distension during CT colonography, and radiologists use it increasingly often for reasons of safety and patient comfort (CO₂ is used in about half of CT colonographies)^[22]. In our survey, the use of CO₂ for CT colonography was not perceived by endoscopists as an incentive to change their practice. As endoscopists become aware of the ease and benefits of CO₂ implementation in an endoscopy suite, the use of CO₂ may be the next logical step to minimize patient discomfort.

Most endoscopists reported that a demonstration (in their endoscopy unit or in a workshop) was likely to change their perception of CO₂ usefulness. This corroborates our previous observation that endoscopists' opinion may significantly change following a demonstration of a particular endoscopy technique^[23]. However, it remains to be seen if intentions translate into actual changes, in particular, because CO₂ benefits are mainly observed after sedation reversal, when many patients are not evaluated by endoscopists.

From a practical standpoint, CO₂ is readily available in centers where laparoscopic surgery is performed (or it can be purchased from various distributors), and endoscopic CO₂ insufflators have recently become more

Table 2 Characteristics of CO₂ insufflators available for gut distension during endoscopy

	CO ₂ -efficient	Olympus keyed ECR	Olympus UCR
Weight (kg)	9.0	26.0	4.9
Size (mm)	254 × 140 × 254	420 × 1049 × 539	130 × 156 × 334
Output of CO ₂ adjustable	No ¹	No	No
Indicator of the amount of gas delivered	Yes	No	No
Indicator of "empty tank"	Yes	Yes	Yes
FDA approved/CE mark	Yes/Yes	Yes/Yes	Yes/Yes
Availability	International	United Kingdom	International
Price (euros)	7400	NA	7000
Manufacturer	Bracco Imaging SPA, San Donato Milanese, Italy	Olympus Keymed, Southend-on-Sea, UK	Olympus, Tokyo, Japan

FDA: Food and Drug Administration; NA: not available. ¹When the vent hole of the insufflation/irrigation valve is not occluded, CO₂ flow decreases from 3 L/min to a managed flow of 0.25 L/min, in order to preserve CO₂ reserves. None of the three models allows selecting between different intensities of CO₂ flow (in contrast with the selection of low/medium/high intensities of air flow with air insufflators).

widely available (Table 2). CO₂ insufflators are electrically powered devices that combine at the minimum a gas pressure regulator, a safety pressure valve to protect against over-insufflation, and connection tubes. When CO₂ is used, the regular air insufflation is inactivated (to prevent endoscopic insufflation with both gases), and endoscope manipulation is unchanged compared to using air for gut distension (CO₂ insufflation is obtained by placing the finger on the vent hole of the insufflation/irrigation valve, and lens cleaning is obtained by firmly pressing this valve). One may also switch from one gas to another during an endoscopic procedure. Contraindications to the use of CO₂ are limited to severe chronic obstructive pulmonary disease (if CO₂ is absorbed at a rate exceeding its respiratory elimination, this leads to CO₂ retention and pulmonary acidosis)^[24]. Provided that this contraindication is observed, Bretthauer *et al*^[3] have shown that, although pCO₂ levels increase during colonoscopy and ERCP (due to the effect of sedative drugs), this increase is no more important with CO₂ than with air insufflation^[4,9].

Finally, the cost of an insufflator was cited as a limiting factor by endoscopists who attempted to implement the system. The cost of an insufflator ranges between 7000 and 7400 euros. The cost of CO₂ gas per colonoscopy is < 1 euro (renting a 2400-L CO₂ tank costs about 50 euros/year, and refilling it costs 25 euros; this volume is sufficient for 800 min of continuous insufflation; a mean of 8.3 L is used per colonoscopy procedure)^[25]. The acquisition cost should be viewed in light of the multiple uses of these systems (e.g. colonoscopy, ERCP, double balloon enteroscopy) and ideally, from a societal perspective. Indeed, if cost calculations of screening colonoscopy took into account total time lost from work for patients undergoing the examination, as well as for the person accompanying the patient, this would increase the cost by about 50%^[26]. A catalyst for CO₂ adoption by endoscopists could be the implementation of CO₂ insufflation capabilities into standard endoscopy processors, as additional costs would be hard to justify in the absence of specific reimbursement. The endoscope manufacturer that would

first take this step would have a competitive advantage.

Our study has several potential limitations, including selection bias and the relatively limited number of responders. However, survey respondents were distributed relatively evenly between different endoscopy practices, and an international audit with a larger panel of individual respondents than reported here is notably difficult to organize^[27,28].

In conclusion, the use of CO₂ for gut distension during endoscopy remains exceptional despite the results of numerous RCTs that have shown the superiority of this technique compared to air. A majority of endoscopists are unaware of this possibility, while those who are aware mostly think that CO₂ implementation in an endoscopy suite is technically difficult or presents few advantages. Greater availability of CO₂ insufflators, more widespread use of CO₂ in competing CT colonography, and better endoscopists' education have the potential to change this situation.

COMMENTS

Background

Carbon dioxide (CO₂) is cleared much more rapidly than air from the bowel and randomized controlled trials have consistently shown that it is superior to air for several gastrointestinal endoscopy procedures. In particular, advantages were demonstrated for colonoscopy (less pain), endoscopic retrograde cholangiopancreatography (less pain), double balloon enteroscopy (deeper bowel intubation), and long, complex, therapeutic procedures (fewer sedative drugs).

Research frontiers

Use of CO₂ is common for colon computed tomography but it does not seem to be widespread in endoscopy practice. Reasons for possible non-adoption of this gas are unknown.

Innovations and breakthroughs

No data about the use of CO₂ by endoscopists have been available for > 20 years. Recently, CO₂ insufflators for endoscopy have become commercially available.

Applications

As a majority of endoscopists were not aware of the possibility to use CO₂ as air replacement during endoscopy, specific endoscopists' education and implementation of CO₂ insufflation capabilities into standard endoscopy processors should be encouraged.

Peer review

The cost of equipment required for CO₂ insufflation during endoscopy is the main barrier to adoption of this technique; it is actually around 7000 euros.

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BRIEF ARTICLES

Positional effect of mutations in 5'UTR of hepatitis C virus 4a on patients' response to therapy

Mostafa K El Awady, Hassan M Azzazy, Ahmed M Fahmy, Sherif M Shawky, Noha G Badreldin, Samar S Yossef, Moataza H Omran, Abdel Rahman N Zekri, Said A Goueli

Mostafa K El Awady, Ahmed M Fahmy, Noha G Badreldin, Samar S Yossef, Moataza H Omran, Department of Biomedical Technology, National Research Center, Dokki, Cairo 12622, Egypt

Hassan M Azzazy, Sherif M Shawky, Department of Chemistry and Yousef Jameel Science and Technology Research Center, the American University in Cairo, Cairo 11511, Egypt

Sherif M Shawky, Structural biology department, Free University of Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

Abdel Rahman N Zekri, National Cancer Institute, Cairo 11796, Egypt

Said A Goueli, Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53711, United States

Author contributions: El Awady MK designed experiment, wrote manuscript; Azzazy HM wrote the manuscript, analyzed data; Fahmy AM, Shawky SM, Badreldin NG, Yossef SS and Omran MH performed research; Zekri ARN and Goueli SA analyzed data.

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Correspondence to: Hassan M Azzazy, PhD, Department of Chemistry and Yousef Jameel Science and Technology Research Center, the American University in Cairo, 113 Kasr El-Aini Street, SSE Rm # 1194, Cairo 11511, Egypt. hazzazy@aucegypt.edu
Telephone: +2-22-6152543 Fax: +2-22-7957565

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Abstract

AIM: To investigate the effects of mutations in domain III of the hepatitis C virus (HCV) internal ribosome entry sequences (IRES) on the response of chronic HCV genotype 4a patients to interferon therapy.

METHODS: HCV RNA was extracted from 19 chronic HCV 4a patients receiving interferon/ribavirin therapy who showed dramatic differences in their response to combination therapy after initial viral clearance. IRES domain III was cloned and 15 clones for each patient were sequenced. The obtained sequences were aligned with genotype 4a prototype using the ClustalW program and mutations scored. Prediction of stem-loop secondary structure and thermodynamic stability of the

major quasispecies in each patient was performed using the MFOLD 3.2 program with Turner energies and selected constraints on base pairing.

RESULTS: Analysis of RNA secondary structure revealed that insertions in domain III altered Watson-Crick base pairing of stems and reduced molecular stability of RNA, which may ultimately reduce binding affinity to ribosomal proteins. Insertion mutations in domain III were statistically more prevalent in sustained viral response patients (SVR, $n = 14$) as compared to breakthrough (BT, $n = 5$) patients.

CONCLUSION: The influence of mutations within domain III on the response of HCV patients to combination therapy depends primarily on the position, but not the frequency, of these mutations within IRES domain III.

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Key words: Hepatitis C virus; Internal ribosome entry sequences; Domain III; Genotype 4a; Ribosomal subunit; Interferon therapy; RNA folding

Peer reviewer: Fritz von Weizsäcker, Professor, Department of Medicine Schlosspark-Klinik, Humboldt University, Heubnerweg 2, Berlin D-14059, Germany

El Awady MK, Azzazy HM, Fahmy AM, Shawky SM, Badreldin NG, Yossef SS, Omran MH, Zekri ARN, Goueli SA. Positional effect of mutations in 5'UTR of hepatitis C virus 4a on patients' response to therapy. *World J Gastroenterol* 2009; 15(12): 1480-1486 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1480.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1480>

INTRODUCTION

The hepatitis C virus (HCV) genome is a 9.5 Kb single stranded RNA molecule of positive polarity. It contains a single open reading frame flanked by 5' and 3' untranslated regions (UTR) of 341 and about 230 nucleotides, respectively^[1]. Both the 5' and 3' UTRs contain conserved RNA structures essential for polyprotein translation and genome replication^[2]. The 5'UTR is uncapped and contains the internal ribosomal

entry site (IRES) which contains highly conserved secondary and tertiary structures that are essential for proper binding and positioning of the viral RNA within the host cell's protein translation machinery^[3,4].

The only FDA approved medication for the treatment of HCV infection is the combination therapy of either standard or pegylated interferon and ribavirin^[5]. However, only 28%-60% of HCV patients respond to the treatment, depending on the viral genotype^[6,7]. Resistance to interferon therapy is believed to be controlled by host and viral factors^[8]; a significant viral factor is the generation of different HCV quasispecies^[9]. The critical role of 5'UTR in initiation of polyprotein translation requires the highest degree of conservation of this region. It contains four highly structured domains numbered I to IV; all are required for recruiting, positioning, and activating/regulating the host protein synthesis machinery^[10]. Translation initiation in HCV starts by binding of the HCV IRES to the 40S subunit and assembly of this binary complex with eukaryotic initiation factor 3 (eIF3). The IRES-40S-eIF3 ternary complex combines with eIF2/GTP/initiator tRNA forming the 48S complex. Progression from 48S to 80S initiation complexes is a slow step during IRES-mediated initiation which may reflect a decrease or absence of factor activity or conformational rearrangements in the IRES leading to sub-unit joining^[11]. The need for flexible rather than rigid binding allows the conformational rearrangement and subsequent efficient sub-unit joining leading to initiation of translation. Therefore low affinity binding of factors due to mutations in binding sites may be associated with more flexibility and efficient initiation of translation.

The 5'UTR can be divided into the 5' and 3' parts. The 5' part is near to a fully single stranded structure and includes domain I. The 3' part is highly structured and is essential for HCV IRES function. It folds into three additional domains II, III and IV. Domain II is a stem with several internal loops. Domain IV is a small hairpin which includes the AUG start codon. The pseudo knot joins domain II with domain III and is base-paired to the sequence directly upstream of domain IV.

The HCV IRES forms an extended structure that binds the 40S subunit by several synergetic interactions, and the domains involved in this binding have been determined by chemical and enzymatic foot-printing experiments^[12]. The segment starting from nucleotide 141 to 279 which comprises domain III, the focus of the present study, is highly conserved at the primary nucleotide sequence as well as at secondary and tertiary structure levels. The basal part of domain III (including principally the pseudo knot and stem-loop III d) includes the elements of secondary structure that determine the binding of the IRES to the 40S subunit. Besides providing affinity for the 40S subunit, the basal part of domain III is thought to be required for correct positioning of the initiation codon in the decoding center of the 40S subunit, as indicated by "toe-printing" experiments^[11]. The apical part of domain III binds eIF3 *via* stem-loop III b and the four-way junction and is required for 40S and eIF3 binding.

In the present study, the positional effect of mutations on the predicted secondary structure and thermodynamic stability of domain III was examined in 19 chronic HCV type 4a patients with initial virological response defined as undetectable viremia after 12 wk from start of treatment. Only 14 patients achieved sustained virological response (SVR) i.e. negative viremia 24 wk after end of treatment. In the remaining 5 patients who suffered virological breakthrough (BT), the predicted stem-loop structures and thermodynamic stability of domain III were compared in both pre- and post-treatment samples.

MATERIALS AND METHODS

HCV patients

Male or female patients ($n = 19$; 18-60 years) with chronic active hepatitis C virus infection were included in this study. Patients were negative for HBsAg and HBsAb but positive for anti-HCV and HCV-RNA by RT-PCR. All had elevated ALT and AST levels and received combined therapy of pegylated IFN α -2b (100 μ g/wk) plus ribavirin (800-1000 mg/d). Patients had normal values for blood counts, other liver functions, auto immune markers, T3, T4 and TSH, renal functions, blood sugar and α -fetoprotein. None of the patients had other causes of liver disease (e.g. α 1 antitrypsin deficiency, Wilson's disease, alcoholic or decompensated liver disease, obesity-induced liver disease, drug-related liver disease), no CNS trauma, or active seizures, no ischemic cardiovascular disease within the last six months or hemochromatosis. None was co-infected with HBV or schistosomiasis.

Cloning and sequencing of the HCV domain III

RNA extraction and reverse transcription-PCR of HCV RNA, using Qiagen single step RT-PCR kit (Qiagen, Inc., Chatsworth, CA, USA), were performed as described previously^[13,14]. Amplification of 266 bp was performed in a single step using primer pair; forward (nt 47-68) 5'-GTGAGGAAGTACTGTCTTCACG-3' and reverse (nt 292-312) 5'-ACTCGCAAGCACCCCTATCAGG-3'. Cloning of amplified products was done with TA cloning kit (Invitrogen Co., Carlsbad, CA). Fifteen clones from each subject were sequenced using the TRUGENE HCV 5-NC genotyping kit, Visible Genetics, Inc. (Toronto, Ontario, Canada) in conjunction with the Open Gene DNA sequencing system. The insert DNA was sequenced by CLIP sequencing which allows both directions of the target amplicon to be sequenced simultaneously in the same tube using two different dye labeled primers (Cy5.0 and Cy5.5) for each reaction. This method provides sequence information for both positive and negative DNA strands from a single reaction. The obtained sequences were then aligned with genotype 4a prototype using the ClustalW program (www.ch.embnet.org/software/ClustalW.html). Among the 15 domain III sequences obtained for each patient only one sequence was found in the majority of the clones, thus representing the major quasispecies of domain III in each patient.

Table 1 Distribution of mutations in different loops of domain III in SVR compared with BT patients

Mutation	Position	Region	No. of patients			Effect on ΔG (kcal/mol)
			SVR ($n = 14$)	BT-PreT ($n = 5$)	BT Post-T ($n = 5$)	
C-T	148	Junction joining stem-loop III a, b, c & d	-	-	1	Increased
G-T	158	Loop III a	-	1	4	Increased
T-A	175	Loop III b	1	-	-	No effect
G & A ins	179		2	-	-	Increased
T ins.	180		1	-	-	Increased
T ins.	181		1	-	-	Increased
C-T	186		-	-	1	No effect
TTT-GGG	194-196		-	1	-	No effect
C ins.	205		1	-	-	No effect
T-C	199		-	-	1	No effect
G-A	243	Junction joining stem-loop III a, b, c & d	5	1	4	No effect
C-T	254	Loop III d	-	1	1	Decreased
A-C	260		1	-	1	Decreased
A-T	260		1	-	-	Decreased
G-T	261		1	-	-	Increased
G-T	268		-	1	1	Decreased
T-C	269		-	1	1	Decreased
G ins.	270		-	1	1	Decreased
A-C	275		2	-	-	Decreased

The distribution of the observed mutations in different loops of domain III in sustained viral response (SVR) and breakthrough (BT) patients is shown. The majority of mutations (14/19) were simple substitutions whereas 5/19 were insertions. Most mutations were found in stem-loops III b and d. The effect of each mutation on the overall thermodynamic stability of the related domain is indicated. Decreased ΔG means increased stability and vice versa. Pre-T: Pre-treatment; Post-T: Post-treatment.

Sequence diversities in each major quasispecies were compared with genotype 4a and various mutation types were scored.

RNA secondary structure and thermodynamic stability

Prediction of stem-loop structure and thermodynamic stability of the major quasispecies in each patient was performed using the MFOLD 3.2 program with Turner energies and selected constraints on base pairing as indicated^[15]. The program was run on EFN server: 1996-2008, Michael Zuker, Rensselaer Polytechnic Institute (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>).

RESULTS

HCV Quasispecies and genotyping

The mean number of domain III quasispecies in pre-treatment samples of SVR and BT states was about 2 quasispecies per patient with no significant difference between both groups. The quasispecies complexity of domain III tripled in post-treatment samples of BT patients. All patients included in the study were genotyped as 4a except for one SVR patient infected with a hybrid 4a/1b genotype.

Domain III sequence diversities in patient study groups

Different types of mutations were compared in both study groups with the prototype sequence of HCV 4a (Table 1). Insertion mutations in domain III were statistically more prevalent in SVR than BT patients. This is expected since insertions are known to dramatically alter the stem-loop structure by changing the Watson-Crick base pairing of stems, thus reducing molecular

stability of the RNA or binding affinity to ribosomal proteins. The percentage of transition mutations was statistically higher in pre-treatment samples of BT than SVR. There were no statistical differences in percentage of transversion mutations between the two patient groups.

Distribution of mutations in different loops of domain III in SVR compared with BT patients

Alignment of domain III sequences derived from study patients revealed the presence of 19 point mutations scattered in stem-loops III a, b, c and d (Table 1). Percentage distribution of the identified mutations in different loops of domain III in SVR and BT patients is presented in Table 2. The majority of mutations, 14/19 (73.7%), were simple substitutions while 5 (26.3%) were insertions. Substitutions included 42.8% transitions and 57.2% transversions. Most of the mutations (84.2%) were localized in 2 stem-loops; III b (nt 175-205) and III d (nt 254-275), with 42.1% of the mutations in each loop. The remaining 15.8% of mutations were located in junction III a, b, c (10.5%) and in loop III a (5.3%). The SVR patients contain specific mutations (Figure 1) that were not detected in either pre-treatment BT groups or post-treatment BT patients (Table 1). These SVR specific mutations comprise 42.1% (8/19) of the total number of mutations detected in the studied patient population (Table 1). Interestingly, approximately two thirds of SVR (5/8) specific mutations were located in loop III b and one third (3/8) in III d with only one exceptional mutational event (nt 243 in junction joining loops III a, b, c, d) that was detected outside loops III b and III d. These two stem-loop structures play critical roles in directing

Table 2 Distribution of the 19 identified mutations in different loops of domain III in SVR and BT patients

Region	Responder (SVR; %)	BT pre treatment (%)	BT post treatment (%)
Stem-loop IIIa (156-171)	-	14.3	10
Stem-loop IIIb (172-227)	50	14.3	20
Stem-loop IIIc (228-238)	-	-	-
Stem-loop III d (253-279)	40	57.1	50
Junction joining stem-loop IIIa, b, c, & d (141-153) & (239-252)	10	14.3	20

Ten mutations, out of the 19 identified point mutations, are found in responders (SVR). Five of these mutations are found in loop IIIb, four in III d, and one in the junction. Seven mutations are found in the BT pre-treatment group (four in loop III d and one in each of loop IIIa, III b, and junction). Ten mutations were detected in BT post-treatment (five in III d, 2 in each of III b and the junction, and one in loop IIIa).

viral protein translation *via* recruiting and regulating ribosomal subunit proteins and cellular initiation factors for accurate positioning of HCV RNA in the translational machinery of the host cell. On the other hand, mutational events in pre-treatment samples of the BT patients were detected in almost all loop structures of domain III, mostly located in loop III d. Most notably loop III d mutations were associated with decreased ΔG value (Table 1) indicating increased thermodynamic stability of the RNA structure, thus explaining resistance to therapy in the BT patient group. It is noteworthy that most of loop III b mutations in SVR were associated with increased ΔG value, indicating decreased thermodynamic stability of the viral RNA, therefore contributing to the multifactorial eradication of viral RNA in the SVR patient group. When comparing the mutational events in pre-treatment BT patients with those observed post-treatment, the number of mutational events known to induce significant elevation in thermodynamic stability (i.e. decreased ΔG in loop III d) did not increase after treatment in BT patients. These results suggest that domain III-associated factors of viral breakthrough are determined by genetic events in the HCV genome before start of treatment rather than being acquired as a result of stress induced by IFN α therapy.

Prediction of thermodynamic stability and effect of mutations on the secondary structure

Changes in the minimum free energy (ΔG) were detected for stem-loops IIIa, b, c and d spanning nucleotides 141 to 282. Predictive stability values were compared for genotype 1b (strain H77), genotype 4a (I.D. Y11604.1) and study samples derived from SVR and BT patients. The ΔG value of genotype 1b was -53.6 kcal/mol; more stable than genotype 4a (ΔG -47.0 kcal/mol) as illustrated in the secondary structure/thermodynamic stability prediction (Figures 2 and 3). As shown in Table 1, 31.5% of the mutations have no effect on the ΔG value, 31.5% of the mutations were associated with increased ΔG i.e. reduced stability of the secondary structure, while 37% of the mutations were associated with decreased ΔG value

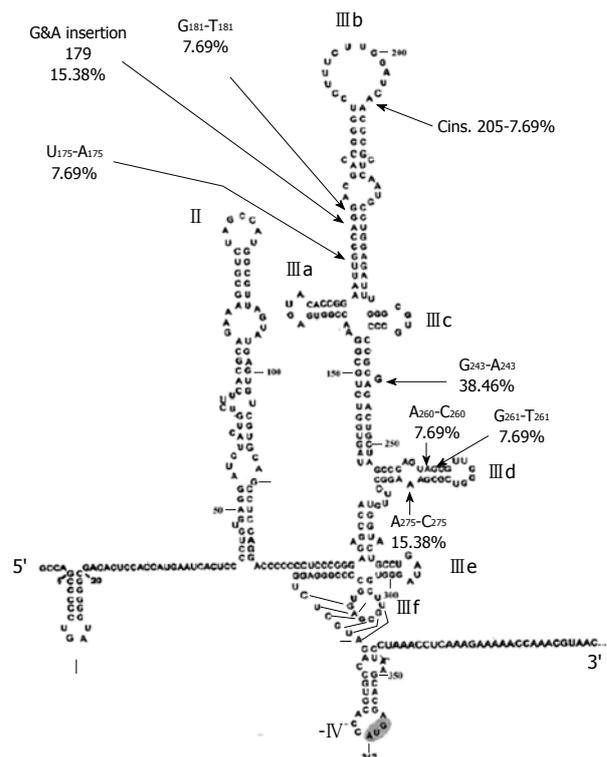


Figure 1 Predicted secondary and tertiary structure of HCV IRES^[23] with mutations observed in SVR patients shown.

i.e. increased stability of the RNA secondary structure. An interesting observation (Table 1 and Figure 1) is that mutations with no effect on ΔG values were located in the apical part of domain III secondary structure (nt 186-243), spanning mostly the upper part of loop III b involved in eIF3 binding. On the other hand, those mutations affecting the stability parameters were localized in the basal part of domain III encompassing loops IIIa/b and III d. Most notably, IIIa/b (nt 158-199) mutations were associated with reduced stability and III d (nt 254-275) mutations were associated with increased stability of the RNA structure. Taken together, the data relating to minimum free energy in SVR and BT patients indicate that the levels of thermodynamic stability are not sufficient parameters to predict response to IFN α treatment and suggest that other parameters involving affinity of RNA binding to ribosomal subunits play important roles in determining response to treatment.

DISCUSSION

The pathway of HCV IRES-mediated initiation of translation is primarily dependent on RNA binding to several cellular proteins, of which ribosomal 40S subunit and eIF3 play pivotal roles in efficient translation of a polyprotein precursor^[10]. Several foot-printing, toe-printing and UV cross linking assays have shown that domain III is the most active part in RNA-protein binding where it folds to form stem-loop structures for high affinity binding to host proteins^[11,17-19]. Although genomic variability in HCV IRES was shown as one mechanism for escaping IFN α effects, there has been

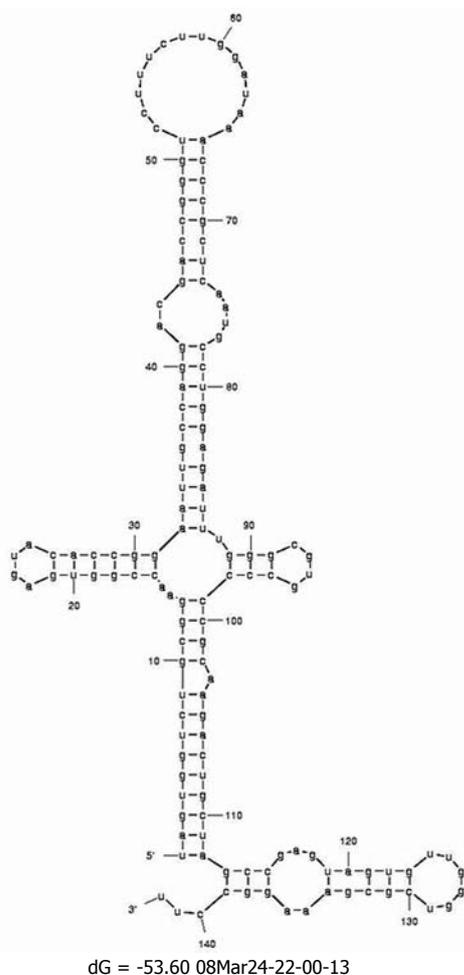


Figure 2 RNA secondary structure and thermodynamic stability of loops III a, b, c, & d from genotype 1b. The minimum free energy value (ΔG) was -53.6 kcal/mol.

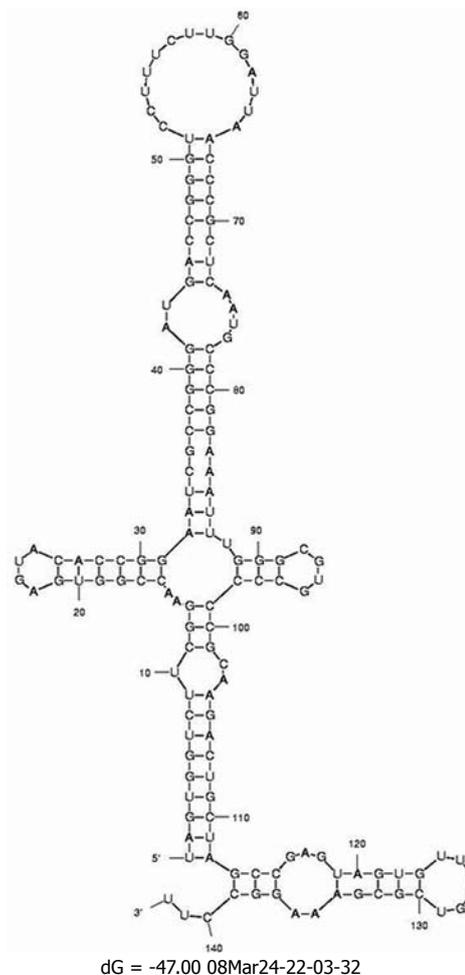


Figure 3 RNA secondary structure and thermodynamic stability of loops III a, b, c, & d from genotype 4a (prototype). The minimum free energy value (ΔG) was -47.6 kcal/mol.

controversy with regard to the predictive value of pre-treatment IRES genomic variations in determining later response to IFN α ^[20]. Although domain III harbors only 22% of the overall IRES mutations in a mixed genotype population^[21], IRES activity seems to depend more on the location of mutant nucleotides which play the most important roles in IRES activity.

We performed a focused study on domain III derived from patients infected exclusively with genotype 4a and presenting a dramatic difference in sustained response after initial viral disappearance. This approach allowed us to minimize quasispecies complexity (mean of 2 variants/patient) compared with 7 or more variants/patient in other studies^[21] and to pinpoint a number of important genomic determinants of response to IFN α . Cloning of domain III and sequencing of 15 clones in each patient allowed us to identify the major variant (identical sequences in 12 clones or more) in each patient so that noise was reduced during extrapolation of the relationship between genomic variation and treatment outcome.

Disruption of base pairing in stem structure significantly inhibits IRES-dependent translation^[22]. In our patient population insertion mutations in domain

III were statistically higher in SVR than in BT patients. These results are expected since insertions are known to dramatically alter the stem-loop structure by changing the Watson-Crick base pairing of stems, thus reducing molecular stability of the RNA or binding affinity to ribosomal proteins. The distributions of domain III mutations in SVR patients were distinct from those of the BT group in the present study. Of the total number of mutations, approximately 42% were exclusive to SVR patients; almost all were located in loops IIIb and III d. These two stem-loop structures were implicated in initiation complex recruitment, positioning and regulation, where III d forms the core of high affinity binding with the 40S subunit^[22] and recruits ribosomal elements involved in positioning of mRNA and tRNA^[10]. Besides binding to eIF3, stem-loop III b interacts with a multisubunit initiation factor involved in subunit assembly and stability of ternary complex^[22].

This explanation cannot simply be taken to resolve mysteries of HCV IRES in light of the apparent controversy in loop III d genomic diversity. All of III d mutations detected exclusively in BT patients (pre- and post-treatment) were associated with increased thermodynamic stability, thus leading to viral persistence;

also most of III_d mutations detected in SVR patients were again associated with increased stability of this RNA. The slow rate of ribosomal subunit transition compared to canonical translation^[11] directs the attention towards the need for flexible rather than rigid binding which allows the conformational rearrangement and subsequent efficient subunit joining, leading to initiation of translation. Therefore mutations affecting binding affinity of factors regardless of thermodynamic stability of RNA structure may be associated with either more flexibility or rigidity which in turn regulates efficiency of translational initiation^[11]. Taken together, the data regarding minimum free energy in SVR and BT patients indicate that the levels of thermodynamic stability are not sufficient parameters to predict response to IFN α treatment and suggest that other parameters involving affinity of RNA binding to ribosomal subunits play significant roles in determining response to treatment. The concept that mutations in BT patients appear only in post-treatment samples and are associated with no effect on RNA stability suggests that viral breakthrough is determined by mutations in domain III before start of treatment rather than being acquired during treatment. Alternatively, the roles of these mutations in viral persistence could be related to fine tuning of the flexibility of RNA structure for binding to cellular factors regardless of its stability. The former view is more plausible since the majority of III_d mutations in pre-treatment were associated with increased RNA stability without change in the frequency of mutations post-treatment. An interesting observation in this study is that the A243G mutation in the III_c/III_d junction was detected both in SVR (5 times) and in pre-treatment BT (2 times) and was more detectable in post-treatment BT patients (4 times). Predictive folding however, revealed no effect of this mutation on the calculated thermodynamic stability. The role of nucleotide 243 in maintaining IRES structure was reported in HCV genotype 1b^[21] and changes at this position were encountered in patients with viral stabilization. In genotype 1b, A243 pairs with U149, which is lacking in 4a, leading to altered pairing and explains the high rate of mutations at this position in our study population regardless of response to IFN α , thus making it more vulnerable to mutational event in genotype 4a.

In conclusion, the RNA structure of domain III in HCV IRES contains several important elements implicated in determining the response to IFN α treatment. The results presented herein demonstrate that domain III structure in SVR patients is different from BT patients. Thermodynamic stability of RNA secondary structure is a significant but not sufficient parameter for prediction of viral stabilization, or response to IFN α . Elements of binding to ribosomal subunit complexes require further studies to unravel the exact role of IRES in HCV stabilization and persistence.

COMMENTS

Background

The hepatitis C virus (HCV) is a major public health problem with about 200 million individuals currently infected with the virus (about 3% of the world's popula-

tion). So far, 11 genotypes and more than 70 subtypes have been identified. The only approved FDA treatment for chronic HCV infection is the combination therapy of pegylated interferon and ribavirin. The variable response of patients to therapy ranges between 28% and 60% and has been proposed to be affected by various host and viral factors. Investigating the effect of mutations within the HCV 5'UTR, the most conserved region in the viral genome, on response to therapy is important because this region is vital for initiation of viral polyprotein translation and the ability of HCV to replicate.

Research frontiers

Although interferon and ribavirin are the only FDA approved drugs to treat HCV, they suffer from several drawbacks including severe side effects (including hematological abnormalities and neuropsychiatric symptoms), very high cost, and most importantly low therapeutic response. Consequently, factors that affect the response of HCV patients to therapy have to be addressed and results could be used for predicting response to therapy before initiation of treatment. Moreover, identification of viral factors that correlate with therapeutic response would contribute to other studies on viral and host factors. This could result in a global view and comprehensive understanding of how host and viral factors affect a patient's response to therapy.

Innovations and breakthroughs

Recent reports, using clinical specimens or HCV replicon systems from different genotypes, have highlighted the effect of mutations in different domains of the viral genome (in particular the HCV 5'UTR) on patients' response to therapy. However, limited studies have been done on genotype 4a. In the present article, The authors focused mainly on genotype 4a which is the predominant genotype in Egypt; found in over 90% of all HCV-infected patients. They showed that the thermodynamic stability of the HCV 5'UTR region is different among responders (sustained viral clearance) and breakthrough patients (who suffer relapse at the end of treatment). Additionally, their results indicate that response to therapy is related mainly to the position of mutations but not their frequency. Finally, thermodynamic stability of IRES was shown to have a direct influence on the binding of the viral genome to the host proteins, which results in initiation of the translation of the viral polyprotein.

Applications

The results of this study suggest that the presence of single nucleotide polymorphisms (SNPs) in certain positions had direct effect on the response of HCV patients to interferon therapy. Taking into consideration the positions of these mutations, different real-time PCR or other assays can be developed for detection of the SNPs to allow the prediction of the response to interferon therapy as a step for identification of patients who are more likely to respond to therapy.

Terminology

5'UTR: non-coding region of HCV RNA; contains the internal ribosomal entry site (IRES); site of initiation of translation. IRES: (internal ribosomal entry site): a structure within the HCV RNA 5'UTR that binds directly to the ribosome to initiate translation. Cap-dependent translation: the mechanism of translation (protein synthesis) predominantly used for cellular proteins. Cap-independent translation: translation via an internal ribosomal entry site (IRES); the mechanism utilized by HCV. Eukaryotic initiation factors: cellular proteins involved in translation. SVR: sustained viral response; patients who show negative HCV PCR results after termination of therapy.

Peer review

The authors present a sequence analysis study of HCV genotype 4a patients undergoing combination therapy. This study is of a substantial potential interest. In this work the authors propose that insertion mutations in domain III of the IRES region are more prevalent in sustained viral response patients compared with breakthrough patients and that such mutations may affect the ability of HCV virus to replicate by decreasing the thermodynamic stability of its RNA.

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Extracolonic findings of computed tomographic colonography in Koreans

Sung Keun Park, Dong Il Park, Sun-Young Lee, Sang Kil Lee, Young-Ho Kim, Soon Jin Lee, Jeong-Sik Byeon, Kyu Chan Huh, Ki-Nam Shim

Sung Keun Park, Dong Il Park, Department of Medicine, Sungkyunkwan University School of Medicine, Kangbuk Samsung Hospital 108, Pyung-dong, Jongro-gu, Seoul 110-746, South Korea

Sun-Young Lee, Department of Internal Medicine, Konkuk University School of Medicine, Seoul 110-746, South Korea

Sang Kil Lee, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 110-746, South Korea

Young-Ho Kim, Departments of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 110-746, South Korea

Soon Jin Lee, Departments of Radiology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 110-746, South Korea

Jeong-Sik Byeon, Department of Internal Medicine, Ulsan University College of Medicine, Asan Medical Center, Seoul 110-746, South Korea

Kyu Chan Huh, Department of Internal Medicine, Konyang University School of Medicine, Daejon 302-718, South Korea

Ki-Nam Shim, Department of Internal Medicine, Ewha Womans University School of Medicine, Seoul 110-746, South Korea

Author contributions: Park SK made out the manuscript; Lee SY played a leading role in designing the study plan; Lee SK, Kim YH, Lee SJ, Byeon JS, Huh KC and Shim KN provided the collection of all study subjects and performed statistical analysis for collected data; Park DI, as corresponding author, designed the study and promoted research.

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Correspondence to: Dong Il Park, MD, PhD, Department of Medicine, Sungkyunkwan University School of Medicine, Kangbuk Samsung Hospital 108, Pyung-dong, Jongro-gu, Seoul 110-746, South Korea. diksmc.park@samsung.com

Telephone: +82-2-20012059 Fax: +82-2-20012610

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Abstract

AIM: To determine the frequency and characteristics of extracolonic lesions detected using computed tomographic (CT) colonography.

METHODS: The significance of extracolonic lesions was classified as high, intermediate, or low. Medical records were reviewed to establish whether further investigations

were carried out pertaining to the extracolonic lesions that were detected by CT colonography.

RESULTS: A total of 920 cases from 7 university hospitals were included, and 692 extracolonic findings were found in 532 (57.8%) patients. Of 692 extracolonic findings, 60 lesions (8.7%) were highly significant, 250 (36.1%) were of intermediate significance, and 382 (55.2%) were of low significance. CT colonography revealed fewer extracolonic findings in subjects who were without symptoms ($P < 0.001$), younger ($P < 0.001$), or who underwent CT colonography with no contrast enhancement ($P = 0.005$). CT colonography with contrast enhancement showed higher cost-effectiveness in detecting highly significant extracolonic lesions in older subjects and in subjects with symptoms.

CONCLUSION: Most of the extracolonic findings detected using CT colonography were of less significant lesions. The role of CT colonography would be optimized if this procedure was performed with contrast enhancement in symptomatic older subjects.

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Key words: Computed tomographic colonography; Extracolonic lesion; Cost; Contrast enhancement; Clinical availability

Peer reviewer: Vito Annesse, MD, Department of Internal Medicine, Unit of Gastroenterology, Hospital, Viale Cappuccini, 1, San Giovanni Rotondo 71013, Italy

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INTRODUCTION

Computed tomographic (CT) colonography allows the visualization of extracolonic organs, thereby permitting

the detection of potentially significant pathologies beyond the colon^[1]. Extracolonic lesions are found in 15%-85% of cases, with some being important lesions, such as extracolonic cancer or aortic aneurysm^[2,3]. However, most of the extracolonic lesions are of minimal importance and lead to further investigations and possibly procedures, with the final diagnosis being simple benign pathology^[2]. Thus, the evaluation and management of extracolonic findings have been found to lead to significant additional cost, and the feasibility and cost-effectiveness of CT colonography needs to be carefully evaluated^[4].

Since multi-section helical CT colonography was first introduced in 1998, improvements such as faster scanning, improved temporal resolution and reduced motion artifacts have been implemented^[5]. However, multidetector CT colonography has been described as a sort of Pandora's box, releasing a cascade of diagnostic events with medicolegal, ethical and economic implications^[6]. Therefore, it would be helpful to clinicians if there were defined strategies for the clinical approach toward the detection of highly significant extracolonic lesions.

To the best of our knowledge, there has been no large multicenter study on extracolonic findings of CT colonography among Koreans. We therefore performed a multicenter study to assess the frequency and characteristics of extracolonic lesions detected with the aid of CT colonography. In addition, we surveyed the factors related to the detection of highly significant extracolonic findings, and analyzed its cost-effectiveness to determine which factors would enhance the potential benefits of CT colonography examination.

MATERIALS AND METHODS

Subjects

The results of CT colonographies performed from January 2005 to December 2006 at the authors' seven university hospitals in Korea were reviewed. Those who were diagnosed as having a malignancy at the time of the CT colonography, those under the age of 16 years and those with ethnicity other than Korean were excluded from the study.

Types and scanning parameters of multidetector array CT colonography are summarized in Table 1. The subjects underwent standard bowel preparation, and a rectal catheter was inserted. Air was used to distend the colon to maximum subject tolerance. Scout image was taken to confirm the adequacy of distention before each examination. Images were taken from the diaphragm to the symphysis with the subject in the supine and prone positions during a breath hold. Medical records were reviewed to establish whether further investigation was carried out pertaining to the extracolonic lesions that were detected by CT colonography during 1 year follow up period. This retrospective study was approved by the institutional review boards which confirmed that the

study was in accordance with the ethical guidelines of the Helsinki Declaration.

Classification of extracolonic lesions

Extracolonic lesions were divided into three categories, according to previous reports^[7,8]. Highly significant lesions include those requiring immediate surgical therapy, medical intervention, and/or further investigation. Examples of highly significant extracolonic lesions include a solid organ mass, adrenal mass greater than 3 cm, aortic aneurysm greater than 3 cm, lymphadenopathy greater than 1 cm, cardiomegaly, pericardial effusion, fistula, abscess and small-bowel infarction.

Lesions of intermediate significance include conditions that do not require immediate therapy but would likely require further investigation, recognition, or therapy at a later time. Examples of such extracolonic lesions include calculi, intermediate cysts, pulmonary fibrosis, inguinal hernia, uterine myoma, endometriosis, pelvic fluid collection, liver cirrhosis, liver hemangioma and bile duct dilatation.

Lesions of low significance include benign conditions that do not require further medical therapy or additional work-up. Examples of such extracolonic lesions include calcifications, granulomas, diverticulosis, simple organ cysts, hernias, pleural thickening, benign prostatic hypertrophy, accessory spleen, benign bony lesion, fatty liver, and renal infarction.

Statistical analyses

Differences between the groups were analyzed using the chi-square test and Student's *t*-test. The age was expressed as mean \pm SD (standard deviation) values. Cost effectiveness was calculated by cost needed for detecting one highly significant lesion (cost of CT colonography \times total number of CT colonography/number of subjects with highly significant extracolonic lesions). Regression analysis was performed to assess the related factors in detecting extracolonic lesions according to their significance. A probability value of $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the subjects

A total of 920 consecutive subjects (men/women = 535/385) were analyzed. Their mean age (\pm SD) was 57.3 \pm 12.8 (range, 34-87). Of these, 692 extracolonic findings were found in 532 (57.8%) subjects (Table 2). Of the 692 extracolonic findings, 60 (8.7%) were highly significant, 250 (36.1%) were of intermediate significance, and 382 (55.2%) were of low significance (Table 3). Data regarding the examination, age, and sex distribution of each group are summarized in Table 4.

Of 920 subjects, 764 and 156 subjects were examined by CT colonography with and without the aid of contrast enhancement, respectively. Extracolonic lesions were found in 459 of the 764 subjects (60.1%)

Table 1 Characteristics of the study population

Hospital	Number of the subjects	Type of CT colonography	kVp	mAs	Pitch	Slice thickness/reconstruction interval for extracolonic finding (mm)
A	278	Sensation 64; Siemens, Erlangen, Germany	120	70	1.5	3/3
B	157	LightSpeed Ultra 8 or 16; GE Medical systems, Milwaukee, WI	120	70	1.35	1.25/1.25
C	152	Sensation 16; Siemens, Erlangen, Germany	120	30	1	5/5
D	135	Brilliance 40-channel MDCT, Phillips Medical System, Netherlands	120	160	1.176	0.5/0.9
E	92	LightSpeed 16; GE healthcare, Milwaukee, Wis	120	200	1.375	1.25/3.75
F	65	MX 8000 IDT 16, Phillips Eindhoven, Netherlands	120	200	1	2/1
G	41	Sensation 16; Siemens, Erlangen, Germany	120	30	1	5/5

Table 2 Results of 920 computed tomographic colonoscopy examinations *n* (%)

Number of extracolonic findings	Number of subjects
0	388 (42.2)
1	403 (43.8)
2	105 (11.4)
3	19 (2.1)
4	5 (0.5)

examined with contrast enhancement, but in only 73 of the 156 subjects (46.8%) examined without contrast enhancement ($P = 0.005$).

Factors related to the clinical significance of extracolonic findings

The mean age was lower in cases without extracolonic findings (Table 4). With regard to indications for CT colonography, gastrointestinal symptoms were more common in those in whom significant lesions were detected (Table 4). Regression analysis revealed that, older age ($P < 0.001$), being female ($P = 0.001$), presence of symptoms ($P < 0.001$), and the use of contrast during CT colonography ($P = 0.003$) were associated with detection of the more significant extracolonic lesions.

Additional evaluation and management of extracolonic findings

Table 5 lists the additional tests performed in each group. It can be seen that, 81.7% of highly significant subjects received further treatment, while such treatment was received in only 20.8% and 2.9% of subjects of intermediate and low significance, respectively.

Cost of finding a highly significant extracolonic lesion

Since each CT colonography procedure costs US \$ 190 (180000 won) in Korea, US \$ 2905 (2760000 won) was needed to detect each highly significant lesion in our study (i.e. cost of CT colonography \times total CT colonography cases/number of subjects with highly significant extracolonic lesions). The following factors were found to be associated with poor cost-effectiveness: patient age below 60 years, lack of symptoms and use of CT colonography without contrast enhancement (Figure 1).

DISCUSSION

In this study, extracolonic lesions were found in 532

Table 3 Proportion of extracolonic lesions according to the clinical significance

Extracolonic findings	Number
Highly significant (<i>n</i> = 60)	
Solid organ mass including malignancy	42 ¹
Cardiomegaly/pericardial effusion	5
Lymphadenopathy greater than 1 cm	3
Peritoneal carcinomatosis	3
Abscess	3
Aortic lesion	2
Small bowel obstruction	2
Intermediately significant (<i>n</i> = 250)	
Benign solid organ lesion	141 ²
Renal stone/hydronephrosis	28
Gall bladder stone/polyp/cholecystitis	22
Liver cirrhosis	13
Bile duct stone/dilatation/hemobilia	9
Small bowel inflammation	8
Vascular lesion (aortic stenosis, varix, etc)	6
Bronchiectasis/emphysema	5
Hepatosplenomegaly	5
Pleural effusion	3
Inguinal hernia	3
Ascites of unknown cause	3
Chronic pancreatitis	2
Mesenteric fat necrosis	1
Spinal stenosis with destruction	1
Lowly significant (<i>n</i> = 382)	
Renal cyst	143
Hepatic cyst	114
Fatty liver	39
Vascular calcification/atherosclerosis	19
Chronic pulmonary disease/pleural thickening	16
Accessory spleen/splenic infarction	15
Hepatic calcification	10
Benign osteolytic lesion	8
Hiatal hernia	6
Benign prostatic hypertrophy	5
Colonic diverticulosis	4
Tiny pancreas cyst	1
Mesenteric calcification	1
Gallbladder sludge	1

¹Liver 9, lung 9, stomach 7, pancreas 3, kidney 3, bladder 3, adrenal gland 2, small bowel 2, bone 1, bile duct 1, psoas muscle 1 and ovary 1. ²Liver 46, kidney 30, uterus 19, ovary 13, lung 9, adrenal gland 8, lymph node 7, muscle 4, pancreas 3, spleen 1 and testis 1.

out of 920 subjects (57.8%), which is consistent with previous studies reporting incidences of between 33% and 85%^[2-4,6,8,9]. A substantial proportion of these lesions were insignificant, which led to further unnecessary workup and, hence, additional cost. Highly significant extracolonic lesions were detected in the present study

Table 4 Baseline characteristics according to the clinical significance of extracolonic lesions *n* (%)

	Highly significant lesion (<i>n</i> = 60)	Intermediately significant lesion (<i>n</i> = 250)	Lowly significant lesion (<i>n</i> = 382)	No extracolonic lesion (<i>n</i> = 388)	<i>P</i> -value
Age (mean ± SD)	58.3 ± 16.4	57.9 ± 13.8	59.0 ± 11.9	54.4 ± 13.1	< 0.001
Male:Female	36:24	116:134	237:145	233:155	0.001
Indication					< 0.001
Screening	16 (26.7)	76 (30.4)	155 (40.6)	160 (41.2)	
Family history	1 (1.7)	4 (1.6)	4 (1.0)	13 (3.4)	
Past history	10 (16.7)	38 (15.2)	55 (14.4)	93 (24.0)	
GI bleeding	5 (8.3)	19 (7.6)	11 (2.9)	17 (4.4)	
IDA	1 (1.7)	7 (2.8)	8 (2.1)	0 (0.0)	
Bowel habit change	6 (10.0)	27 (10.8)	44 (11.5)	38 (9.8)	
Abdominal pain	17 (28.2)	58 (23.2)	78 (20.4)	56 (14.4)	
Others	4 (6.7)	21 (8.4)	27 (7.1)	11 (2.8)	
CT with enhancement ¹	53 (88.3)	225 (90.0)	320 (83.7)	305 (78.6)	0.001
Hospital					< 0.001
A (<i>n</i> = 313)	23 (38.4)	61 (24.4)	77 (20.2)	151 (38.9)	
B (<i>n</i> = 214)	9 (15.0)	86 (34.4)	86 (22.6)	33 (8.5)	
C (<i>n</i> = 171)	5 (8.3)	24 (9.6)	60 (15.7)	81 (20.9)	
D (<i>n</i> = 149)	2 (3.3)	31 (12.4)	70 (18.3)	45 (11.6)	
E (<i>n</i> = 104)	10 (16.7)	20 (8.0)	44 (11.5)	30 (7.7)	
F (<i>n</i> = 73)	5 (8.3)	16 (6.4)	12 (3.1)	40 (10.3)	
G (<i>n</i> = 59)	6 (10.0)	12 (4.8)	33 (8.6)	8 (2.1)	

¹Computed tomography with pre- and post-contrast images enhanced by intravenous contrast. SD: Standard deviation; GI: Gastrointestinal; IDA: Iron deficiency anemia.

Table 5 Further managements according to the clinical significance of extracolonic lesions *n* (%)

	Highly significant lesion (<i>n</i> = 60)	Intermediately significant lesion (<i>n</i> = 250)	Lowly significant lesion (<i>n</i> = 382)
Diagnostic intervention			
US	6 (10.0)	108 (43.2)	31 (8.1)
CT	17 (28.3)	65 (26.0)	21 (5.5)
MRI	4 (6.7)	14 (5.6)	0 (0.0)
Biopsy	8 (13.3)	3 (1.2)	2 (0.5)
Endoscopy	6 (10.0)	6 (2.4)	1 (0.3)
Other tests	18 (30.0)	25 (10.0)	10 (2.6)
Not done	1 (1.7)	29 (11.6)	317 (83.0)
Therapeutic intervention	49 (81.7)	52 (20.8)	11 (2.9)

US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging.

in only 60 of 920 subjects (6.5%), which is slightly lower than the incidences found in previous studies. This discrepancy might be due to differences in the study population (ours included only Koreans), the definition of highly significant lesion used and the CT colonography conditions used. In our study, a solid organ mass suspicious of malignancy was detected in 42 of 920 (4.6%) subjects. Considering that substantial numbers of subjects undergoing CT colonography are found to have clinically important extracolonic findings, this would have positive effects on health care by undergoing additional evaluations^[10]. The cost of a CT colonography in Korea, i.e. US \$190 (180000 won), is only US \$ 53 (50000 won) more expensive than colonoscopy. Therefore, CT colonography might be more attractive in Korea, since it is less expensive when compared with US^[11,12]. Several studies have reported

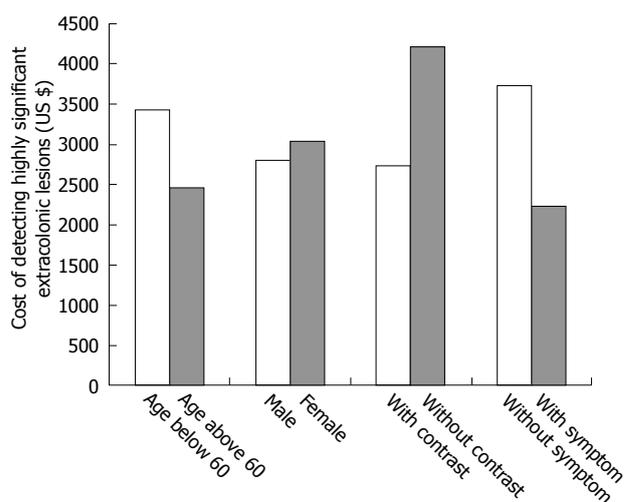


Figure 1 Cost of detecting highly significant extracolonic lesions. Cost-effectiveness was assessed using the following calculation for each group. Poor cost-effectiveness in the detection of highly significant lesions was observed for subjects aged below 60 year-old (US \$ 3442), subjects without symptoms (US \$ 3737), and CT colonography performed without contrast enhancement (US \$ 4221).

on a prospective cost-benefit analysis of diagnostic CT colonography^[10,13]. Some reported low clinical relevant disease in average-risk asymptomatic adults^[14], while others revealed higher proportion of colon cancers in subjects with colonic symptoms^[13]. We further tried to identify the factors associated with the more effective use of CT colonography by analyzing the cost of detecting highly significant extracolonic lesions. As expected, the prevalence of significant extracolonic lesions was higher in older subjects and those with gastrointestinal symptoms. Since our results suggest that significant extracolonic lesions can be anticipated at a higher

frequency in this population than in an asymptomatic younger population, they also contribute toward a better understanding of the selection of subjects who would benefit more effectively from CT colonography.

Apart from age and clinical symptoms, contrast enhancement was found to be advantageous in identifying extracolonic lesions on CT colonography. This demonstrates that some important extracolonic lesions might have been overlooked in non-contrast enhanced cases. The inability of low-radiation dose CT colonography to accurately define lesions in other organs also raises important medico-legal considerations^[2]. Based on our findings, age, the presence of gastrointestinal symptoms and the use of contrast enhancement must be taken into account when deciding when to use CT colonography in routine clinical practice.

In the present study, 221 of 250 (88.4%) subjects with extracolonic lesions of intermediate significance were referred for further investigations, of which 52 (20.8%) received treatment, while 65 of 382 (17.0%) subjects with extracolonic lesion of low significance were referred for further investigations, of which only 11 (2.9%) received treatment. Because symptomatic subjects were included in our study, CT colonography was performed as a diagnostic evaluation as well as a screening tool. This would explain why further investigations frequently followed CT colonography. Our results indicate that further investigations pertaining to extracolonic lesions, other than those of high significance, benefit only a few and result in additional and unnecessary cost as a result of unnecessary workups.

The limitation of our study is that there were some differences due to inhomogenous settings. Different participating institutions used such relevant differences in study protocols: Slice thickness varies between 0.5 and 5 mm and mAs varies between 30 and 200. The radiation dose was in the range of 1.7-8.8 mSv, with a median of 3.9 mSv. It was comparatively larger than simple X-ray or plain abdomen with approximately 0.1 mSv. For example, the hospitals D, E and F used almost standard dose contrast, while slice thickness were less than 1 mm for extracolonic lesions at the hospitals B, D and F. However, when considering that the proportions of normal extracolonic findings were highest in hospital F (54.5%) > A (48.2%) > C (47.3%) > D (30.2%) > E (28.8%) > B (15.4%) > G (13.6%), slice thickness and standard dose are not predictive factors of the presence of extracolonic lesions. Another limitation concerns the number of false positive and false negative results of the exam. Since study populations have not been followed up periodically, correct false positivity and negativity could not be evaluated. However, subjects diagnosed as having significant extracolonic lesions received full evaluation and treatment for their lesions. Accordingly, false positivity of significant extracolonic lesions was nearly zero.

In conclusion, most of the extracolonic lesions detected by CT colonography were of low significance, and resulted in additional costly investigations. However,

CT colonography may demonstrate asymptomatic malignant disease requiring immediate treatment in older subjects and among those with symptoms, particularly when performed with contrast enhancement. Based on these results, CT colonography should be performed with contrast enhancement in symptomatic older subjects.

COMMENTS

Background

Currently, computed tomographic (CT) colonography is widely used in the clinical field to visualize colon and extracolonic lesions. Extracolonic lesions occur in 15%-85% of cases, with some being important lesions, such as extracolonic cancer or aortic aneurysm. The utilization of CT colonography will increase in clinical field, and research for availability, detection rate and cost-effectiveness of CT colonography is necessary.

Research frontiers

Early detection of extracolonic lesions is an aim of CT colonography. In particular, the detection of significant lesions is very important. However, the incidence rates of significant extracolonic lesions vary from country to country, and most reports relate to the Western population. This is the first study focusing on the Asian population, where the incidence rate of colorectal disease is lower than in the Western population.

Innovations and breakthroughs

In recent reports, cost-effectiveness of CT colonography was calculated in US dollars, because most studies were carried out in the USA. However, the cost of CT colonography varies according to different countries. This study evaluated its cost-effectiveness taking into consideration the specific medical system of the country. In addition, optimal methods to detect significant extracolonic lesions were evaluated. This study showed that the selective use of CT colonoscopy (for symptomatic elderly and with contrast enhancement) shows a good cost-effectiveness.

Applications

Use of CT colonography is currently rising due to its various functions. However, cost of CT colonography is comparatively high, and clinical availability is being evaluated. This study is helpful to clinicians to determine the best way to use the CT colonography for detecting highly significant extracolonic lesions.

Terminology

Multi-section helical CT colonography was first introduced in 1998. There have many improvements such as faster scanning, improved temporal resolution, and reduced movement artifacts. Various approaches were tried to increase the effectiveness of CT colonography, and contrast enhancement is recommended as a good strategy if it is applied to an ideal case.

Peer review

The authors examined the cost-effectiveness of CT colonography for various Korean patients, and proposed how to optimize the use of CT colonography. Considering the rising application of CT colonography to the medical field, this study will provide a good basis to guide its use.

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Hirschsprung's disease: Is there a relationship between mast cells and nerve fibers?

Amit Kumar Yadav, Kiran Mishra, Anup Mohta, Sarla Agarwal

Amit Kumar Yadav, Department of Pathology, University College of Medical Sciences, New Delhi 110095, India

Kiran Mishra, Department of Pathology, University College of Medical Sciences, New Delhi 110095, India

Anup Mohta, Department of Paediatric Surgery, Chacha Nehru Baal Chikitsalya, New Delhi 110095, India

Sarla Agarwal, Department of Pathology, University College of Medical Sciences, New Delhi 110095, India

Author contributions: Yadav AK and Mishra K contributed equally to this work; Yadav AK, Mishra K and Agarwal S designed research; Yadav AK, Mishra K and Mohta A performed research; Yadav AK, Mishra K, Agarwal S and Mohta A contributed new reagents/analytic tools; Yadav AK and Mishra K analyzed data; Yadav AK and Mishra K wrote the paper.

Correspondence to: Amit Kumar Yadav, Department of Pathology, University College of Medical Sciences, Dilshad Garden, New Delhi 110095, India. amityadav7284@yahoo.co.in
Telephone: +91-11-22592971-79

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Indian children than that reported in Western literature. Their role in HD needs further research. Morphometry of S-100 stained nerve fibers is a useful adjunct to conventional methods for diagnosis of HD.

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Key words: Hirschsprung's disease; Mast cells; S-100; Acetylcholinesterase

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Abstract

AIM: To define the topography of mast cells and their numbers in cases of Hirschsprung's disease (HD) and non-HD, assess neural hypertrophy using imaging software and to study the relationship between mast cells and nerve fibers.

METHODS: HE stained sections of 32 cases of chronic constipation in the age group of 0-14 years were reviewed for ganglion cells. AChE staining was performed on frozen sections of colonic and rectal biopsies. Based on their findings cases were divided into HD and non-HD and mast cells stained by toluidine blue were evaluated. Image analysis by computerized software was applied to S-100 stained sections for assessment of neural hypertrophy.

RESULTS: Difference between number of mast cells in HD group (mean = 36.44) and in non-HD group (mean = 14.79) was statistically significant. Image analysis morphometry on S-100 stained sections served as a useful adjunct. The difference between number, size, and perimeter of the nerve fibers between HD and non-HD group was statistically significant.

CONCLUSION: Mast cells are significantly increased in HD and their base line values are much higher in

INTRODUCTION

Constipation is a common presenting complaint in children attending the pediatric outpatient department^[1]. Some 13%-15% cases of constipation are due to aganglionosis of bowel segment or Hirschsprung's disease (HD). HD is a serious disorder and can be fatal if left untreated. The disease is however, surgically correctable, underscoring the need to identify these cases.

HD has an incidence of 1 in 5000 neonates in Western literature and a male to female predominance of 4:1. Although there are no statistical figures available from India, a large number of studies in Indian literature suggest that the disorder is not uncommon in our country^[2-4].

Of late there has been a lot of interest in the role of mast cells (MC) in the pathogenesis of Hirschsprung's disease. Kobayashi *et al*^[5] have found an increased number of mast cells in aganglionic bowel segments and in those with intestinal neuronal dysplasia. The exact role of mast cells in HD is not known. Therefore we undertook this study to evaluate the relationship between mast cells and nerve fibers in HD cases to shed some light on this issue. We have used image analysis morphometry to analyze nerve fibers stained with S-100 and correlated the findings with mast cell numbers.

There are only a couple of studies on mast cells in

HD in the literature^[5-7]. There is however no study from India on mast cells in children with HD or constipation. As children in a developing country like India are more likely to be exposed to inflammatory stimuli like bacterial and viral antigens and pollutants/allergens as compared to their Western counterparts, an increase in mast cells was expected in the intestines. The cells were assessed in children with constipation, with an emphasis on HD, to ascertain their distribution in the colonic and rectal biopsies.

MATERIALS AND METHODS

The study was conducted in the departments of Pathology and Surgery, University College of Medical Sciences and GTB Hospital, New Delhi.

Selection criteria

Thirty two cases of chronic constipation (defined as decreased frequency of bowel movements “fewer than three each week” or a difficulty in defecation perceived by the parents as a problem that requires medication or manual intervention^[1]) in the age group of 0-14 years were enrolled for the study. Cases of constipation due to local causes like anal fissure, anal stenosis and anterior perineal anus were excluded from the study.

The criterion used for diagnosis of HD in all of the HD cases was absence of ganglion cells upon evaluation of Haematoxylin & Eosin (HE) stained sections of colonic and rectal biopsies. Acetylcholinesterase (AChE) stain for the nerve fiber pattern was carried out in 12 cases. Based on the findings of HE and AChE they were divided into two groups: Group I - HD and Group II -non-HD. Group I -HD included 11 rectal biopsies and 7 resection specimens. Group II -non-HD included 14 rectal biopsies. Rectal biopsies obtained were rectal punch biopsies. Mucosa and submucosa was available for review in all the cases. Muscularis propria was available in resection specimens only.

Toluidine blue stain was performed on paraffin sections of all the cases to evaluate mast cells. Immunohistochemical stain for S-100 was also performed on 12 HD cases & 8 non-HD cases.

Acetylcholinesterase stain

Ten micrometer thick sections cut on the cryostat were fixed by dipping in buffered formalin for 1-2 min, preserved by wrapping them in aluminium foil and stored at -20°C in a deep freezer till the time of staining. Stock solutions of various ingredients of incubation medium were kept at 4°C and the incubation medium was prepared just before staining. AChE stain was performed using modified Karnovsky and Roots method as described previously^[8]. Incubation medium was prepared by mixing 0.1 mol/L sodium hydrogen maleate buffer 6.5 mL (pH 6.0), 0.1 mol/L sodium citrate 0.5 mL, 30 mmol/L copper sulphate 1.0 mL, 5 mmol/L potassium ferricyanide 1.0 mL, water 1.0 mL and acetylthiocholine iodide 5 mg. The sections were incubated at 37°C for 20 min in the incubation medium. Thereafter, the incubation medium

was drained off and re-incubation with rubanic acid solution was performed for 10 min. Rubanic acid solution was prepared by mixing absolute alcohol 10 mL, sodium acetate 6.55 g, rubanic acid 10 mg and distilled water 40 mL. Thereafter, the sections were counterstained with haematoxylin, dehydrated, cleared and mounted in DPX.

Toluidine blue

Toluidine blue staining on paraffin sections was performed using a simple toluidine blue method that required incubation of sections in 1% aqueous solution of toluidine blue and mounting in a water based medium. Mast cells were counted in five successive high power fields ($\times 400$) in the submucosa.

S-100 Stain

Immunohistochemical stain for S-100 (Dako Corporation) was performed on HD (12 cases) and non-HD (8 cases) by the standard immunoperoxidase technique. The nerve fibers that stained positive for S-100 were counted in five high power fields ($\times 400$) in the submucosa.

Image analysis

Images of relevant areas in S-100 stained slides were captured using a digital camera. They were subsequently analyzed using the Scion image analysis software (www.scioncorp.com). The size and perimeter of the thickest nerve fiber in the submucosa was measured. The number, size and perimeter of nerve fibers were correlated with mast cell number in both the groups.

Statistical analysis

Statistical analysis was performed by the chi-square and student *t*-test and correlation assessed by Pearson's correlation coefficient.

RESULTS

A total of 32 cases of chronic constipation in children below 14 years were studied. Based on the findings of HE and AChE stained sections of colonic and rectal biopsies, they were divided into two groups: Group I - HD and Group II -non-HD. Group I -HD included 18 cases and Group II -non-HD included 14 cases.

The age of the patients ranged from 1 mo to 84 mo in the HD group (mean ± 2 SD = 26.78 \pm 36.72 mo). Maximum numbers of cases were found in the age group 36-47 mo in this group. In the non-HD group, age ranged from 6 d to 96 mo (mean ± 2 SD = 26.64 \pm 49.60 mo), maximum cases in this group being in the age group of 0-11 mo. Male to female ratio was 17:1 in the HD group and 4:3 in the non-HD group.

There was statistically no significant difference in the age distribution between the two groups ($P = 0.8$). The difference in sex distribution between the two groups was statistically significant, the patient population in the HD group having a distinct male bias ($P = 0.01$).

Mast cells

The data obtained after evaluation for mast cells are

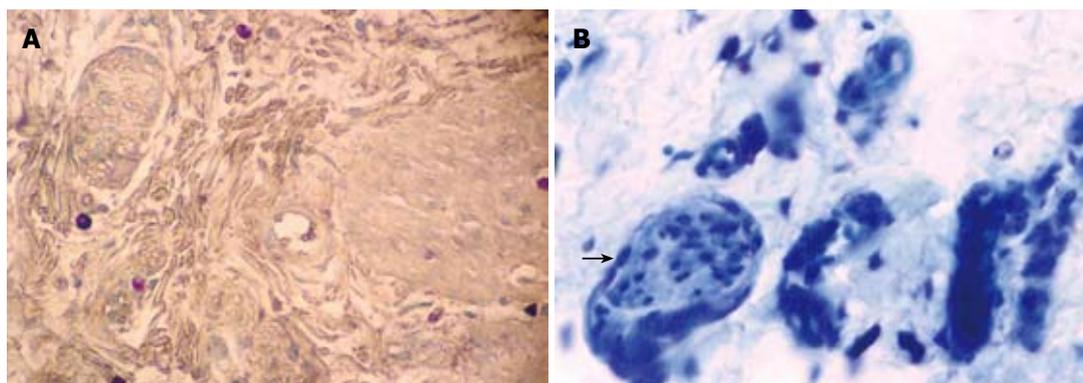


Figure 1 Perineural and intraneural mast cells ($\times 200$) are increased in HD patients (A) compared to non-HD patients (B). (Toluidine blue, $\times 200$).

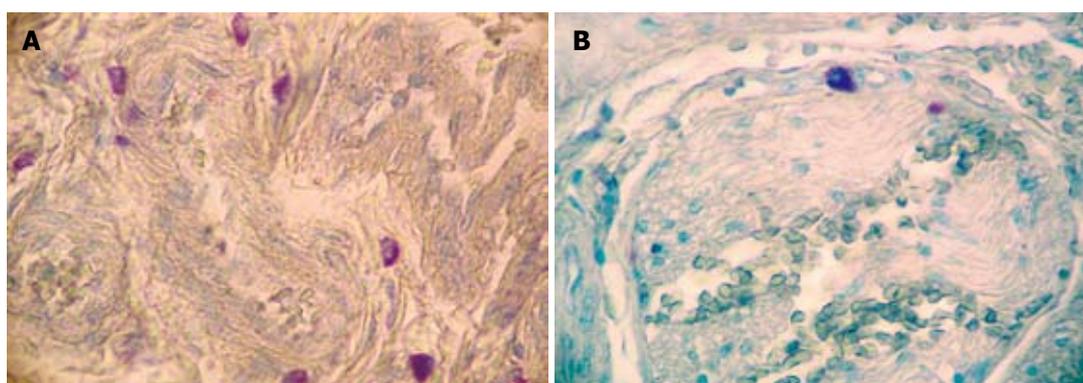


Figure 2 Increased number of mast cells in perivascular distribution ($\times 400$) in HD case (A) as compared to non-HD case (B) showing an occasional mast cell ($\times 400$) only. (Toluidine blue).

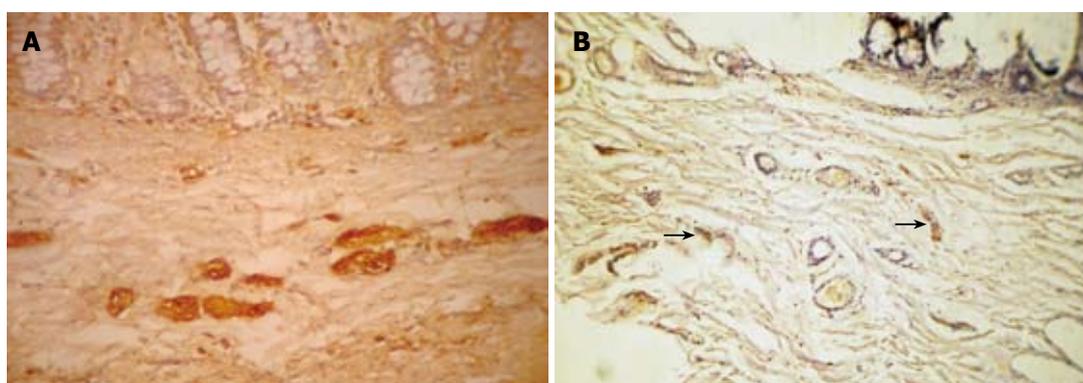


Figure 3 Hypertrophic nerve fibers in submucosa ($\times 100$) in HD case (A) as compared to small and thin nerve fibers (black arrows, $\times 100$) in non-HD case (B). (S-100).

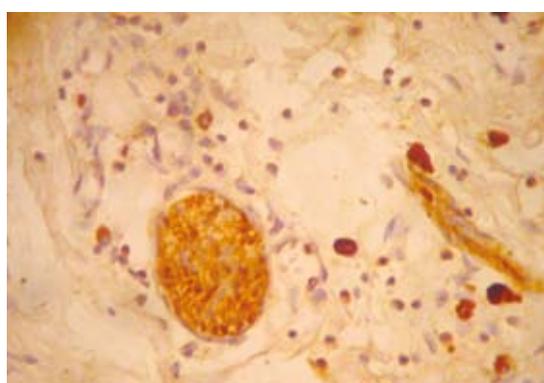


Figure 4 Hypertrophic nerve fiber in submucosa surrounded by mast cells. (S-100, $\times 400$).

summarized in Table 1. There was a statistically significant difference in the number of MC between HD (mean ± 2 SD = 36.44 ± 36.02) and non-HD (mean ± 2 SD = 14.79

± 19.8) ($P = 0.0001$). The mast cells were distributed transmurally (An observation in resection specimens of HD cases. Statistical analysis could not be done due to small sample size) and were notably present around the nerve fibers (Figure 1) and perivascularly (Figure 2). The number of mast cells in HD as well as in non-HD cases was significantly higher than previous studies (Table 2).

S-100

S-100 immunohistochemistry was performed on 20 cases consisting of 12 HD and 8 non-HD cases (Table 3). The number of nerve fibers in the submucosa (Figure 3) of HD (mean ± 2 SD = 11.17 ± 6.18) cases was higher than in non-HD (mean ± 2 SD = 5.13 ± 7.66) cases. The difference between the two groups was statistically significant ($P = 0.0001$). S-100 also stained the mast cells (Figure 4).

Image analysis

Size of the nerve fibers in HD group was mean ± 2 SD

Table 1 Mast cells in Hirschsprung's disease (HD) and non-HD cases

S. No.	HD cases			S. No.	Non-HD cases		
	Age (mo)	Sex	No. of mast cells		Age (mo)	Sex	No. of mast cells
1	36	M	35	1	30	F	1
2	36	M	40	2	24	M	15
3	12	F	35	3	36	M	25
4	24	M	35	4	36	F	5
5	3	M	10	5	4	M	10
6	36	M	30	6	48	M	3
7	5	M	50	7	1	M	1
8	5	M	20	8	1	M	25
9	36	F	70	9	96	M	15
10	24	F	20	10	1	F	32
11	84	M	45	11	2	M	15
12	36	M	45	12	30	F	25
13	84	M	80	13	36	M	15
14	18	F	30	14	48	M	20
15	36	M	45				
16	48	M	26				
17	48	M	13				
18	12	F	27				

HD (mean \pm 2 SD = 36.44 \pm 36.02) and non-HD (mean \pm 2 SD = 14.79 \pm 19.8) [(HD vs non-HD) $P = 0.0001$].

= 57.18 \pm 24.86 μm and in non-HD group it was mean \pm 2 SD = 31.03 \pm 39.08 μm . The difference between the two was statistically significant ($P = 0.0001$). The perimeter of nerve fiber in HD group was mean \pm 2 SD = 201.89 \pm 88.78 μm and in non-HD group was mean \pm 2 SD = 113.26 \pm 141.18 μm . This difference between the two groups was also statistically significant ($P = 0.0001$) (Table 2).

Mast cells and nerve fibers

We correlated each of the parameters obtained by image analysis morphometry of the nerve fibers with MC number. In the HD group it was observed that maximum correlation was seen with number of nerve fibers (correlation coefficient = 0.467). No correlation was seen with size of nerve fibers (correlation coefficient = -0.131) and some correlation was observed with the perimeter of nerve fibers (correlation coefficient = 0.274). In the non-HD group the respective values were 0.406, 0.304 and 0.157.

DISCUSSION

Recently there has been a lot of interest in the role of mast cells in HD. Kobayashi *et al*^[5] described an increased number of mast cells in the aganglionic segment of the colon in patients with HD. The number of these cells in transitional segments was significantly less compared with ganglionic segments in HD patients and controls. Similar findings were reported by Demirbilek *et al*^[6].

In the present study, mast cells were evaluated in rectal biopsies as well as in resected specimens from cases of HD and non-HD. Although the mast cells can be roughly estimated after HE staining, an exact count is obtained by

Table 2 Comparison of mast cells in submucosa of HD and non-HD cases with previous studies

Mast cells per 5 hpf in submucosa		Kobayashi <i>et al</i> ^[5]	Demirbilek <i>et al</i> ^[6]	Present study
HD cases	Aganglionic segment	23.9 \pm 6.6	18.2 \pm 3.3	36.36 \pm 39.58
	Ganglionic segment	8.5 \pm 3.9	1.7 \pm 0.4	38 \pm 16.66
Non-HD cases		7.2 \pm 3.4	5.4 \pm 1.2	14.8 \pm 5.3

Aganglionic segment (Kobayashi *et al* vs Present study, $P = 0.0001$; Demirbilek *et al* vs Present study, $P = 0.0001$); Ganglionic segment (Kobayashi *et al* vs Present study, $P = 0.0001$; Demirbilek *et al* vs Present study, $P = 0.0001$); Non HD cases (Kobayashi *et al* vs Present study, $P = 0.0001$; Demirbilek *et al* vs Present study, $P = 0.0001$).

special histochemical methods. Other authors^[5] have used immunohistochemical detection of anti-MC antibody to demonstrate MC. In our study, these were studied by a simple and effective toluidine blue staining which requires a time as short as one minute incubation. A significant increase was noticed in the number of mast cells in HD as compared with the non-HD cases. The HD cases showed a transmural distribution of these cells as described in the previous two studies by Kobayashi *et al*^[5] and Demirbilek *et al*^[6]. However, in a recent study Hermanowicz *et al*^[7] found them to be increased in the mucosa and lamina propria but the increase in mast cells in the submucosa, muscularis propria and serosa was not statistically significantly changed.

The mast cells were characteristically distributed around the nerves and blood vessels in addition to being randomly scattered. In our study the baseline number of the mast cells (i.e. number of mast cells in non-HD cases) was much higher than previous studies^[5,6] (Table 2) and few mast cells were also seen in the ganglionic segment. This may be due to the response of the mast cells to infectious agents or allergens rather than their association with aganglionosis. An increased number of mast cells is also reported in various other gastrointestinal disorders such as acute appendicitis, ulcerative colitis, celiac disease and gluten enteropathy^[9,10]. They are seen to be in apposition to the nerves^[11] and are known to secrete substances like nerve growth factor^[12].

We also correlated each of the parameters obtained by image analysis morphometry of the nerve fibers with MC number. It was observed that maximum correlation was seen with number of nerve fibers (correlation coefficient = 0.467). No correlation was seen with size of nerve fibers and some correlation was observed with the perimeter of nerve fibers (correlation coefficient = 0.274). This correlation has not been shown in any previous study. This suggests that mast cells via their mediators may cause increased number of nerve fibers and affect size of nerve fibers to some extent. The interactive role of mast cells, their nerve growth factor secretion and neural hypertrophy as suggested by morphology is not explained by the studies to date.

Table 3 Number, size and perimeter of nerve fibers in HD and non-HD cases

S. No.	HD cases					Non-HD cases				
	Age (mo)	Sex	No. of nerve fibers	Size of nerve fibers (μm)	Perimeter of nerve fibers (μm)	Age (mo)	Sex	No. of nerve fibers	Size of nerve fibers (μm)	Perimeter of nerve fibers (μm)
1	42	M	13	40.37	230.48	36	M	3	42.5	215.7
2	12	F	8	68.0	195.9	1	M	2	11.2	37.0
3	36	M	11	65.4	172.8	5	M	4	15.3	47.3
4	84	M	7	47.3	135.4	1	M	4	15.0	51.8
5	48	M	16	63.5	288.75	1	F	3	40.6	135.56
6	36	M	10	77.38	237.79	30	F	3	25.0	126.67
7	48	M	12	57.5	256.11	4	M	9	28.8	83.2
8	36	M	14	40.7	149.3	13	M	13	69.9	208.09
9	12	M	6	55.5	190.03					
10	24	M	11	47.6	184.08					
11	1	M	15	49.6	178.18					
12	60	M	11	73.4	203.9					

Number of nerve fibers (HD vs non-HD), $P = 0.0001$; Size of nerve fibers (HD vs non-HD), $P = 0.0001$; Perimeter of nerve fibers (HD vs non-HD), $P = 0.0001$.

The exact role of these cells in the pathogenesis of HD needs further research with focus on the enteric nervous system, its development and the role of MC in cases where ganglion cells are absent.

The neural hypertrophy in the colonic submucosa is associated with aganglionosis and is a surrogate marker for the disease. The thickness of submucosal nerve fibers has been measured by various authors in previous studies^[13,14]. However, they have used less objective methods for measurement such as image graticules. In this study, we analyzed the status of the nerve fibers after S-100 immuno-staining and examination using an objective technique provided by image analysis software. The number, size and perimeter of the nerve fibers in the colonic submucosa of HD and non-HD cases were measured and results compared between HD and non-HD cases. The difference between the two groups was statistically significant for all the three parameters. Thus image analysis can be a useful adjunct to the available tools for the diagnosis of HD.

In conclusion, mast cells appear to have a significant role in the pathogenesis of Hirschsprung's disease. Their increased baseline number in Indian children may be a response to additional factors like allergens or antigenic components of infectious agents. The role of anti-mast cell reagents like sodium cromoglycate needs to be explored with regard to constipation in Indian children. The fact that Indian children with HD present at a later age is also significant and emphasizes the need for a different approach to disease in various geographical regions.

COMMENTS

Background

Hirschsprung's disease is an important cause of constipation in children. It is a serious disorder and can be fatal if left untreated. Recently, there has been a lot of interest in the role of mast cells in the pathogenesis of Hirschsprung's disease. A few studies have found increased number of mast cells in aganglionic bowel segments of Hirschsprung's disease cases. However the exact role of mast cells in Hirschsprung's disease is not known.

Research frontiers

Mast cells are normally present in small numbers in various organs of the body.

They have been reported to be increased in a number of conditions other than Hirschsprung's disease. Regarding the role of mast cells in Hirschsprung's disease, the research hotspot is to elucidate the exact role played by them in the pathogenesis of Hirschsprung's disease and the relationship between mast cells and the neural hypertrophy observed in Hirschsprung's disease.

Innovations and breakthroughs

Previous studies on the role of mast cells in the pathogenesis of Hirschsprung's disease have described an increased number of mast cells in the aganglionic segment in patients with Hirschsprung's disease. Apart from one article, other studies have found mast cells to be increased transmurally. In the present study a significant increase was also noticed in mast cells in Hirschsprung's disease. However their number was significantly higher than previous studies. This may be due to the response of the mast cells to infectious agents or allergens rather than their association with aganglionosis. The authors also performed image analysis morphometry and correlated each of the parameters obtained with mast cell number. It was observed that maximum correlation was seen with number of nerve fibers. No correlation was seen with size of nerve fibers and some correlation was observed with the perimeter of nerve fibers. This correlation has not been shown in any previous study. This suggests that mast cells via their mediators may cause increased number of nerve fibers and affect size of nerve fibers to some extent.

Applications

The study suggests mast cells appear to have a significant role in the pathogenesis of Hirschsprung's disease. Whether this fact can be exploited to open novel therapeutic options in the management of this disease is something which needs to be looked at in the future.

Terminology

Hirschsprung's disease is a congenital condition characterized by dilatation and hypertrophy of colon due to absence (aganglionosis) or marked reduction (hypoganglionosis) of ganglion cells of the myenteric plexus of the rectum and a varying but continuous length of colon above the rectum. Mast cells are connective tissue cells with cytoplasmic coarse metachromatic granules and their normal function is unknown.

Peer review

This article only contributes minimal new information to the literature (morphometry or S-100 stained nerve fibers), but it reinforces the observation that mast cells are increased in patients with HD and reports this finding in Indian children for the first time.

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Expression of phosphatase regenerating liver 3 is an independent prognostic indicator for gastric cancer

Ni Dai, Ai-Ping Lu, Cheng-Chao Shou, Ji-You Li

Ni Dai, Ai-Ping Lu, Ji-You Li, Department of Pathology, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

Cheng-Chao Shou, Department of Biochemistry & Molecular Biology, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

Author contributions: Li JY, Shou CC and Lu AP designed the research; Dai N and Lu AP performed the research; Shou CC provided the antibody; Dai N and Lu AP analyzed data; Dai N and Li JY wrote the paper.

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Correspondence to: Ji-You Li, MD, Department of Pathology, Peking University School of Oncology, Beijing Cancer Hospital, 52 Fucheng Road, Haidian District, Beijing 100036, China. lijyou@263.net

Telephone: +86-10-88196291 Fax: +86-10-88122450

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Abstract

AIM: To investigate the prognostic significance of phosphatase regenerating liver 3 (PRL-3) protein expression in gastric cancer.

METHODS: PRL-3 expression in paraffin-embedded tumor specimens from 293 patients with gastric cancer was studied retrospectively by immunohistochemistry. Monoclonal antibody specifically against PRL-3, 3B6, was obtained with hybridoma technique.

RESULTS: Positive PRL-3 expression was detected in 43.3% (127 of 293) of gastric cancer cases. High expression of PRL-3 was positively correlated with tumor size, depth of invasion, vascular/lymphatic invasion, lymph node metastasis, high TNM stage and tumor recurrence. Patients with positive PRL-3 expression had a significantly lower 5-year survival rate than those with negative expression (28.3% vs 51.9%, $P < 0.0001$). Patients who received curative surgery, and with positive PRL-3 expression had a significant shorter overall survival and disease-free disadvantage over patients with negative expression (hazard ratio

of 16.7 and 16.6, respectively; $P < 0.0001$ for both). Multivariate analysis revealed that PRL-3 expression was an independent prognostic indicator for overall and disease-free survival of gastric cancer patients, particularly for survival in TNM stage III patients.

CONCLUSION: PRL-3 expression is a new independent prognostic indicator to predict the potential of recurrence and survival in patients with gastric cancer at the time of tumor resection.

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Key words: Phosphatase regenerating liver 3; Gastric cancer; Prognosis; Recurrence; Antibody

Peer reviewers: Harry HX Xia, PhD, MD, Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936-1080, United States; Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies in the world with a high incidence and death rate. In China, it remains the most frequent cancer and the second cancer-related cause of death with a high case fatality^[1]. TNM staging system is used worldwide to predict prognosis and direct therapeutic decisions of patients with GC. The 5-year survival rate in patients with stage I GC is close to 90% and around 10% for patients with stage IV GC^[2]. However, the prognoses of patients with stage II and III GC are more heterogeneous and less predictable by staging criteria. Therefore, finding molecular markers that are able to predict the potential of tumor recurrence and prognosis of patients is extremely important for appropriate individualized therapy. Phosphatase regenerating liver 3 (PRL-3) (also known as PTP4A3) belongs to a newly

discovered group of phosphatase regenerating liver family which is implicated in oncogenic and metastatic processes^[3,4]. The PRL family represents a protein tyrosine phosphatase superfamily possessing a unique COOH-terminal prenylation motif and a protein tyrosine phosphatase-active site signature sequence CX5R^[5,6]. Three PRLs (PRL-1, -2, and -3) are highly homologous with similar amino acid sequence of 76%-87%^[7-9]. Recent reports found that PRL-3 was consistently expressed at higher level of metastasis in liver compared to normal colorectal epithelia and primary cancer tissues^[10,11]. A growing body of evidence showed that an excess of PRL-3 phosphatase is a key alteration contributing to the acquisition of metastatic properties of the tumor cells. For example, nontumorigenic or low metastatic cell lines transfected with wild type PRL-3 displayed higher cell motility and invasiveness and could induce metastatic tumor formation in mice, while cells expressing catalytically inactive mutant PRL-3 significantly reduced the migratory capability^[12,13]. Knockdown of endogenous PRL-3 in cancerous cells using small interfering RNA or phosphatase inhibitors can abrogate cell motility and the ability to form metastasis-like tumors in mice^[12-15]. PRL-3 was further demonstrated to be a useful indicator for tumor recurrence and patient outcome in several human cancers including colorectal cancer and breast cancer^[16-19]. In gastric cancer, PRL-3 was found to be highly expressed in tumor metastatic lymph nodes and closely associated with the peritoneal metastasis^[20-23], but the prognostic impact of PRL-3 expression in gastric cancer still remains to be further investigated.

In this study, we detected the expression of PRL-3 in GC tissue samples by immunohistochemistry using a PRL-3 specific monoclonal antibody 3B6 to investigate PRL-3 protein expression in GC tissues and whether PRL-3 could be applied as a prognostic indicator for GC to predict the potential of tumor recurrence and patient outcome.

MATERIALS AND METHODS

Patients

This retrospective study enrolled patients who underwent clinical surgery for primary gastric cancer at the Department of Surgery, Beijing Cancer Hospital, Peking University School of Oncology between July 1994 and December 2000. Patients with inadequate histologic specimens or missing clinical information were excluded. A total of 293 patients were finally included. There were 194 males and 99 females, with ages ranging from 25 to 82 (mean \pm SD, 58 \pm 17.1 years). Two hundred (68.3%) patients received curative resection (R0) with radical lymph node dissection; the remaining 93 (31.7%) patients with microscopic or macroscopic tumor residues were given palliative resection (R1/R2). Site distribution of the primary tumor was 153 at antrum, 52 at cardia or fundus, and 88 at corpus. Tumor size ranged from 5 to 120 mm (mean, 43.8 mm).

Histology

Data were collected from clinical case report record and follow-up database. Tumor staging was based on the clinical evaluation and postoperative pathological reports. TNM staging was on the basis of the 1997 fifth edition of AJCC/UICC TNM staging criteria for gastric cancer^[24]. The tumors were histologically classified according to the WHO classification criteria.

Follow-up

None of these patients had received radiotherapy or chemotherapy preoperatively. All the patients were followed up at regular intervals of 6 mo after surgery until June 2006 with a minimum of five years. Tumor recurrence was clinically defined as the reappearance of tumor after curative surgery. The overall survival time was calculated from the date of surgery to the date of last visit or death and the disease-free survival time from the date of resection to relapse.

Immunostaining of PRL-3

Tumor tissue specimens from the 293 patients were routinely fixed in 10% formalin and embedded with paraffin. Paraffin embedded tissue samples were cut into 4 μ m sections. The sections were put in an oven at 60°C for 5 h and cooled down overnight before they were deparaffinized in xylene. The sections were then dehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwaving the sections in 1 mmol/L EDTA (PH 8.0) for 15 min. PRL-3 monoclonal antibody 3B6 (a generous gift from Prof. Shou, Beijing Institute for Cancer Research, China)^[25] was used as the primary antibody at a dilution of 1:100 overnight at 4°C. The Powervision two-step histostaining reagent PV-6002 (Dako, Glostrup, Denmark) was applied as the secondary antibody. The sections were visualized with diaminobenzidine and counterstained with hematoxylin. Each incubation step was followed by washing with phosphate-buffered saline. For negative control, the primary antibody was omitted from the reaction sequence. Sections of liver metastasis from colon cancer with known strong PRL-3 immunoreactivity were used as positive controls. The number of tumor cells with cytoplasm staining of PRL-3 was counted without knowledge of the clinicopathological data, and > 5% positive tumor cells were defined as positive PRL-3 expression^[19].

Statistical analysis

Statistical analyses were performed with SAS 8.1 software. The χ^2 test was used to analyze the association between PRL-3 expression and clinicopathological features of GC. Cumulative survival rates and differences in survival curves were estimated by Kaplan-Meier method with the log-rank test. The effect of PRL-3 on survival was analyzed using the Cox proportional hazard regression model adjusted for clinical and histopathologic features. Two-sided *P* values

Table 1 Association between PRL-3 expression and clinicopathological features

Factors	Patients	PRL-3 expression		P value
		Positive (n = 127)	Negative (n = 266)	
Gender				
Male	194	85	109	NS
Female	99	42	57	
Age (yr)				
< 60	149	66	83	NS
≥ 60	144	61	83	
Tumor size (cm)				
≥ 5	118	63	55	0.004
< 5	175	64	111	
Depth of invasion				
T1	22	5	17	0.008 ^a
T2	46	15	31	0.009 ^b
T3	175	77	98	0.042 ^c
T4	50	30	20	
Lymph node metastasis				
N0	82	30	52	< 0.0001 ^d
N1	120	42	78	
N2	51	33	18	
N3	40	22	18	
Distant metastasis				
Present	42	21	21	NS
Absent	251	106	145	
Vascular/lymphatic invasion				
Present	162	89	73	< 0.0001
Absent	131	38	93	
Differentiation				
Poor	194	91	103	NS
Well/Moderate	99	36	63	
Surgical curability				
Curative	200	79	121	NS
Not curative	93	48	45	
Recurrence ^e				
Absent	177	63	114	0.002
Present	23	16	7	
TNM stages				
I	42	13	29	0.007 ^f
II	52	17	35	0.008 ^g
III	99	43	56	
IV	100	54	46	

NS: Not significant; ^aT1-T2 vs T3-T4; ^bT1-T3 vs T4; ^cT1 vs T2-4; ^dN0-N1 vs N2-N3; ^e200 cases received curative surgery; ^fI - II vs III-IV; ^gI - III vs IV.

of less than 0.05 were considered to be statistically significant.

RESULTS

Patient outcome

Forty-two patients were classified as stage I, 52 as stage II, 99 as stage III and 100 as stage IV. A total of 194 cases were poorly differentiated, 69 cases were moderately differentiated and the remaining 30 cases were well differentiated.

The follow-up period for survivors ranged from 2 to 120 mo (median, 31 mo). The 5-year overall survival rate was 41.7% in the entire cohort of patients, 92.9% in stage I, 72.5% in stage II, 32.5% in stage III and 12.3% in stage IV patients. One hundred and five patients remained alive and disease-free, 15 patients were alive with disease. One hundred and seventy patients died

of GC, and 3 patients died of other causes. Among the 200 patients who received curative surgery, 23 patients had tumor recurrence with 3 in peritoneum, 3 in lymph node, 7 in liver, 3 in other organs (2 ovarian, 1 lung), 4 in multiple organs and 3 in remnant stomach.

PRL-3 expression in GC and its relation with clinicopathological features

PRL-3 immunostaining was predominantly localized in the cytoplasm of normal or tumor epithelial cells. PRL-3 stained cells in normal epithelia were mainly observed in the neck of gastric glands (Figure 1). Among the 293 GC specimens analyzed, 127 (43.3%) tumors had positive PRL-3 expression. The rate of positive PRL-3 expression was significantly higher in stage III and IV than in stage I and II (48.7% vs 31.9%, $P = 0.007$). High expression of PRL-3 was correlated closely with large tumor size, depth of invasion in gastric wall, lymph node metastasis, vascular/lymphatic invasion and recurrent frequency. No significant correlation was observed between PRL-3 expression and sex, age, distant metastasis, grade of differentiation and surgical curability (Table 1).

Univariate survival analysis of prognostic impact of PRL-3 expression

Kaplan-Meier method with log-rank test revealed that patients with positive PRL-3 expression had a significantly lower cumulative 5-year overall survival rate than those with negative expression (28.3% vs 51.9%, $P < 0.0001$). Among the 99 patients with stage III GC, those with positive PRL-3 expression had a lower survival rate than those with negative expression (18.6% vs 43.2%, $P = 0.0004$, Figure 2). Among the 200 patients who received curative surgery, patients whose tumor had positive PRL-3 expression had worse disease-free status and poorer overall survival (hazard ratio, 16.6 and 16.7 respectively; $P < 0.0001$ for both) than those with negative expression (Figure 3). Among patients who received palliative resection or patients in stages other than stage III, PRL-3 showed no significant correlation with prognosis.

Multivariate survival analysis of prognostic impact of PRL-3 expression

Multivariate analysis by extended Cox regression model revealed that PRL-3 expression remained an independent prognostic factor after adjusting for sex, age, tumor location, tumor size, depth of invasion, lymph node metastasis, distant metastasis, TNM staging, vascular/lymphatic invasion, and surgical curability. PRL-3 expression was a significantly independent prognostic factor for the overall survival of all 293 GC patients. For the 200 patients who received curative resection, PRL-3 expression was found to be an independent prognostic factor for both disease-free and overall survival. The results are shown in Table 2.

DISCUSSION

In this study, we detected the protein expression

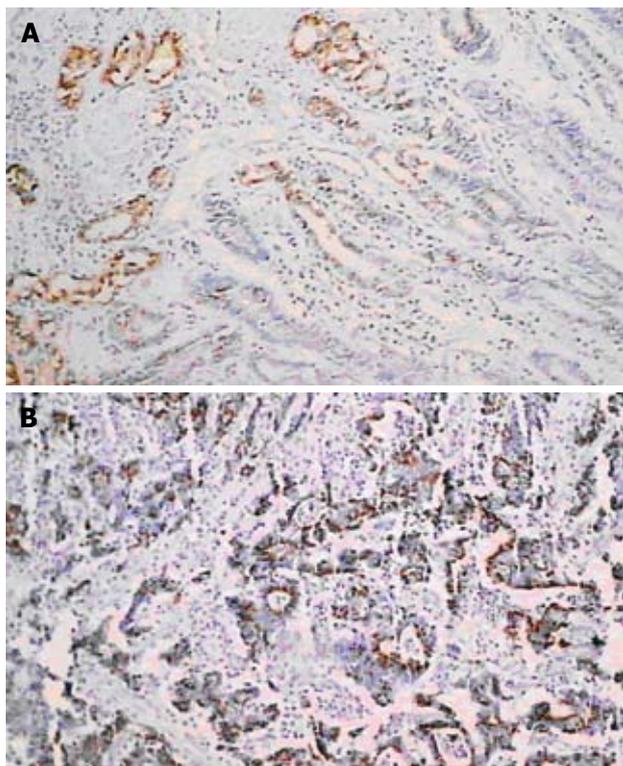


Figure 1 Immunohistochemical staining. A: PRL-3 is negative or weak in adjacent (3 cm away from the tumor) normal gastric epithelial mucosa ($\times 40$); B: In positive cases, PRL-3 expression in cancer cell cytoplasm is strong ($\times 200$).

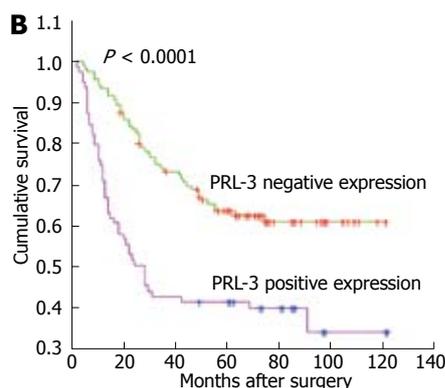
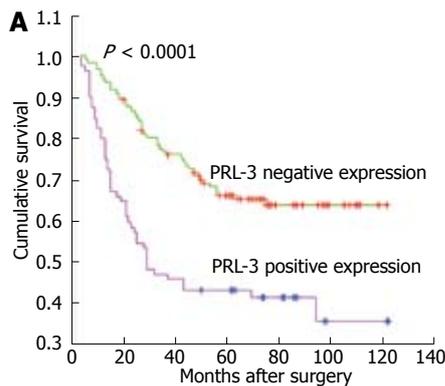


Figure 3 Patients who underwent curative surgery. A: Overall survival; B: Disease-free survival. Significant differences were observed between the PRL-3 negative and positive groups.

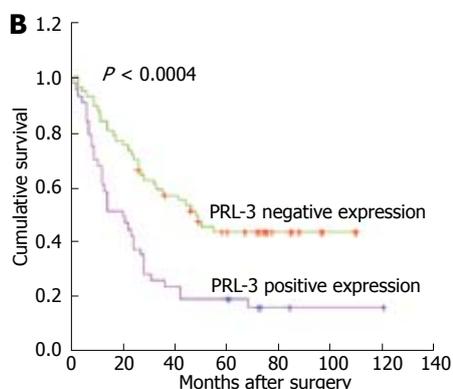
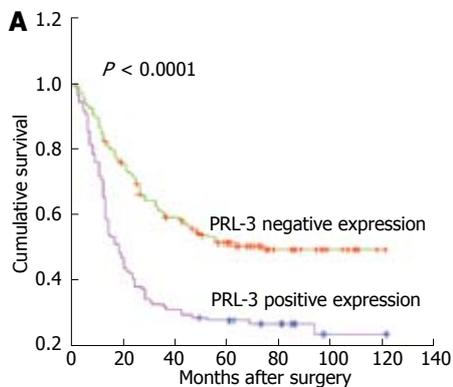


Figure 2 Overall survival curve. A: Entire cohort of 293 patients; B: Patients with stage III. Significant differences were observed between the two groups with PRL-3 negative and positive expression.

of PRL-3 in GC tissues using the highly specific monoclonal antibody 3B6 prepared by Peng *et al.* PRL-3

had higher rates of positive expression in advanced stages and PRL-3 expression was positively correlated with tumor size, depth of invasion, and lymph node metastasis vascular/lymphatic invasion at the time of surgery and recurrence. These results suggest that PRL-3 may play a crucial role in invasion, progression and metastasis of GC. The present analyses revealed that PRL-3 was an independent prognostic indicator for overall and disease-free survival of GC. Among patients with advanced TNM stages especially stage III, patients with positive PRL-3 expression have more frequent recurrence and poorer survival, adjuvant therapies such as radiotherapy and chemotherapy may be necessary after curative surgery. Evaluation of PRL-3 expression status may identify a subset of patients with GC who require more intensive treatment.

Miskad *et al*^[20] reported that PRL-3 was highly expressed in metastatic lymph nodes of GC and high expression of PRL-3 was closely associated with tumor stage. Wang *et al*^[21] found that the high expression of PRL-3 in lymph node metastases had a negative impact on the prognosis of patients with GC. Li *et al*^[22-23] reported that PRL-3 expression was correlated with peritoneal metastasis and poor prognosis in GC patients. The superiority of our study may be the use of antibody specifically against PRL-3 and the relatively extensive clinical data which facilitated the analysis from multiple angles. One limitation of our study is that the relatively small sample hindered the analysis in stage I and stage II patients.

Table 2 Multivariate analysis of PRL-3 expression by Cox proportional hazard model

Factors	Overall survival						Disease-free survival		
	All patients (n = cases)			Curatively resected (n = 200)			Curatively resected (n = 200)		
	P	Relative risk	95% CI	P	Relative risk	95% CI	P	Relative risk	95% CI
PRL-3	< 0.001	1.76	1.30-2.40	< 0.001	2.35	1.54-3.59	< 0.001	2.46	1.62-3.73
T	< 0.001	3.16	1.79-5.58	0.003	3.04	1.47-6.29	0.004	2.76	1.38-5.52
N	< 0.001	3.29	1.93-5.60	< 0.001	3.99	2.21-7.20	< 0.001	4.67	2.59-8.43
D	0.04	1.38	1.01-1.88	0.32	1.23	0.82-1.87	0.015	1.69	1.11-2.57
S	< 0.001	2.24	1.54-3.26	-	-	-	-	-	-

PRL-3 expression, positive *vs* negative; T: Depth of invasion, T3-4 *vs* T1-2; N: Lymph node metastasis, present *vs* absent; D: Tumor size, ≥ 5 cm *vs* < 5 cm; S: Surgical curability, palliative *vs* curative.

Attributed to the high sequence similarity of three PRLs and the wide expression of PRL-1 and PRL-2 in normal tissues and cancer cell lines, commercial polyclonal antibody against PRL-3 used in previous studies could potentially cross-react with PRL-1 and PRL-2. Monoclonal antibody specifically reacting with PRL-3 is extremely important to exclude the interference of PRL-1 and PRL-2 and therefore allows us to accurately evaluate the prognostic implication of PRL-3 expression^[25,26]. To prepare specific PRL-3 monoclonal antibody, Peng *et al*^[25] obtained the monoclonal antibody 3B6 with hybridoma technique, and confirmed its specificity with ELISA and Western blotting assays. High specificity of the monoclonal antibody 3B6 against PRL-3 was demonstrated. The applicability of the monoclonal antibody has been further confirmed by two other studies investigating the prognostic impact of PRL-3 expression in colorectal cancer and breast cancer^[16,19].

PRL-3 has been confirmed to be an important metastatic instrumental molecule. Although the actual signal transduction pathways in which PRL-3 is implicated are largely unknown, Rho signaling pathway molecules which are regulators of motility and invasion have been identified as potential candidate targets of PRL-3. PRL-3 transfectants displayed altered extracellular matrix adhesive property and up-regulated integrin-mediated cell spreading efficiency^[14,27]. Peng *et al*^[28] recently found that PRL-3 activates the mitogen-associated protein kinase pathway by binding a cell membrane protein in cell migration and invasion. PRL-3 was also found to be associated with membrane structures including ruffles, protrusions, and some vacuolar-like membrane extensions which have been demonstrated to play a role in cell movement and invasion^[29-31]. Besides, PRL-3 may be involved in triggering angiogenesis and establishing microvasculature *in vitro*^[32-34]. These findings suggest that PRL-3 may be implicated with the key steps of tumor metastasis including tumor cell invasion and survival in circulation and vasculature formation.

In addition to its role in predicting tumor recurrence and prognosis, PRL-3 has a potential value of being a candidate for metastasis tailored therapies. Since primary tumors can be surgically resected, the metastatic tumors are the main cause responsible for a high case fatality. PRL-3 was highly expressed in tumor metastasis and

found to play a key role in tumor metastatic process^[10-21]. PRL-3 may serve as a potential therapeutic target for cancer metastases. Inhibition of PRL activity might be carried out using phosphatase inhibitors targeting the consensus phosphatase motif, farnesyltransferase inhibitors, interference RNA or monoclonal antibody as well^[9,35-37]. Recent progress in active recombinant PRL-3 production and findings on PRL-3 structure will undoubtedly facilitate the development of PRL-3 inhibitors^[38-40]. Detection of PRL-3 expression would be able to provide supportive information for anti-cancer therapy.

In conclusion, PRL-3 is closely associated with tumor invasion and lymphatic metastasis and is identified as a new prognostic indicator to predict tumor recurrence and patient survival in GC.

COMMENTS

Background

It is established that phosphatase regenerating liver 3 (PRL-3) is consistently expressed in liver metastasis of colon cancer. A recent study reported that PRL-3 expression was related to peritoneal metastasis of stomach cancer.

Research frontiers

In a few studies, the association of PRL-3 expression with prognosis of cancers was investigated and demonstrated that this is related to poor prognosis of breast and stomach cancer. The antibody used in many studies was commercial polyclonal antibody that could cross-react with PRL-1 and PRL-2.

Innovations and breakthroughs

A recent study showed that PRL-3 was expressed in 70.4% of 637 GCs. PRL-3 expression was correlated with peritoneal metastasis. Patients with PRL-3 negative expression had a better survival rate than those with positive PRL-3 at all stages. In this study, PRL-3 was found to express in 43.3% (127/293) of primary tumor tissues of GC. PRL-3 protein expression was demonstrated to be an independent predictor for poor prognosis in GC. In addition, a specific monoclonal antibody to PRL-3 was used, allowing us to accurately evaluate the prognostic significance of PRL-3 expression in GC.

Applications

Defection of PRL-3 protein expression in primary tumor tissues of GC might be helpful in identification of GC patients with poor prognosis who should receive more intensive treatment. In addition, PRL-3 may serve as a potential therapeutic target for GC.

Terminology

PRL-3 (also known as PTP4A3) is a member of phosphate of regeneration liver (PRL) family including PRL-1, PRL-2 and PRL-3 which are implicated with oncogenic and metastatic processes of tumors. The excess of PRL-3 phosphates is an alteration contributing to the acquisition of metastatic properties of tumor cells. The monoclonal antibody 3B6 used in this study is a specific monoclonal antibody to PRL-3 generated by Peng *et al*, Beijing Institute for Cancer Research.

Peer review

The authors examined the expression of PRL-3 protein in primary tumor tissues of 293 cases of GC. It revealed that patients with negative PRL-3 expression had a higher 5-year survival rate than those with positive PRL-3 expression. PRL-3 is an independent prognostic indicator for GC. The findings are of clinical significance.

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BRIEF ARTICLES

Intrahepatic transplantation of hepatic oval cells for fulminant hepatic failure in rats

Chen-Xuan Wu, Qi Zou, Zheng-Yan Zhu, Ying-Tang Gao, Yi-Jun Wang

Chen-Xuan Wu, Qi Zou, Zheng-Yan Zhu, Ying-Tang Gao, Yi-Jun Wang, Department of Hepatobiliary, The Third Center of Affiliated Hospital of Tianjin Medical University; Key Laboratory of Artificial Cells of Tianjin, Tianjin 300170, China
Author contributions: Wang YJ, Wu CH and Zou Q designed the research; Zou Q, Zhu ZY, Gao YT performed the research; Zou Q analyzed the data; Wang YJ and Wu CX wrote the paper. Supported by Tianjin Science Committee, Grant No. 05SSYSYJC02600 and Tianjin Health Bureau, Grant No. 05KYR01

Correspondence to: Yi-Jun Wang, Department of Hepatobiliary, The Third Center of Affiliated Hospital of Tianjin Medical University; Key Laboratory of Artificial Cells of Tianjin, Tianjin 300170, China. wangyijuncarl@yahoo.com.cn

Telephone: +86-22-84112205 Fax: +86-22-84112205

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Abstract

AIM: To evaluate the effect of intrahepatic transplantation of hepatic oval cells (HOC) on fulminant hepatic failure (FHF) in rats.

METHODS: HOC obtained from rats were labeled with green fluorescent protein (GFP) or 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). Cell fluorescence was observed under fluorescent microscope at 6, 24, 48 and 72 h after labeling. CFDA-SE labeled HOC (5×10^6 cells each rat) were injected into livers of rats with FHF induced by D-galactosamine. Serum albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) levels were measured at different time points. Liver function of rats was examined on days 3, 7, 14 and 21 after HOC transplantation.

RESULTS: The positive rate of GFP and CFDA-SE labeled HOC was 10% and 90%, respectively, with no significant change in cell viabilities. The survival rate was higher in HOC transplantation group than in control group, especially 48 (9/15 vs 6/15) and 72 h (9/15 vs 4/15) after HOC transplantation. The serum ALT, AST and TBil levels were decreased while the serum Alb level was increased after HOC transplantation. Fluorescence became faded and diffused in liver tissues, suggesting that proliferation and differentiation occur in transplanted HOC.

CONCLUSION: CFDA-SE is superior to GFP in labeling HOC, although fluorescence intensity is decreased progressively with cell division. HOC transplantation can improve the liver function and increase the survival rate of recipients.

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Key words: Liver; Stem cells; Hepatic oval cells; Fluorescence labeling; Transplantation; Fulminant hepatic failure

Peer reviewers: Ezio Laconi, MD, PhD, Professor of General Pathology, Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4-IV Piano, 09125-Cagliari, Italy; Dr. Yukihiko Shimizu, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan

Wu CX, Zou Q, Zhu ZY, Gao YT, Wang YJ. Intrahepatic transplantation of hepatic oval cells for fulminant hepatic failure in rats. *World J Gastroenterol* 2009; 15(12): 1506-1511 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1506.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1506>

INTRODUCTION

Hepatic oval cells (HOC) are liver stem cells with a self-renewal capacity and a high proliferation potential^[1]. Transplantation of HOC cultured *in vitro* can restore damaged liver function, thus providing more opportunities for patients with terminal-stage liver diseases^[2,3]. In this study, we established a rat HOC proliferation model by feeding 2-acetylaminofluorene (2-AAF) and resecting 2/3 liver. HOC were isolated, purified and labeled with 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), a fluorescence agent, before they were transplanted into the rats with fulminant hepatic failure (FHF). Then, we detected the fluorescence distribution in the recipient liver and a few laboratory indexes, trying to find the effect of HOC transplantation on FHF.

MATERIALS AND METHODS

Animals and reagents

Wistar rats were provided by the Animal Experimental Center of the Radiation Institute, Chinese Academy of

Sciences (Beijing, China). 2-AAF was purchased from Sigma and D-galactosamine (D-GalN) was provided by Chongqing Medical University (Chongqing, China). *E. coli* strain harboring plasmids carrying the GFP gene, Pmax-GFP, was produced by Amaxa. Fugene HD transfection reagent was from Rocheand. CFDA-SE was from Molecular Probes.

Establishment of a rat HOC proliferation model

Twenty healthy Wistar rats, weighing 180-220 g, received intra-gastric 2-AAF, 15 mg/kg per day, for 4 d. On day 5, the rats were anesthetized with 1% sodium pentobarbital and their left and middle liver lobes (about 2/3 of the liver volume) were resected. From day 6, the rats were given 2-AAF, 15 mg/kg per day, for additional 10 d to induce a rat HOC proliferation.

Isolation, cultivation and identification of HOC

Hepatic cells were separated from the rat model by the improved *in situ* perfusion of Seglen collagenase^[4]. HOC were purified from the separated hepatic cells by Percoll density gradient centrifugation and inoculated in a DMEM/F12 culture medium at a concentration of 1×10^6 /mL. The cells were cultured at 37°C in an atmosphere containing 50 mL/L CO₂, and half of the medium was changed every two days. Cell morphology and expansion were regularly observed under an inverted microscope. The cells were passaged when necessary and observed under an electronic microscope. OV-6, AFP, ABL and PCNA, expressed on cells were tested by immunohistochemical assay.

Fugene HD-mediated transfection of HOC with the GFP gene

HOC were transfected with Fugene HD transfection reagent following its manufacturer's instructions. Briefly, HOC at passage 1 were seeded onto 12-well plates at a density of 2×10^5 /mL. One day later, transfection compounds at different ratios [transfection agent (μL): plasmid (μg) = 3:2, 4:2, 5:2, 6:2, 7:2 and 8:2] were added to the culture medium and shaken for 30 s at a low speed to ensure a homogeneous mixture. Then, the cells were incubated at 37°C in a humidified atmosphere containing 50 mL/L CO₂. After 6, 12, 24, 48 and 72 h, samples were taken from three random sections in each well and observed under an inverted confocal microscope and 100 cells were counted. GFP expression in these cells was observed under a fluorescence microscope with the excitation wavelength at 488 nm and the emission wavelength at 507 nm. The transfection rate was calculated according to the following equation: transfection rate (%) = number of green fluorescent cells in dark field/number of cells in bright field. The cell proliferation was determined by 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) colorimetry and growth curves were plotted.

HOC labeling by fluorochrome CFDA-SE

Passage 1 HOC, reaching an approximate confluence of

80%, were adjusted to 1×10^6 /mL in serum-free PBS with 5 μmol/L CFDA-SE and incubated at 37°C for 10 min. After the same volume of complete medium was added to terminate the staining, cells were separated by centrifugation and the staining was repeated three times before incubation. After 0, 6, 24 and 72 h, the cells were observed under a fluorescence microscope at 488 nm. Cell growth activity was also determined by MTT colorimetry and growth curves were plotted.

HOC transplantation for FHF in rats

Thirty Wistar rats were intra-peritoneally injected with a 10% D-GalN solution at a dose of 1400 mg/kg to induce FHF. One day after FHF induction, rats were divided into transplantation group ($n = 15$) and control group ($n = 15$). Rats in HOC proliferation model were anaesthetized at the supine position, and a 1.5 cm incision was made at the middle of the upper abdomen to expose the liver. The number of fluorescence labeled HOC was adjusted to 1×10^7 /mL for transplantation. Rats in the transplant group were injected with 0.5 mL CFDA-SE labeled HOC suspension in the left lobe of liver, while rats in the control group were given the same volume of culture medium. After 1, 2, 3, 5 and 7 d of transplantation, blood sample was taken from the rat tail and liver function was determined with an automatic biochemical analyzer. Albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) levels were measured. After 3, 7, 14 and 21 d, the animals were killed and their livers were removed for pathological examination. Frozen liver tissue around the injected site was cut into sections to observe the distribution of fluorescence labeled cells in the liver tissue under a fluorescent microscope.

Statistical methods

All data were analyzed by SPSS 13.0. Two sets of sample means (mean ± SD) were compared by *t*-test and the percentages were compared by χ^2 -test. $P < 0.05$ was considered statistically significant.

RESULTS

HOC incubation

The freshly separated rat HOC adhered to the wall after 12 h of incubation were spindle or polygonal in shape. After about 7 d, the cells grew into colonies. The HOC, 10 d after passage, grew into a single-layer flagstone which did not change 14 d after passage (Figure 1).

Morphological and phenotypic identification of the cells

The expression of OV-6, AFP and ALB in HOC was detected by immunohistochemistry. Positive staining of OV-6 and AFP was detected in isolated HOC while no ALB expression was found in HOC. Electron microscopy showed short and tiny microvilli-like protuberances on the surface of HOC. The cell nuclei were oval with dispersed and homogenous nuclear chromatin, small nucleoli, little cytoplasm, great nucleus-cytoplasm ratio

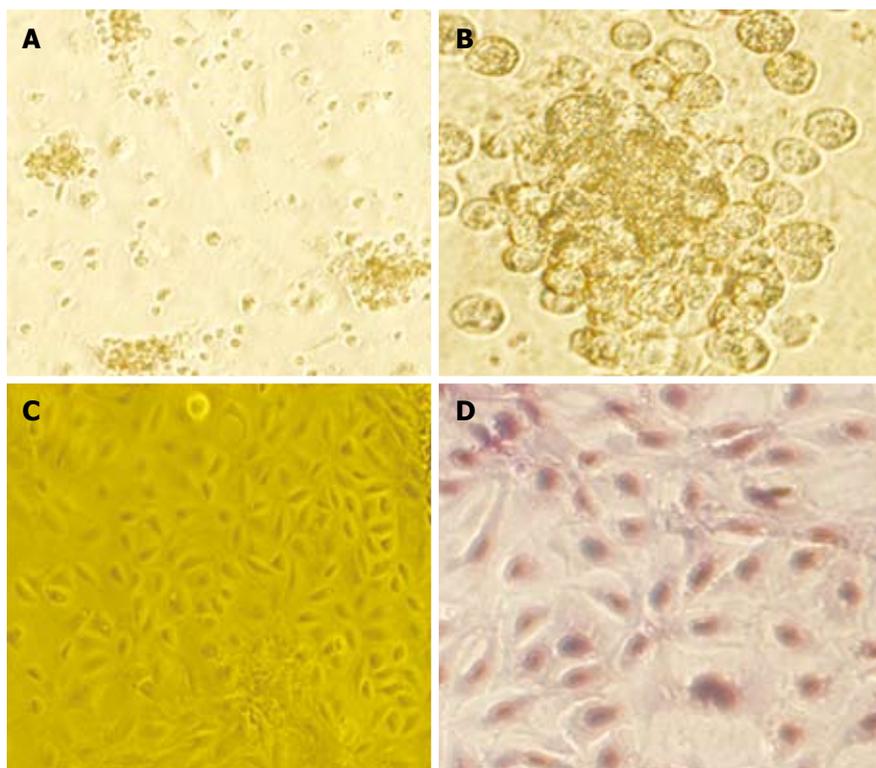


Figure 1 HE stained HOC 10 h (A, $\times 100$), 7 (B, $\times 400$), 10 (C, $\times 100$), and 14 d (D, $\times 200$) after incubation.

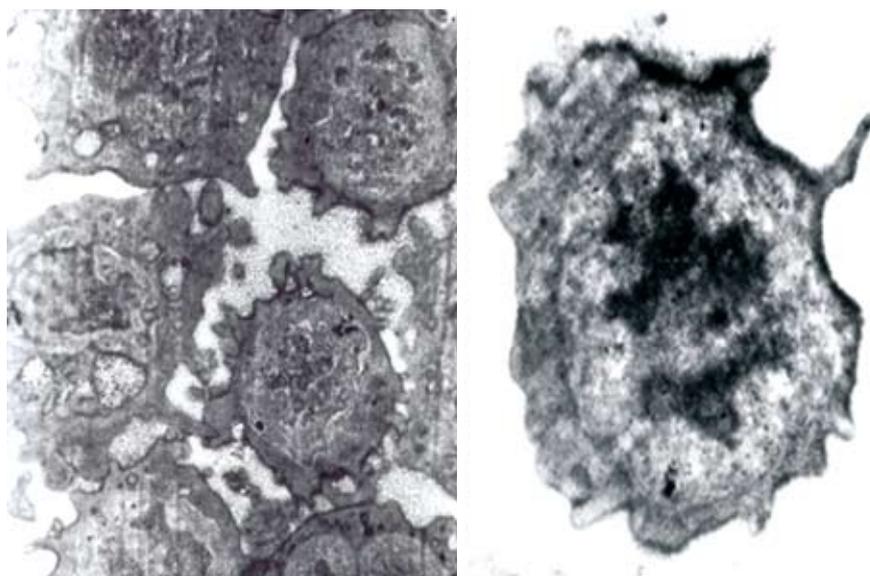


Figure 2 Ultra-structure of rat HOC under electron microscope ($\times 4000$).

and underdeveloped endoplasmic reticulum, mitochondria and ribosome (Figure 2), indicating that incubated HOC are primitive, naive and undifferentiated.

HOC labeling

Six hours after transfection with the GFP gene, a low GFP expression level in some HOC could be observed under a fluorescent microscope. The GFP gene was expressed both in nuclei and in cytoplasm. Its expression increased significantly after 24 h, reached its peak at 48 h and maintained till 72 h. A higher transfection efficiency (about 10.0%) could be achieved at a transfection reagent-plasmid ratio of 5:2. The fluorescent intensity of transfected cells was gradually reduced and disappeared after 5-7 generations. Green fluorescence could be

observed immediately after CFDA-SE labeling, with a labeling rate of 90%. The fluorescence intensity decreased slightly after 6 h and significantly after 24 h. However, the fluorescence intensity was almost the same at 72 h and 24 h (Figure 3).

Liver functions and survival of FHF rats after HOC transplantation

The rats in HOC transplantation group slightly restored their general conditions, food taking and movements 48 h after transplantation. On the contrary, the conditions of most rats in control group were further exacerbated. The serum ALB, AST, ALT and TBiL levels in rats of both groups are listed in Table 1. Death occurred in rats of both groups around 6 h after transplantation.

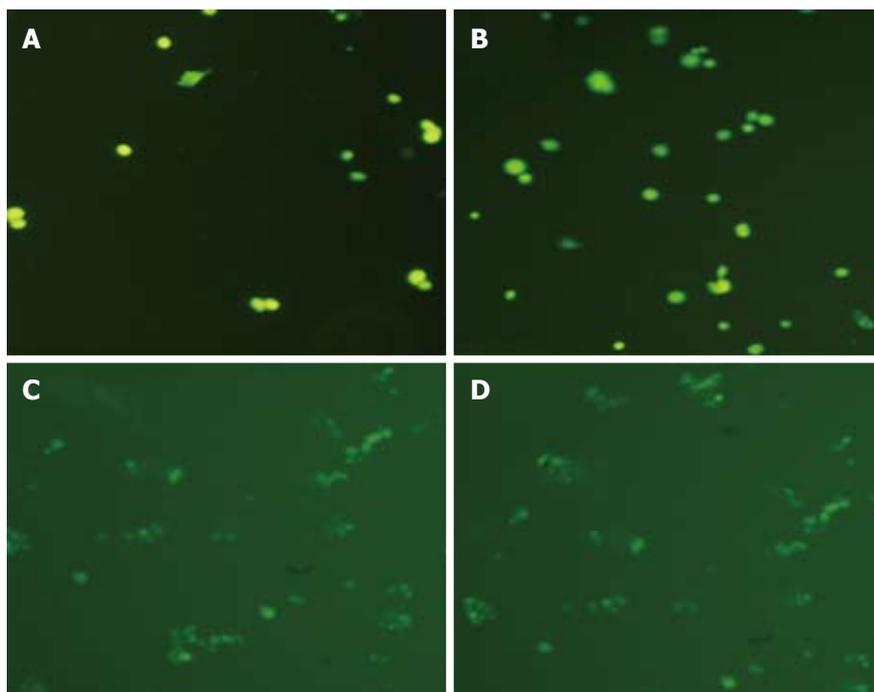


Figure 3 CFDA-SE stained HOC at 0 h (A, $\times 200$), 6 h (B, $\times 200$), 24 h (C, $\times 100$), and 72 h (D, $\times 100$).

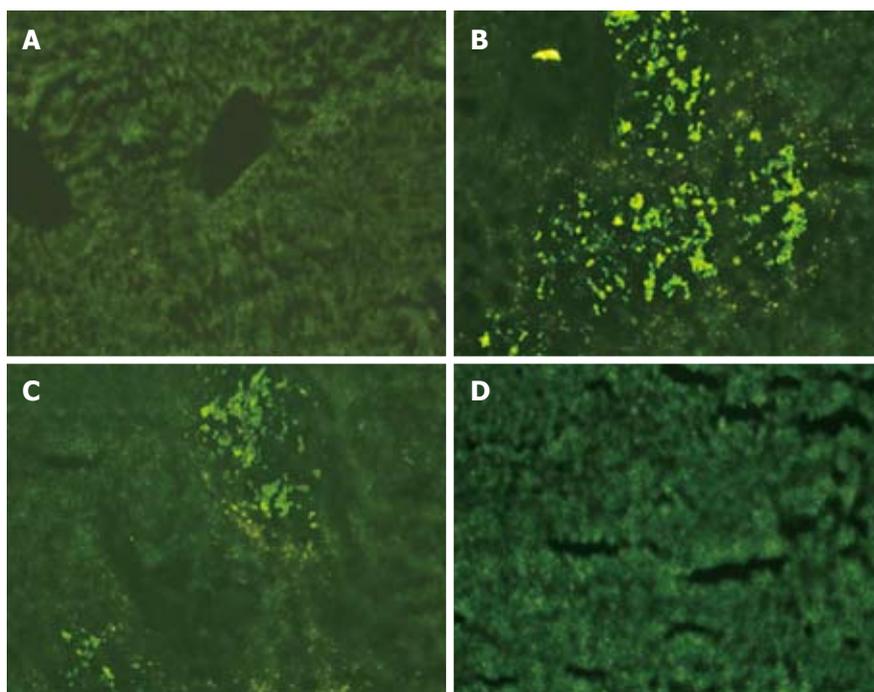


Figure 4 Fluorescent microscopy showing colonies in control group (A), and 3 (B), 7 (C) and 14 d (D) after HOC transplantation ($\times 100$).

However, no rat died in transplantation group 72 h after transplantation. The survival rate for rats in transplantation group and control group was 60% (9/15) and 26.7% (4/15), respectively.

Fluorescent microscopy after HOC transplantation

Green fluorescent cell colonies could be seen in sections of frozen rat liver tissue three days after transplantation. Most fluorescent colonies were located at the injection site with a strong fluorescent intensity. The fluorescent intensity decreased seven days after transplantation but the number of colonies increased with a wider distribution. No fluorescent cell was detected in the liver

sample 14 d after HOC transplantation and afterwards (Figure 4).

DISCUSSION

The Solt-Farber model^[4] is the most commonly used model for HOC proliferation, and has been used in studying the relationship between local disease and liver cancer or between nodules and liver cancer. Collagenase perfusion, proposed by Howard *et al.*^[5] and improved by Berry and Seglen *et al.*^[6,7], is often used in detection of HOC separation. In this study, Solt-Farber model was used to detect HOC proliferation, and a large

Table 1 Changes of ALB, ALT, AST and TBil levels in rat liver tissue after HOC transplantation (mean \pm SD)

	Groups (survival)	ALB (g/L)	ALT (U/L)	AST (U/L)	TBil (μ mol/L)
Transplantation 0 d	Control (15)	14.6 \pm 1.9	757.3 \pm 47.2	348.0 \pm 66.5	55.4 \pm 7.1
	Transplantation (15)	15.2 \pm 2.4	736.3 \pm 58.1	357.4 \pm 42.3	50.6 \pm 4.6
1 d after transplantation	Control (10)	13.4 \pm 2.5	789.6 \pm 27.5	384.6 \pm 73.3	56.6 \pm 7.1
	Transplantation (10)	14.2 \pm 1.8 ^a	803.3 \pm 62.4 ^a	375.3 \pm 49.2 ^a	61.6 \pm 19.2 ^a
2 d after transplantation	Control (6)	11.6 \pm 1.6	873.5 \pm 43.2	409.0 \pm 31.8	60.3 \pm 6.5
	Transplantation (9)	20.3 \pm 1.3 ^b	649.0 \pm 90.3 ^b	263.3 \pm 28.2 ^b	53.0 \pm 4.2 ^a
3 d after transplantation	Control (4)	9.8 \pm 0.6	896.6 \pm 44.8	434.3 \pm 25.4	46.3 \pm 3.7
	Transplantation (9)	26.3 \pm 0.9 ^b	430.0 \pm 28.3 ^b	124.6 \pm 21.6 ^b	23.7 \pm 6.9 ^b
5 d after transplantation	Control (3)	13.5 \pm 1.2	774.6 \pm 26.7	326.6 \pm 15.5	45.8 \pm 4.3
	Transplantation (8)	27.8 \pm 2.6 ^b	377.3 \pm 29.4 ^b	106.0 \pm 15.3 ^b	19.5 \pm 5.2 ^b
7 d after transplantation	Control (3)	19.7 \pm 1.6	564.2 \pm 43.2	246.3 \pm 26.7	32.3 \pm 5.0
	Transplantation (8)	31.5 \pm 2.6 ^b	333.3 \pm 36.4 ^b	89.3 \pm 13.2 ^b	6.9 \pm 1.8 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control group.

amount of small proliferation focuses were found in portal area. The HOC were oval or oblong in shape, and their size was much smaller than that of hepatic cells (about 1/6-1/3 of the normal size). Moreover, immunohistochemical staining showed positive OV-6 and AFP expression, consistent with the traits of HOC. The positive PCNA staining showed that HOC were at their proliferating stage and that HOC proliferation in liver of adult rats could be induced by 2-AAF injection and 2/3 liver resection. After the proliferation model was established, suspended hepatic cells were prepared by two-step collagenase perfusion. The cells were purified by density gradient centrifugation and observed under an electron microscope. The purified cells were primitive, naive and undifferentiated. Immunohistochemical staining of OV-6 and AFP in the freshly separated cells was similar to that of proliferated cells in the model. The cells showed a certain proliferation capacity in culture and were heterogenous as previously reported^[8-10].

The therapeutic effect of HOC transplantation on FHF has been proved both in animal models and in clinical trials^[11,12]. Matsusaka *et al*^[13] transplanted hepatic cells with a large number of HOC into the spleen of rats, and found that hepatic cells can significantly proliferate compared to those without HOC. Yasui *et al*^[14] transplanted HOC into the liver of Nagase rats (a family of rats with inherited serum albumin deficiency), and showed that the serum albumin level maintained high in these rats for 10 wk, indicating that HOC have differentiated into mature and functional hepatic cells. In this study, the rat FHF model was induced by D-GalN, into which rat HOC were transplanted. Biochemical assay showed the liver functions and pathological lesions of rats were slightly improved 48 h after transplantation. Moreover, the ALB and ALT levels were decreased in the following days, indicating that the transplanted HOC can survive in rats with FHF, and proliferate and differentiate to replace the damaged hepatic cells. The effect of HOC transplantation on FHF is related to the strong proliferation and differentiation of HOC into mature hepatic cells and biliary epithelial cells, which consequently benefit rat survival. In addition, liver failure elicits liver regeneration and up regulation of hepatocyte

growth factors. These cytokines, forming a suitable microenvironment, are necessary for the survival, growth and proliferation of transplanted HOC. Thus, newly regenerated hepatocytes compensate the damaged liver function, their robust activity may interact with adjacent cells and rescue some damaged liver cells with reversible pathologic lesions.

It is essential to appropriately label the transplanted cells to track their location and function in receptor. Both GFP and CFDA-SE are fluorescent labels for *in vivo* cell transplantation, but CFDA-SE showed superior properties in this study. CFDA-SE, a fluorescent dye, has been applied in various fields of immunology due to its stability and long duration. When cells divide, CFDA-SE is equally divided into two daughter cells, leading to an exponential decrease in fluorescence intensity with cell proliferation and division^[15]. In this study, CFDA-SE labeled HOC were transplanted in rats with FHF. Seventy-two hours after transplantation, green fluorescent colonies could be observed in sections of frozen liver tissue. The fluorescence intensity was strong but the colonies were only found near the injection site. On day 7, the fluorescence intensity of the transplanted cells decreased but transplanted cells were widely distributed, indicating that HOC have proliferated and differentiated into hepatic cells. Therefore, multiple green fluorescent colonies could be observed.

In conclusion, labeled HOC transplantation exerts its effects on FHF by improving the serum levels of ALT, AST, and TBil. However, since fluorescence intensity of CFDA-SE decreases with cell division, it is still not the ideal label for cell transplantation. Further study is needed on the location and distribution of transplanted HOC.

COMMENTS

Background

Fulminant hepatic failure is a serious clinical disease and may threaten the life of patients. However, because of the damage of mass liver cells, the organ function is often irreversible due to the liver cell degeneration, swelling, or apoptosis. Thus, to supply new sources of functional liver cells is a valuable choice for these patients.

Innovations and breakthroughs

The cultured hepatic oval cells (HOC) can provide cells for liver cell transplantation and even for biological artificial liver, thus solving the problem of liver donor shortage. In this study, a rat HOC proliferation model was established and the HOC were isolated, purified, labeled with CFDA-SE (a fluorescence agent), and transplanted into rats with fulminant hepatic failure (FHF). Then the authors detected the fluorescence distribution in the receptor liver to observe the role of HOC transplantation in FHF treatment.

Applications

The study indicated that transplantation of hepatic oval cells was a potential therapeutic strategy for the treatment of fulminant hepatic failure.

Terminology

HOC: liver stem cells with a self-renewal capacity and a high proliferative potential. FHF is usually defined as the severe impairment of hepatic functions in the absence of preexisting liver disease. 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE): a fluorescence agent

Peer review

The manuscript reports a therapeutic potential of transplantation of hepatic oval cells for fulminant hepatitis. Although liver transplantation is the most effective therapy for fulminant hepatitis at present, cell-based therapy could be an alternative treatment modality. The data presented are encouraging and promising.

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BRIEF ARTICLES

Lung tissue flap repairs esophagus defection with an inner chitosan tube stent

Gang Chen, Wen-Jun Shi

Gang Chen, Wen-Jun Shi, Department of Thoracic Surgery, Shengjing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China

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Correspondence to: Dr. Wen-Jun Shi, Department of Thoracic Surgery, Shengjing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China. xy7224@sina.com
Telephone: +86-24-83955064 **Fax:** +86-24-83955064

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Abstract

AIM: To repair the partial esophagus defect with a chitosan stent, a new esophageal prosthesis made of pulmonary tissue with vascular pedicle.

METHODS: Fifteen Japanese big ear white rabbits were divided into experimental group ($n = 10$) and control group ($n = 5$). Esophagus defect in rabbits of experimental group was repaired using lung tissue flap with a chitosan tube stent, gross and histological appearance was observed at week 2, 4 and 8 after operation, and barium sulphate X-ray screen was performed at week 10 after operation. Esophagus defect of rabbits in control group was repaired using lung tissue flap with no chitosan tube stent, gross and histological appearance was observed at week 2, 4 and 8 after operation, and barium sulphate X-ray screen was performed at week 10 after operation.

RESULTS: In the experimental group, 6 rabbits survived for over two weeks, the lung tissue flap healed esophageal defection, and squamous metaplasia occurred on the surface of lung tissue flap. At week 10 after operation, barium sulphate examination found that barium was fluent through the esophagus with no stricture or back stream, the creeping was good. In the control group, 4 rabbits survived for two weeks, the lung tissue flap healed esophageal defection with fibrous tissue hyperplasia,

barium sulphate examination found that barium was fluent through the esophagus with a slight stricture or back stream, and the creeping was not good at week 10 after operation.

CONCLUSION: Esophagus defect can be repaired using lung tissue flap with an inner chitosan tube stent.

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Key words: Lung; Tissue flap; Chitosan; Stent; Esophagus reconstruction

Peer reviewer: James D Luketich, MD, Professor and Chief, Division of Thoracic and Foregut Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, United States

Chen G, Shi WJ. Lung tissue flap repairs esophagus defection with an inner chitosan tube stent. *World J Gastroenterol* 2009; 15(12): 1512-1517 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1512.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1512>

INTRODUCTION

Esophagus disease is one of the common digestive tract diseases in our country. After excision, the esophagus needs to be reconstructed to restore the digestive tract continuity. At present, the commonly used esophagus reconstruction substitutes mainly include musculo-cutaneous flap with vessel peduncle^[1], platysma musculo-cutaneous flap^[2,3], periosteum intercostal muscle flap^[4] and others^[5-8] from stomach, colon and jejunum. Plastic tube, metallic pipe, teflon tube, silica gel tube, *etc*, are also used in reconstruction of artificial esophagus. However, their effects are not really ideal. The ideal substitute should be nontoxic, absorbable, without repulsive response and carcinogenicity, and easy to gain.

At present, various esophagus reconstruction techniques are available^[9-11] and each has its own particular advantages and disadvantages. Irrespective of the kind of reconstruction, reducing scar formation and preventing stricture are still the key points. Different flaps from the lung tissue petal have been successfully applied in reconstruction of trachea^[12]. Application of

lung tissue petal, inside lining metal and silica gel pipe stent in animals is also successful, but it is unable to overcome foreign matter response. In this study, we used inside lining metal, a new absorbable biological material-chitosan tube, to repair partial esophagus defect by preventing post-surgery stricture. Some questions concerning its application were discussed.

MATERIALS AND METHODS

Experimental animals and main materials

Fifteen healthy Japanese big ear white rabbits, weighing 3 kg, were provided by China Medical University Shengjing Hospital Animal Center. The rabbits were divided into experimental group ($n = 10$) and control group ($n = 5$). Chitosan tube stent, 15 mm long, 4 mm in inside diameter, was purchased from Shandong Province, China. Antiseptic glutaric dialdehydel were independently developed by the authors. TKR-200C micro-organism life-support machine was provided by Jiangxi Province, China. Rabbit surgery table, chest surgery instruments, infusion instruments, 20% urethane vein anesthetics, ketamine anaesthetics, 1% procaine, laryngoscope, 3.0-4.0 model trachea intubation were bought from Jiangxi Province, China. Tooth pad, medical adhesive plaster, digital camera (Sony, DSC-T10), and optical microscope (Olympics CH-20) were used in this study.

Experimental techniques

Before surgery, rabbits did not receive any medicine. Twenty percent of urethane vein anesthetics (5 mL/kg) was injected into abdominal cavity. Three minutes after anesthesia, the breath of rabbits was slow and changed to shallow and abdominal breath with corneal reflex. Ketamine (1 mg/kg) was intramuscularly injected to reduce the pain. The anaesthetized rabbits were fixed on the operation table at a supine position, and the assistant pulled in the flank with the bandage to draw in the front tooth and lower jaw. The mouth was pulled open with 1% procaine spraying. The throat was superficially anaesthetized. The operator stood in the rabbit head side, set the laryngoscope from one side of mouth, pushed away the tongue from tip to root. Epiglottis was exposed behind the tongue root resembling the white soft bone. Sometimes, vocal cord could not be found, but air bubbles could be observed. The trachea was gently pulled in intubation. The air current in the trachea pipe could be heard. When the inspiration was sent in gently, rapid vertical insertion was performed. Trachea intubation and tooth pad were fixed 13 cm away from the front teeth. When the air exhaled from the trachea intubation could be felt with hands, the life-support machine connected with oxygen was adjusted to a low current capacity.

Surgery method

The left side and barrier height of rabbit decubitus were exposed in the chest cavity, with the four limbs of rabbits fixed and the right flank prepared for

operation of the chest. The first step was to cut open pleural membrane. When lung collapse was observed, the rabbits were given machinery ventilation, and the breath frequency was adjusted to 30 times/min, and then adjusted according to the lung inflation. A stomach tube was inserted into esophagus to support it, and the center-section was searched for its dissociation, and slung with a spun yarn cloth strip. The excision of central esophagus was an esophagus wall, 3 mm in diameter, to make a animal model of partial esophagus wall damage. In the experimental group, a chitosan tube was placed in breakage of the esophagus and fixed with a needle. The esophagus was wrapped by the nearby lung tissue petal to form an encystation in the breakage place, and the edge of esophagus breakage was sutured continuously with a 3-0 silk suture. Then, the stomach tube was withdrawn to release the stress and return the esophagus. The control group did not need any inside lining chitosan tube. No chest internal hemorrhage and lung air leakage were found. Transition to ventilation was performed several times before the last needle was inserted into the pleural membrane. At end of the inspiration, pleural cavity was closed. The rabbits received fluid diet and anti-inflammation treatment, fresh milk and normal diet a week after operation.

Observation of target

Observation of ordinary circumstances: Survival, feed, body weight, and complication were observed after operation.

Observation of body: If the experimental rabbits died in the observation period, prompt postmortem examination was performed to find the cause of death. The animals were killed at weeks 2, 4 and 8 after surgery, respectively. Scar formation and chitosan tube were observed.

Observation of histology: The surviving animals were executed at week 2, 4 and 8, respectively. The damaged patching lung tissue was stained with H&E. The growth of lung tissue petal was observed under optical microscope.

Barium meal: Ten weeks after operation, esophagus of survived rabbits was observed by barium meal to see whether the esophagus was unobstructed.

RESULTS

Animal survival

After operation, 6 animals in experimental group, and 4 animals in control group survived, respectively. Five animals and 1 animal died on the same day after operation, 1 animal died due to anesthesia 1 d after operation, 1 animal died of unhealed fistula on day 4 after operation, 1 animal died of infusion accident on day 7 after operation, and 1 animal died of malnutrition at week 2 after operation.

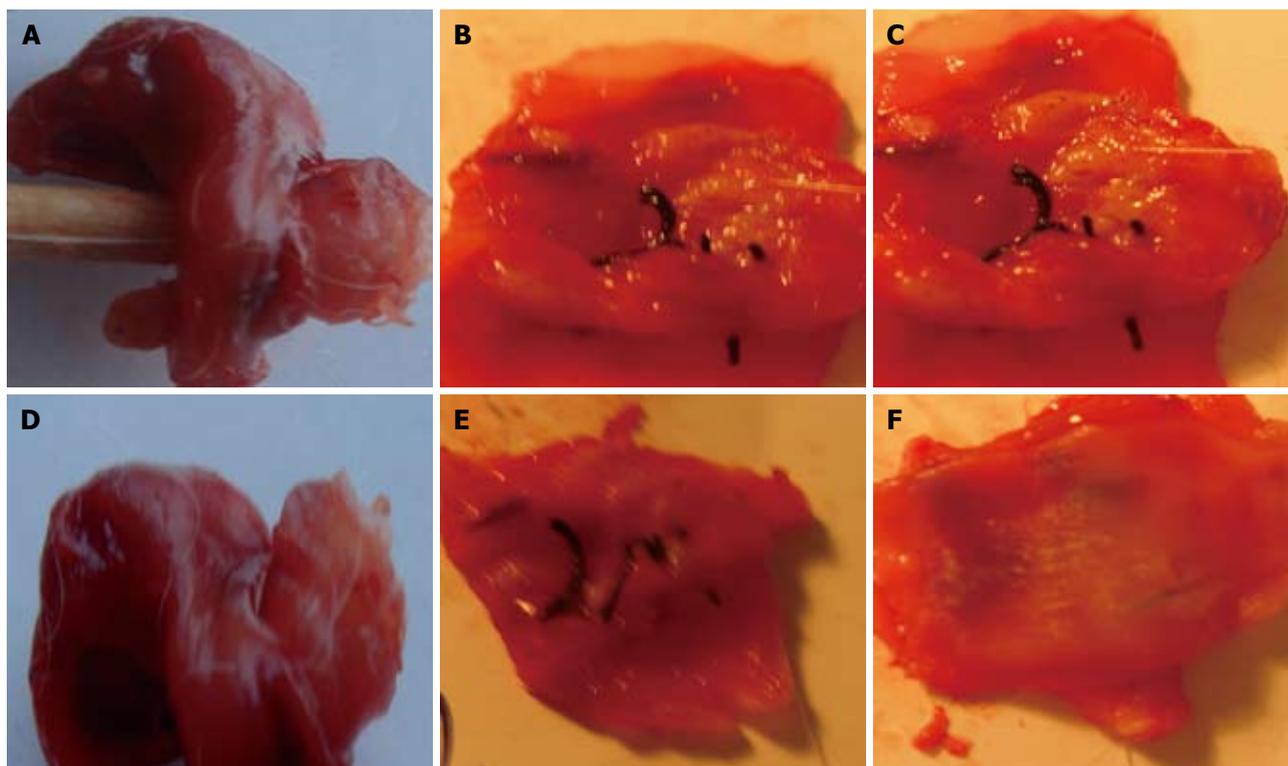


Figure 1 Lung tissue flap change in control group (A-C) and experiment group (D-F) after operation.

Observation of ordinary circumstances

Five days after operation, animals in experimental and control groups could have oral diet. The animals were provided with a small amount of food 5 d after operation and normal diet 10 d after operation. During this period, no obvious feed barrier was observed in animals of the experimental group. Three weeks after operation, animals in the control group had a poor appetite. One week after operation, the body weight of all animals decreased about 1-2 kg. The body weight of animals in the control group was still lighter than before operation.

Observation of body

Two weeks after operation, the esophagus damage was observed, the esophagus breakage edge and lung tissue petal were tightly united, accompanying dropsy. The chitosan tube could be touched under the substitute lung tissue petal and was soft, and the lumen surface had membranous contamination. Four weeks after operation, the damage was completely repaired, the suture was not absorbed. In the experimental group, the internal lumen surface of esophagus substitute was smooth, the blood circulation was rich, and the chitosan tube was partly decomposed. In the control group, the damage was red in color, hyperplasia was found around the damage, accompanying dropsy. In the experimental group, eight weeks after operation, the esophagus defect was covered by the white thick membranous substance. No dropsy, necrosis and ulcer, obvious stricture, or chitosan cast were found in the pipe wall. In the control group, scar

formation and hyperplasia were found on tissue petal surface (Figure 1).

Two weeks after operation, the lung structure of rabbits in experimental and control groups was observed. Pulmonary alveoli were withered and collapsed, and the denatured pulmonary alveolus cells were tumescent with acute inflammation response. Four weeks after operation, atypical pulmonary alveolus structure was observed in the experimental group. The inflammatory response was weakened accompanying a few neutral granular cells and lymphocytes, but no obvious fiber proliferation was observed. In the control group, the central pulmonary alveolus structure was atypical accompanying fiber cells and a few inflammatory cells. Eight weeks after operation, a large number of squamous epidermis cells were observed on the surface of lung tissue petal, and the chronic inflammatory response was significantly decreased in the experimental group. The control group had chronic inflammation response, accompanying obvious fiber proliferation but no superficial squamous metaplasia (Figure 2).

Barium meal

Ten weeks after operation, esophagus barium test was performed. In the experimental group, barium meal went smoothly through the esophagus. No obvious stricture, reverse flow, anastomotic stoma leakage and expansion were observed in the esophagus. The creeping motion was good. Mild stricture was observed in control group. The anastomotic stoma was healed with expansion, and the barium meal went through smoothly with general creeping motion (Figure 3).

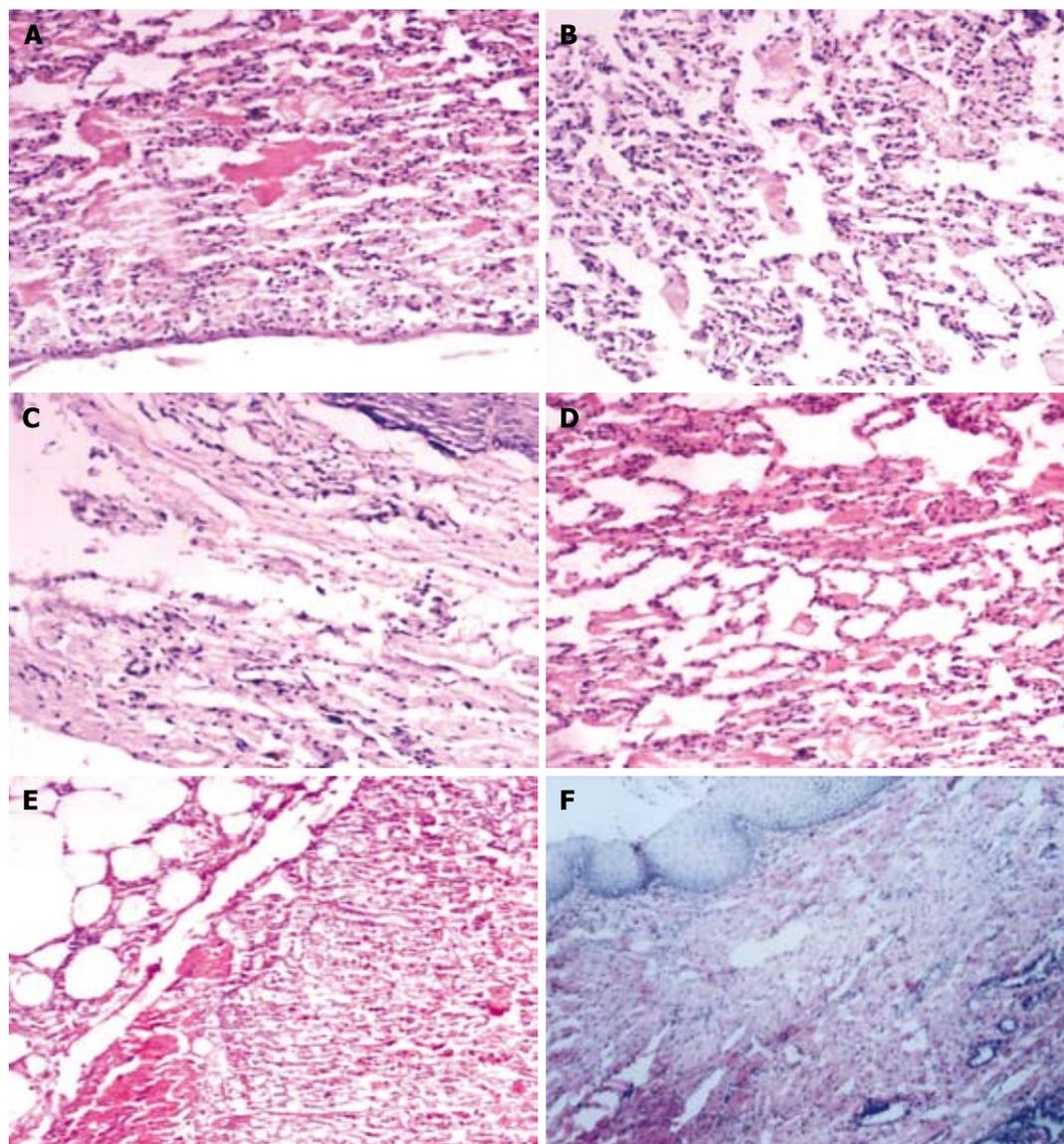


Figure 2 Lung tissue flap change under optical microscope in control group (A-C) and experimental group (D-F) after operation (HE, $\times 10$).

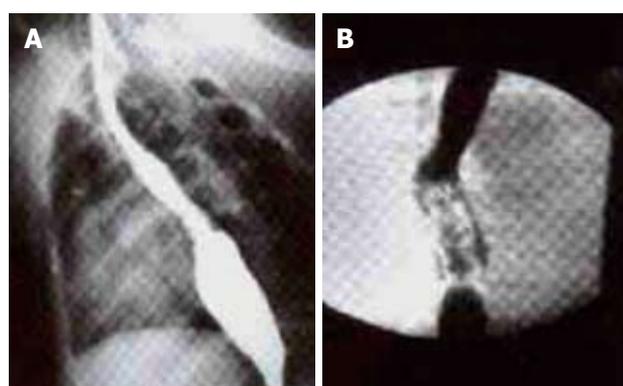


Figure 3 Results of esophagus barium test in control group (A) and experimental group (B) ten weeks after operation.

DISCUSSION

Esophagus excision and reconstruction are important in esophagus surgery. In recent years, biological substitutes have been extensively studied^[13-21]. Zhi *et al*^[13] reported that biological artificial esophagus can be used

to repair esophagus defect. In their study, esophagus substitution test was performed in 30 experimental dogs, showing that esophagus substitution can repair 93.3% of esophagus defects. Zhang *et al*^[14-17] excised chest esophagus at the right chest flank of 30 Chinese hybrid dogs, and an 8 cm long biological artificial esophagus was used to reconstruct esophagus. At present, we still face the problems of fistula, stricture, and length of implants, which need further research. Based on the biological esophagus substitute, biodegradable and non-biodegradable materials have been developed for making artificial esophagus^[18,19]. Meanwhile, the degradation speed of biodegradable material matches that of non-degradation material, which exerts the supporting function and prolongs the supporting time of biological materials, and finally regenerates esophagus to completely substitute the artificial esophagus. The project of digestion tube has made certain progress. However, its effect is unknown. Further study is needed on implantation and production of epithelial and muscle cells, gland and nerve plexus regeneration, scar reduction, speeding up repair and regeneration of

structure, *etc*^[20].

Dr. Shi first used lung tissue petal substitution to repair trachea defect successfully^[21], which is a new direction to repair esophagus defect with lung tissue petal and reconstruct esophagus. In the earlier experiment, we wrapped the damage spot using the lung tissue petal to make the artificial esophagus, in order to prevent stricture after repair of esophagus defect^[22,23]. Based on previous experiment results, we chose a chitosan material to make absorbable tube stent to overcome the rejection, and further explored the feasibility of this new method. The blood circulation of lung tissue petal was good, which was confirmed by pathology and electron microscopy. The compatibility of repair material was good, thus avoiding foreign matter rejection and forming reliable scars of fibers. The lung tissue petal has certain ductility, and different lobes of the lungs can be selected to make different lengths of lung tissue petal. In brief, the selection of lung tissue petal is convenient, the compatibility is good and the blood circulation is rich, with a high scar formation ability and good anti-infectiousness, and good environment for esophagus epidermis.

Chitin is the only high polymer material with widespread biodegradation^[24]. Acetyl-escaped product, also known as chitosan^[25], has the good biological compatibility with animal organs and cells, and degrades the low molecular oligosaccharide with no accumulation of products *in vivo* and no immunogenic ability. The tube we made of it is elastic and tough, slightly soft when it meets water, and can suppress inflammation response, prevent adhesion and scar formation in the breakage site. It was reported that chitosan membrane can prevent the adhesion to peritoneum and thus can be used in clinical practice^[26]. It was also reported that chitosan can insert into nerve tube stent^[27,28]. Chitosan tube stent is degraded gradually and absorbed *in vivo*, with no toxicity, stimulation and rejection. It has been shown that epidermis of the esophagus has certain degree of stricture but it is not serious enough to cause feed barrier in esophagus of dogs^[29]. Long-term survival and delayed chronic inflammation have been achieved using metallic pipe and silica gel tube as an inner lining support, but foreign matter rejection occurs. We used chitosan as a support to prevent scar formation and stricture of esophagus by making use of its compatibility and degradability. When stricture is formed, chitosan tube is degraded *in vivo*, and its product is not toxic and has no side effects. Thus, it is worthy to be further studied. The most serious complication is fistula, which occurs 5-7 d after operation and is related with infection, anastomotic techniques and blood circulation, *etc*. Infection is the most important factor for the occurrence of complication, because esophagus patching is a pollutant. Since we performed the surgery under strict asepsis, we solved the problem. After operation, the rabbits in experimental group were fasted for 5 d, and then received venous transfusion and anti-inflammation treatment. Because the resistance of rabbits was lower than that of dogs, the mortality rate of rabbits was

30%. Since this study was to verify the new method and explore experimental conditions, a large sample size of slightly bigger animals, like dogs, pigs, *etc*, should be used in the study. We used big white rabbits to establish esophagus partial damage model, and repaired esophagus wall partial damage using chitosan tube with lung tissue petal as its inside lining. Based on results of this experiment, pigs and dogs and other bigger animals, may be further tested for the replacement of chitosan tube in the entire esophagus. Our experiment did not operate chest of rabbits. Compared with big animals, rabbits are docile, convenient, inexpensive, and easy to obtain. The choice of support is the key to the maintenance of unobstructed lumen, protection of the surface granulation tissue of lung from adhesion, formation of diverticulum and false passage. The metal lattice support is widely applied in treatment of esophagus stenosis, mainly because of its good support effect. Therefore, we used the chitosan tubular support, which functions as a support, reduces inflammation response, and can be absorbed and degraded by organisms, and can be used as a substitute of esophagus.

Further study involving smooth muscle, nerve plexus and gland regeneration is needed^[30]. Creeping motion restoration, long esophagus reconstruction, *etc*, can be achieved in clinical practice.

COMMENTS

Background

At present, esophagus reconstruction techniques are available, but each of them has its advantages and disadvantages. Reducing scar formation and stricture is still the key to esophagus reconstruction.

Research frontiers

In this study, chitosan tube was used as a support to prevent post-operation stricture.

Innovations and breakthroughs

Trachea was reconstructed using chitosan and silica gel pipe stent. A chitosan material was used to make absorbable support tubes to overcome the rejection and prevent stricture of esophagus.

Applications

Chitosan tubes provide a new surgery method for patients in whom substituting esophagus with cavity internal organs is impossible.

Terminology

Lung tissue petal: A lung lobe closing the segment of trachea.

Peer review

It is an interesting animal experiment about the use of chitosan stent with a lung tissue flap to repair esophageal perforation.

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BRIEF ARTICLES

Biotransformation of aesculin by human gut bacteria and identification of its metabolites in rat urine

Wei-Jun Ding, Yun Deng, Hao Feng, Wei-Wei Liu, Rong Hu, Xiang Li, Zhe-Ming Gu, Xiao-Ping Dong

Wei-Jun Ding, Yun Deng, Wei-Wei Liu, Rong Hu, Xiang Li, Xiao-Ping Dong, Chengdu University of Traditional Chinese Medicine, Chengdu 610075, Sichuan Province, China

Hao Feng, Zhe-Ming Gu, XenoBiotic Laboratories, Inc. 107 Morgan Lane. Plainsboro, NJ 08536, United States

Author contributions: Ding WJ, Dong XP, Gu ZM and Deng Y designed the research; Ding WJ, Deng Y, Liu WW, Feng H, Hu R and Li X performed the research; Hu R, Deng Y and Feng H analyzed the data; Ding WJ, Gu ZM and Deng Y wrote the paper.

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Correspondence to: Dr. Wei-Jun Ding, Laboratory of Microbiology, Chengdu University of Traditional Chinese Medicine, 37#, Shier Qiao Street, Chengdu 610075, Sichuan Province, China. ding2004wj@yahoo.com.cn

Telephone: +86-28-61800219 Fax: +86-28-87763471

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6-O-methyl-7-gluco-coumarin (M5) and 6-O-methyl-7-sulf-coumarin (M6). Of which, M2 and M6 were novel metabolites.

CONCLUSION: Aesculin can be transferred into aesculetin by human gut bacteria and is further modified by the host *in vivo*. The diverse metabolites of aesculin may explain its pleiotropic pharmaceutical effects.

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Key words: Aesculin; Biotransformation; Human gut bacteria; Rat urine; Sulfated derivatives; LC/ESI-MS; Aesculetin

Peer reviewer: Zhiheng Pei, MD, PhD, Assistant Professor, Department of Pathology and Medicine, New York University School of Medicine, Department of Veterans Affairs, New York Harbor Healthcare System, 6001W, 423 East 23rd street, New York NY 10010, United States

Abstract

AIM: To observe the biotransformation process of a Chinese compound, aesculin, by human gut bacteria, and to identify its metabolites in rat urine.

METHODS: Representative human gut bacteria were collected from 20 healthy volunteers, and then utilized *in vitro* to biotransform aesculin under anaerobic conditions. At 0, 2, 4, 8, 12, 16, 24, 48 and 72 h post-incubation, 10 mL of culture medium was collected. Metabolites of aesculin were extracted 3 × from rat urine with methanol and analyzed by HPLC. For *in vivo* metabolite analysis, aesculetin (100 mg/kg) was administered to rats *via* stomach gavage, rat urine was collected from 6 to 48 h post-administration, and metabolite analysis was performed by LC/ESI-MS and MS/MS in the positive and negative modes.

RESULTS: Human gut bacteria could completely convert aesculin into aesculetin *in vitro*. The biotransformation process occurred from 8 to 24 h post-incubation, with its highest activity was seen from 8 to 12 h. The *in vitro* process was much slower than the *in vivo* process. In contrast to the *in vitro* model, six aesculetin metabolites were identified in rat urine, including 6-hydroxy-7-gluco-coumarin (M1), 6-hydroxy-7-sulf-coumarin (M2), 6, 7-di-gluco-coumarin (M3), 6-glc-7-gluco-coumarin (M4),

Ding WJ, Deng Y, Feng H, Liu WW, Hu R, Li X, Gu ZM, Dong XP. Biotransformation of aesculin by human gut bacteria and identification of its metabolites in rat urine. *World J Gastroenterol* 2009; 15(12): 1518-1523 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1518.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1518>

INTRODUCTION

Aesculin, a 6, 7-dihydroxy derivative of coumarin with pleiotropic pharmacological and biochemical properties, has recently been analyzed for its biochemical activity^[1]. Kaneko and colleagues^[2] found that aesculetin and its 6-glycoside, aesculin, can inhibit oxidative DNA damage and formation of aberrant crypt foci and tumors. Furthermore, this compound shows an inhibitory effect on BOP-induced oxidative DNA damage and carcinogenesis in a hamster pancreatic tumor model^[2], as well as chemo-preventive^[3] and anti-tumor activity on cancer^[4]. Aesculin and aesculetin have strong antioxidative and photo-protective activities, by scavenging 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and superoxide anions from the xanthine/xanthine oxidase system and inhibiting oxidation of 5-(6-)chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate^[5]. Aesculetin increases apoptosis of 3T3-L1 adipocytes in a time- and

dose-dependent manner^[6], and inhibits cell growth and cell cycle progression by inducing G1 phase arrest in HL-60 leukaemia cells, which is a direct result of the inhibition of retinoblastoma protein phosphorylation^[7]. Aesculetin also displays multiple immunomodulatory effects on murine lymphocytes and peritoneal macrophages in rat liver^[8], including anti-inflammatory activity, inhibition of lipoxygenase and tyrosinase activity, scavenge of hydroxyl radicals and suppressing lipid peroxidation.

However, the biotransformation progress of aesculin and its derivatives has not been completely determined, let alone their complex structure-activity relationships. Aesculetin is one of the simplest coumarins with two hydroxyl groups at carbons 6 and 7 that serve as targets for O-methylation or O-glycosylation. It has been reported that biotransformation of aesculetin with *E. coli* expressing O-methyltransferase (POMT-9) generates scopoletin, isoscapoletin, and scoparone^[9]. The growth of *E. coli* is inhibited by aesculetin, but not by aesculin^[10]. Hairy roots of medicinal morning glory (*Pharbitis nil*) show a potent glucosylation activity against aesculetin, especially at 7-hydroxyl group^[11]. As one of the metabolites of caffeic acid oxidation, aesculetin observed in perfused rat liver may be responsible for its biological effects observed *in vivo*^[12]. Aesculetin is a substrate of mushroom polyphenol oxidase and horseradish peroxidase enzyme resulting in the oxidization of aesculetin into its o-quinone^[13]. Despite these accumulated data, we still do not fully understand the structural basis and dynamic pattern underlying the pleiotropic biological effects of aesculetin and there are few reports describing its derivatives or metabolites.

Therefore, in the present work, we analyzed the biotransformation process of aesculin in response to human gut microflora in a rat model, in order to identify its novel metabolites, which may enrich our understanding of the complicated structure-activity relationships between aesculin and its metabolites.

MATERIALS AND METHODS

Collection of representative human gut bacteria

Twenty healthy volunteers (10 males and 10 females) at the age of 20-24 years, were recruited from students of Chengdu University of Traditional Chinese Medicine. Their faeces were collected and intestinal flora was identified as normal with an automatic system of bacteria identification (BD, USA). Then, fecal samples were pooled. The bacterial concentration was adjusted to 2×10^7 CFU/mL in culture medium, and 1 mL of aliquots was stored at -80°C until use.

Preparation of anaerobic culture medium

Culture medium for anaerobic gut bacteria, GAM, was prepared according to the following formula: 3 g of soybean phytone, 10 g of peptone, 13.5 g digested blood serum powder, 5 g yeast extract, 2.2 g extractum carnis, 1.2 g beef liver extract, 3 g glucose, 5 g soluble starch, 2.5 g KH₂PO₄, 3 g NaCl, 0.3 g L-cysteine, 0.3 g sodiumthioglycollate, were added into 800 mL of dH₂O. The pH was adjusted to 7.4 with 1 mol/L NaOH and the

final volume was adjusted to 1000 mL with dH₂O. The medium was sterilized by autoclaving at 121°C for 20 min and stored at 4°C for further use.

Chemicals

Aesculin (6-hydroxy, 7-glycoside coumarin), with an estimated purity of 98%, was purchased from Aldrich Chemical Co. (Milwaukee, WI). For the preparation of standard aesculin, 250 mg of aesculin was added to 25 mL of saline (0.9% NaCl solution) and dispensed into the solution by sonication. The mixture was then boiled for 10 min to dissolve aesculin. This stock solution of aesculin (10 mg/mL) was stored in the dark at -20°C until use.

Aesculetin (6, 7-dihydroxycoumarin) was also purchased from Aldrich Chemical Co. (Milwaukee, WI). The stock solution of aesculetin (10 mg/mL) was prepared as above and stored under the same condition for aesculin.

Exposure of aesculin/aesculetin to human gut bacteria

Twenty millilitre of aesculin stock solution was added to 179 mL of GAM in a 500 mL glass flask, followed by addition of 1 mL of human gut bacteria. The final concentration of aesculin was 1 mg/mL. The mixture was incubated at 37°C in DY-II anaerobic incubator containing 80% N₂, 10% CO₂, and 10% H₂ (Yiwu Co. Ltd, Zhejiang Province, China). Ten mL of the solution was removed at 0, 2, 4, 8, 12, 16, 24, 48 and 72 h post-incubation, respectively, and 10 mL of methanol was then added to at each time point. The solutions were mixed well with hands for 5 min and the upper phase was transferred into a fresh flask. This extraction process was repeated two times and all the upper phase fractions were pooled for HPLC analysis.

The same protocol was employed to incubate aesculetin with human gut bacteria.

HPLC analysis of cultured aesculin/aesculetin

Preparation of control stock solution: Methanol was used as a solvent to prepare the aesculin and aesculetin stock solutions. The final concentration of aesculin and aesculetin was 10.08 mg/mL and 6.120 mg/mL, respectively. The solutions were kept in the dark at 4°C for up to one week.

HPLC was performed on a Waters Separation Module 2695 at a detection wavelength of 340 nm, at a screen wavelength of 210-400 nm, at a flow rate of 1.0 mL/min, at a sample size of 10 µL, and at a column temperature of 25°C, with mobile phase A set at 5% acetic acid : methanol (78:22), mobile phase B at 5% acetic acid: methanol gradient elution (0-20 min, 80:20; 20-35 min, linearity changed from 80:20 to 40:60), and mobile phase C at 5% acetic acid: methanol gradient elution (0-20 min, 90:10; 20-35 min, linearity changed from 90:10 to 50:50), respectively.

Preparation of urinary metabolites

Twelve adult SD rats (6 males and 6 females), weighing 200 ± 5.6 g, were raised under specific pathogen-free conditions at the University Laboratory Animal Service

Table 1 Quantitative biotransformation efficiency of human gut bacteria for aesculin

Incubation time (h)	0	2	4	8	12	16	24	48	72
Aesculin Conc. (mg/mL)	0.886	0.848	0.832	0.873	0.166	0.043	ND	ND	ND
Aesculetin Conc. (mg/mL)	ND	ND	ND	ND	0.332	0.418	0.434	0.466	0.462
Transformation ratio (%)	0	0	0	0	79.20	94.90	100	100	100

"ND" indicates that nothing was tested.

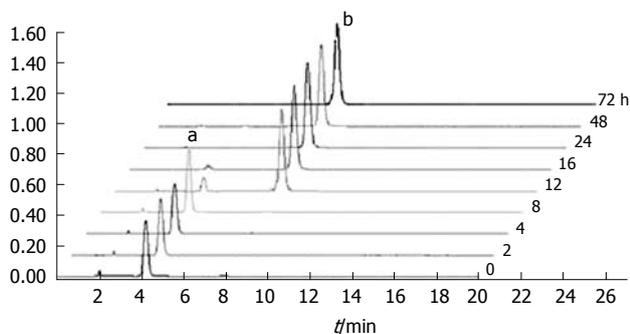


Figure 1 Biotransformation of aesculin by anaerobic human gut bacteria. The gut bacteria comprised 20 pooled fecal samples from healthy volunteers. Incubation was performed under anaerobic conditions (80% N₂, 10% CO₂, 10% H₂) at 37°C for defined time periods. Ten millilitre of samples was collected at 0, 2, 4, 8, 12, 16, 24, 48 and 72 h, respectively post-incubation. Aesculin and metabolites were then extracted with methanol and analyzed by HPLC. The vertical axis shows the relative values for the concentration of aesculin and/or its metabolite. The horizontal axis indicates the HPLC overflow time.

Center of Chengdu University of Traditional Chinese Medicine, China. Each rat was raised in a solitary metabolic cage, with free access to normal food in a 12 h light and dark cycle. After adaptive nurture for one week, the aesculetin solution was administrated at a dosage of 100 mg per kilogram. Rat urine produced from 6 to 48 h after administration of aesculetin solution was collected for analysis. Five millilitre of urine sample from each rat was mixed for HPLC analysis.

Identification of aesculin metabolites in rat urine by LC/MS

Aesculin metabolites were identified in rat urine by XenoBiotic Laboratories, Inc., Plainsboro, NJ, USA. Control solutions of aesculetin and aesculin, and rat urine sample were analyzed by LC/ESI-MS and MS/MS in the positive and negative modes. The actual instrument conditions were modified to optimize chromatography and instrument sensitivity.

HPLC: HPLC System: Waters Separation Module 2695. Column: Ace 3, C18, 3 μm, 150 mm × 4.6 mm; guard column: Ace 3, C18, 10 × 3.2 mm; column temperature: 25°C; autosampler temperature: 4°C; mobile phase A: 0.4% HCOOH in H₂O; mobile phase B: CAN.

MS: Mass spectrometer: Finnigan LCQ™ mass spectrometer. Data system: ThermoQuest Xcalibur Version 1.3; ionization mode: positive or negative electrospray ion modes [(+)/(-) ESI]; ion spray (IS): 4.5 kV; capillary temperature: 240°C; sheath gas flow: N₂,

~80 units; auxiliary gas flow: N₂, ~20 units; collision gas: helium.

RESULTS

Human gut bacteria converted aesculin into aesculetin

HPLC analysis revealed that our representative human gut bacteria degraded the glycoside of aesculin, thus completely converting it into aesculetin (Figure 1). The biotransformation process occurred between 8 and 24 h post-culture. After 24 h, aesculin was almost transferred into aesculetin. No other metabolite was observed, indicating that human gut bacteria cannot further modify aesculetin. No conversion of aesculin to aesculetin was observed in the absence of human gut bacteria 72 h after incubation (data not shown), indicating that human gut bacteria are a prerequisite for the biotransformation of aesculin.

The biotransformation efficiency of aesculin was quantitatively determined according to the following equation: Transformation ratio = (aesculetin concentration/178)/(aesculetin concentration/178 + aesculin concentration/340), where 178 represents the molecular weight of aesculetin and 340 represents the molecular weight of aesculin. Almost 80% of aesculin biotransformation occurred from 8 to 12 h post-incubation (Table 1).

The mixture of human gut bacteria did modify aesculetin under the same culture conditions (data not shown), indicating that human gut bacteria can only biotransform aesculin but not further modify its molecules.

Identification of aesculetin metabolites in rat urine

Six metabolites of aesculin were detected in rat urine (Figure 2). The primary characteristics of each metabolite were obtained by CAD-MS/MS analysis (Figure 3). One or two hydrogens in these metabolites [6-hydroxy-7-gluco-coumarin (M1), 6-hydroxy-7-sulf-coumarin (M2), 6, 7-di-gluco-coumarin (M3), 6-glc-7-gluco-coumarin (M4), 6-O-methyl-7-gluco-coumarin (M5) and 6-O-methyl-7-sulf-coumarin (M6)] of the hydroxyl groups of aesculetin were modified by glucosylation, glycoside, sulfation, and/or methylation. In the present study, M2 and M6 were found to be novel aesculin metabolites compared with the reported data^[1,2].

Compared with the reference standards for aesculetin and aesculin, six aesculetin metabolites in rat urine were clearly observed on HPLC UV-chromatogram (Figure 4). The spectral data of aesculetin using LC/(+)ESI-MS and

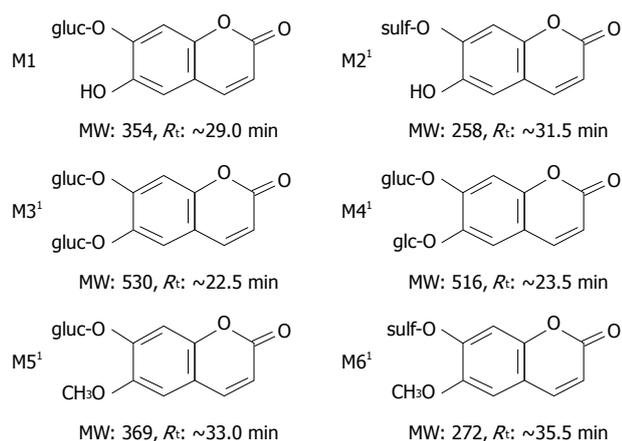


Figure 2 Summary of aesculetin metabolites identified in rat urine. ¹The position(s) of methylation and/or conjugation(s) may be exchangeable on the two phenols.

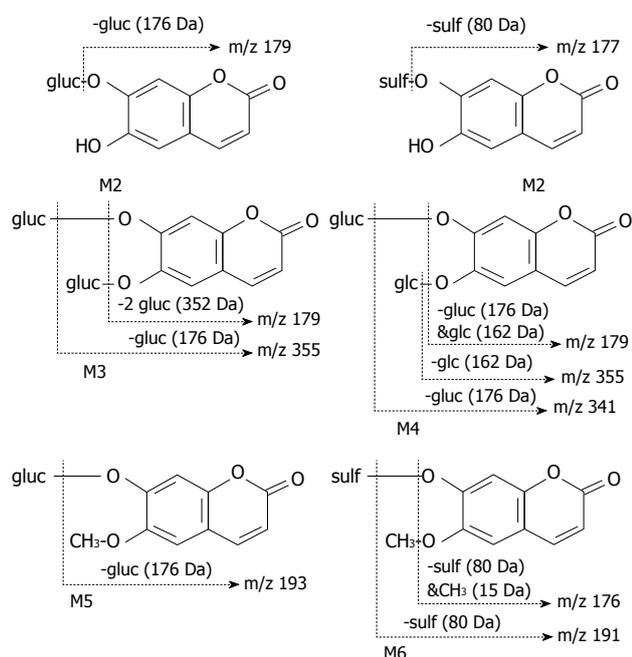


Figure 3 Proposed characteristic (-) CAD-MS/MS fragmentations for aesculetin metabolites.

MS/MS are shown in Figure 5. By comparing the data, we could identify the substitution position of aesculetin metabolites in rat urine *in vivo*.

Unlike gut microflora, mammalian physiology could further modify aesculin and the pharmaceutical effects of aesculin might be a result of these metabolic modifications.

DISCUSSION

Natural coumarins, widely distributed in plants, fungi and bacteria, have pleiotropic biological effects. Coumarins, made of fused benzene and α -pyrone rings, have phenolic hydroxyl groups in their structures that can be easily replaced and/or modified, forming various metabolites or derivatives. Aesculin, one of the simplest

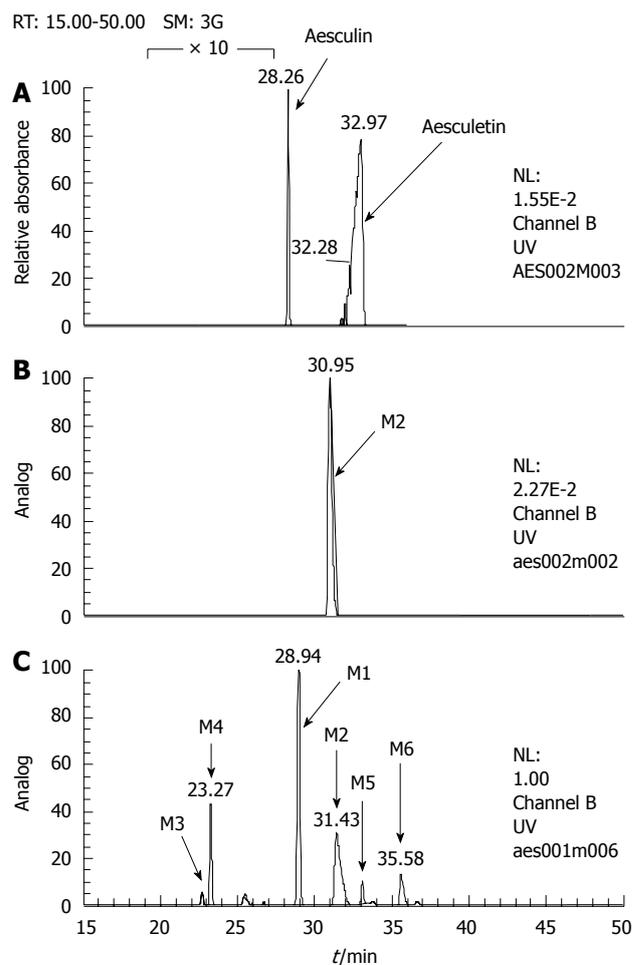


Figure 4 HPLC UV-chromatograms of reference standards for aesculetin and aesculin (A), M2 isolated from rat urine (B), and a rat urine sample dosed with aesculetin with assignments of aesculetin urinary metabolites (C).

coumarins known to have multiple pharmacological and biochemical activities, is an established inhibitor of lipoxygenase and cyclo-oxygenase and shows scavenging effects on ROS^[13]. It has been reported that aesculetin has chemo-preventive and anti-tumor activities *in vivo* against cancer^[5,14] and also induces apoptosis in several types of human cancer cells by diverse pathways^[1,6,7,15,16]. Hence, it is a great challenge to determine the complex structure-activity relationships between aesculin/aesculetin and their metabolites. The aesculin metabolites in rat urine were identified both *in vitro* and *in vivo*.

The results of this study show that human gut bacteria could completely biotransform aesculin into aesculetin. About 80 % of aesculin was converted into aesculetin in less than 12 h post-incubation. The reason why the *in vitro* biotransformation speed of aesculin was so slow remains unclear. The universal time lag of our bacterial culture conditions might be a major reason, since human gut bacteria must first synthesize enough enzymes in order to adapt to any new environment. Since the starting concentration (10^5 CFU/mL) of human gut bacteria was markedly lower than that of human gut flora (about 10^{12} CFU/gram), some additional time is necessary for the exponent growth of seeded bacteria in order to reach the

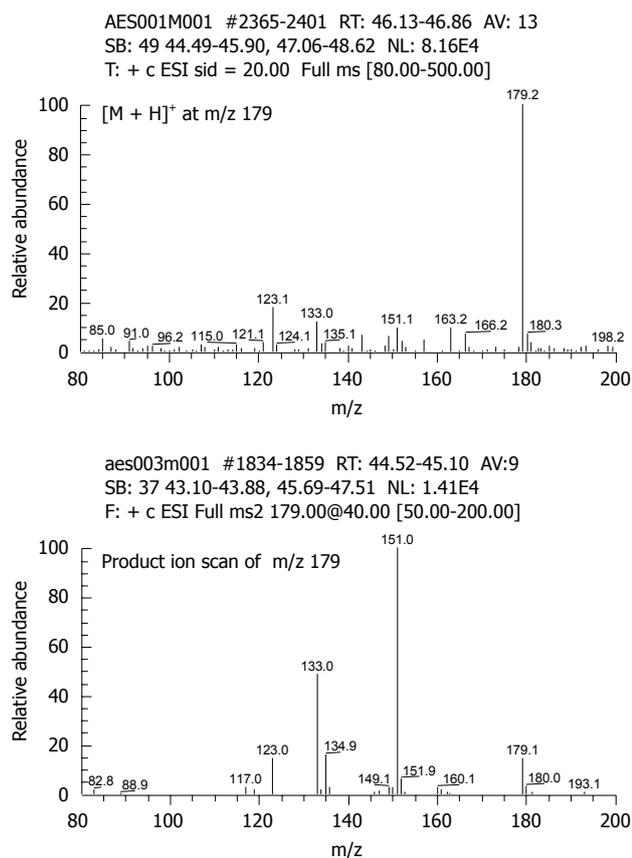


Figure 5 Aesculetin LC/(+)ESI-MS and MS/MS spectral data.

maximum bacterial concentration in the culture medium. In addition, we only employed anaerobic gut bacteria for the biotransformation of aesculin, which should be somewhat slower than *in vivo* conditions, since both anaerobic and aerobic bacteria may be involved in the *in vivo* aesculin biotransformation process.

The results of aesculin transformation suggest a simple but vivid example of host-gut bacteria cooperation in the dynamic utilization and subsequent modification of xenobiotics. Human gut bacteria degrade the glycoside of aesculin to facilitate its absorption in intestine, then modification of aesculetin into various derivatives with the potential of having multiple biological activities, occurs in the host. It was reported that the distal human intestine represents an anaerobic bioreactor programmed with a large population of bacteria, providing us with genetic and metabolic attributes, including the ability to harvest otherwise inaccessible nutrients and to metabolize diverse xenobiotics^[17-19]. Microbiome is still a largely under explored regulator of drug metabolism and bioavailability^[17-19]. Our data present here provide additional evidence for the metabolism of aesculin by human gut microbiota.

In this study, two novel sulfated aesculetin metabolites were identified in rat urine, which might represent very interesting modifications of coumarins. As aesculetin has two hydroxyl groups at carbons 6 and 7, it can serve as targets for O-methylation or O-glycosylation. It has been reported that O-methylated products of

aesculetin are scopoletin (6-O-methyl aesculetin), isoscapoletin (7-O-methyl aesculetin), and scoparone (6, 7-O-dimethyl aesculetin), which possess antimicrobial, immunosuppressive, and hypolipidemic activities^[7,9]. The hydroxyl groups of aesculetin, especially the 7-hydroxyl group, can also be glucosylated in plants such as hairy roots of medicinal morning glory^[11]. In addition, mushroom polyphenol oxidase (PPO) and horseradish peroxidase (POD) can oxidize aesculetin and generate its o-quinone^[13]. However, as far as we are know, no sulfated derivative of aesculetin has been identified. Indeed, sulfation is one of the most important modifications in many natural compounds. As their sulfate groups accumulate negative charges, sulfated coumarins can interact with certain molecular domains that usually have positive charges, resulting in compounds with anti-viral, anti-tumor and anti-oxidative effects^[12]. Therefore, these novel derivatives identified in rat urine offer new evidence for the biological activities ascribed to aesculetin.

COMMENTS

Background

Aesculin and its metabolites have pleiotropic pharmacological and biochemical properties, such as antioxidative, photo-protective and multiple immunomodulatory effects. However, their biotransformation has not been extensively studied, let alone their complex structure-activity relationships.

Research frontiers

To uncover the dynamic progress and identify the pharmacological metabolites of natural products is one of the hotspots in development of traditional Chinese herbal drugs.

Innovations and breakthroughs

The biotransformation of aesculin was investigated in this study. Human gut bacteria could completely convert aesculin into aesculetin *in vitro*. Six aesculetin metabolites were identified in rat urine, 2 of which were first found. These novel sulfated aesculetin metabolites represent one of the most important modifications of coumarins. As their sulfate groups accumulate negative charges, sulfated coumarins can interact with certain molecular domains that usually have positive charges, resulting in compounds with anti-viral, anti-tumor and anti-oxidative effects. Therefore, these novel derivatives offer new evidence for the biological activities ascribed to aesculin.

Applications

The results of this study can direct the development of anti-viral, anti-tumor and/or anti-oxidative natural herbal drugs, and display a useful design for retrieval of the complex biotransformation process of ceterin natural compounds.

Terminology

LC/ESI-MS stands for Liquid chromatography-electrospray ion trap mass spectrometry; CFU indicates that colony forming units.

Peer review

The authors studied the biotransformation of aesculin by gut bacteria in rats and showed that aesculin had a wide range of biological activities that may have important pharmaceutical applications. Hence, knowledge about its metabolism is essential for understanding its therapeutic effects. The findings are interesting. Aesculin was converted to aesculetin in an *in vitro* bacterial culture system and 6 aesculetin metabolites were identified in rat urine, 2 of which were first observed.

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CASE REPORT

A case of Noonan syndrome and Whipple's disease in the same patient

Sundip S Karsan, Hetal A Karsan, Anand S Karsan, James Ian McMillen

Sundip S Karsan, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Room 5512, Los Angeles, California, CA 90048, United States

Hetal A Karsan, Emory University School of Medicine and Atlanta Gastroenterology Associates, Atlanta, GA 30322, United States

Anand S Karsan, University of California, San Diego, CA 92093, United States

James Ian McMillen, Atlanta Clinical Care and Atlanta Specialty Research, Atlanta, GA 30322, United States

Author contributions: Karsan SS, Karsan HA, Karsan AS contributed equally to this work and were responsible for the review of the literature and initial preparation of the paper; Karsan SS and Karsan HA wrote the paper and had final approval of the version to be published; McMillen JI contributed to revising it critically for important intellectual content.

Correspondence to: Sundip S Karsan, MD, MPH, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Room 5512, Los Angeles, California, CA 90048, United States. sundip.karsan@cshs.org

Telephone: +1-650-9068387 Fax: +1-650-4946112

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Abstract

We report the first known case of both Noonan syndrome and Whipple's disease occurring in the same patient. A 36-year-old female with history of Noonan syndrome developed fatigue, anorexia, arthritis of the knees and hands with a diffuse hyperpigmented rash, night sweats, and an unintentional fifteen pound weight loss over 4 mo. Small bowel enteroscopy demonstrated mild edematous yellowish mucosa without friability. Random small bowel biopsies revealed extensive periodic acid-Schiff positive material within the foamy macrophages. She was treated with a 12 mo course of trimethoprim-sulfamethoxazole DS with clinical improvement to baseline status.

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Key words: Whipple's disease; Noonan syndrome; *Tropheryma whipplei*; Periodic acid-schiff stain; *PTPN11* gene

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of Gastroenterology, Medical School of Lisbon, Av Prof Egas Moniz, 1649-028 Lisbon, Portugal

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INTRODUCTION

We report the first known case of both Noonan syndrome and Whipple's disease (WD) occurring in the same patient. Noonan syndrome is an autosomal dominant genetic disorder that causes abnormal development, originally labeled as the "male Turner syndrome." It affects at least 1 in 2500 male and female children and is thought to be due to a genetic mutation of *PTPN11* gene, first discovered in 2001. Signs and symptoms include webbing of the neck, changes in the sternum (pectus excavatum), facial abnormalities (low-set or abnormally shaped ears, ptosis, hypertelorism, epicanthal folds, antimongoloid palpebral slant, micrognathia), cubitus vulgaris, congenital heart disease (especially pulmonary stenosis, and/or atrial septal defect), and variable hearing loss. Mild mental retardation is present in approximately 25% of cases. WD is a rare systemic infection caused by a non-acid fast gram positive bacillus, *Tropheryma whipplei* (*T. whipplei*). Thus far fewer than 1000 reported cases have been described with an annual incidence of approximately 30 cases per year since 1980. In 1907, Whipple first reported this syndrome in an original case report. Invasion or uptake of the bacillus is widespread throughout the body, including the intestinal epithelium, macrophages, capillary and lymphatic endothelium, colon, liver, brain, heart, lung, synovium, kidney, bone marrow, and skin. Advancement in diagnosis has only recently occurred with the first successful culture of *T. whipplei* in the year 2000; nearly a full century after the disease entity was first described.

CASE REPORT

A 36-year-old female with history of Noonan syndrome developed fatigue, anorexia, arthritis of the knees and hands with a diffuse rash, night sweats, and an



Figure 1 Computerized tomography scan image displaying diffuse lymphadenopathy and small bowel wall thickening.

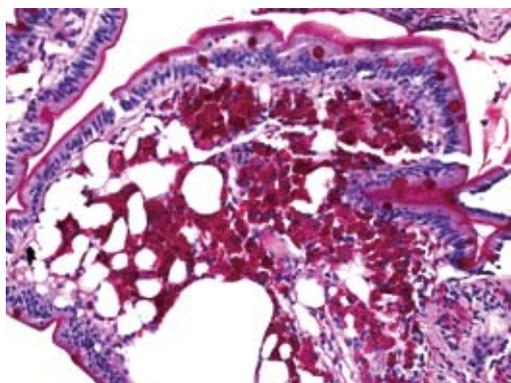


Figure 2 A periodic acid-Schiff (PAS) stain showing extensive PAS positive material within the foamy macrophages (small intestinal biopsy).

unintentional fifteen pound weight loss over the past 4 mo. One year prior, she had an endoscopic workup performed for iron deficiency, which was significant for erosive esophagitis. Of note, ileal and duodenal biopsies were histologically normal. Prior to this consultation, she had recently seen a rheumatologist and was placed on prednisone and Plaquenil for arthritis thought to be of rheumatoid origin. She then developed an episode of acute right lower quadrant abdominal pain and an abdominal computerized tomography (CT) scan was ordered. This revealed lymphadenopathy and diffuse thickening of the small bowel (Figure 1). The patient was then referred for gastroenterological consultation at which time her chief complaints were fatigue, arthralgias, and anorexia. She denied abdominal pain, diarrhea, hematochezia, fevers, chills, nausea, or vomiting.

She was 5'2" feet tall and weighed 117 pounds. Physical examination was significant for arthritis of the knees and hands with mild diffuse hyperpigmented skin. Laboratory tests were significant for sedimentation rate of 33, hemoglobin 9.6, and a white blood cell count of 10.4 without a shift. At this time, concern for lymphoma warranted further investigation. Thus, enteroscopy was performed in May 2006. The only significant endoscopic findings were a mild edematous yellowish mucosa without friability in the

distal duodenum and jejunum. Random small bowel biopsies revealed intestinal mucosa in which the lamina propria was markedly expanded due to an infiltrate of histiocytic-appearing cells with foamy cytoplasm and a periodic acid-Schiff (PAS) stain showing extensive PAS positive material within the foamy macrophages; AFB stain was negative (Figure 2). These findings were consistent with a diagnosis of WD. Due to the patient's inability to psychologically tolerate intravenous therapy and an allergy to penicillin, she was placed on an oral regimen of trimethoprim-sulfamethoxazole double-strength twice daily for a 12 mo course.

At follow up in December 2006, she had a remarkable clinical improvement and returned to her baseline clinical status. That is, she had a marked improvement in weight and complete resolution of anemia, arthritis, rash, and bowel wall thickening and lymphadenopathy on CT scan.

DISCUSSION

We report the first known case of both Noonan syndrome and WD occurring in the same patient. Noonan syndrome is an autosomal dominant genetic disorder that causes abnormal development, originally labeled as the "male Turner syndrome." It affects at least 1 in 2500 male and female children and is thought to be due to a genetic mutation of *PTPN11* gene, first discovered in 2001.

WD is a rare systemic infection caused by a non-acid fast gram positive bacillus, *T. whipplei*. Thus far, fewer than 1000 reported cases have been described^[1], with an annual incidence of approximately 30 cases per year since 1980. In postmortem studies, the frequency of the disease is less than 0.1%^[2]. In 1907 Whipple first reported this syndrome in an original case report^[3]. The ability to diagnose WD was advanced in 1949 with PAS staining which identified granules within macrophages that likely represented degenerating bacterial forms^[4]. Invasion or uptake of the bacillus is widespread throughout the body, including the intestinal epithelium, macrophages, capillary and lymphatic endothelium, colon, liver, brain, heart, lung, synovium, kidney, bone marrow, and skin. The chronic, insidious nature of WD may at least be in part due to the long doubling time of 17 d. Advancement in diagnosis has only recently occurred with the first successful culture of *T. whipplei* in the year 2000; nearly a full century after the disease entity was first described. One year later, in 2001, the first phenotypic characterization of the Whipple bacillus occurred, resulting in the renaming of the bacterium to *T. whipplei*^[5].

Clinical manifestations include four cardinal findings: arthralgias, weight loss, diarrhea, and abdominal pain. Neurological involvement (dementia, supranuclear ophthalmoplegia, nystagmus, and myoclonus) has been recognized in up to 40% of patients, either as initial manifestations or during the course of the disease. Less common symptoms include fever and skin

hyperpigmentation. There may also be symptoms or signs related to cardiac disease (dyspnea, pericarditis, culture-negative endocarditis), pleuropulmonary disease (pleural effusions) or mucocutaneous disease.

Traditionally, the disease has been described in two stages: a prodromal stage and a much later steady-state stage. The time between these stages varies; however, it typically averages 6 years^[6]. The prodromal stage is predominantly characterized by nonspecific symptoms including arthralgias and fatigue. More specific findings such as weight loss, diarrhea, and multi organ involvement occur in the steady state stage. Approximately 15% of patients with WD do not present with classic signs and symptoms of the disease; therefore, the diagnosis should be considered in a broad range of clinical scenarios^[7,8]. Furthermore, immunosuppressed patients can display a more rapid progression of the disease^[9,10].

The clinical manifestations of the disease are believed to be caused by infiltration of *T. whipplei* into various tissues. The patient's immune system reacts by incorporating the bacteria into tissue macrophages. Serum studies typically have presented nonspecific findings, therefore biopsy of the appropriate tissue is essential for diagnosis. Tissue samples from the small bowel show expanded villi containing PAS staining macrophages. After the discovery of PAS staining in 1949, the detection of bacteria in macrophages in 1961 further contributed to our understanding of WD^[4,11,12]. However, the only specific diagnostic tests for WD include determining the presence of *T. whipplei* DNA through molecular amplification of the 16S rRNA of *T. whipplei* by polymerase-chain-reaction (PCR) and cell culture of the organism^[13-15]. Subsequently, this finding led to electron microscopy, which in turn led to DNA testing for *T. whipplei*. Most recently, in 2003 the full sequencing of two genomes from two different strains of *T. whipplei* has been reported^[6,16,17].

In conjunction with clinical signs and symptoms, endoscopy is an important diagnostic modality in WD. Endoscopic examination of the postbulbar region of the duodenum and jejunum includes findings consisting of pale yellow and shaggy mucosa alternating with eroded, erythematous, or mildly friable mucosa^[18]. Several biopsy samples should be studied because the lesions can be sparse and focal^[6].

In the era before routine access to antibiotics, WD was a universally fatal disease. The discovery of antibiotics has resulted in antibiotics as the mainstay of therapy for WD. Treatment regimens are tailored for severity of disease, however, lack of well-controlled trials limits recommendations to reports of experience. The first reported efficacy of antibiotic treatment with chloramphenicol occurred in 1952^[19]. With the progressive discovery of safer antibiotics, tetracycline at one time was the mainstay of therapy for many years. However, a comprehensive review by Keinath *et al*^[20] subsequently revealed an overall relapse rate of 35% with

a high CNS relapse rate among patients treated primarily with tetracycline^[21,22]. As a result, the current standard of therapy includes an initial phase of intravenous antibiotics known to penetrate the blood brain barrier followed by 12 mo of oral maintenance treatment. A typical course often includes ceftriaxone or penicillin for two weeks followed by trimethoprim-sulfamethoxazole double strength twice per day for one year. Depending on allergies to medications, antibiotics can be substituted as needed.

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CASE REPORT

Successful isolation of *Helicobacter pylori* after prolonged incubation from a patient with failed eradication therapy

Yan Yin, Li-Hua He, Jian-Zhong Zhang

Yan Yin, Li-Hua He, Jian-Zhong Zhang, Department of Diagnosis, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

Author contributions: Yin Y, Zhang JZ and He LH designed the research; Yin Y and He LH performed the research; Yin Y, He LH and Zhang JZ analyzed the data; Yin Y and Zhang JZ wrote the paper.

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Correspondence to: Jian-Zhong Zhang, Professor, Department of Diagnosis, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping, Beijing 102206, China. zhangjianzhong@icdc.cn

Telephone: +86-10-61739319 Fax: +86-10-61730233

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Abstract

Helicobacter pylori (*H pylori*), a gastric pathogen, is a major cause of chronic gastritis and peptic ulcer disease, and is an important risk factor for the development of gastric malignancies. Culture of the bacterium from gastric biopsy is essential for the determination of drug resistance of *H pylori*. However, the isolation rates of *H pylori* from infected individuals vary from 23.5% to 97% due to a number of factors such as biopsy preparation, cultural environment, medium and the method adopted. In the present case, we found that a prolonged incubation period of up to 19 d allowed successful isolation of *H pylori* from a patient who received triple therapy that failed to eradicate the bacterium.

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Key words: *Helicobacter pylori*; Isolation; Eradication

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a gastric pathogen, which is present in approximately half of the world's population. It is a major cause of chronic gastritis and peptic ulcer disease, and is an important risk factor for the development of gastric malignancies^[1-4]. Although accurate non-invasive methods such as the urea breath test, the stool antigen test, and serology are available, biopsy-based invasive techniques, including the rapid urease test, histology and culture, are required to confirm the infection. Moreover, culture of the bacterium from gastric biopsy is essential for the determination of drug resistance of *H pylori* and thus for the subsequent treatment strategy after failed eradication therapy. However, the isolation rates of *H pylori* from infected individuals vary from 23.5% to 97%^[5,6] due to a number of factors, such as biopsy preparation, cultural environment, medium and the method adopted. The duration of incubation for isolation of *H pylori* has been recommended to be 2 to 7 d. Here we reported our observation that a prolonged incubation period of up to 19 d allowed successful isolation of *H pylori* from a patient who received triple therapy that failed to eradicate the bacterium.

CASE REPORT

A patient (female, 59 years old) with an *H pylori* positive duodenal ulcer received two consecutive trials of 7-d triple regimens in a regional hospital. The regimen consisted of Metronidazole, Clarithromycin, and Cimetidine. Four weeks after the second trial, the patient was still positive for a ¹³C urea breath test. She then came to Beijing for a solution. To obtain the drug resistance profile of the *H pylori* strain, the patient underwent an upper gastrointestinal endoscopy, and four biopsy specimens were taken from gastric antrum. The biopsies were placed directly into transport medium at room temperature and processed for culture within 2 h. Biopsy samples were smeared on *H pylori* selective Dent Columbia agar plates (Oxoid Ltd., London, England) supplemented with 8% sheep blood (Hengzhaoxiang Science & Technology Co., Beijing), and incubated in a microaerophilic environment (5% O₂, 10% CO₂, and 85% N₂) at 37°C. The plates were scheduled to be checked on days 3, 6, 8, and 10. However, there was no colony growing after 10 d. We decided to incubate the plates further, and check the plates every 3 d. On day 19, one suspected *H pylori*-like colony appeared

on one of the plates. This colony was subcultured in non-selective 2 Columbia agar plates containing 8% sheep blood in the same environment mentioned above, for three days. The isolate was confirmed to be *H pylori* based on its typical colony morphology, negative Gram's stain, and positive urease, catalase, and oxidase tests.

The antimicrobial susceptibilities of the isolate to metronidazole, clarithromycin, amoxicillin, ampicillin, levofloxacin, and rifampicin were determined by the E-test (AB Biodisk, Solna, Sweden). Briefly, a bacterial suspension (2 McFarland standard) was prepared with brain heart infusion (Oxoid Ltd., London, England) containing 10% heat-treated serum^[7]. After the bacterial suspension was swabbed onto the entire Columbia plates, sterile E-test strips impregnated with the above antibiotics were placed on the agar surface of corresponding plates. Minimal inhibitory concentration (MICs) were determined according to the manufacturer's instructions after three to four days of incubation. The isolate exhibited high-level resistance to metronidazole (MIC > 256 µg/mL) and clarithromycin (MIC > 48 µg/mL), but was susceptible to amoxicillin, ampicillin, levofloxacin, and rifampicin (all MIC < 0.016 µg/mL), which explained the failure of the triple regimens containing metronidazole and clarithromycin.

DISCUSSION

Culture has been considered the "gold standard" in confirmation of the diagnosis of *H pylori* infection. Moreover, the isolation and identification of strains is important for the investigation of profiles of bacterial virulence and, particularly, drug resistance. Due to the gradually rising prevalence of *H pylori* resistance to many antibiotics commonly used in triple regimens, the determination of antibiotic susceptibility of individual isolates is of particular importance. However, primary isolation of *H pylori* from gastric biopsies is rather demanding, and is affected widely by the culture conditions in addition to the biopsy-related factors^[8]. Our report also indicates that a prolonged incubation is necessary for some strains, especially those enduring hostile environment or a period of antibiotic force. It was reported that a longer incubation of 11 d is helpful for isolating *H pylori* strains from long-term-frozen specimens^[9], but this is the first report of the bacteria recovered after 19 d incubation. Indeed, the isolate requiring 19 d recovery later exhibited normal growth characteristics of *H pylori* strains when compared to another strain, NCTC11637, indicating its unusually long incubation requirement was a temporary predicament.

It has been demonstrated *in vitro* that *H pylori* cells can transform from a cultivatable spiral-shaped form to a non-cultivable coccoid form, in which the recovery of the bacterium is very difficult by routine culture methods^[10]. We would propose that during the period of eradication therapy, some organisms transform into the so-called "uncultivable form" with the propagation being stopped under the antimicrobial pressure in the local environment.

However, these organisms, which may have been selectively resistant to the used antimicrobials, survive, possibly with some suppressed metabolic activities^[11]. Once released from the medication at the end of the trial, these organisms gradually restore their normal growing features after prolonged incubation in an optimal environment and eventually become cultivatable. Therefore, these "uncultivable form" organisms might contribute, at least partially, to treatment failures and the development of antimicrobial resistance. In the meantime, we suggest that a new *H pylori* culture after a first attempt to eradicate *H pylori* needs to be postponed, probably by four weeks or even longer. It is noticeable that the patient was positive for the ¹³C urea breath test four weeks after completion of treatment, indicating that there are a number of organisms that are able to produce urease activities after release from antimicrobial pressure after four weeks. The coccoid *H pylori* can produce urease, though at a decreased level^[12], suggesting its potential pathogenicity.

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DNA-guided hepatitis B treatment: Viral load is insufficient with few exceptions

Pankaj Jain

Pankaj Jain, Consultant Gastroenterology, Apollo Modi Hospital, Swami vivekanand nagar, Kota-324010, Rajasthan, India

Author contributions: Jain P reviewed, analyzed and wrote the paper.

Correspondence to: Pankaj Jain, MD, DM, Consultant Gastroenterology, Apollo Modi Hospital, Swami vivekanand nagar, Kota-324010, Rajasthan, India. panj2007@rediffmail.com
Telephone: +91-744-2473501 Fax: +91-744-2473500

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Abstract

In DNA-guided hepatitis B treatment, viral load is insufficient, and requires other viral markers for treatment of hepatitis B patients as in patients with acute exacerbation of chronic hepatitis B, end-stage renal disease on dialysis, human immunodeficiency virus co-infected patients. There are exceptions to this rule: a residual level hepatitis B virus (HBV) DNA at 24 wk predicts beneficial outcome and reduced resistance at 1 year. The genotypic viral resistance to antiviral agents and occult HBV infection can be determined by HBV-DNA levels.

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Key words: DNA; Hepatitis B; Viral load

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TO THE EDITOR

We read with interest the article "DNA-guided hepatitis B treatment: viral load is essential, but not sufficient" by Barcena Marugan *et al*^[1]. We agree that viral load is

essential but requires other viral marker for treatment of hepatitis B patient.

Patients with exacerbation of chronic hepatitis B, requiring treatment, can be differentiated from acute hepatitis B based on hepatitis B virus (HBV) DNA^[2], but the sensitivity and specificity increase with addition of IgM anti-HBc. Low or undetectable DNA levels were seen in acute hepatitis^[3], whereas HBV DNA levels became detectable during reactivation of chronic hepatitis^[4]. Kumar *et al*^[5] in their study showed a low level of HBV DNA (< 0.5 pg/mL) in about 96% of patients with acute infection, as opposed to 13% in those with exacerbation of chronic hepatitis. The sensitivity and specificity of low levels of HBV DNA for identifying an acute infection are 96% and 86.6%, respectively, which increase to 100% and 97.9% respectively with high titers of IgM anti-HBc.

Tong *et al*^[6] applied the four criteria (European Association for the Study of the Liver, a treatment algorithm by an independent panel of hepatologists in the United States, an Asian-Pacific consensus statement and the practice guidelines from the American Association for the study of liver disease) to treat 369 HBsAg-positive patients with antiviral therapy. Using these criteria for antiviral therapy as stated by the guidelines, only 20%-60% of hepatocellular carcinoma (HCC) patients and 27%-70% of patients who died of non-HCC were identified for antiviral therapy. If the criteria were broadened with baseline serum albumin 3.5 gm/dL or less or platelet counts of 130 000 mm³ or less, 89%-100% of deaths from non-HCC liver-related complications and 96%-100% HCC patients would be identified for antiviral therapy.

In patients with end-stage renal disease on dialysis with HBV infection, it remains very difficult to predict the severity and outcome of liver disease based on the HBV-DNA level *per se*^[7]. Liver biopsy appears to be the only definitive and reliable means to establish the activity of liver disease in patients on dialysis. It is recommended before starting antiviral therapy and undergoing kidney transplantation. Weisberg *et al*^[8] have shown that the estimated 5-year survival rates in patients with end stage renal disease, chronic persistent hepatitis, chronic active hepatitis and chronic active hepatitis with cirrhosis due to hepatitis B are 97%, 86% and 55%, respectively.

In human immunodeficiency virus (HIV) infected patients with HBV, there is an increased risk of cirrhosis,

end-stage liver disease and death from liver disease, especially in patients with a low CD4 cell count or concomitant alcohol use^[9]. The treatment of HBV patients co-infected with HIV depends on HBV-DNA levels, histological evidence of active and /or advanced disease (Metavir > A2 and/or \geq F2) and CD4 counts whether < or \geq 500/mm³. So, HBV-DNA levels cannot be used alone in co-infected patients with HIV. A CD4 count < 500/mm³ requires HAART regimen including tenofovir and lamivudine or emtricitabine. A CD4 count \geq 500 mm³ can be treated with entecavir, interferon or adefovir^[10].

HBV-DNA load is essential but not sufficient and has few exceptions. Keeffe *et al*^[11] showed that complete virologic response (no detectable residual HBV DNA) at 24 wk in patients on anti-viral drugs, and the likelihood of HBeAg sero-conversion and maintenance of an undetectable level of HBV DNA are high, and resistance unlikely occurs. So the residual level HBV DNA at 24 wk can be used as a predictor of beneficial outcome and reduced resistance at 1 year.

The genotypic viral resistance to antiviral agents can be determined by \geq 1 log₁₀ IU/mL increase in serum HBV DNA. Virological breakthrough or secondary antiviral treatment failure is usually defined as reappearance or \geq 1 log₁₀ IU/mL increase after initial lack of detection or initial \geq 1 log₁₀ IU/mL reduction of serum HBV DNA^[12]. Virological breakthrough is usually followed by biochemical response^[13]. So, a change of serum HBV DNA can be an earliest predictor of viral resistance to antiviral agents. All patients commencing antiviral therapy should have quantitative HBV DNA measurements at baseline and three months after starting therapy^[14]. It helps identify response and primary treatment failure in patients on lamivudine.

Occult HBV infection is defined as the detection of HBV-DNA in the serum or liver tissue of patients with negative hepatitis surface antigen^[15]. Occult HBV infection has low HBV DNA levels less than 10000 in the serum and 0.01-0.1 copy per liver cell^[16]. The likelihood of antiviral therapy benefit is low as most patients with occult HBV infection have very low levels of HBV DNA. Serum HBV DNA levels fluctuate in cryptic HBV carriers, repeating the HBV test over time is a useful tool in identifying the occult HBV status^[17].

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Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Nikolaus Gassler, Professor

Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Anna S Gukovskaya, Professor

VA Greater Los Angeles Health Care System, University of California, Los Angeles, 11301 Wilshire Blvd, Los Angeles 91301, United States

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Dipl-Biol, Medizinische Klinik & Poliklinik I, Uni-Klinik Bonn, Sigmund-Freud Str. 25, 53105 Bonn, Germany

Serhan Karvar, MD, Assistant Professor of Medicine

University of Southern California, Keck School of Medicine, Division of Gastrointestinal & Liver Diseases, 2011 Zonal Avenue, HMR 101, Los Angeles, CA 90089, United States

Leonidas G Koniaris, Professor

Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

John M Luk, Associate Professor

Department of Surgery, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong, China

Henry Thomson Lynch, MD

Department of Preventive Medicine, Creighton University School of Medicine, 2500 California Plaza, Omaha NE 68178, United States

Mercedes Susan Mandell, MD, PhD

Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

Roberto Mazzanti, MD, Professor, Chair of Medical Oncology

Department of Internal Medicine, University of Florence, viale Morgagni, 85-50134 Florence, Italy

Stephan Menne, Assistant Professor of Virology

Department of Clinical Sciences/GI Unit, College of Veterinary Medicine, Cornell University, C2-005 Veterinary Medical Center, Ithaca, NY 14853, United States

Satoshi Osawa, MD

First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, 431-3192, Japan

Kostas Pantopoulos, Associate Professor

Department of Medicine, McGill University, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada

Silvia Pastorekova, PhD, DSc., Associate Professor

Centre for Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dubravská cesta 9, Bratislava 845 05, Slovakia

Alberto Piperno, Professor

Department of Clinical Medicine and Prevention, Clinical Medicine, San Gerardo Hospital, Via Pergolesi 33, 20052, Monza, Italy

Raymund R Razonable, MD

Division of Infectious Diseases, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, United States

Qin Su, Professor

Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

Eddie Wisse, Professor

Irisweg 16, Keerbergen 3140, Belgium

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.apdwcgress.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

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- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

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Statistical data

Write as mean \pm SD or mean \pm 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 ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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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 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; 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 23243641.

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

^[3]Passed away on June 14, 2008



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EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No.62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
 Telephone: +86-10-59080039
 Fax: +86-10-85381893
 E-mail: wjg@wjgnet.com
 http://www.wjgnet.com

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Hepatitis C virus and type 2 diabetes

Francesco Negro, Mahnaz Alaei

Francesco Negro, Mahnaz Alaei, Division of Gastroenterology, Hepatology and Clinical Pathology, University Hospitals, 24 rue Micheli-du-Crest, 1211 Geneva 14, Switzerland

Francesco Negro, Division of Clinical Pathology, University Hospitals, 24 rue Micheli-du-Crest, 1211 Geneva 14, Switzerland

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Correspondence to: Francesco Negro, MD, Associate Professor, Division of Gastroenterology, Hepatology and Clinical Pathology, University Hospitals, 24 rue Micheli-du-Crest, 1211 Geneva 14,

Switzerland. francesco.negro@hcuge.ch

Telephone: +41-22-3795800 Fax: +41-22-3729366

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Abstract

This review focuses on the relationship between hepatitis C virus (HCV) infection and glucose metabolism derangements. Cross-sectional and longitudinal studies have shown that the chronic HCV infection is associated with an increased risk of developing insulin resistance (IR) and type 2 diabetes (T2D). The direct effect of HCV on the insulin signaling has been analyzed in experimental models. Although currently available data should be considered as preliminary, HCV seems to affect glucose metabolism *via* mechanisms that involve cellular pathways that have been implicated in the host innate immune response. IR and T2D not only accelerate the histological and clinical progression of chronic hepatitis C, but also reduce the early and sustained virological response to interferon-alpha-based therapy. Thus, a detailed knowledge of the mechanisms underlying the HCV-associated glucose metabolism derangements is warranted, in order to improve the clinical management of chronic hepatitis C patients.

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Key words: Hepatitis C; Fibrosis; Insulin resistance; Insulin signaling; Type 2 diabetes

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INTRODUCTION

Hepatitis C virus (HCV) infection is a frequent cause of acute and chronic hepatitis, and leads to the development of cirrhosis and hepatocellular carcinoma. It is estimated that about 150 to 200 million people have been in contact with HCV worldwide, and approximately 85% are chronically infected. The spectrum of severity of liver disease associated with HCV varies widely, as does the rate of progression towards the cirrhotic stage. The latter seems to depend on several, mostly host-related cofactors, such as age, sex, level of alcohol consumption, overweight, immune status and co-infections^[1,2]. One of these cofactors is type 2 diabetes (T2D), which has been recognized to modify the course of hepatitis C even at the stage of insulin resistance (IR), a condition that precedes the development of T2D^[3,4]. Although individuals may develop IR independently of HCV, a considerable amount of clinical and experimental data suggest that HCV contributes to its pathogenesis. This aspect is important, because IR seems not only to accelerate the course of chronic hepatitis C, but also to influence the response to antiviral therapy^[5]. The scope of this review is to discuss the current level of evidence in favor of a causal association between HCV and T2D/IR, its clinical impact, and some directions for management.

ASSOCIATION BETWEEN HCV AND T2D

T2D is a common complication of all liver diseases, independently of the etiology, especially at the advanced stage. However, clinical and experimental data suggest a direct role of HCV in the perturbation of glucose metabolism. The first observation that cirrhotic patients infected with HCV may present with T2D more often than patients with cirrhosis of other

etiology was reported in 1994 by Allison *et al*^[6]. Most studies using a cross-sectional design and comparing the prevalence of T2D in a population of chronic hepatitis C patients with that of a comparator group have confirmed these preliminary observations^[7-14], with rare exceptions^[15]. Comparator groups have included patients with chronic liver disease^[7-9,12-14], drug users^[10] or human immunodeficiency virus (HIV) mono-infected patients^[11]. It can be argued that, in these studies, the two populations of patients-i.e. HCV-infected and uninfected-may have differed by relevant risk factors for T2D, notably age, gender distribution and stage of liver disease. It is noteworthy that, in the HIV-co-infected cohorts studied by Visnegarwala *et al*^[11], the association between HCV and T2D was significant, as assessed by multivariate analysis, only among subjects < 50 years old. Similarly, in the study by Lecube *et al*^[12], glucose abnormalities, including impaired fasting glucose (IFG), were significantly more prevalent (i.e. about three-fold) among HCV-positive patients attending a liver unit, compared to HCV-negative patients, only when they were at a pre-cirrhotic stage. These observations suggest that HCV interferes with glucose metabolism independently of age and stage of liver disease. At a later, cirrhotic stage, however, multiple factors contributing to IR may prevail and partially or completely mask the HCV-related effect. Further evidence has come *via* case-control studies in which all cases were represented by HCV-infected individuals^[16-24], although in most cases, the prevalence of T2D among HCV-infected individuals compared to matched controls was, in general, lower than that seen in previous studies.

Several investigators have approached this issue from a different point of view, i.e. measuring the prevalence of HCV markers among populations of diabetic patients^[8,15,25-33]. Most controlled studies have suggested a significant association, the proportion of HCV-positive persons among diabetics being two- to seven-fold compared to controls^[8,15,29,32]. The prevalence of HCV markers among patients with T2D reported by uncontrolled studies was also claimed to be higher than that observed in the general population taken as a reference^[25,27,30]. However, the study by Sotiropoulos *et al*^[26] reported a rather low HCV prevalence (1.65%), especially if one considers that a field survey in the Greek general population gave a HCV seroprevalence of 1.25%^[34]. Other controlled studies from Italy^[28], Nigeria^[31] and Turkey^[33] have failed to find an excess prevalence of HCV infection among patients with T2D. The data have therefore proven inconclusive. It has been suggested that patients with T2D are at risk of blood-borne infections *via* repeated use of finger stick devices. However, a single study from France, evaluating the prevalence of HCV antibodies in 259 patients with T2D seen during 1998 at a diabetic unit, has failed to confirm this hypothesis^[35]. One cannot exclude that iatrogenic transmission of HCV among diabetic patients may however have been significant in previous decades.

The potential ascertainment bias that may occur in

clinic-based studies that target a specific disease group has been overcome in a vast (and hitherto unsurpassed) study conducted in the general population, the Third National Health and Nutrition Examination Survey (NANHES-III)^[36]. This study, which included 9841 subjects aged ≥ 20 years, showed that persons who were anti-HCV-positive and aged ≥ 40 years had an odds ratio of 3.77 (95% CI: 1.80-7.87), after adjusting for sex, body mass index (BMI) and ethnicity, of having T2D compared to anti-HCV-negative individuals.

Thus, clinic-based studies and the general population-based NANHES-III study came to similar conclusions, which reinforce the hypothesis of a causal association between HCV infection and T2D. As a result of the cross-sectional nature of all these surveys, however, as hinted before, a temporal relationship between HCV infection and T2D cannot be established. This issue, i.e. did the HCV infection come before the occurrence of T2D or *vice versa*, has been addressed by longitudinal studies. A prospective, case-cohort study, performed in the United States, analyzed whether persons who developed T2D were more likely to have had precedent HCV infection when enrolled in a community-based cohort of 1084 persons aged 44-65 years (the Atherosclerosis Risk in Communities Study)^[37]. The prevalence of HCV in this population was 0.8%. A total of 548 subjects developed *de novo* T2D over 9 years of follow-up. Prior to entry, subjects had been categorized as low-risk or high-risk for T2D based on age and BMI. Among those at high risk for T2D, persons with HCV infection were more than 11 times as likely as those without HCV infection to develop T2D (relative hazard, 11.58; 95% CI: 1.39-96.6). Among those at low risk, the incidence of T2D was not increased among HCV-infected subjects. The conclusion of this important survey was that pre-existing HCV infection may increase the incidence of T2D in persons with known risk factors. The second study^[38], a community-based cohort survey performed in southern Taiwan, enrolled 4958 persons aged ≥ 40 years, without T2D at entry. This study included 3486 seronegative persons, 812 anti-HCV-positive patients, 544 individuals with the hepatitis B surface antigen (HBsAg) and 116 with hepatitis B virus (HBV)/HCV co-infection. Over a follow-up of 7 years, 474 cases of incident T2D were recorded: overall, 14.3% of anti-HCV-positive, 7.5% of HBsAg-positive, and 8.6% of seronegative individuals developed T2D during the study. Compared to anti-HCV-negative individuals, anti-HCV-positive persons had a higher cumulative incidence of T2D ($P < 0.0001$). By multivariate analysis, the fact of being anti-HCV-positive, co-infection with HBV and HCV, overweight, obesity, and increasing age were all significantly associated with T2D, while sex and alcohol consumption, among other factors, were not. Interestingly, when patients were stratified by age and BMI, the risk of developing T2D among anti-HCV-positive individuals increased when age decreased and BMI levels increased. This study concluded that HCV infection is an independent predictor of T2D. The risk

was higher in patients with elevated BMI, but, at variance with the previous study, seemed to decrease with age.

Thus, cross-sectional and longitudinal studies both seem to converge towards the same conclusion, i.e. there exists an excess T2D risk in HCV-infected persons compared to controls infected with HBV, which suggests a direct role of HCV in inducing derangement of glucose metabolism. A recent, large meta-analysis, the first of this kind, has reached the same conclusion^[39].

An additional, strong case in favor of an association between HCV and T2D comes from longitudinal studies performed in patients having received a liver or kidney transplant. T2D is a common complication of liver transplantation (LT). Apart from isolated negative reports^[40], there is accumulating evidence that HCV is a strong predictor of new-onset T2D after LT^[41]. A first study from Toronto, Canada^[42], analyzed the prevalence of T2D among 278 LT recipients, whose indication for transplantation was liver failure caused by HCV (110 patients), HBV (53 patients) or cholestatic liver disease (115 patients). Multivariate analysis revealed that HCV-related cirrhosis ($P = 0.002$), pre-LT T2D ($P < 0.0001$) and male gender ($P = 0.019$) were independent predictors of the presence of T2D 1 year after LT. The high prevalence of T2D persisted among HCV-positive persons, with 41% being diabetic at 5 years. This observation was subsequently confirmed by other studies. In a series from Harvard^[42], which compared 47 HCV-positive to 111 HCV-negative cases, HCV infection was an independent risk factor for the development of T2D after LT (hazard ratio 2.5, $P = 0.001$). These data were repeatedly confirmed by later studies^[43-49], with one exception from the [University of California, Los Angeles (UCLA)] series, in which the lack of association may have been a consequence of the excess representation of HCV-positive patients^[50]. Several predisposing factors were identified across the studies: impaired fasting glucose and a maximum lifetime BMI over 25 kg/m²^[49], age and male gender^[48], serum HCV RNA level after LT^[51], and use of tacrolimus^[45] or steroid boluses^[43]. On the other hand, use of cyclosporine^[49] and rapid discontinuation of steroids^[52] seem to reduce the incidence of T2D among HCV-positive persons.

A similarly increased risk of T2D has been reported after kidney transplantation (KT). After two early reports, underlining a rather strong association between ongoing HCV infection and post-KT T2D^[53,54], a major retrospective analysis on 427 kidney recipients without T2D before KT^[55] showed that, by multivariate logistic regression, HCV (adjusted OR 5.58; 95% CI: 2.63-11.83; $P = 0.0001$), weight at transplantation (adjusted OR 1.028; 95% CI: 1.00-1.05; $P = 0.001$), and tacrolimus (adjusted OR 2.85; 95% CI: 1.01-5.28; $P = 0.047$) were associated with newly onset T2D after KT. In this study, a significant interaction ($P = 0.0001$) was found between presence of HCV and use of tacrolimus, since in the HCV-positive group, T2D occurred more often in tacrolimus-treated than cyclosporine A-treated patients (57.8% *vs* 7.7%; $P < 0.0001$)^[55]. Most subsequent studies

confirmed this robust association^[56-63], with some exceptions^[21,64-66]. Thus, in a recent meta-analysis of 10 studies, the pooled relative risk for post-KT T2D was 2.73 (95% CI: 1.94-3.83)^[67]. When only two large studies were considered, the pooled relative risk was still 1.36 (95% CI: 1.21-1.54). The existing publication bias did not change the results in a meaningful way, after a sensitivity analysis was performed^[67]. In addition to ongoing HCV infection, risk factors for developing T2D after KT are family history of T2D^[55,60], age^[57,59,61,62], use of tacrolimus^[55,59,60,62,63], smoking^[61], overweight/obesity^[62,63], African-American ethnicity^[62] and pre-transplantation impaired fasting glucose^[63]. Thus, there exists a significant increase of the risk of post-KT T2D in HCV-positive recipients, especially in the first 2 mo after transplantation^[57]. Since T2D and its complications are a leading cause of mortality after KT, it is easy to understand that every effort should be made to clear HCV with antiviral therapy in the pre-KT period, whenever this is feasible.

Thus, HCV and T2D are associated more than just by chance, suggesting that HCV may alter glucose homeostasis by its direct action, or *via* indirect mechanisms such as through cytokine stimulation (see below). The association between HCV infection and glucose abnormalities holds true if, instead of looking at the occurrence of overt T2D, one considers pre-diabetes conditions, such as impaired glucose tolerance (IGT) or IR. The latter is defined as a condition in which higher than normal insulin concentration are needed to achieve normal metabolic responses or, alternatively, normal insulin concentration are unable to achieve normal metabolic responses^[68]. It has to be stated clearly, however, that it is not clear whether IR associated with HCV infection invariably evolves towards T2D in all infected persons, especially those without other risk factors of T2D. There is a clear need of longitudinal studies that may clarify this issue.

In a classical paper, Hui and collaborators^[4] compared fasting levels of serum insulin, C-peptide and IR [measured as homeostasis assessment (HOMA) score] in 121 HCV patients with stage 0 or 1 liver fibrosis and 137 healthy volunteers matched by sex, BMI, and waist-to-hip ratio. Results showed that such HCV-infected persons, notwithstanding their early stage of liver disease, had higher levels of insulin, C peptide, and HOMA scores compared with controls. Besides, this study was the first to suggest that genotype 3 may have significantly lower HOMA scores than other genotypes (which were comparable when adjusted for the remaining independent predictors of IR). Thus, this work showed how HCV may induce IR irrespective of the stage of advancement of the underlying liver disease, an effect that seemed to be genotype specific. In a similar, more recent paper, Moucari *et al.*^[69] analyzed 600 consecutive patients (500 with chronic hepatitis C and 100 controls with chronic hepatitis B). IR was less frequent in chronic hepatitis B than in matched chronic hepatitis C cases (5% *vs* 35%, respectively, $P < 0.001$), again irrespective of the stage of liver disease (patients were divided

according to the presence or absence of liver cirrhosis). Furthermore, IR was associated with genotypes 1 and 4 and high serum HCV RNA levels, even suggesting a trend, among patients without features of the metabolic syndrome, between HCV replication level and HOMA score. These data further corroborated the hypothesis that HCV may have a direct involvement in glucose metabolism derangement. A correlation between HCV RNA levels and HOMA score has been reported also by other studies^[70-72], especially in genotype 1^[71] or after adjustment for age, gender and visceral adipose tissue area^[72]. These results are not, however, confirmed by all investigators. In a recent paper, Anty *et al*^[73] reported that lean patients with non-3 genotypes had higher glycemia and lower adiponectin levels than controls, at closer look it was evident that, considering only the 52 patients with F0/F1, then the HOMA scores were comparable to those of 22 controls (1.7 ± 1.6 vs 1.4 ± 1.5 , $P = \text{NS}$). Negative results have also been reported from Japan, where two studies failed to identify HCV infection as independent predictor of IR^[14,74]. Thus, further work is warranted in this field, and, more importantly, a thorough analysis, at the population level, of HCV sequences that may be directly involved in stimulating IR. Furthermore, it is impossible to determine whether HCV replication is responsible for increased IR or whether HCV replication is favored by hyperinsulinemia, as suggested by some *in vitro* data^[75], and/or by the increased serum levels of free fatty acids^[76] typically observed in IR and T2D^[77]. Finally, the poor correlation between HCV RNA levels and HOMA score may also be caused by the fact that the overall level of IR also depends on the contribution from the adipose tissue and muscle, two extrahepatic compartments not infected by HCV.

Finally, if HCV is increasing the level of IR or predisposes to the development of glucose metabolism disturbances, including T2D, in high-risk individuals, then curing HCV should result in amelioration of the HOMA score and in a decreased incidence of T2D after the end of therapy. Kawaguchi *et al*^[78], in their study on 89 patients, showed that eradication of HCV improved the HOMA score and the intrahepatic expression of the insulin receptor substrate (IRS) 1 and 2, two cellular transducers of the insulin signal (see below). Similar results have been reported in a cohort of 181 genotype 4 patients from Egypt^[79]. Regarding the incidence of glucose metabolism derangements after sustained virological response (SVR), Romero-Gómez *et al*^[80] assessed the effect of SVR and other host and viral factors on the incidence of impaired fasting glucose and T2D in 1059 patients with chronic hepatitis C treated with pegylated interferon (IFN)- α 2a and ribavirin. Their data show that SVR reduces by half the incidence of T2D and/or IFG during a post-therapy follow-up of 27 ± 17 mo (range, 9.3-67 mo). Similar data have been reported in 234 patients followed in Barcelona for at least 3 years after the end of therapy^[81]. However, in a cohort of 202 patients with a significantly longer follow-

up (8.0 years, range 5-16)^[82], the benefit of SVR (if any) was not observed, even after adjustment for several baseline risk factors of T2D.

In conclusion, HCV seems to increase the risk of incident T2D in predisposed individuals. As a result, the association between HCV and T2D is more evident among patients who are older and have higher BMI. When measuring IR before T2D has occurred, some HCV-infected patients are clearly less insulin sensitive than controls, matched for risk factors of T2D and stage of liver disease. This effect is probably associated with specific HCV sequences and/or subtypes, and shows some dose-dependence, i.e. may be correlated with HCV replication level. Curing HCV seems to have beneficial effects on the level of insulin sensitivity, although this may not be the rule. In the next chapter we will analyze the potential mechanisms of interference with the insulin signaling brought about by HCV.

MECHANISMS OF HCV INTERFERENCE WITH INSULIN SIGNALING

Experimental data are compatible with direct interference of HCV with the insulin signaling cascade. This was first suggested by a study in which liver specimens obtained from 42 non-obese, non-diabetic, HCV-infected individuals and 10 non-HCV-infected subjects matched for age and BMI were exposed *ex vivo* to insulin, and examined for the contents and phosphorylation/activation status of some insulin signaling molecules^[83]. Insulin-stimulated IRS-1 tyrosine phosphorylation was decreased by two-fold in HCV-infected patients compared to non-HCV-infected ones, and this was paralleled by significant reductions in IRS-1/p85 phosphatidylinositol 3 (PI3)-kinase association, IRS-1-associated PI3-kinase enzymatic activity and insulin-stimulated Akt phosphorylation^[83]. It was concluded that, in patients with chronic hepatitis C, direct interactions between HCV and insulin signaling components occur that may result in IR, which in turn, may progress to T2D in at-risk individuals. In the transgenic mouse model^[84], the core-encoding region of HCV is sufficient to induce IR. This effect was reversed by treatment with anti-tumor necrosis factor (TNF)-antibodies, which suggested an increased level of serine phosphorylation of IRS-1 as induced by TNF- α . Thus, the core protein may induce IR indirectly *via* stimulation of the secretion of TNF- α . However, *in vitro* models suggest otherwise, hinting at a direct interaction of the core protein with the insulin signaling pathway. An increased proteasomal degradation of the IRS-1 and -2 *via* the activation of the suppressor of cytokine signaling (SOCS)-3 has been reported after transient expression of the core protein^[85]. Direct but genotype-specific mechanisms have been advocated in another study^[86], in which down-regulation of peroxisome proliferator-activated receptor- γ (PPAR- γ) and up-regulation of SOCS-7 was observed in cells transfected with the core protein of genotype 3,

whereas the core protein of genotype 1b activated the mammalian target of rapamycin, findings that were confirmed by using agonists for PPAR- γ (rosiglitazone) or short interfering RNAs for SOCS-7^[87]. Among the indirect mechanisms, an increased endoplasmic reticulum stress has also been described that may lead to IR^[87]. More recently, the role of c-Jun N-terminal kinase (JNK) has been emphasized^[88]. The HCV core protein-mediated Ser (312) phosphorylation of IRS-1 was inhibited by a JNK inhibitor in an *in vitro* infection assay using cell-culture-grown HCV^[88].

Studies on chronically infected patients have suggested that increased oxidative stress and intrahepatic inflammation may also play a role. Mitsuyoshi *et al*^[89] evaluated 203 chronic hepatitis C patients with HCV genotypes 1 or 2 infection. HOMA and serum levels of thioredoxin, a marker of oxidative stress, were significantly correlated with each other, even after adjustment for BMI. However, in the human model, the indirect role of inflammatory mediators, such as TNF- α , seems more likely, in keeping with the transgenic mouse model. In fact, in chronic hepatitis C patients, an increased intrahepatic TNF- α response, which results in IR and a higher risk of developing T2D, has been described^[90,91]. Further work is necessary in this field, and the availability of genotype-specific replicon assays may pave the way to more in-depth mechanistic analyses.

CLINICAL CONSEQUENCES OF IR/T2D IN CHRONIC HEPATITIS C

The clinical consequences of IR and T2D on chronic hepatitis C are dual: accelerated fibrogenesis and reduced response to IFN-based therapy. Since one of the most frequent consequences of IR/T2D on the liver is steatosis, many data can be inferred indirectly looking at past studies in which the impact of non-virus- and non-alcohol-induced fatty liver on fibrosis progression was evaluated^[2]. In fact, in these cases, the most likely cause of fatty liver was IR, and this, rather than steatosis, seems to predict the stage of fibrosis and its progression over time^[4]. More generally, accelerated liver fibrogenesis should be considered in the complex of the consequences of the metabolic syndrome on the liver. This view allows one to consider several pathogenetic mechanisms other than IR, such as oxidative stress, increased secretion of pro-inflammatory adipokines and cytokines, and the peculiar susceptibility to apoptosis that has been associated with steatosis. High serum glucose^[92], hyperinsulinemia^[93] and IR^[4,71,94-97] are all associated with increased fibrosis in chronic hepatitis C, and more rapid progression of hepatitis C in diabetics has been reported also after LT^[98] and KT^[99]. However, claiming that the sole pathogenetic mechanism that underlies accelerated fibrogenesis in patients with chronic hepatitis C and IR is the hyperglycemic/hyperinsulinemic state is an oversimplification. First, it is not unknown whether patients with virus-induced

IR alone, i.e. without the other components of the metabolic syndrome (especially in the absence of the visceral obesity and the inflammatory state associated with it), share the same risk of increased liver disease progression compared to patients with overt metabolic syndrome. Second, patients with central obesity have not only increased IR but also altered levels of a whole array of pro-inflammatory cytokines and adipokines, which may exert their unwanted effects on the liver and other extra-adipose tissues independently of the action of insulin. The relative contribution of these cytokines to liver fibrosis in chronic hepatitis C is starting to be unraveled, but it is far from being fully understood.

In non-alcoholic steatohepatitis, hyperglycemia/hyperinsulinemia may be directly stimulating hepatic stellate cells to produce connective tissue growth factor (CTGF), which leads to increased collagen fiber deposition^[100]. Increased intrahepatic levels of CTGF have been reported to occur in chronic hepatitis C^[101]. The reduction of IR consequent to body weight reduction and increased physical activity may lead to reduced fibrosis score over time and a diminished number of activate hepatic stellate cells^[102].

Several pro-inflammatory cytokines and adipokines may be involved in the pathogenesis of liver injury in chronic hepatitis C. However, their relative contribution is under debate. A large, careful study has evaluated the role of TNF- α , interleukin 6, leptin and adiponectin in the pathogenesis of HCV-associated liver injury^[103]. Only TNF-levels seemed to correlate with severity of portal and periportal inflammation, but none of the cytokines considered in this study were correlated with liver fibrosis. Several other studies have failed to pinpoint a clear correlation between the severity of fibrosis and serum levels of leptin^[94,104-106], with only one positive report^[107]. The role of adiponectin is quite controversial^[103]. In addition, recent data have suggested a potential involvement of resistin in the pathogenesis of liver fibrosis^[108]. However, these latter data await independent confirmation. Finally, increased liver cell apoptosis has been reported to be correlated with steatosis^[109]. Hepatocyte apoptosis can be measured by caspase activity in serum^[110]. In the presence of steatosis, apoptosis is correlated with activation of stellate cells and increased stage of fibrosis, in keeping with the hypothesis that a steatotic liver is more vulnerable to liver injury, and suggesting another mechanism of liver disease progression in patients with fatty liver and the metabolic syndrome^[109].

Increasing levels of IR are associated with reduced rates of initial virological response^[111-113] as well as SVR in chronic hepatitis C patients treated with a combination of pegylated IFN- α and ribavirin^[114-119]. This negative association has been reported not only in patients infected with the HCV genotype 1^[114,116,119], but also in those with the so-called “easy-to-treat” genotypes 2 and 3^[118]. Furthermore, the negative impact of IR on the early response to anti-HCV therapy has been

recently confirmed among HIV-infected patients^[120]. The molecular link between IR and lack of responsiveness to IFN- α seems to lie in the increased levels of SOCS-3 in the liver^[117,121]. Interestingly, SOCS-3, as stated above, is not only promoting the proteasomal degradation of IRS-1, which leads to impaired insulin signaling and IR^[85], but, together with other members of the SOCS family, is also a negative regulator in the transduction of the IFN- α signaling^[122]. Thus, it is not too unlikely that HCV may have developed, from the evolutionary standpoint, the ability to activate SOCS-3 or other members^[86] of the SOCS family as a mechanism to inhibit the IFN- α signaling, one of the main arms of the host innate immune response, simultaneously impairing the insulin signaling. This view seems to be supported by the recent finding that HCV may also activate the protein phosphatase 2A, again with the dual effect of interfering with the insulin^[87] and IFN- α ^[123] signaling pathways. Whether these mechanisms may be exploited pharmacologically, i.e. with drugs aimed at reducing IR while improving the responsiveness to IFN- α , remains to be fully explored (see below).

PERSPECTIVES FOR CLINICAL MANAGEMENT

The treatment of IR and T2D in chronic hepatitis C patients has two goals, as far as the underlying liver disease is concerned: to reduce fibrogenesis (hence liver disease progression) and to increase the response to IFN-based therapy. As pointed out above, it is not known whether IR invariably increases liver fibrosis, i.e. in the context of the metabolic syndrome or in cases of purely virus-induced IR, without the remaining constellation of cytokine changes that accompany the metabolic syndrome. This distinction is important also when antiviral therapy has to be undertaken, because here therapy should be aimed at correcting IR based on the underlying molecular mechanisms, which may differ according to the viral genotype and the presence or absence of metabolic syndrome. At present, however, the approach that is being followed is rather empirical.

A single study^[102] has analyzed the biochemical and histological consequences of a 3-mo program that comprises body weight reduction and increased physical activity. In 19 subjects with steatosis and chronic hepatitis C, the weight loss was paralleled by progressive reduction of serum alanine aminotransferase levels and of mean fasting insulin. In patients with paired liver biopsies, steatosis decreased, together with the fibrosis score and the number of activated stellate cells, despite the persistence of HCV. The authors concluded that weight reduction may provide an important adjunct management strategy for patients with chronic hepatitis C^[102]. Lifestyle changes are the single most important measure to reduce the incidence of T2D in those at risk^[124] and of the metabolic syndrome in patients with IGT^[125]. Moreover, the metabolic syndrome may even

regress following such intervention^[125], more often than among patients treated with metformin. Therefore, lifestyle changes (weight reduction and increased physical activity) should constitute the mainstay of the clinical management of patients with chronic hepatitis C and initial glucose metabolism derangements (IR and IGT), with the aim of reducing their progression to overt T2D and possibly, their impact on liver fibrogenesis.

Alternatively, insulin sensitizing agents have been tested with the specific aim of improving the rate of response to IFN- α -based therapy. As said above, IR reduces the rate of response to antivirals in chronic hepatitis C. Thus, it was suggested that IR should be corrected in patients with chronic hepatitis C not responding to IFN- α -based treatment, in order to improve response upon re-treatment. The modalities of this intervention, however, have not been established. In addition, the optimal HOMA score to be reached has not been identified. The preliminary data from four independent studies^[126-129] have not been encouraging. A first prospective, multicenter study aimed at investigating the efficacy and safety of the insulin sensitizer pioglitazone, 15 mg *qd*, added to pegylated IFN-2a, 180 g *qw*/ribavirin, 1000-1200 mg *qd* combination therapy in chronic hepatitis C patients who were previously non-responders to a pegylated IFN- α /ribavirin combination^[126]. All patients had a baseline HOMA > 2, because this was the threshold that discriminated responders from non-responders in previous studies^[114,118]. None of the first five patients enrolled into the trial had a sufficient virological response after 12 wk to warrant continuation of the trial, which was therefore prematurely terminated. Data from three additional trials have been presented at the 2008 meeting of the American Association for the Study of Liver Diseases. In an interim analysis of one of them, 30 mg *qd* pioglitazone was given for 4 wk as monotherapy, and then added for the first 4 wk of standard therapy of treatment-naïve, non-diabetic, chronic hepatitis C patients. The authors showed that the triple regimen that contained pioglitazone increased significantly the rate of virological response after 4 wk therapy, compared to pegylated IFN- α /ribavirin combination alone^[127]. However, long-term data are awaited before any conclusion can be drawn, and some caution is required. In fact, in another randomized, double-blind, placebo-controlled study, adding pioglitazone 30 mg *qd* simultaneously to standard care increased the early and end-of-treatment virological response, but failed to increase the SVR^[128]. Further data are needed before insulin sensitizers can be added to the panoply of drugs to treat hepatitis C.

Furthermore, the effects of PPAR agonists on serum lipids and their potential consequences on the HCV life cycle should be investigated in more detail. It is also unclear whether the treatment with the insulin sensitizer should be started at the same time as the antiviral retreatment or precede it, in order to start the pegylated IFN- α /ribavirin combination only when the HOMA

score has decreased to a level predictive of an increased SVR^[114,118]. It is not clear whether the best approach is to use a PPAR agonist (and at what dose) or a biguanide such as metformin, whose mechanism of action is specifically directed against the hepatic AMP-activated protein kinase^[130]. The final results of the TRIC-1 study^[129] show that adding metformin to pegylated IFN- α /ribavirin combination afforded a marginal, non-significant gain as to the SVR rate, despite an increased rapid virological response after 4 wk of triple therapy. Thus, further clinical trials aimed at reducing the IR in chronic hepatitis C *via* different pharmacological interventions are warranted.

CONCLUSION

HCV and IR/T2D are associated to an extent that cannot be merely explained by chance, which suggests that HCV interferes directly (through one or more of its proteins) and/or indirectly (by modulating the production of specific cytokines, like TNF- α) with glucose metabolism. Independently of the mechanism, IR and T2D have important effects on the hepatitis C progression and response to antivirals, which warrants specific and effective measures to correct such metabolic anomalies. Although lifestyle interventions are certainly indicated in patients with chronic hepatitis C and the metabolic syndrome, in order to reduce the cardiovascular morbidity and mortality, it remains to be fully explored whether these measures will also have an impact on the underlying liver disease. Insulin sensitizers are currently being evaluated in clinical trials, but available data do not warrant their use in all chronic hepatitis C patients with IR, with the specific aim of increasing response to antivirals, at least outside of clinical trials.

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Efficacy of tricyclic antidepressants in irritable bowel syndrome: A meta-analysis

Roja Rahimi, Shekoufeh Nikfar, Ali Rezaie, Mohammad Abdollahi

Roja Rahimi, Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran
Shekoufeh Nikfar, Food & Drug Affairs, and Food & Drug Laboratory Research Center, Ministry of Health & Medical Education, Tehran 1314715311, Iran

Ali Rezaie, Faculty of Medicine, University of Alberta, Edmonton T6G 2V2, Canada

Mohammad Abdollahi, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Author contributions: Rahimi R collected data and drafted the manuscript; Nikfar S conducted meta-analysis, reviewed data and the manuscript; Rezaie A completed searches and reviewed the manuscript; Abdollahi M supervised whole study and edited the manuscript.

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Correspondence to: Mohammad Abdollahi, Professor, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran. mohammad@tums.ac.ir

Telephone: +98-21-66959104 Fax: +98-21-66959104

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Abstract

We aimed to evaluate the efficacy of tricyclic antidepressants (TCAs) as a therapeutic option for irritable bowel syndrome (IBS) through meta-analysis of randomized controlled trials. For the years 1966 until September 2008, PubMed, Scopus, Web of Science, and Cochrane Central Register of Controlled Trials were searched for double-blind, placebo-controlled trials investigating the efficacy of TCAs in the management of IBS. Seven randomized, placebo-controlled clinical trials met our criteria and were included in the meta-analysis. TCAs used in the treatment arm of these trials included amitriptyline, imipramine, desipramine, doxepin and trimipramine. The pooled relative risk for clinical improvement with TCA therapy was 1.93 (95% CI: 1.44 to 2.6, $P < 0.0001$). Effect size of TCAs *versus* placebo for mean change in abdominal pain score among the two studies was -44.15 (95% CI: -53.27 to -35.04, $P < 0.0001$). It is concluded that low dose TCAs exhibit clinically and statistically significant control of IBS symptoms.

antidepressants; Irritable bowel syndrome; Efficacy; Clinical response; Abdominal pain

Peer reviewers: Ian D Wallace, MD, Shakespeare Specialist Group, 181 Shakespeare Rd, Milford, Auckland 1309, New Zealand; Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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EPIDEMIOLOGY AND PATHOGENESIS OF IRRITABLE BOWEL SYNDROME (IBS)

IBS is a prevalent functional gastrointestinal (GI) disorder characterized by chronic or recurrent abdominal pain or discomfort associated with altered bowel habits^[1]. Up to 20% of the North American population are affected by IBS^[2]. A community-based study carried out in Birmingham, UK estimated the prevalence of IBS to be 10.5%^[3]. IBS is commonly diagnosed between the ages of 15 and 45 years and affects women twice as often as men^[3,4]. IBS places a significant burden on health economy in terms of using more health care services even for non-gastrointestinal symptoms comparing to general population^[5].

Environmental factors (psychological disturbances and stress), genetic links, recent infection, bacterial overgrowth, food intolerance, altered bowel motility and/or secretion, visceral hypersensitivity, altered central nervous system sensory processing, disturbed autonomic nervous system regulation, and serotonin dysregulation are all proposed as possible etiological factors for IBS^[2-4,6,7].

MANAGEMENT OF IBS AND CURRENT PLACE OF TRICYCLIC ANTIDEPRESSANTS (TCAs)

In addition to non-pharmacological strategies such as diet and psychotherapy, various pharmacological agents are used for the management of IBS including bulking

agents, antidiarrheal agents, laxatives, antispasmodics, antidepressants, serotonergic agonists or antagonists, antibiotics and probiotics^[8-12]. The rationale of using antidepressants in IBS is that these agents may alter pain perception by a central modulation of visceral afferents, treat comorbid psychologic symptoms, and alter GI transit. Different classes of antidepressants likely act by different combinations of mechanisms^[1]. Two classes of antidepressants frequently used for the treatment of IBS are selective serotonin reuptake inhibitors (SSRIs) and TCAs. In a meta-analysis done in 2007^[8], the efficacy of SSRIs in IBS was reported. In the present paper, the efficacy of TCAs in IBS has been reviewed by meta-analysis of all randomized controlled trials.

EVALUATION OF STUDIES

PubMed, Scopus, Web of Science, and Cochrane Central Register of Controlled Trials were searched for studies investigated the efficacy of TCAs in IBS. Data were collected for the years 1966 to 2008 (up to September). The search terms were: “tricyclic antidepressants”, “amitriptyline”, “amoxapine”, “clomipramine”, “desipramine”, “dothiepin”, “doxepine”, “imipramine”, “iprindole”, “lofepramine”, “nortriptyline”, “opipramol”, “protriptyline”, or “trimipramine” and “irritable bowel”, “functional bowel diseases” or “irritable colon”. Search was restricted to English literature. Reference lists of the retrieved articles were also reviewed for additional applicable studies.

All controlled trials investigating the efficacy of TCAs in patients with IBS were considered. “Global improvement of symptoms” and “adequate relief of pain and discomfort” were the key outcomes of interest for assessment of efficacy. We evaluated all published studies as well as abstracts presented at meetings. Three reviewers independently examined the title and abstract of each article to eliminate duplicates, reviews, case studies, and uncontrolled trials. Trials were disqualified if they were not placebo-controlled or their outcomes did not consider efficacy. Reviewers independently extracted data on patients’ characteristics, therapeutic regimens, dosage, trial duration, and outcome measures. Disagreements, if any, were resolved by consensus.

Jadad score, which evaluates studies based on their description of randomization, blinding, and dropouts (withdrawals), was used to assess the methodological quality of the trials^[13]. The quality scale ranges from 0 to 5 points with a low quality report of score 2 or less and a high quality report of score at least 3.

Data from selected studies were extracted into 2 × 2 tables. All included studies were weighted and pooled. Data analysis was done using StatsDirect (2.7.2). Relative risk (RR) and 95% confidence intervals (95% CI) were calculated using Mantel-Haenszel and effect size (weighted mean difference) meta-analysis was performed using Mulrow-Oxman method. The Cochran *Q* test was used to test heterogeneity. The event rate in the experimental (intervention) group against the event rate

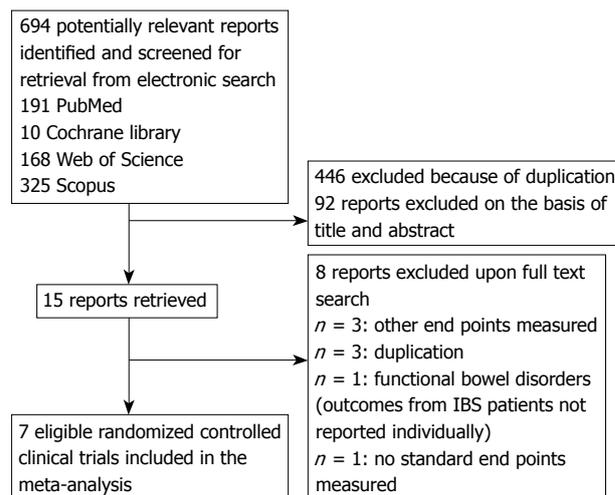


Figure 1 Flow diagram of the study selection process.

in the control group was calculated using L'Abbe plot as an aid to explore the heterogeneity of effect estimates. In case of homogeneity, fixed effect model was used for meta-analysis; otherwise random effect model was applied. In addition to Kendall's *t* test^[14], funnel plots were used as an indicator for publication bias^[15].

FINDINGS

The electronic searches yielded 694 items; 191 from PubMed, 10 from Cochrane Central, 168 from Web of Science, and 325 from Scopus. Of these, 15 trials were scrutinized in full text and 7 trials^[16-22] were included in the analysis (Figure 1). Of these 7 studies, 6^[16-21] obtained a Jadad score of 3 or more and the remaining one^[22] gained a Jadad score of 2 (Table 1). Regarding the Cochran *Q* test for heterogeneity, it was found that this study did not cause heterogeneity in our meta-analysis and thus, it was not excluded. Patients’ characteristics, IBS subtype, TCA subclass, dosage, duration of treatment/follow up for each study are reported in Table 2. All subtypes of IBS (diarrhea-predominant, constipation-predominant and alternating) were incorporated in the included studies. This meta-analysis included 257 IBS patients randomized to receive either TCA or placebo. The efficacy of various TCAs has been investigated including amitriptyline (3 trials), imipramine (1 trial), desipramine (1 trial), doxepin (1 trial) and trimipramine (1 trial). Duration of treatment/follow up ranged between 4 and 12 wk. Definition of clinical response and mean change in abdominal pain score in each study are reported in Table 3.

Cochrane *Q* test suggested that the studies are homogeneous ($P = 0.3284$, Figure 2B) therefore, a fixed effect model was used for meta-analysis. Regression of normalized effect *versus* precision for all included studies for clinical response among TCAs *vs* placebo therapy was 2.40 (95% CI: -1.14 to 5.95, $P = 0.14$). Funnel plot was suggestive of publication bias (Figure 2C); however, Kendall's *t* test was not indicative of such a bias ($\tau = 0.05$, $P > 0.9999$). Pooled RR for clinical response in 7 trials^[16-22]

Table 1 Jadad quality score of randomized controlled trials included in the meta-analysis

Study	Factors and Jadad score			
	Randomization	Blinding	Withdrawals and dropouts	Total Jadad score
Vahedi <i>et al</i> ^[16] , 2008	1	1	1	3
Talley <i>et al</i> ^[17] , 2008	2	2	0	4
Morgan <i>et al</i> ^[18] , 2005	1	1	1	3
Rajagopalan <i>et al</i> ^[19] , 1998	1	2	0	3
Vij <i>et al</i> ^[20] , 1991	1	1	1	3
Greenbaum <i>et al</i> ^[21] , 1987	1	1	1	3
Tripathi <i>et al</i> ^[22] , 1983	1	1	0	2

Table 2 Characteristics of papers included in the meta-analysis

Study	Mean age	Sex		IBS subtype	Type of TCA	Daily dosage	Duration of treatment/follow up (wk)
		Female	Male				
Vahedi <i>et al</i> ^[16] , 2008	36	21	29	D-IBS	Amitriptyline	10 mg	8
Talley <i>et al</i> ^[17] , 2008	ND	21	13	D-IBS, C-IBS, Alt-IBS	Imipramine	2 wk: 25 mg; Thereafter to the end: 50 mg	12
Morgan <i>et al</i> ^[18] , 2005	39	22	0	D-IBS, C-IBS, Alt-IBS	Amitriptyline	First week: 25 mg; Thereafter to the end: 50 mg	4
Rajagopalan <i>et al</i> ^[19] , 1998	34.8	11	11	ND	Amitriptyline	First week: 25 mg; 2nd week: 50 mg; Thereafter to the end: 75 mg	12
Vij <i>et al</i> ^[20] , 1991	32.5	14	36	D-IBS, C-IBS, Alt-IBS	Doxepin	75 mg	6
Greenbaum <i>et al</i> ^[21] , 1987	45.2	18	11	D-IBS, C-IBS	Desipramine	First week: 50 mg; 2nd week: 100 mg; Thereafter to the end: 150 mg	6
Tripathi <i>et al</i> ^[22] , 1983	37	ND	ND	ND	Trimipramine	30 mg	5

IBS: Irritable bowel syndrome; D: Diarrhoea predominant; Alt: Alternating; C: Constipation predominant; TCA: Tricyclic antidepressant.

Table 3 Response to treatment

Study	Definition of response	Response		Change in abdominal pain score (No. of patients)	
		TCA	Placebo	TCA	Placebo
Vahedi <i>et al</i> ^[16] , 2008	Complete loss of symptoms (total score= 0) at the end of the study or at least two scores with a decrease in the number of symptoms	17/25	10/25	-	-
Talley <i>et al</i> ^[17] , 2008	Adequate relief of IBS symptoms over 50% of the weeks	10/18	9/16	-45.3 ± 26.3 (18)	-7.4 ± 46.9 (16)
Morgan <i>et al</i> ^[18] , 2005	Improvement of IBS symptoms determined by patients	13/22	5/22	-	-
Rajagopalan <i>et al</i> ^[19] , 1998	Global well-being: patients were asked to estimate at the post-treatment interview how much better or worse they were on the whole (in percentage) as compared to the pretrial period	7/11	3/11	-	-
Vij <i>et al</i> ^[20] , 1991	Improvement of 50% or above in IBS symptoms	11/21	5/23	-	-
Greenbaum <i>et al</i> ^[21] , 1987	Global assessment of improvement	15/28	5/28	-58.96 ± 19.37 (28)	-13.93 ± 17.76 (28)
Tripathi <i>et al</i> ^[22] , 1983	Improvement of 50% or above in IBS symptoms	7/25	4/25	-	-

was 1.93 (95% CI: 1.34 to 2.6, $P < 0.0001$, Figure 2A).

Studies that considered abdominal pain score as an outcome showed homogeneity using Cochrane Q test ($P = 0.61$). Regression of normalized effect *vs* precision for all included studies for mean change in abdominal pain score could not be calculated because of too few strata.

Using a fixed effect model, effect size of TCAs *versus* placebo for mean change in abdominal pain score among the two studies^[17,21] was -44.15 (95% CI: -53.27 to -35.04, $P < 0.0001$, Figure 3).

DISCUSSION

Visceral hypersensitivity and dysregulation of central pain perception in the brain-gut axis is considered to play a pivotal role in the pathophysiology of IBS. IBS

patients have a lower sensory threshold to colonic and rectal balloon distention and electrical stimulation^[23]; therefore, beneficial effects of antidepressants can be explained by partial increment in central pain threshold. Other mechanisms by which antidepressants might express their effect include anticholinergic effects, regulation of GI transit and peripheral antineuropathic effects^[24,25]. The results from the current meta-analysis show that TCAs induce clinical response and reduce abdominal pain score in patients with IBS.

Other meta-analysis studies that considered the effects of antidepressants in functional gastrointestinal diseases have essential differences with the present study: O'Malley *et al*^[26] pooled all functional diseases including IBS, functional dyspepsia, headache, fibromyalgia, and chronic fatigue. Jackson *et al*^[27]

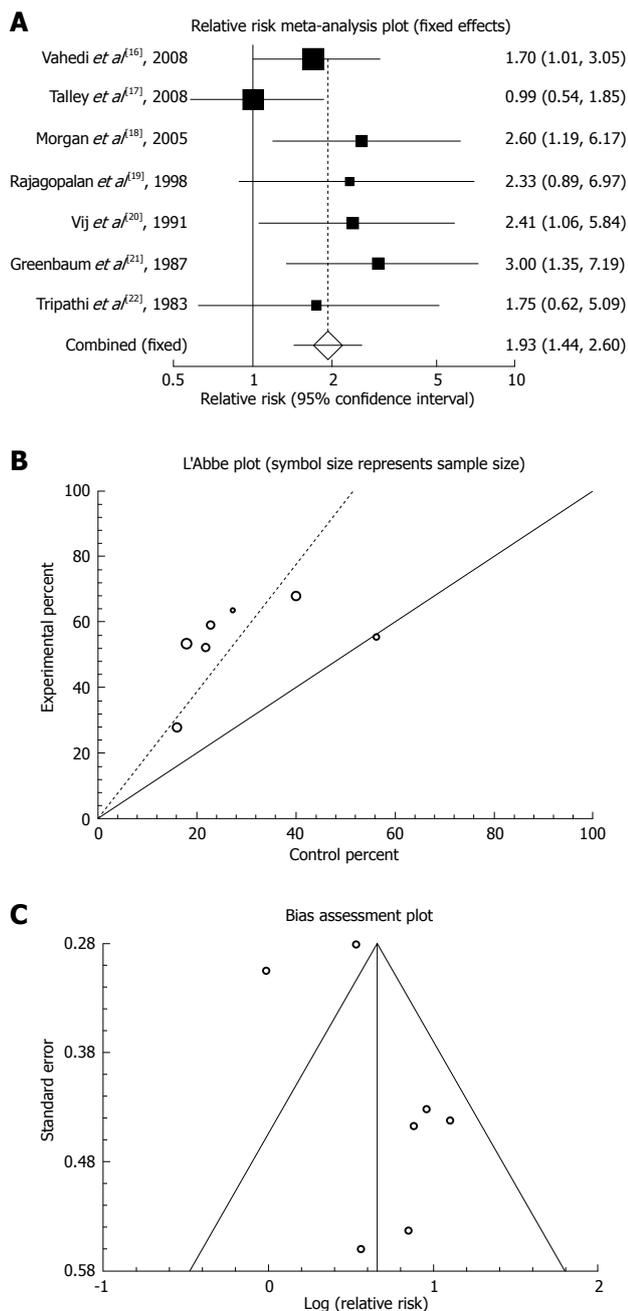


Figure 2 Outcome of “clinical response” in the studies considering TCAs vs placebo therapy. A: Individual and pooled relative risk; B: Heterogeneity indicators; C: Publication bias funnel plot.

included all the functional gastrointestinal disorders and found a statistically significant effect for TCAs (OR 4.2; 95% CI: 2.3 to 7.9). Quartero *et al*^[8] included 4 studies for “global improvement of symptoms” and 2 studies for “abdominal pain” and demonstrated no benefit for antidepressants. Lesbros-Pantoflickova *et al*^[28] demonstrated a favorable effect for antidepressants (OR 2.6; 95% CI: 1.9 to 3.5). Studies with visual analogue outcome^[29] and functional dyspepsia patients were included in their analysis. None of these reviews meta-analyzed the newer evidence that has surfaced in the literature^[16-18].

Unfortunately, conduction of a randomized controlled trial in the field of antidepressants and IBS

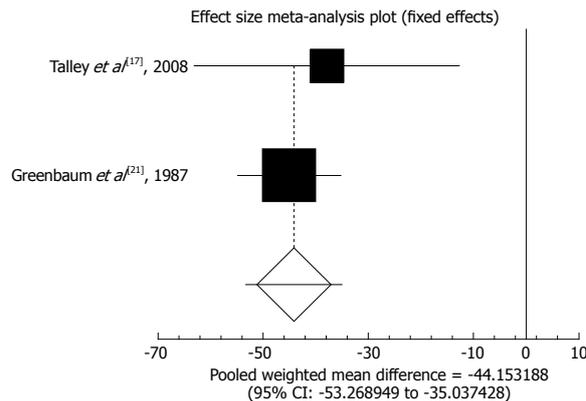


Figure 3 Pooled weighted mean difference for the outcome of “mean change in abdominal pain score” in the studies considering TCAs vs placebo.

is challenging. High placebo response in IBS affects study trials and the stigma of antidepressants disturbs the compliance rate. Randomization is elusive as TCAs have immediate noticeable side effects for patients. As mentioned in Table 1, the majority of trials are of a medium quality. A well-designed trial has been conducted by Drossman *et al*^[30] although not included in our study because of the recruitment of all functional bowel disorders. Drossman *et al*^[30] conducted a large randomized 12-wk placebo-controlled trial evaluating the efficacy of desipramine in treating moderate to severe IBS (80% of the patients), functional constipation and functional chronic abdominal pain. Desipramine was shown to have statistically significant benefit over placebo in the per protocol analysis after non-compliant and drop-out patients were excluded (responder rate 73% *vs* 49%). 11% of the patients were proven to be non-adherent with non-detectable blood levels. This underlines the fact that previous studies might have underestimated the effect of antidepressants by the inclusion of non-adherent cases.

The choice of antidepressants in IBS patients remains controversial. Head to head trials comparing different classes or subclass formulations of antidepressants are lacking in the literature. In a recent meta-analysis^[9], we concluded that on current evidence, SSRIs do not improve abdominal pain, abdominal bloating or other IBS symptoms. Three studies have compared the effects of TCAs with SSRIs and all together they depict a non-conclusive picture^[17,31,32]. Talley *et al*^[17] compared imipramine with citalopram during a 12-wk trial. Clinical response was seen in 56% of imipramine group, 47% of citalopram group and 56% of placebo arm. Neither imipramine nor citalopram significantly improved global IBS endpoints over placebo. Forootan *et al*^[31] compared the effects of nortriptyline, amitriptyline, and fluoxetine. The results demonstrated improvement of abdominal pain, flatulence, and general performance in all subgroups. Amitriptyline and nortriptyline improved frequency of defecation in both diarrhea- and constipation-predominant IBS, while fluoxetine improved GI transit of constipation- predominant IBS. Differential effects of amitriptyline and fluoxetine on

anorectal motility and visceral perception were assessed by Siproudhis *et al*^[32] and the results demonstrated that both antidepressants similarly relax the internal anal sphincter, but only amitriptyline relaxed the external anal sphincter.

Generally, management of IBS requires lower doses of TCAs compared to doses used to treat depression; reflecting the fact that modulation of the brain-gut axis rather than treating concomitant depression is the target in IBS patients. Myren *et al*^[29] in a large 8-wk trial demonstrated no dose-related response with various dosing regimens of trimipramine.

CONCLUSION

This review has some limitations. Funnel plot is suggestive of publication bias with lack of negative small RCTs. However, a firm conclusion about bias is elusive to reach as the asymmetry of the funnel plot is minimal and Kendall's T is not suggestive of publication bias. In addition funnel plots can show asymmetry for various reasons other than publication bias^[33]. Paucity of small negative or neutral studies has been brought to attention in other functional disorders^[26] which might encompass IBS as well. Therefore, our pooled OR might be an overestimate of the true effect. Some other limitations that can be numbered for this meta-analysis are usage of various TCA formulations and doses, dissimilar duration of treatment, and different diagnostic criteria for IBS.

TCAs exhibit clinically and statistically significant control of IBS symptoms; however, given their abundant side effects they should be reserved for moderate to severe cases. Subjects should be started on subtherapeutic doses for depression and choice of drug should be tailored for each individual. We suggest using TCAs with the least anticholinergic effects (i.e. doxepin and desipramine) for elderly patients or constipation-predominant IBS and imipramine or amitriptyline for diarrhea-predominant IBS and patients with insomnia. Larger comparative trials with strict surveillance on compliance are needed to elaborate the role of antidepressants in standard practice.

In addition, new evidence suggests that IBS is very similar to IBD in pathogenesis but different in severity. Recent meta-analyses have indicated the benefit of antibiotics, probiotics, and anti-tumor necrosis factor agents^[34-42] in IBD and thus the effects of these drugs on IBS remain to be elucidated in the future.

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, Series Editor

***Clostridium difficile* associated infection, diarrhea and colitis**

Perry Hookman, Jamie S Barkin

Perry Hookman, Jamie S Barkin, University of Miami Miller School of Medicine; Division of Gastroenterology, Mount Sinai Medical Center, 4300 Alton Road, Miami Beach, FL 33140, United States

Author contributions: Hookman P and Barkin JS equally contributed to this work.

Correspondence to: Perry Hookman, MD, Associate Professor of Medicine, Division of Gastroenterology, Mount Sinai Medical Center, 4300 Alton Road, Miami Beach, FL 33140, United States. hookman@hookman.com

Telephone: +1-561-2418550 Fax: +1-561-2418554

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Abstract

A new, hypervirulent strain of *Clostridium difficile*, called NAP1/BI/027, has been implicated in *C. difficile* outbreaks associated with increased morbidity and mortality since the early 2000s. The epidemic strain is resistant to fluoroquinolones *in vitro*, which was infrequent prior to 2001. The name of this strain reflects its characteristics, demonstrated by different typing methods: pulsed-field gel electrophoresis (NAP1), restriction endonuclease analysis (BI) and polymerase chain reaction (027). In 2004 and 2005, the US Centers for Disease Control and Prevention (CDC) emphasized that the risk of *C. difficile*-associated diarrhea (CDAD) is increased, not only by the usual factors, including antibiotic exposure, but also gastrointestinal surgery/manipulation, prolonged length of stay in a healthcare setting, serious underlying illness, immune-compromising conditions, and aging. Patients on proton pump inhibitors (PPIs) have an elevated risk, as do peripartum women and heart transplant recipients. Before 2002, toxic megacolon in *C. difficile*-associated colitis (CDAC), was rare, but its incidence has increased dramatically. Up to two-thirds of hospitalized patients may be infected with *C. difficile*. Asymptomatic carriers admitted to healthcare facilities can transmit the organism to other susceptible patients, thereby becoming vectors. Fulminant colitis is reported more frequently during outbreaks of *C. difficile* infection in patients with inflammatory bowel disease (IBD). *C. difficile* infection with IBD carries a higher mortality than without underlying IBD. This article

reviews the latest information on *C. difficile* infection, including presentation, vulnerable hosts and choice of antibiotics, alternative therapies, and probiotics and immunotherapy. We review contact precautions for patients with known or suspected *C. difficile*-associated disease. Healthcare institutions require accurate and rapid diagnosis for early detection of possible outbreaks, to initiate specific therapy and implement effective control measures. A comprehensive *C. difficile* infection control management rapid response team (RRT) is recommended for each health care facility. A communication network between RRTs is recommended, in coordination with each country's department of health. Our aim is to convey a comprehensive source of information and to guide healthcare professionals in the difficult decisions that they face when caring for these oftentimes very ill patients.

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Key words: *Clostridium difficile*; Colitis; Diarrhea; Gastroenterology; Nosocomial infection; Iatrogenic infection

Peer reviewer: Hugh J Freeman, Professor, Department of Medicine, University of British Columbia, UBC Hospital 2211 Wesbrook Mall, Vancouver, BC V6T 1W5, Canada

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INTRODUCTION

In our two previous reviews^[1,2], we joined those who have written about the new more virulent strain of *Clostridium difficile* that was described in December 2005 in the National Institutes of Health (NIH)/Center for Disease Control and Prevention (CDC) Morbidity and Mortality Weekly Report. This CDC report emphasized that, in the past, *C. difficile*-associated diarrhea (CDAD) usually affected hospitalized inpatients, but now was appearing in relatively healthy adults, including some

who had not even been exposed to a hospital setting.

Loo *et al.*^[3] and McDonald *et al.*^[4] have indicated that, not only is the rate of disease associated with *C. difficile* increasing, but a previously uncommon strain of *C. difficile* has been identified. This strain of *C. difficile*, which has variations in its toxin genes, is more resistant to fluoroquinolones than prior strains. This newer and more virulent organism has emerged as a cause of geographically dispersed outbreaks of antibiotic-associated diarrhea (AAD), specifically *C. difficile* diseases, CDAD and *C. difficile*-associated colitis (CDAC).

CDAD has also become a more severe disease, and more often has progressed to toxic megacolon (TM). More severe CDAC and CDAD have started to increase in incidence and severity. *C. difficile* also accounts for an increasing percentage of community-acquired diarrhea cases. Fluoroquinolones, especially C-8-methoxy fluoroquinolones, such as moxifloxacin and gatifloxacin, have been incriminated in CDAD epidemics in different health care facilities. This current review attempts to provide an update on this new virulent organism that causes very severe CDAD and CDAC, and emphasizes the importance of early recognition of its complications and its treatment.

Typing of bacterial outbreaks characterize *C. difficile* as a Gram-positive, anaerobic, spore-forming bacillus that is spread indirectly *via* the fecal-oral route through spores left on surfaces. It produces two cytotoxins, which bind to receptors on intestinal epithelial cells, leading to inflammation and diarrhea. The toxins loosen the junctions of the epithelial cells that line the colon, allowing for penetration between epithelial cells^[5]. This begins a cascade of tissue-damaging inflammatory processes that involve the release of destructive leukotrienes and cytokines.

Colonization of *C. difficile* is facilitated by the disruption of normal intestinal flora as a result of antimicrobial therapy. The antibiotics most frequently implicated in CDAD are clindamycin, penicillins, cephalosporins and fluoroquinolones^[6].

There has been a dramatic increase in the frequency, severity and refractoriness of *C. difficile* as seen in multiple outbreaks, not only in North America, but around the world. These factors are attributed to this hypervirulent strain, NAP1/BI/027.

Bartlett documented that, over the first 5 years in which CDAD was acknowledged to exist, 1978 to 1983, the most common cause of CDAD was previous use of clindamycin^[7]. The standard diagnostic test was a cytotoxin assay. Standard management was to withdraw the implicated antibiotic and treat with oral vancomycin. Most patients responded well, but 25% relapsed when vancomycin was withdrawn.

Over the next 20 years (1983-2003), the most commonly implicated antibiotics were the cephalosporins, which reflected their increased rates of use. Fluoroquinolones now are the major inducing agents, along with cephalosporins, a phenomenon which presumably reflects newly-acquired *in vitro* resistance and the escalating rates of use^[8].

Between 2003 and 2006, *C. difficile* has become more frequent, more severe, more refractory to standard therapy, and more likely to relapse than in previous years. This pattern has been seen throughout the United States, Canada and Europe, and is now attributed to a new strain of *C. difficile*, alternatively designated as BI, NAP1, or ribotype 027 toxinotype III (all synonymous terms). Although this strain had been isolated as far back as 1984, it has recently emerged as a public concern with the development of fluoroquinolone resistance in our current era of widespread fluoroquinolone use.

The emergence of this hypervirulent *C. difficile* strain has vastly altered the face of the disease, with increased nosocomial outbreaks and concomitant morbidity. In 2007, Blossom and McDonald^[9] reported on the increasing incidence and severity of *C. difficile*-associated disease attributable to this hypervirulent strain. This strain produces increased levels of toxins A and B, as well as an extra toxin, known as 'binary toxin', which accounts for its increased toxicity. This previously uncommon strain now has become epidemic, and has been reported in populations that previously had been thought to be at low risk, including peripartum women and healthy persons living in the community. Individuals with low or undetectable levels of antibody against *C. difficile* toxins are more likely to develop diarrhea than those with detectable antibody against the toxin. Careful adherence to infection control policies is critical to the control of *C. difficile*, especially at nursing facilities, long-term care and rehabilitation facilities and hospitals, as well as in the community. CDAD primarily occurs in hospitals, where exposure to antimicrobial drugs (the major risk factor for CDAD) and environmental contamination by *C. difficile* spores are more common^[10].

Outbreaks of CDAD due to the new, highly-virulent strain of *C. difficile* have been recognized throughout European health care facilities, including 75 hospitals in England, 16 hospitals in the Netherlands, 13 healthcare facilities in Belgium, and nine healthcare facilities in France. In Germany, the first cases of the highly-virulent *C. difficile* strain, reported in 2007 and characterized as PCR ribotype 027, were associated with high mortality^[11]. Larger outbreaks of *C. difficile* have been reported in northern France in particular^[12]. These outbreaks are very difficult to control, and preliminary results from case-control studies indicate a correlation with the administration of fluoroquinolones and cephalosporins.

Seroprevalence increased in Denmark with increasing age in both 1990 and 1998. Unfortunately, the increase was about four times higher in 1998 than in 1990, which suggests a higher rate of exposure to *C. difficile* in the general Danish adult population^[13].

In Dublin, Ireland, *C. difficile* is a major cause of infectious diarrhea in hospitalized patients^[14]. Between August 2003 and January 2004, there was an appreciable increase in the incidence of *C. difficile*-associated disease, peaking at 21 cases per 1000 patient admissions. Of the *C. difficile* isolates recovered, 85 (95%) were identical toxin A-negative and toxin B-positive strains, corresponding to

toxintype VIII and PCR ribotype 017. All clonal isolates were resistant to multiple antibiotics, including ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin [minimum inhibitory concentrations (MICs) > 32 µg/mL] and erythromycin, clarithromycin and clindamycin (MICs > 256 µg/mL). Recurrent *C. difficile*-associated disease occurred in 26 (36%) of the patients. At least 10 of these 26 patients (14%) developed *C. difficile* colitis. The authors found that careful attention to improving infection control interventions was the most important means of controlling this nosocomial pathogen.

Reported mortality rates from *C. difficile*-associated disease in the United States increased from 5.7 per million population in 1999 to 23.7 per million in 2004. These increased rates also may be caused by the emergence of a highly virulent strain of *C. difficile*. *C. difficile* infection, according to Schroeder^[15], is now responsible for approximately 3 million cases of diarrhea and colitis annually in the United States, and has a mortality rate of 1%-2.5%. Zilberberg *et al*^[16] have reviewed a sample of more than 36 million annual discharges from non-governmental US hospitals, and have concluded that 2.3% of the cases of *C. difficile*-related disease were fatal, amounting for roughly 5500 deaths. That was nearly double the percentage that resulted in death in 2000.

In Canada, Pépin *et al*^[17,18] have documented that, since 2002, an epidemic of CDAD caused by the same hypervirulent strain previously found in the United States, the United Kingdom and the Netherlands, has spread to as many as 30 hospitals in Quebec. More than half (55%) of the patients with CDAD at the investigators' own hospital had received fluoroquinolones within the preceding 2 mo. Moreover, the excessive use of proton pump inhibitors might have facilitated this epidemic. This CDAD was associated with a very high case-fatality rate and with a 30-d mortality rate of 23.0% (37/161) compared with 7.0% (46/656) of matched control subjects ($P < 0.001$). Twelve months after diagnosis, mortality was 37.3% (60/161) among patients with CDAD *vs* 20.6% (135/656) among controls ($P < 0.001$), for a cumulative absolute attributable mortality of 16.7% [95% confidence interval (CI) 8.6%-25.2%]. Each case of nosocomial CDAD led, on average, to 10.7 additional hospital days. These investigators documented especially high attributable mortality among elderly patients with CDAD, mostly caused by this hypervirulent strain, which represents a dramatic change in the severity of this infection. Kuijper *et al*^[19] have estimated that the financial impact of CDAD on the healthcare system is 5-15000 Euros/case in England and \$1.1 billion/year total expenditures in the USA. Assuming a European Union (EU) population of 457 million, the potential cost of CDAD in the EU can be estimated to be 3 billion Euros/year, and this is expected to almost double over the next four decades.

In Zimbabwe, *C. difficile* was isolated from 29.0% of 100 chicken feces samples and from 22.0% of 100 soil samples. Some of the *C. difficile* isolates from chickens (89.7%) and soil (95.5%) were toxigenic. All

of the isolates were resistant to cefotaxime, gentamicin, ciprofloxacin, norfloxacin and nalidixic acid. The results of this study suggest that broiler chickens sold at marketplaces can be an important source of *C. difficile*, and may infect humans through consumption^[20].

The incidence of CDAD in Singapore has remained relatively low, with isolates remaining susceptible to metronidazole and vancomycin^[21].

CHARACTERISTICS OF AN INCREASINGLY PATHOGENIC *C. DIFFICILE*

The new, hypervirulent strain, NAP1/BI/027, has been implicated as the responsible pathogen in selected *C. difficile* outbreaks since the early 2000s. The epidemic strain is resistant to fluoroquinolones *in vitro*, a characteristic which was an infrequent observation in *C. difficile* strains prior to 2001. Five main characteristics of this strain contribute to the clinical and epidemiological observations. (1) The epidemic strain produces a binary toxin, an additional toxin that is not present in other *C. difficile* strains^[22,23]. (2) Binary toxin is related to the iota-toxin found in *Clostridium perfringens*, and its role in *C. difficile* pathogenesis is not fully understood^[24,25]. (3) The epidemic strain produces substantially larger quantities of toxins A and B *in vitro* than other *C. difficile* strains^[26]. (4) Toxin production by an emerging strain of *C. difficile* has been associated with outbreaks of severe disease in North America and Europe^[27]. The epidemic strain is toxinotype III; most other *C. difficile* strains are toxinotype 0^[28]. Toxinotyping is based on analysis of the pathogenic locus (PaLoc) of the *C. difficile* genome, the region that includes the genes for toxin A (*tcdA*), toxin B (*tcdB*), and neighboring regulatory genes. (5) The epidemic strain has a partial deletion of *tcdC*, a gene in PaLoc that is responsible for down-regulation of toxin production^[29].

C. difficile produces at least two distinct toxins^[30]. These have been labeled toxin A and toxin B. Although initially thought to have distinctive actions, both now appear to be cytotoxic and enteropathic. Previous animal experiments have suggested that only toxin A mediates diarrhea and enterocolitis, even though *C. difficile* releases two structurally similar exotoxins. But when toxin A-negative/toxin B-positive strains of *C. difficile* are isolated from patients with AAD and colitis, this indicated that toxin B also may also be pathogenic in humans. *C. difficile* toxin B, like toxin A, has been found to be a potent inflammatory enterotoxin in the human intestine^[31].

Both toxins disrupt the actin cytoskeleton of intestinal epithelial cells by uridine diphosphate-glucose dependent glycosylation of Rho and Ras proteins^[32]. Stabler *et al*^[33] have reported that toxin B from 027 strains may have a different binding capacity than their less-virulent counterparts and may, in addition to the mutated *tcdC* regulator, be responsible for the increased virulence of the 027 strains.

The most widely used laboratory assays for *C. difficile* infection involve toxin A and/or toxin B detection, and

both are usually detected if diarrhea is present. Atypical toxin variant strains that may cause symptoms have also been described in Asia^[34].

Kuijper *et al.*^[19] have claimed that *C. difficile* has more than 150 PCR ribotypes and 24 toxinotypes, and has a PaLoc with genes that encode for enterotoxin A (tcdA) and cytotoxin B (tcdB). Genes for the binary toxin are located outside the PaLoc. The recently completed genome sequence of *C. difficile* 630 has revealed a large proportion (11%) of mobile genetic elements, mainly in the form of conjugative transposons.

Drudy *et al.*^[35] have reported on several *C. difficile* outbreaks due to PCR ribotype 027 (PCR-027) associated with a mutation in *gyrA* that is associated with high-level resistance to fluoroquinolones. This strain type, which contains genes for the binary toxin, has an 18-bp deletion and a frameshift mutation in *tcdC*, which results in deregulated expression of toxins A and B. These strains can produce up to 16 times more toxin A and 23 times more toxin B *in vitro* than toxinotype 0 strains. The strain demonstrates universally high-level resistance to fluoroquinolones, in contrast to PCR 027 isolates that were collected before 2001. Mutations at the active site or the quinolone resistance determining region of DNA gyrase and topoisomerase IV have been associated with increased resistance to fluoroquinolones in several bacteria. In *Escherichia coli*, amino acid substitutions that occur at Ser-83 in *gyrA* have also been associated with fluoroquinolone resistance. Thus, the emergence of the hypervirulent NAP1/O27 *C. difficile* strain, also known as BI NAP1, has vastly altered the face of the disease, with increased nosocomial outbreaks and concomitant morbidity in countries worldwide.

In an epidemic of *C. difficile*-associated disease in the Canadian province of Quebec, Warny *et al.*^[26] documented that the dominant strain produced higher amounts of toxins A and B than those produced by non-epidemic strains. The epidemic strain was characterized as toxinotype III, North American PFGE type 1, and PCR-ribotype 027 (NAP1/027). This strain carried the binary toxin gene *cdtB* and an 18-bp deletion in *tcdC*. The authors isolated this strain from 72 patients with *C. difficile*-associated disease. Peak median (IQR) toxin A and toxin B concentrations produced *in vitro* by NAP1/027 were 16 and 23 times higher, respectively, than those measured in isolates representing 12 different PFGE types, known as toxinotype 0 [toxin A, median 848 µg/L (IQR 504-1022) *vs* 54 µg/L (23-203); toxin B, 180 µg/L (137-210) *vs* 8 µg/L (5-25); $P < 0.0001$ for both toxins]. Thus, the severity of *C. difficile*-associated disease caused by NAP1/027 appears to be the result of hyper-production of toxins A and B. The dissemination of this strain across North America and Europe has led to dangerous changes in the epidemiology of *C. difficile*-associated disease.

A nationwide epidemiological study conducted in Korea has revealed that tcdA(-)tcdB(+) *C. difficile* strains already have spread extensively throughout the country. The use of enzyme immunoassays capable of detecting

TcdA and TcdB is strongly recommended for the diagnosis of CDAD in microbiology laboratories, in order to control the spread of the tcdA(-)tcdB(+) strains of *C. difficile*^[36]. Sixty to 80% of *C. difficile* isolates in Korea have been reported to be toxigenic. Endoscopy, performed on 55/106 patients, revealed 29 with pseudomembranous colitis (PMC), five with colitis, 14 with other colon diseases, and seven normal colons. Among the 29 PMC cases, 21 (72.4%) were associated with tcdA-tcdB + strains ($P = 0.0016$). These results reveal the emergence of tcdA-tcdB+ *C. difficile* strains in Korea, and these variant strains could evoke a higher rate of PMC than tcdA + tcdB + strains^[37].

Toxin damage

C. difficile toxin A elicits intestinal fluid secretion and neutrophil infiltration by both mast cell-dependent and -independent pathways, and substance P participates in both pathways^[38].

Extensive mitochondrial damage occurs within 15 min in cells exposed to toxin A. Diminished ATP concentrations and increased oxygen radicals contribute to cytotoxicity from this bacterial toxin^[39].

The toxins damage the tight junctions of the intestinal epithelium. Tight junctions are crucial determinants of barrier function in intestinal epithelia, and are regulated by Rho guanosine triphosphatase. Rho kinase (ROCK) is a downstream effector of Rho. ROCK inhibition in calcium switch assays has shown that ROCK is necessary for the assembly of tight and adherens junctions. ROCK also is critical for assembly of apical junctional proteins and F-actin cytoskeleton organization during junctional formation^[40].

C. difficile toxicity and the immune response processes

C. difficile toxins A and B are glucosyltransferases, which catalyze the inactivation of Rho proteins. *C. difficile* toxins act *via* translocation into target cells, and do their damage through autocatalytic processes by inactivating low-molecular-mass GTP-binding proteins of the Rho GTPase family involved in cellular signaling. This leads to cytotoxicity, including depolymerization of the target cell's actin cytoskeleton. Thus, these toxins glycosylate members of the Rho GTPase family, and this GTPase inactivation leads to depolymerization of the cell's actin cytoskeleton and, ultimately, cell death^[41]. In addition, the *C. difficile* toxins further damage the intestine's target cells by initiating massive cellular immune responses; i.e. neutrophilic infiltration with up-regulation and release of cytokines, such as interleukin (IL)-8, IL-6, IL-1β, leukotrienes B4 and interferon-γ.

Part of the mammalian immune response falls to the innate immune system called defensins and, specifically, human α-defensins produced by leukocytes, mucosal epithelial cells, and skin. Defensins, one of evolution's major groups of antibiotic peptides, have broad-spectrum antibiotic activity against Gram-positive and Gram-negative bacteria, fungi, and viruses^[42-44]. Defensins are characterized by a conserved 6-cysteine array. Each cysteine has intra-molecular disulfide bonds

that are essential to protection against proteolysis^[45]. Defensins also are known to contribute to wound healing, chemotaxis, and cytokine function^[46,47]. Defensins are part of two major groups of antimicrobial peptides: defensins and cathelicidins. These groups of human defensins consist in part of alpha, beta and omega defensins, human neutrophil protein (HNP)-1, HNP-3, cathelicidin LL-37 and enteric human defensin (HD)-5. These peptides play a role in the innate immune response, by deactivating various microbial pathogens, as well as specific bacterial exotoxins.

The antibiotic activity of both HNPs and HD5 is well documented in host defenses against enteric pathogens^[48,49]. HD5 and HD6 are produced and stored in Paneth cell secretory granules^[50], along with a variety of additional Paneth cell products demonstrated to have antimicrobial and immune activity^[51-55]. The impact of defensins on *C. difficile* disease has been described by Giesemann *et al*^[56] and others^[57-60].

Giesemann *et al*^[56] have studied the effects of α -defensin HNP-1, HNP-3, and enteric HD-5 on the activity of *C. difficile* toxins A and B. They found that the treatment of cells with human α -defensins caused a loss of cytotoxicity of toxin B, but not of toxin A. In this study, only α -defensins, but not β -defensin-1 or cathelicidin LL-37, inhibited toxin B-catalyzed *in vitro* glucosylation of Rho GTPases in a competitive manner. This indicates that human α -defensins interact with high affinity for *C. difficile* toxin B. Defensins thereby provide a defense mechanism against clostridial glucosylating cytotoxins. At high concentrations, defensins (HNP-1 \geq 2 μ mol/L) also cause high-molecular-mass aggregates of *C. difficile* toxins, thus further decreasing their toxic effects on target cells.

C. difficile has been found in approximately 3% of normal adults and up to 40% of hospitalized patients^[7]. However, as Salzman emphasizes: "only about one third of patients harboring *C. difficile* develop colitis, whereas the rest remain asymptomatic^[61]". Giesemann *et al*^[56] have shown that α -defensins inhibit *C. difficile* toxin B, which offers insight into the possibility of different inflammatory responses in patients who develop CDAC *versus* others who do not. Salzman feels that " α -defensins show an additional antitoxin activity, in which HD5 is more effective; i.e. the stimulation of toxin aggregation." Giesemann has shown that HD5, used at concentrations that normally can be found in the small intestine, is effective at causing aggregation of toxin B, thus effectively preventing the toxin's ability to enter cells and interact with its target. These findings suggest an additional mechanism of antitoxin activity by α -defensin HD5.

This ability of HD5 to cause toxin B aggregation may provide an explanation for both the asymptomatic carriage of this pathogen and the frequency of patient relapse following antibiotic treatment, especially if the small intestine is a reservoir for *C. difficile* carriage in the gut. Salzman postulated that *C. difficile* is able to maintain colonization of the small intestine, but unable to cause colitis, because the high concentration of HD5 at this site neutralizes the secreted exotoxin.

In summary, Salzman feels that, in the small intestine, high concentrations of HD5 result in toxin B aggregation and therefore, the prevention of intoxication. While, in the large intestine, inadequate amounts of α -defensin are present to aggregate or inhibit toxin B, resulting in epithelial intoxication, inflammation, and neutrophilic infiltration.

Usually, *C. difficile* that transits through the large bowel will be prevented from finding a niche by the normal colonic microbiota. Yet, if the microbial ecology of the colon is disrupted, perhaps through antibiotic treatment, *C. difficile* can colonize the large intestine. Salzman postulates that, under these conditions, HD5 concentration is reduced by diffusion and dilution; thus, *C. difficile* exotoxins become free to interact with colonic enterocytes, resulting in intoxication, inflammatory responses, and infectious colitis.

The carrier state

Many patients are colonized with *C. difficile*, but have no symptoms. Perhaps *C. difficile* is harbored in the small intestine, where its toxic effects are well neutralized. Lawrence has claimed that about 20% of hospitalized adults are *C. difficile* carriers; and, in LTCFs, the carriage rate may approach 50%^[62]. Although asymptomatic, these individuals shed pathogenic organisms and serve as a reservoir for environmental contamination. About 3% of healthy adults and 20%-40% of hospitalized patients are colonized with *C. difficile*, which in healthy persons is metabolically inactive in the spore form. Many patients have *C. difficile* as an asymptomatic organism in their intestine on hospital admission, and it only becomes a problem after they are treated with antibiotics, if, in fact, it ever induces symptoms. Exposure to antibiotics that disrupt the colonic microbial flora appears to be the most important risk factor for CDAD.

Treatment of asymptomatic carriers

Asymptomatic colonized patients can act as a reservoir for the transmission of CDAD. Data, however, are limited regarding whether the treatment of these asymptomatic carriers leads to a decrease in the nosocomial transmission of *C. difficile*. Thirty asymptomatic *C. difficile* carriers were randomly assigned to one of three treatment groups: oral vancomycin 125 mg four times daily; metronidazole 500 mg orally twice daily; or placebo. Johnson *et al*^[63] have found that nine of 10 patients receiving vancomycin became culture-negative during and immediately after treatment, compared to three of 10 receiving metronidazole and two of 10 receiving placebo. However, this decolonization was transient, as most patients became re-colonized within weeks. Thus, metronidazole does not appear to be effective for the treatment of asymptomatic carriers. In the setting of a hospital outbreak in which temporary elimination of the organism is felt necessary to reduce horizontal transmission, vancomycin may be a useful tool^[63].

Riggs *et al*^[64] have reported on molecular typing of *C. difficile* performed on asymptomatic carriers using pulsed-field gel electrophoresis. They found that 35 (51%)

of 68 asymptomatic patients were carriers of toxigenic *C. difficile*, and 13 (37%) of these patients carried epidemic strains. They have also reported that 87% of isolates found in skin samples and 58% of isolates found in environmental samples were identical to concurrent isolates found in stool samples. Spores on the skin of asymptomatic patients were transferred easily to the investigators' hands, again accounting for spread to persons in contact. This might be an explanation for the McFarland *et al*^[65] observation that nosocomial CDAD frequently is transmitted between hospitalized patients, and that the organism often is present on the hands of hospital personnel caring for such patients. Kyne *et al*^[66] have studied prospectively *C. difficile* infections in hospitalized patients who were receiving antibiotics, and identified no evidence of immune protection against repeat colonization by *C. difficile*. However, after colonization, there is an association between a systemic anamnestic response to toxin A, as demonstrated by increased serum levels of IgG antibody against toxin A, and asymptomatic carriage of *C. difficile*.

PRESENTATION OF *C. DIFFICILE* INFECTION

C. difficile infection causes diarrhea, often watery, rather than bloody, and it generally develops within 48-72 h of infection. In some, the symptoms may be delayed for 2-3 mo, usually after an antimicrobial agent has been administered. In some, only a single antibiotic tablet may lead to severe disease. Over time, the clinical spectrum has become better appreciated, with illness severity noted to be broad-ranging, from an asymptomatic carrier state (without detectable toxin) to severe and life-threatening pseudomembranous colitis with toxic megacolon^[67].

The clinician must be ever on the alert to make an early diagnosis of *C. difficile*-related disease in the setting of new-onset loose stools or symptoms of abdominal distension and/or leukocytosis, since unexplained leukocytosis in hospitalized patients, even in the absence of diarrhea, may reflect underlying *C. difficile* infection^[68]. In a prospective study, Bulusu *et al*^[69] found that, of 60 patients with unexplained leukocytosis (with a white blood cell count > 15000/ μ L), a positive stool *C. difficile* toxin was observed more frequently in cases than in controls (58% *versus* 12%, respectively). Age over 75 years and immunosuppression were associated with a poor outcome. Earlier surgical consultation is warranted in severe cases to consider potentially life-saving colectomy, as well as alterations in the hospital-based standard of care for prevention.

Usually, the disease affects the colon and, in many cases, is made evident by the presence of colonic pseudomembranes. However, in patients with underlying Crohn's or ulcerative colitis, pseudomembranous changes may not occur; therefore, typical endoscopic findings of *C. difficile* may not be present, and the colonic mucosa will reflect only the underlying inflammatory bowel disease.

C. difficile infection may present with an acute abdomen but either absent or mild diarrhea, as described by

Triadafilopoulos and Hallstone^[70] in 1991. Plain abdominal radiographs revealed megacolon in these patients. This was combined with small and large bowel dilation in one who exhibited a volvulus-like pattern, and isolated small-bowel ileus in another. Diagnosis was revealed by emergency colonoscopy. All patients had positive results for *C. difficile*, and two tested positive for cytotoxicity. All were treated with IV metronidazole, resulting in the resolution of all symptoms and abdominal findings.

An unusual manifestation of CDAC was described in 1981 by Dansinger *et al*^[71]. They reported that up to half of patients with indolent *C. difficile* infection develop manifestations of protein-losing enteropathy, including ascites, peripheral edema, and hypoalbuminemia. Inflammation of the bowel may allow leakage of albumin into the lumen, causing colonic loss of albumin with inadequate compensatory hepatic synthesis. As a result, serum albumin levels may drop below 20 g/L (20 g/L)^[71,72]. Older patients may present with pedal edema, and be mistakenly diagnosed with CHF.

Rubin *et al*^[73] studied patients who had developed a more aggressive form of CDAD *versus* those who developed milder disease. They found that 21 of 710 patients (3%) either required intensive care unit (ICU) admission or died as a result of their infection. The factors predisposing to the development of severe *C. difficile* colitis included concurrent malignancy, chronic obstructive pulmonary disease, immunosuppressive or anti-peristaltic medications, renal failure, and the administration of clindamycin ($P < 0.05$ for all). Patients with severe *C. difficile* colitis were more likely to have abdominal pain, tenderness and distention, peritonitis, hemoconcentration (> 5 points), hypoalbuminemia (< 30 mg/L), and an elevated (> 25000) or suppressed (< 1500) white blood cell count ($P < 0.05$ for all). Therefore, we must initiate aggressive diagnostic and therapeutic modalities in this patient group.

Extra-colonic features may occur in CDAD patients^[74]. These include small bowel involvement in those patients with previous small bowel surgery, and visceral abscesses, primarily in the spleen, and less commonly in the pancreas. Other features include a reactive polyarticular arthritis, cellulitis, necrotizing fasciitis, osteomyelitis, and prosthetic device infections. Arthritis after *C. difficile* was further characterized by Birnbaum as being an asymmetric oligoarthritis^[74]. *C. difficile* colitis has also been reported associated with intra abdominal hypertension and abdominal compartment syndrome^[75].

POPULATIONS AT INCREASED RISK

In 2004 and 2005, the CDC emphasized that the risk of CDAD is increased in certain susceptible populations (Table 1).

Drug exposure

Although the antibiotics most frequently implicated in predisposition to *C. difficile* infection are fluoroquinolones, clindamycin, cephalosporins and penicillins, virtually all

Table 1 Populations at increased risk for *C. difficile*

Patients who take the following drugs
Antibiotics
Proton pump inhibitors
Valacyclovir
Patient characteristics
IBD
Serous underlying illness-comorbidities
Gastrointestinal surgery/manipulations
Advanced age
Immune-compromising conditions (post transplantation)
Peri-partum
Environment
Prolonged stay in health-care settings
Laboratory factors
Hypoalbuminemia
Low levels of anti-toxin and B antibodies

antibiotics, including metronidazole and vancomycin, can predispose to *C. difficile*. De Andrés *et al*^[76] reported a case of *C. difficile* colitis associated with valacyclovir treatment.

The risk of CDAD in hospitalized patients receiving antibiotics may be compounded by co-existing disorders that require treatment with PPI therapy, which inhibits one's defenses against ingested bacteria by virtually eliminating gastric acid^[77]. Dial *et al*^[78] estimated an adjusted risk ratio for *C. difficile*-associated disease with the current use of PPIs as 2.9 (95% CI: 2.4-3.4); and with H2-receptor antagonists, the rate ratio was 2.0 (95% CI: 1.6-2.7). The authors also uncovered an elevated rate of CDAD in patients on non-steroidal anti-inflammatory drugs (rate ratio, 1.3; 95% CI: 1.2-1.5). Thus, the consumption of drugs other than antibiotics may put one at increased risk for community-acquired *C. difficile*.

PPI therapy is also associated with an increased risk of recurrent *C. difficile* colitis. Patients receiving PPIs have been found to be 4.17 times as likely to have recurrence as their counterparts not receiving them^[79]. This relationship between PPI therapy and *C. difficile* was elucidated by Jump who found that the survival of vegetative *C. difficile* in gastric contents obtained from patients receiving PPIs was also increased at a pH of > 5^[80].

Peripartum

The incidence of severe CDAD is increasing in peripartum women. A PubMed search identified 24 recorded cases of peripartum *C. difficile* infection. Most patients (91%) had received prophylactic antibiotics during delivery or for treatment of bacterial infections. Two cases without known risk factors were found, by polymerase chain reaction analysis, to be infected with an epidemic and hypervirulent *C. difficile* strain. These cases demonstrate the need for clinicians to consider *C. difficile* infection in pregnant and peripartum patients with diarrhea, even if they do not have the traditional risk factors for *C. difficile* infection, such as antibiotic use or concurrent hospitalizations^[81].

Co-morbidities

The Agency for Healthcare Research and Quality

(AHRQ) is the lead US Federal agency charged with improving the quality, safety, efficiency, and effectiveness of health care. AHRQ data make clear that one of the challenges in accurately diagnosing CDAD is that it is not unusual for patients who acquire *C. difficile* to have multiple co-morbidities. Thus, multiple co-morbidities put patients at risk for *C. difficile* infection. AHRQ found that hospitalized patients with CDAD had over 10 diagnoses, *versus* six diagnoses among patients without CDAD^[82]. According to recent AHRQ data, four out of the top 20 most common principle diagnoses observed with CDAD are infections (sepsis, pneumonia, urinary tract infection, and skin infection), where antibiotic use would be difficult to avoid^[82].

Post-transplantation patients

Sixteen patients, representing an incidence rate of 0.16%, developed a *C. difficile* infection after total joint arthroplasty (TJA) at one institution. Those at risk for developing CDAD after TJA were patients with deteriorated physical status and those who had received more than one antibiotic postoperatively^[83].

In addition, *C. difficile* is now considered to be a significant cause of diarrhea in heart transplant recipients, and the post-transplantation period is now considered one of greater risk^[84]. With *C. difficile* infection, CDAC prior to 2000 was a rare complication in this patient group; but 38 of the 43 reported cases of CDAC in these patients occurred after 2000. Therefore, *C. difficile* is now also one of the most common causes of diarrhea in patients who have undergone solid organ transplantation^[85]. Another group of patients at increased risk are post orthotopic liver transplant patients. Testing for *C. difficile* toxins among orthotopic liver transplant patients with nosocomial diarrhea revealed that 63% of samples are toxin-positive^[86].

The development of life-threatening toxic megacolon secondary to CDAC now must be considered in solid organ recipients. Toxic megacolon was reported in five patients by Stelzmueller *et al*^[85].

Post-surgery

The risk of *C. difficile* infection was 14.9 cases per 1000 surgical procedures among patients who received preoperative prophylaxis (PAP) during the period 2003-2005, which is a significant increase compared with 0.7 cases per 1000 surgical procedures during the period 1999-2002 ($P < 0.001$). Independent risk factors associated with *C. difficile* infection in patients given PAP alone, were older age, the administration of cefoxitin (rather than cefazolin) alone or in combination with another drug, and the year of surgery. Thus, in the context of a large epidemic of *C. difficile* infection associated with the emergence of a novel strain of organism, 1.5% of patients who had received PAP as their sole antibiotic treatment developed *C. difficile* infection. In situations in which the only purpose of PAP is to prevent infrequent and relatively benign infections, the risks of PAP may outweigh its benefits, especially in elderly patients^[87].

Unfortunately, the incidence of *C. difficile* infection is increasing in US surgical patients even without PAP, and infection with *C. difficile* is most prevalent after emergency operations and among patients who have undergone intestinal tract resections^[88].

IBD as a risk factor for CDAC

IBD patients are at greater risk than the general population for acquiring *C. difficile* infection^[89]. Issa *et al*^[90] performed a retrospective, observational study in IBD patients to evaluate the impact of *C. difficile*. They found that the rate of *C. difficile* infection had increased from 1.8% of IBD patients in 2004 to 4.6% in 2005 ($P < 0.01$). The proportion of IBD patients within the total number of *C. difficile* infections at their institution increased from 7% in 2004 to 16% in 2005 ($P < 0.01$). In 2005, IBD colonic involvement was found in the vast majority (91%) of *C. difficile*-infected patients, a clear majority (76%) had contracted infection as an outpatient, and antibiotic exposure was identified in 61% of IBD patients with *C. difficile* infection. Over the period 2004-2005, more than half of the infected IBD patients required hospitalization, and 20% required colectomy. Univariate and multivariate analyses identified maintenance immunomodulator use and colonic involvement as independent risk factors for *C. difficile* infection in IBD. The authors also reported a nationwide doubling in the rate of *C. difficile* infection among hospitalized UC patients between 1998 and 2004. The pathologic/endoscopic features of pseudomembranous colitis CDAC varies as a spectrum, with some patients exhibiting only mild inflammatory changes confined to the superficial epithelium, and typical pseudomembranes and crypt abscesses may not be present. The more severe cases demonstrate marked mucin secretion, and more intense inflammation. Intense necrosis of the full thickness of the mucosa, with a confluent pseudo-membrane, can become more prominent as disease severity increases.

The association between IBD and *C. difficile* may be due to a variety of factors, including antibiotic use for treatment of other gastrointestinal pathogens and frequent hospitalizations for the management of IBD flares. Many of these patients are taking immunosuppressive medications that may confer additional risk of *C. difficile* infection. *C. difficile*, and specifically its toxins, have been implicated as a risk factor for the exacerbation of the inflammatory process in up to 5% of patients with ulcerative colitis or Crohn's disease. A severe clinical course may result from *C. difficile* infection superimposed on IBD, including the precipitation of toxic colitis and toxic megacolon.

CDAC in patients with IBD carries a higher mortality than in patients with *C. difficile* without underlying IBD. On multivariate analysis, patients in the *C. difficile*-IBD group had a four times greater mortality than patients admitted to hospital for IBD alone (AOR = 4.7, 95% CI: 2.9 to 7.9) or *C. difficile* alone (AOR = 2.2, 95% CI: 1.4 to 3.4), and stayed in the hospital for 3 d longer (95% CI: 2.3 to 3.7 d). Significantly higher mortality, endoscopy and surgery rates were found in patients with ulcerative colitis compared

with Crohn's disease ($P < 0.05$) who had associated *C. difficile*^[91]. The median times from admission to a positive *C. difficile* test result for non-IBD was much longer than in Crohn's disease and ulcerative colitis patients (4.0, 0.8, and 0.5 d, respectively). *C. difficile* infections in IBD are confirmed predominantly within 48 h of admission, suggesting most were acquired before hospitalization. CDAD rates approximately doubled in Crohn's disease (9.5 to 22.3/1000 admissions) and tripled in ulcerative colitis (18.4 to 57.6/1000). Length of stay was similar among the groups. For all years combined, the adjusted odds ratios for CDAD in all IBD, Crohn's disease, and ulcerative colitis admissions were 2.9 (95% CI: 2.1-4.1), 2.1 (1.3-3.4), and 4.0 (2.4-6.6), respectively^[92].

Patients with severe *C. difficile* infection, especially IBD patients, require prompt diagnosis and management, since failure to diagnose the infection can lead to inappropriate treatment with glucocorticoids or immunosuppressive therapy. Furthermore, *C. difficile* may be difficult to distinguish from an IBD relapse, given the similar symptoms of diarrhea, abdominal pain, and low-grade fever. Thus, a high index of suspicion is required when evaluating IBD patients with apparent flares, especially those who recently have received antibiotics and/or have been hospitalized.

Thus, speedy diagnosis largely requires the use of laboratory tools, since endoscopy may not be helpful early, because IBD patients may not develop pseudomembranes. Given the underlying colonic pathology, patients with IBD who develop *C. difficile* colitis frequently require colectomy (20 percent in one series)^[90].

CLINICAL DIAGNOSIS

Delays in both diagnosing and treating both initial and recurrent CDAD^[93] are due to the fact that CDAD can mimic the more common 'benign' antibiotic-associated diarrhea that is not caused by *C. difficile*^[94]. Thus, the diarrhea from *C. difficile* will be ascribed to other causes; e.g. food poisoning, viral infection, or other causes. *Klebsiella pneumoniae*, *Candida* species and *Staphylococcus aureus* have been identified as potential causative organisms in *C. difficile* negative AAD patients^[95].

Patients can be infected with this microorganism and may have no symptoms of colitis. They, therefore, may not be tested for *C. difficile* infection (see section on presentation). These asymptomatic carriers, who are admitted to healthcare facilities and hospitals, become vectors during outbreaks and can transmit the organism to other susceptible patients. Most cases of CDAD occur at 4-9 d after discontinuation of antibiotic therapy, according to Schroeder^[15]; however, CDAD can occur up to 8 wk after the discontinuation of antibiotics.

Sigmoidoscopy/colonoscopy for the diagnosis of CDAD

Lower endoscopy is a useful tool for the diagnosis of *C. difficile*. This is especially when: (1) there is a high level of clinical suspicion for *C. difficile*, despite a negative laboratory assay; (2) prompt *C. difficile* diagnosis is needed before laboratory results can be obtained; (3) *C. difficile*

infection fails to respond to antibiotic therapy; or (4) when there is an atypical disease presentation, and *C. difficile* is suspected, as with ileus, acute abdomen, leukocytosis or diarrhea.

Endoscopy is not indicated in patients with classic clinical findings and a positive stool toxin assay. Conversely, endoscopy may be contra-indicated, especially in the setting of fulminant colitis, due to the risk of perforation.

Endoscopic findings: Pseudomembranes are pathognomonic for CDAC, but are not found in all areas of the colon, even in severe cases; thus, findings may be patchy. Pseudomembranes may be absent in the rectosigmoid area, but may be visualized more proximally with colonoscopy. This is true in patients with co-existing IBD. Pseudomembranes are raised yellow or off-white plaques, up to 2 cm in diameter, which are randomly scattered over the colorectal mucosa with normal intervening mucosa, and that cannot be removed by lavage. The pseudomembranes form when *C. difficile* toxin-induced cytoskeleton disruption causes shallow ulcerations on the intestinal mucosal surface. It is postulated that ulcer formation allows for the release of serum proteins, mucus, and inflammatory cells, which appear grossly on the colorectal mucosal surface as pseudomembranes. Light and scanning electron microscopy after exposure to either of the *C. difficile* toxins reveal patchy damage and exfoliation of superficial epithelial cells, while crypt epithelium remains intact. Fluorescent microscopy of phalloidin-stained sections shows that both toxins cause the disruption and condensation of cellular F-actin^[96].

Other colonic mucosal findings include bowel-wall edema, erythema, friability, and inflammation, with or without pseudomembranes. This manifests on the abdominal CT scan as thickening of the colonic wall.

Colonoscopic findings among 16 patients with histologically-proven antibiotic-associated PMC or CDAC were described by Seppälä *et al*^[97]. Pseudomembranes were found in only five of 16 (31%) patients by sigmoidoscopy, but were found in 11 of 13 patients (85%) in whom colonoscopy also was performed. These findings suggest the importance of colonoscopy in the early diagnosis of CDAC, because the typical endoscopic changes of pseudomembranes are limited to the colon above the rectosigmoid area in most patients. Consequently, colonoscopy should be performed, instead of sigmoidoscopy, at least in clinically suspected CDAC cases^[98].

Complications of *C. difficile* colitis

C. difficile colitis is usually associated with a mild/moderate course, but may progress to fulminant colitis. Fulminant colitis develops in 3%-8% of patients. The manifestations of fulminant colitis typically include severe lower quadrant or diffuse abdominal pain, diarrhea, abdominal distention, fever, hypovolemia, lactic acidosis, and marked leukocytosis (up to 40 000 white blood cells/microL or higher). Diarrhea may be

less prominent in patients with prolonged ileus, due to pooling of secretions in the dilated, atonic colon. Other potential complications of fulminant colitis include toxic megacolon and bowel perforation^[73].

Toxic megacolon is a clinical diagnosis based upon the finding of colonic dilatation (> 7 cm in its greatest diameter) accompanied by severe systemic toxicity. Abdominal plain films also may demonstrate small-bowel dilatation, air-fluid levels (mimicking an intestinal obstruction or ischemia), and 'thumb printing' (scalloping of the bowel wall) due to submucosal edema. Toxic megacolon may be complicated by bowel perforation.

This latter complication presents with abdominal rigidity, involuntary guarding, diminished bowel sounds, rebound tenderness, and severe localized tenderness in the left or right lower quadrants. Abdominal radiographs may demonstrate free intra-abdominal air. Thus, patients with toxic megacolon must be followed with daily upright abdominal X-rays to ascertain if perforation has occurred. Patients with toxic megacolon should be evaluated for surgical resection. Once fulminant colitis is diagnosed, subtotal colectomy with ileostomy usually is required. In these patients who develop a marked leukocytosis or bacteremia, surgery is advisable, because the leukocytosis frequently precedes hypotension. The requirement for vasopressor therapy carries a poor prognosis, according to Shen *et al*^[99].

Lamontagne *et al*^[100] has documented that emergency colectomy reduces mortality in patients with fulminant CDAD. The independent predictors of 30-d mortality in their study were leukocytosis $\geq 50 \times 10^9/L$ (AOR, 18.6; 95% CI: 3.7-94.7); serum lactate ≥ 5 mmol/L (AOR, 12.4; 95% CI: 2.4-63.7); age ≥ 75 years (AOR, 6.5; 95% CI: 1.7-24.3); immunosuppression (AOR, 7.9; 95% CI: 2.3-27.2); and shock requiring vasopressor therapy (AOR, 3.4; 95% CI: 1.3-8.7). After adjusting for these confounders, patients who had an emergency colectomy were less likely to die than those treated medically. Colectomy also seemed more beneficial in patients 65 years or older; in those who were immune-competent; and those with leukocytosis $\geq 20 \times 10^9/L$ or a serum lactate level between 2.2 and 4.9 mmol/L.

Small-bowel involvement with *C. difficile* enteritis is unusual^[101]. Potential risk factors for small-bowel involvement with *C. difficile* enteritis include prior gastrointestinal surgery (including colonic resection) and advanced age^[102]. Such patients may present with ileitis and high ileostomy output and may be at increased risk for fulminant disease.

Small-bowel involvement with *C. difficile* infection enteritis has been described increasingly since 2000. Usually, this occurs in patients with a history of a prior colectomy or total procto-colectomy for severe and extensive IBD. The ileal mucosa appears to be at increased risk for inflammatory disease in the specific subset of patients who have undergone a prior colectomy^[67]. Serious post-colectomy concerns, like severe ileostomy dysfunction with high ileostomy volumes and marked diarrhea, have been known to occur after pan-procto-

colectomy and restorative ileo-anal pouch formation. They are almost always due to a non-*C. difficile* enteritis. This non-CDAD post colectomy enteritis can be life threatening; fortunately it is steroid/ immunosuppressive responsive, according to Gooding *et al*^[103]. This picture can be mimicked by *C. difficile* infection.

Lundeen *et al*^[104] reported that high ileostomy volumes may result from *C. difficile* enteritis in patients who have undergone colectomy for ulcerative colitis. All of the ileostomy output was positive for *C. difficile* toxins. These patients responded to metronidazole and/or vancomycin, in contrast to subjects with the former, non-CDAD entity.

Refractory or treatment-resistant pouchitis also may occur with *C. difficile* infection^[105]. *C. difficile* infection involving ileal pouch-anal anastomosis is common, and occurs with or without the previous receipt of antibiotics^[99]. Diagnosing recurrent *C. difficile* infection can be difficult in this group of patients, especially in the 20% without diarrhea.

Laboratory confirmation

All health care facilities must develop rapid communication between the laboratory and the treating physician. At the Mayo Medical School, the time between electronic medical record reporting of a positive result for a test for *C. difficile* toxin in stool and the ordering of antimicrobial therapy was compared during consecutive periods when results were not telephoned ($n = 274$) and when results were telephoned ($n = 90$) to the clinical service^[106]. The mean times to the ordering of antimicrobial therapy were 11.9 and 3.6 h, respectively ($P < 0.001$). The clinical implications of this 8-h delay may be important, especially in patients with severe disease. Early recognition of CDAD caused by NAP1/027, followed by the initiation of rapid treatment, can help to prevent complications and further spread of the bacterium^[107].

Current laboratory testing lacks a single assay that is sensitive, specific, and rapid. Peterson *et al*^[108] used clinical criteria that required at least three loose stools in one day, as part of the reference standard for a positive test result supporting CDAD (Table 2). They found that real-time PCR and anaerobic culture assays were significantly more sensitive than the enzyme immunoassay ($P < 0.01$ to $P < 0.05$). Real-time PCR has an assay turnaround time of < 4 h, and is both more sensitive than, and as rapid as enzyme immunoassay. They feel that it is a feasible laboratory option to replace enzyme immunoassay for toxigenic *C. difficile* detection in clinical practice, as well as for use during the development of new therapeutic agents.

Tests for the presence of *C. difficile* and its toxins are imperfect, and false positives and false negatives are not uncommon. McFarland^[30] found that false-negative results occur in 29%-56% of cases. False-negative results may occur when specimens are not promptly tested or not kept refrigerated until testing is performed. Also, there is a relatively high false-negative rate, due to the fact that 100-1000 pg of toxin must be present for an EIA test to be positive. Utilizing up to three serial EIA tests may increase the diagnostic yield by as much

Table 2 Laboratory diagnosis of *C. difficile*

Test	Sensitivity (%)	Specificity (%)	PPV	NPV
Enzyme immunoassay	73	98	73	98
Real-time PCR	93	97	76	99
Cell culture assay	77	97	70	98
Anaerobic culture for toxigenic <i>C. difficile</i> strains	100	96	68	100

Peterson LR, Manson RU, Paule SM, Hacek DM, Robicsek A, Thomson RB Jr, Kaul KL. Detection of toxigenic *Clostridium difficile* in stool samples by real-time polymerase chain reaction for the diagnosis of *C. difficile*-associated diarrhea. *Clin Infect Dis* 2007; 45(9): 1152-1160.

Table 3 Diagnosis of *C. difficile*

Enzyme immunoassay for toxins A & B - 80% sensitive Use 3 samples Cytotoxicity assay-more sensitive and specific, but takes 24-48 h

as 10 percent, if the initial test is negative. If CDAD is suspected, despite negative initial testing, submission of multiple specimens and verifying that the laboratory is testing for both the A and B toxins is mandatory (Table 3).

Enzyme immunoassays are labor-intensive tests, requiring several hours of technician time and an assay reader. The batching of specimens increases cost efficiency, but may delay the reporting of results, especially if tests are not done every day. Rapid enzyme immunoassay is more costly for each test performed but, for laboratories that process only occasional samples, it appears to provide prompt, reliable, and cost-effective results.

Enzyme immunoassay rapid cards have been evaluated, in terms of their ability to detect *C. difficile* toxins A and B. For one such card, the EIAPrem, the positive predictive value (PPV) was 75/85 samples (88.2%; CI: 79% to 94%) and the negative predictive value (NPV) was 360/361 samples (99.7%; CI: 98% to 99%). For a review of all card performances, see references^[109-112].

Killgore *et al*^[113] compared the results of analyses done with seven *C. difficile* typing techniques: multi-locus variable-number tandem-repeat analysis (MLVA); amplified fragment length polymorphism; surface layer protein A gene sequence typing; PCR-ribotyping; restriction endonuclease analysis (REA); multi-locus sequence typing; and pulsed-field gel electrophoresis (PFGE). All techniques appeared to be capable of detecting outbreak strains; but only REA and MLVA exhibited sufficient discrimination to distinguish strains from different outbreaks.

Rapid laboratory tests

Comparison of four enzyme immunoassays (Bartels Prima System *C. difficile* Toxin A EIA, Cambridge Biotech Cytoclone A+B EIA, Meridian Diagnostics Premier *C. difficile* Toxin A EIA, and TechLab *C. difficile* Tox-A Test EIA) found that, although enzyme immunoassays

were less sensitive than cytotoxin assay, they provide same-day results and may be useful in laboratories without tissue culture facilities^[114].

ELISA Toxin A+B is a reliable method with 100% specificity and sensitivity in the rapid diagnosis of *C. difficile*. Its results can be utilized until culture results are obtained. The specificity of the Toxin A latex test is 100%; however, its use alone as a primary rapid diagnostic test is not recommended, because of its low (30.7%) sensitivity. This was shown when all of the culture positive samples underwent testing by ELISA Toxin A+B method and were found to be 100% positive, but only four of these positive culture samples (30.7%) yielded positive results with the Toxin A latex test^[115].

Overall, the new-generation assays still are less sensitive than the cytotoxin assay; however, their advantages are that they provide same-day results; they can be used as a screening test; and they may be useful in laboratories without tissue-culture facilities. Results from a study by Vanpoucke *et al*^[111] could not recommend one single assay over the other for the diagnosis of CDAD.

Therefore, the cytotoxin assay test (CYTA) is highly sensitive and specific, but it is difficult to perform, and results are not available for 24-48 h^[115]. What further complicates efforts to determine if toxin was present on admission is that *C. difficile* toxin is very unstable. The toxin degrades at room temperature and may be undetectable within 2 h after collection of a stool specimen. Given the cost and complexity of culture and cytotoxicity assays, most laboratories rely on tests for toxin A detection only. Moreover, enzyme immunoassays generally are available at lower cost and provide more rapid results, usually within 4 h. Their sensitivity generally ranges from 60% to 90%, and specificity from 75% to 100%. Testing of a single diarrheal stool generally is sufficient to make the diagnosis of CDAD; but unfortunately, doing so misses a substantial proportion of cases. Therefore, testing only should be performed on three loose stool specimens.

The cytotoxin assay test, though the 'gold standard' for assaying *C. difficile* toxins A and B, is labor-intensive, requires tissue-cultured cells and an inverted microscope, and needs overnight incubation before results can be read.

TREATMENT OF THE NEW VIRULENT STRAIN OF CDAD

Recent experience has not altered the principles of management for the individual patient, but it does serve to emphasize the need for: (1) recognition of clinical characteristics that indicate severe CDAD (Table 4); (2) early recognition of *C. difficile*; (3) improved methods to manage severe relapsing disease; and (4) greater attention to infection control and antibiotic restraint. Previously published *C. difficile* infection management is available: [Fekety "Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis" *Am J Gastroenterol*, 1997; 92(5): 739-750] and the CDC's own

Table 4 CDAD severe disease

Patient characteristics
Older patients (> 65 yr)
Presence of comorbid conditions
Immune compromising conditions
Systemic immune response syndrome
Organ failure
Renal
Respiratory
Hypotension
Laboratory markers
Marked leukocytosis > 15 000
Renal failure Cr > 2.3 mg/L
Hypoalbuminemia
Extent of disease
Pancolitis by imaging modalities
Complications
Ileus
Toxic megacolon
Intestinal perforation

Any one of the above calls for classification as 'severe disease', using the authors' approach.

guidelines found at http://www.cdc.gov/ncidod/dhqp/id_CdiffFAQ_HCP.html and at <http://www.cdc.gov/ncidod/dhqp/pdf/isolation2007.pdf>.

The efficacy of metronidazole or vancomycin prophylaxis to prevent *C. difficile* infection in patients who are receiving other antimicrobials is unproven, and treatment with these agents is ineffective against *C. difficile* in asymptomatic carriers^[116].

The usual treatment for *C. difficile*-associated disease has been to stop antibiotics being given for other purposes and immediately start treatment with metronidazole or vancomycin. Patients who remain on antibiotics while undergoing treatment of CDAD have a high likelihood of treatment failure with metronidazole^[117].

In 1983, before the virulent *C. difficile* epidemics, metronidazole and vancomycin were shown to have equivalent efficacy and relapse rates, and to be tolerated to a similar extent by patients with *C. difficile*-related diarrhea and colitis, but metronidazole was considerably more economical. Metronidazole was favored because the pharmacy cost for the dosage used was \$387.48 to \$520.00 for vancomycin and \$11.84 for metronidazole^[118].

Findings from another study suggest that metronidazole and vancomycin are equally effective for the treatment of mild CDAD, but that vancomycin is superior for treating patients with severe CDAD. Among the patients with mild CDAD, treatment with metronidazole or vancomycin resulted in clinical cure in 90% and 98% of the patients, respectively ($P = 0.36$). On the other hand, among the patients with severe CDAD, treatment with metronidazole or vancomycin resulted in clinical cure in 76% and 97% of the patients, respectively ($P = 0.02$). Clinical symptoms recurred in 15% of the patients treated with metronidazole and 14% of those treated with vancomycin^[119].

In order to reduce vancomycin resistance, current guidelines still recommend the first-line use of

metronidazole over vancomycin. However, the new strain of *C. difficile* may not respond as well to treatment with metronidazole, despite the absence of laboratory evidence of metronidazole resistance.

Comparison of the clinical and microbiological effects of vancomycin and metronidazole reveal that vancomycin-treated patients are more likely to develop undetectable levels of *C. difficile* (adjusted hazard ratio, 3.99; 95% CI: 1.41-11.3; $P = 0.009$) and to have resolution of diarrhea (adjusted hazard ratio, 4.17; 95% CI: 1.53-11.40; $P = 0.005$) during the first 5 d of therapy^[120].

Recent studies demonstrate a high rate of failure of metronidazole, due either to infection with NAP-1 or to the presence, in hospitals, of older and sicker adults who previously have been treated with many broad-spectrum antibiotics. This raises the question as to what drug should be used as the initial therapy of *C. difficile* infection. The standard of care seems to be shifting towards using vancomycin first, if one is facing either a virulent organism or if risk factors for severe disease or several risk factors are present, like advanced age, immune deficiency, or pre-existing IBD (Table 4).

In addition, the cure rate seems to be significantly higher with vancomycin than metronidazole (97% versus 76%). In clinical practice, there is a shift toward using oral vancomycin as initial therapy for severe CDAD; and some clinicians are endorsing vancomycin as the preferred therapy for moderate to severe disease caused by this new epidemic strain. Currently, the treatment for hypervirulent *C. difficile* strains appears to be no different than for other *C. difficile* infections, and includes oral vancomycin^[121].

Failure with metronidazole treatment may be attributable to a slower and less consistent microbiological response than that with oral the next sentence is deleted because it is repeated exactly from a previous paragraph. Vancomycin-treated patients are more likely to develop undetectable levels of *C. difficile* (adjusted hazard ratio, 3.99; 95% CI: 1.41-11.3; $P = 0.009$) and to have resolution of diarrhea (adjusted hazard ratio, 4.17; 95% CI: 1.53-11.40; $P = 0.005$) during the first 5 d of therapy^[120].

Freeman *et al.*^[122] found that duration of cytotoxin production by *C. difficile* ribotype 027 markedly exceeds that of ribotype 001. These findings may help to explain the increased severity of symptoms and higher case-fatality ratio associated with infections with *C. difficile* ribotype 027. The authors also found that sub-optimal gut concentrations of metronidazole, possibly due to inactivation by components of normal gut flora, are associated with continued toxin production. The persistence of *C. difficile* spores suggests that additional strategies to restore the normal colonic microflora also may be beneficial^[123]. However we must take this paradigm change from metronidazole to vancomycin as initial therapy with caution. Pépin *et al.*^[17,18] reported a large epidemic of CDAD in Quebec that included large numbers of patients with severe and complicated disease. They examined the relative efficacy of metronidazole and vancomycin in the wake of this hypervirulent strain. Pépin *et al.*^[17,18] described a greater incidence of severe

Table 5 Therapeutic approach to patients with severe *C. difficile* infection

Oral vancomycin, 500 mg <i>q.i.d.</i>
Substitute intracolonic vancomycin infusion if ileus and add metronidazole 500 mg <i>q.i.d.</i> , IV
Consider IV immunoglobulin therapy (400 mg/kg)
Surgical evaluation for acute abdomen

complications associated with CDAD (defined as 30-d mortality, sepsis, toxic megacolon, emergent colectomy, or intestinal perforation) with the coincident emergence of NAP1/027 in Quebec in 2003. They observed an overall 79% decrease in progression to severe complicated CDAD in patients initially treated with vancomycin, rather than metronidazole, between 1991 and 2003. They also noted that marked leukocytosis or renal failure predicted a significant risk of complications and mortality. In 2004, this led to a change in guidelines in Quebec, which recommended that oral vancomycin be used as initial treatment in patients with these markers of severity. In some cases, rectal vancomycin (0.5-1 g dissolved in 1-2 L of isotonic saline) can be given as a single 60-min retention enema every 4-12 h. Rifaximin administered as a 'chaser', after control of acute *C. difficile* infection with a standard 10-14-d course of vancomycin, appeared to prevent recurrence in seven of eight patients, even though they were rifaximin resistant^[124].

An albumin level < 2.5 g/L and ICU stay are predictors of failure of metronidazole therapy for CDAD. These patients may benefit from oral vancomycin therapy at the outset^[125].

Regardless of what therapy is used, patients should be monitored carefully to ensure that they are responding to therapy, and not developing complications. If deterioration is suspected, or if the patient fits the criteria for very severe disease or is immunosuppressed or elderly, it may be wise to utilize vancomycin initially (Table 5). Our approach to patients with suspected or known *C. difficile* infection is based on the severity of their illness (Figures 1 and 2).

Recurrent *C. difficile* infection

Twenty percent of *C. difficile* infection patients relapse, despite adequate therapy. Risk factors for relapse are presented in Table 6. Diagnosing recurrent *C. difficile* infection can be difficult, especially in the 20% without diarrhea. The usual treatment for recurrent *C. difficile* infection is a repeat course of metronidazole, unless the patient has severe disease. Tapered and pulsed dosing schedules of vancomycin have been investigated for the treatment of *C. difficile* infection that recurs after an initial course of vancomycin (Table 7). An example of an oral vancomycin taper schedule is as follows: 125 mg *qid* × 10-14 d; 125 mg *bid* × 7 d; 125 mg daily × 7 d; 125 mg once every 2 d × 8 d; and 125 mg once every 3 d × 15 d^[126]. The treatment of recurrent *C. difficile* infection with various vancomycin daily doses (2 g/d, 1 g/d, and 500 mg/d) and administration schedules (daily vancomycin followed by tapered or pulsed dose

Table 6 Risk factors for relapse (occurs in 10%-25% of cases¹)

Prolonged antibiotic usage
Prolonged hospitalization
Age > 65 yr
Diverticulosis
Comorbid medical condition(s)

¹Increased risk of relapse with increased number of relapses. Kelly CP, Lamont JT. Up-to-date May 2008.

Table 7 Therapeutic approach to patients with recurrent *C. difficile* infection

Second course of initial antibiotic, if the patient has mild/moderate disease; if severe disease, begin vancomycin
If recurrence after vancomycin, re-evaluate and treat with oral vancomycin and add tapering vancomycin regime and <i>s. boulardii</i>
If recurrence despite above, consider
Rifampicin
Cholestyramine
Fecal bacteriotherapy

vancomycin therapy) was reported by McFarland *et al*^[123]. They found that tapered and pulsed dosing schedules of vancomycin result in significantly better *C. difficile* infection cure rates than traditional vancomycin dosing.

Wenisch *et al*^[127] conducted a prospective, randomized study to compare the efficacy of the oral drugs fusidic acid, metronidazole, vancomycin, and teicoplanin in the treatment of CDAD. Treatment resulted in clinical cure greater than 90% with all the agents: 94% vancomycin, 96% teicoplanin, 93% fusidic acid, and 94% metronidazole. However, recurrent clinical symptoms occurred in 16% of patients treated with vancomycin or metronidazole, 7% of those treated with teicoplanin, and 28% of those treated with fusidic acid. There was asymptomatic carriage of *C. difficile* toxin in 13% of patients treated with vancomycin, 16% with metronidazole, 4% with teicoplanin and 24% with fusidic acid. No adverse effects related to therapy were observed with vancomycin or teicoplanin. Considering the costs of treatment, their findings suggest that metronidazole is the drug of choice for CDAD, and that glycopeptides should be reserved for patients who cannot tolerate metronidazole or who do not respond to treatment with this drug.

Probiotics

Studies on probiotics for *C. difficile* infection have been inconclusive and conflicting, with respect to treatment benefit. Nonetheless, the use of probiotics is becoming more widespread.

Pillai and Nelson conducted a meta-analysis to assess the potential therapeutic effects of probiotics for *C. difficile* infection^[128]. Randomized, prospective studies (1966-2007) using probiotics alone or in conjunction with conventional antibiotics for the treatment of documented *C. difficile* colitis were eligible for inclusion. Ultimately, four studies met the inclusion criteria and were included in the review. The four studies examined the use of probiotics

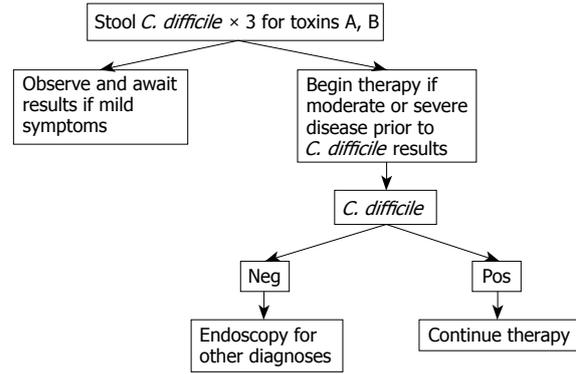


Figure 1 Approach to patients with suspected *C. difficile* infection.

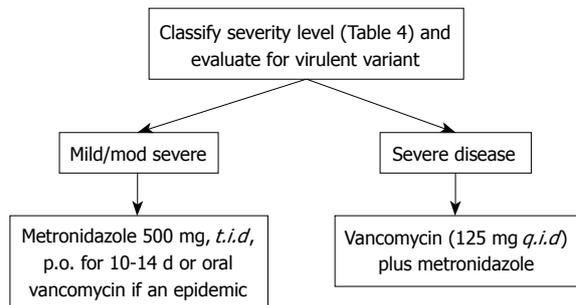


Figure 2 Initial therapeutic approach to patients with *C. difficile* infection.

in conjunction with conventional antibiotics (vancomycin or metronidazole) for the treatment of recurrence or the initial episode of *C. difficile* colitis in adults. All of the studies were small and had methodological issues. A statistically-significant benefit of probiotics combined with antibiotics was detected in only one study. The authors concluded that, overall, there is insufficient evidence to recommend probiotic therapy as an adjunct to antibiotic therapy for *C. difficile* colitis. There also is no evidence to support the use of probiotics alone in the treatment of *C. difficile* colitis.

In 1994, McFarland *et al*^[129] reported that patients receiving *Saccharomyces boulardii* were significantly less likely than patients receiving placebo to experience a recurrence of *C. difficile* diarrhea (RR 0.59; 95% CI: 0.35 to 0.98). Consequently, in a later meta-analysis, he compared the efficacy of probiotics for the prevention of AAD and the treatment of CDAD. Across 25 randomized controlled trials (RCTs), probiotics significantly reduced the relative risk of AAD (RR 0.43, 95% CI: 0.31 to 0.58, *P* < 0.001)^[130]. Across six randomized trials, probiotics had significant efficacy for CDAD (RR 0.59, 95% CI: 0.41 to 0.85, *P* = 0.005).

This time, McFarland *et al*^[129] concluded that a variety of different types of probiotic show promise as effective therapies for these two diseases. Again using meta-analysis, three types of probiotics (*S. boulardii*, *Lactobacillus rhamnosus* GG, and probiotic mixtures) were found to significantly reduce the development of AAD. Only *S. boulardii* was effective for CDAD.

Treatment of recurrent *C. difficile* infection with high-dose vancomycin plus *S. boulardii* is the only treatment

combination that has been evaluated in a prospective, randomized, controlled trial and found to generate a significant trend toward reduced recurrent *C. difficile* infection^[131]. *Lactobacillus* spp. have been evaluated for use in recurrent *C. difficile* infection, but data on regimens containing these organisms are poorly derived and conflicting. Fungemia with its administration has been reported in immunocompromised hosts. Therefore, its use is not appropriate in this group^[132].

Fecal bacteriotherapy especially for relapsing (recurrent) CDAD

Relapse of *C. difficile* occurs in 10%-25% of patients treated with metronidazole or vancomycin. Furthermore, multiple relapses may occur in the same patient. An alternative approach to patients with recurrent CDAD involves the administration of the entire fecal flora from a healthy individual, which is referred to as fecal bacteriotherapy. Borody *et al*^[133] reviewed 84 fecal transplantation therapies for severe cases of relapsing, or recurrent *C. difficile* infection (*via* various routes of administration). They found that 80% resulted in a good clinical response, resolution, or cure^[133]. A review of eight reports on the infusion of feces or fecal bacteria revealed an optimistic cure rate, without recurrence in most patients^[134]. In a study involving 18 patients treated with healthy donor stools *via* a nasogastric tube, 15 patients were recurrence-free at 90 d (two died of unrelated causes and one experienced recurrence)^[135]. The patients described in these reports^[133,136-144] included those with symptomatic relapse after receiving multiple courses of antibiotics; e.g. vancomycin, and/or metronidazole, and/or rifampicin together with cholestyramine. Case series have suggested a clinical benefit of fecal bacteriotherapy in patients with severe or recurrent CDAD who have failed to respond to standard approaches. Although the data are limited to case series, fecal bacteriotherapy has been used successfully to treat relapsing *C. difficile* infection. The precise mechanisms for the benefits of fecal bacteriotherapy are unclear. The reappearance of *Bacteroides* species after treatment suggests that *Bacteroides* species may be involved in the restoration of the presumably antibiotic-damaged flora in the colon.

Successful treatment with two or more fecal enemas has been described in other reports, according to Borody, Leis, & Gerald Pang (www.Up-to-date.com2008), involving a total of 23 patients with PMC who were refractory to antibiotic therapy, or who had experienced multiple relapses. In one study of 16 patients with severe, refractory disease treated over an 18-year period, 13 responded dramatically with decreases in diarrhea, temperature and leukocytosis. In a report describing nine patients, the single administration of a fecal enema (5-10 gm homogenized stool in pasteurized cow's milk) was effective in seven. In another case report, according to Borody, Leis, & Gerald Pang (www.Up-to-date.com), the one-time administration of bacteriotherapy was effective when 500 mL of fecal infusion in saline was delivered throughout the colon *via* a colonoscope. The authors hypothesized that the greater area of re-colonization by

fecal bacteria created a greater capacity to inhibit spore formation proximal to the splenic flexure. The use of the colonoscope to deliver fecal bacteria has an added theoretical advantage of permitting delivery of the active flora components to the distal small bowel, where *C. difficile* can reside. In addition, the colonoscope may permit the proximal delivery of flora in patients with a dilated colon, although colonoscopy must be performed extremely cautiously in this setting, because of the risk of perforation. One of the current authors (JSB) has utilized this modality with similar results.

Aas *et al*^[135] reported on 18 subjects who received donor stool by nasogastric tube for recurrent *C. difficile* infection over a 9-year period at a single institution. During the period between the initial diagnosis of *C. difficile* colitis and the stool treatments, the 18 subjects received a total of 64 courses of antimicrobials (range, 2-7 courses; median, three courses). During the first 90 d after receipt of treatment with stool, two patients died of unrelated illnesses. Only one of the 16 survivors experienced a single recurrence of *C. difficile* colitis over the 90-d follow-up. No adverse effects associated with stool treatment were observed. Patients with recurrent *C. difficile* colitis may benefit from the introduction of stool from healthy donors *via* a nasogastric tube.

Lund-Tønnesen *et al*^[140] reported on 18 patients with CDAD who were given homologous feces from one healthy donor. In 17 patients, feces were instilled *via* a colonoscope, and in one patient *via* a gastric stoma. Fifteen patients were clinically cured, and no relapses were observed; however, it is important to note that three patients with severe colitis did not respond to the treatment.

In recalcitrant, recurrent *C. difficile* infection, one should attempt initially to use probiotics that have been shown to be effective in published studies. Subsequently, in patients who remain seriously ill from recurrent *C. difficile* infection, fecal bacteriotherapy may be used when other approaches have been unsuccessful^[133,145]. The above-mentioned study by Lund-Tønnesen *et al*^[140], in which three patients with severe colitis did not respond to the treatment, while only the remaining less-severely ill patients were clinically cured, and no relapses were observed may indicate that this may serve as rescue therapy for patients with recurrent *C. difficile*. But its role in patients with severe *C. difficile* infection remains unproven.

Suggested protocol for fecal bacteriotherapy: Barody's protocol is as follows. (a) Donor stool and blood are screened for pathogens and viruses before infusion. CBC, serological testing for hepatitis A, B, and C; HIV-1 and HIV-2 and syphilis, stool culture for enteric bacterial pathogens, and light microscopy examination of stool sample for parasites and ova are performed. (b) The donor is clinically well, with the passage of normal, daily stools, and has had no intake of antibiotics for the last 6 mo. (c) The donor should not be a close relative, living in the same household such as a husband or child, theoretically to avoid use of flora from a silent carrier

of the same pathogen. (d) The recipient is evaluated for HIV and hepatitis markers to avoid future questions about transmission. (e) Oral vancomycin (500 mg twice daily for 7 d) is administered, and then followed by a single oral lavage with 3-4 L of polyethylene glycol with electrolyte purgative (such as GoLYTELY). (f) Although the lavage is skipped in patients too ill to tolerate it, vancomycin pretreatment is used, whenever possible. (g) 200-300 gm of donor stool suspended in 200-300 mL of sterile normal saline (homogenized briefly in a kitchen blender to a liquid consistency) is administered *via* an enema within 10 min of preparation, and this is repeated daily for 5 d. (h) Initial infusion may be filtered and infused *via* colonoscopy, preferably into the terminal ileum to address known ileal presence of *C. difficile*. (i) At least five consecutive days of rectal enemas are administered, using donor stools. (j) The enema should be retained for at least 6 h (loperamide pretreatment may help), followed by a high-fiber meal and overall diet. (k) Although some patients are unable to retain the enema initially for prolonged periods, it appears that coating of the mucosa by the infusate is adequate. (l) Adverse effects have been transient and mild, and have consisted primarily of abdominal gurgling, gas and borborygmi-expected post-enema symptoms. Recurrence has not been observed with follow-up of 1-3 years in most patients, even though a number of patients subsequently have required antibiotics for unrelated infections.

In summary, patients who develop a second episode of *C. difficile* infection after successful treatment of the first episode may be at increased risk for developing complications. Although different drugs and regimens have been used, vancomycin may be the best option; and the combination of high-dose vancomycin plus *S. boulardii* is the only treatment combination that has been evaluated in a prospective, randomized, controlled trial to demonstrate a significant trend toward reduced recurrent *C. difficile* infection. Fecal bacteriotherapy seems promising and is undergoing further testing at this time

NEWER ALTERNATIVE THERAPIES

Other therapeutic options for CDAD are being developed, and drugs used for other infections are being studied as alternatives to metronidazole and vancomycin.

Nitazoxanide, a nitrothiazolide and metabolic precursor of tizoxanide, has broad-spectrum activity against helminths and protozoa, as well as bacterial enteric pathogens, including *C. difficile*. It is marketed in the US and has been widely used throughout the world to treat parasitic diseases of the gastrointestinal tract; several million children have been treated with this drug over the past decade. Nitazoxanide is a US FDA approved drug that is used as an anti-protozoal agent for oral administration in pediatric patients, aged 1-11 years, with diarrhea. The drug acts by interfering with anaerobic metabolic pathways, and it has been shown to have excellent *in vitro* activity against *C. difficile*. An ongoing double-blind study comparing metronidazole with nitazoxanide for *C. difficile* infection involved the treatment

of 16 patients. The response rate for nitazoxanide was recently shown to be comparable to metronidazole for CDAD treatment in a prospective, randomized, double-blinded clinical trial^[146-148]. It is associated with fewer side effects than metronidazole, which should improve compliance.

Tinidazole is a structural analogue of metronidazole, with similar bioavailability (100%) and fewer drug-related adverse effects, but similar *in vitro* activity against *C. difficile*^[149,150].

OPT-80, previously known as tiacumicin B, and with the proposed name difimicin, is a novel 18-membered macrocycle antibiotic. It has little or no systemic absorption after oral administration, and a narrow activity spectrum against Gram-positive aerobic and anaerobic bacteria, and has tested well in patients with *C. difficile* infection^[151,152].

Rifalazil and rifaximin are rifamycin derivatives. Rifalazil is an orally-absorbed systemic antibiotic with a broad spectrum of activity that has been shown to prevent and treat CDAD recurrence in a hamster model. Rifaximin, a non-systemic antibiotic approved by the US FDA for travelers' diarrhea, currently is under evaluation for the treatment of CDAD^[153,154].

In unresponsive cases (e.g. those who have had no improvement after 3 d on metronidazole), one should add oral vancomycin, 500 g four times daily and intracolonic vancomycin (500 mg of IV vancomycin in 100 mL of normal saline per rectal Foley catheter, clamping for 60 min, repeating every 6 h). In addition, if there is an ileus, metronidazole can be given intravenously. While there is still no significant experience with nitazoxanide or rifaximin, these would be reasonable choices. As well, Pullman *et al*^[155] report that ramoplanin, a poorly-absorbed glycolipodepsipeptide that has been evaluated for the prevention of vancomycin-resistant enterococci, has good *in vitro* activity against *C. difficile*.

Teicoplanin may be a good choice, because some empirical evidence suggests that it is better than vancomycin for bacteriologic cure. It has borderline superior effectiveness in terms of symptomatic cure, but it is not readily available in the United States.

Therefore, in addition to nitazoxanide, bacitracin, teicoplanin, and fusidic acid, agents that have published efficacy, are several drugs, like rifaximin and PAR-101, which currently are under investigation. Other therapies, including polymers that bind *C. difficile* toxin, monoclonal antibodies to toxins, and preventative measures like toxoid vaccines, also are under study.

A role for monoclonal antibodies?

Taylor *et al*^[156] examined the safety and pharmacokinetics of a novel neutralizing human monoclonal antibody against *C. difficile* toxin A (CDA1) in 30 healthy adults whose median age was 27.5 years. While there were no serious adverse events related to its use, 21 of 30 reported non-serious adverse events were possibly related to CDA1. These included transient blood pressure changes requiring no treatment, nasal congestion, headache, abdominal cramps, nausea, and self-limited diarrhea. The authors concluded that, at least in healthy subjects, the

administration of CDA1 as a single intravenous infusion is safe and well tolerated.

Anion-binding resins

The importance of toxin production in the pathophysiology of *C. difficile* diarrhea has prompted consideration of anion-binding resins as a possible alternative to antimicrobial therapy. An advantage of resin therapy is that the bowel flora is not altered, as occurs with antibiotics (e.g. vancomycin or metronidazole), which may allow for more rapid reconstitution of normal colonic flora. Anion-exchange resins bind vancomycin as well as toxins; thus, the resin must be taken at least 2 h or 3 h apart from the vancomycin. Suggested regimens are colestipol (5 g every 12 h) or cholestyramine (4 g three or four times daily) for 1-2 wk, usually in conjunction with vancomycin.

Tolvamer, a novel toxin-binding polymer, has been developed to ameliorate *C. difficile*-associated disease without adversely affecting normal flora. Tolvamer has been tested for its ability to neutralize clostridial toxins produced by the epidemic BI/027 strains, thereby preventing toxin-mediated tissue culture cell rounding. The titers of toxin-containing *C. difficile* culture supernatants were determined using confluent cell monolayers, and then the supernatants were used in assays containing dilutions of tolvamer to determine the lowest concentration of drug that prevented $\geq 90\%$ cytotoxicity. Tolvamer neutralized toxins in the supernatants of all *C. difficile* strains tested. Specific antibodies against the large clostridial toxins TcdA and TcdB also neutralized the cytopathic effect, suggesting that tolvamer specifically neutralizes these toxins, and that the binary toxin (whose genes are carried by the BI/027 strains) is not a significant source of cytopathology against tissue culture cells *in vitro*^[157].

However, tolvamer is not FDA approved or commercially available, to date. Castanospermine has been identified as an inhibitor of the Rho/Ras-glucosylating *Clostridium sordellii* lethal toxin and *C. difficile* toxin B. Microinjection of castanospermine into embryonic bovine lung cells prevents the cytotoxic effects of toxins. The inhibitor binds in a conformation that brings its four hydroxyl groups and its N atom almost exactly into the positions of the four hydroxyls and the ring oxygen of the glucosyl moiety of UDP-glucose, respectively^[158]. It is in its early stage of development.

Vaccination

Testing the feasibility of active vaccination against *C. difficile* and its toxins in high-risk individuals currently is ongoing^[159]. *C. difficile* toxoid vaccine has induced immune responses to toxins A and B in patients with CDAD, and has been associated with resolution of recurrent diarrhea. This parenteral *C. difficile* vaccine, which contains toxoid A and toxoid B, has been reported to be safe and immunogenic in healthy volunteers. Three patients with multiple episodes of recurrent CDAD were vaccinated. Two of the three exhibited an increase in serum IgG antitoxin A antibodies (three- and four-fold

increases), and in serum IgG antitoxin B antibodies (52 and 20-fold). Both individuals also developed cytotoxin-neutralizing activity against toxins A and B. Prior to vaccination, the subjects had required nearly continuous treatment with oral vancomycin for 7, 9, and 22 mo, respectively, to treat recurrent episodes of CDAD. After vaccination, all three subjects discontinued treatment with oral vancomycin without any further recurrence. Thus, *C. difficile* toxoid vaccine induced immune responses to toxins A and B in patients with CDAD, and was associated with resolution of recurrent diarrhea.

Vaccination with a partially-purified preparation of inactivated toxins A and B is also undergoing current study. Several studies have shown that the humoral immune response of the host to *C. difficile* toxins A and B influences the clinical course of CDAD, as well as the risk of relapse^[160-162].

Another vaccine, containing toxoids A and B, has been shown to induce adequate antibody responses in healthy volunteers^[163]. The efficacy of this vaccine subsequently was evaluated in an open-label study involving three patients with recurrent *C. difficile* colitis^[159]. Following four intramuscular inoculations over an 8-wk period, all three patients discontinued antibiotic treatment without further recurrence over 6 mo of follow-up.

Immunoglobulin therapies

A retrospective review was performed on 264 *C. difficile* toxin-positive patients (November 2003-January 2005), which documented 14 patients with severe, refractory, recurrent *C. difficile* diarrhea who were treated with intravenous immunoglobulin (Flebogamma, 150-400 mg/kg)^[164]. Patients received a median of three (range, 1-5 g/L) courses of vancomycin or metronidazole before receiving intravenous immunoglobulin. All had hypoalbuminemia (median, 22 g/L; range, 18-33 g/L) and raised C-reactive protein (median, 47 mg/L; range, 25-255 g/L) at the time of infusion. The median white cell count was $15.3 \times 10^9/L$ (range, 4-24 g/L). Eight patients had evidence of pancolitis on abdominal imaging, suggesting severe *C. difficile* diarrhea. All patients tolerated intravenous immunoglobulin without side effects. Nine (64%) responded with bowel habits normalizing in a median of 10 (range, 2-26) d; one patient received two doses. One patient had a partial response from two doses, but died 2 mo later after a recurrence. Thus, intravenous immunoglobulin may be effective for severe, refractory, or recurrent *C. difficile* diarrhea after failed conventional treatment.

Surgery

In patients with *C. difficile* colitis, a progressive, systemic inflammatory state may develop that is unresponsive to medical therapy; some cases ultimately will progress to colectomy or death. *C. difficile* colitis is a significant and increasingly common cause of death. Surgical treatment of *C. difficile* colitis has a high death rate once the fulminant expression of the disease is present^[165]. These authors reviewed 2334 hospitalized patients with *C. difficile* colitis from January 1989 to December 2000.

In the setting of CDAD before the predominance of the hypervirulent strain, 64 patients died or underwent colectomy for pathology-proven *C. difficile* colitis. Unfortunately, those patients who underwent colectomy for *C. difficile* colitis had an overall death rate of 57%. Significant predictors of death after colectomy were preoperative vasopressor requirements and older age.

Fulminant *C. difficile* colitis is associated with a high mortality rate. As in the former study, Hall *et al*^[166] found that the development of a vasopressor requirement and the need for intubation are ominous signs which should lead to rapid surgical intervention. From 1998 to 2006, they studied a total of 3237 consecutive patients with *C. difficile* cytotoxin-positive stool samples. Commonly referenced indicators for surgical intervention were gathered on the day of surgery. The preoperative characteristics of patients surviving subtotal colectomy were compared with those who did not survive. They found that 36 patients underwent colectomy. Twenty-three patients (64%) were discharged from the hospital alive. Preoperative intubation and vasopressor requirement were risk factors for in-hospital mortality (OR: 7.15; 95% CI: 1.28-39.8 and OR: 6.0; 95% CI: 1.08-33, respectively). Patients who had a recent surgical procedure experienced a lower in-hospital mortality rate (OR: 0.11; 95% CI: 0.02-0.52).

In the setting of CDAD due to the hypervirulent strain, some patients have progressed from severe disease to death in less than 48 h. Emergency colectomy has prevented mortality in some patients with fulminant CDAD. The decision to perform an emergency colectomy remains largely empirical^[100]. In a retrospective observational cohort study of 165 cases of CDAD, among those patients who required ICU admission or prolongation of ICU stay between January 2003 and June 2005 at two tertiary care hospitals in Quebec, 53% died within 30 d of ICU admission, and almost half (44%) within 48 h of ICU admission. The independent predictors of 30-d mortality were: leukocytosis $\geq 50 \times 10^9/L$ (AOR: 18.6; 95% CI: 3.7-94.7), lactate ≥ 5 mmol/L (AOR: 12.4; 95% CI: 2.4-63.7), age ≥ 75 years (AOR: 6.5; 95% CI: 1.7-24.3), immunosuppression (AOR: 7.9; 95% CI: 2.3-27.2) and shock requiring vasopressors (AOR: 3.4; 95% CI: 1.3-8.7). After adjustment for these confounders, patients who had an emergency colectomy were less likely to die (AOR: 0.22; 95% CI: 0.07-0.67, $P = 0.008$) than those treated medically. Surgical intervention is indicated in the setting of peritoneal signs, severe ileus, or toxic megacolon; but colectomy also seems more beneficial in patients aged 65 years or older, in the immune competent, and in those with a leukocytosis $\geq 20 \times 10^9/L$ or serum lactate between 2.2 and 4.9 mmol/L.

The standard of care for patients undergoing emergency surgical intervention for CDAD is a total colectomy (with preservation of the rectum) and ileostomy, since primary anastomosis is not feasible acutely due to the pancolitis associated with severe disease. However, after colonic inflammation has subsided,

Table 8 Indications for emergency colectomy

Based upon
30-d mortality
Leukocytosis $\geq 20 \times 10^9/L$
Lactate ≥ 5 mmol/L
Age ≥ 75 yr
Immunosuppression
Shock requiring vasopressors
Especially in the presence of:
Toxic megacolon
Multi-organ system failure

Kelly CP, Lamont JT. Up-to-date May 2008.

closure of the ileostomy and ileorectal anastomosis can be performed (Table 8).

CONCLUSION

CDAD has increased in frequency and severity throughout North America and Europe over the last several years, largely due to the emergence of the NAP1 epidemic strain. This transformation of a formerly mild disease into one that can cause severe morbidity and mortality within a few days has challenged the entire approach to this suddenly serious infection. Institutions require accurate and rapid diagnostics for early detection of cases and possible outbreaks, in order to initiate specific therapy and implement early and effective infection control^[167].

Aggressive diagnostic and therapeutic interventions are warranted in the setting of *C. difficile* infection. Bedside sigmoidoscopy or colonoscopy may be performed to make a presumptive diagnosis of *C. difficile* infection, by evaluating for the presence of pseudomembranes. Given the risk of perforation, care should be taken to introduce minimal amounts of air to avoid exacerbating ileus or distention. The choice of initial drug therapy depends on severity of illness, co-morbidities, and strain suspicion. Prompt surgical consultation is warranted to assess the requirement for colectomy^[100].

C. difficile infection is a global problem. A comprehensive *C. difficile* infection control management rapid response team (RRT) is recommended for each health care facility throughout the world. A communications network between RRTs also is recommended, in coordination with each country's Department of Health. It is only through the implementation of the new approaches to its diagnosis, therapy and presentation that we can help to reduce the morbidity and mortality caused by this infection.

ADDENDUM I

Contact precautions: For patients with known or suspected *C. difficile*-associated disease

We must address environmental reservoirs to help to limit transmission. *C. difficile* has been cultured not only from patient bathrooms and bedpans, but from stethoscopes, blood pressure cuffs, and hospital furniture.

The initial step is identifying possible *C. difficile* patients, especially in long-term care facilities (LTCFs). Quinn *et al.*^[168] determined that only 111 facilities (42.2%) had a protocol to identify residents with *C. difficile* infection, and most (77.5%) did not test for *C. difficile* unless a resident had severe diarrhea. Only 58.5% of the facilities placed residents with *C. difficile* infection in private rooms, and 60.9% cohorted residents infected with *C. difficile* with other residents with *C. difficile* colonization or infection. Only 66 facilities (25.1%) had a program to control the use of antimicrobial agents.

Findings suggest that asymptomatic carriers of epidemic and non-epidemic *C. difficile* strains have the potential to contribute significantly to disease transmission in long-term care facilities. Thirty-five (51%) of 68 asymptomatic patients were carriers of toxigenic *C. difficile*, and 13 (37%) of these patients carried epidemic strains. Compared with non-carriers, asymptomatic carriers had higher percentages of skin (61% *vs* 19%; $P = 0.001$) and environmental contamination (59% *vs* 24%; $P = 0.004$). Eighty-seven percent of isolates found in skin samples and 58% of isolates found in environmental samples were identical to concurrent isolates found in stool samples. Spores on the skin of asymptomatic patients were transferred easily to investigators' hands. Previous *C. difficile*-associated disease ($P < 0.001$) and previous antibiotic use ($P = 0.017$) were associated with asymptomatic carriage, and the combination of these two variables was predictive of asymptomatic carriage (sensitivity, 77%; specificity, 58%; PPV, 66%; NPV, 70%)^[64].

In a prospective study of 27 patients with *C. difficile*-associated disease, it was found that *C. difficile* frequently contaminated multiple skin sites, including groin, chest, abdomen, forearms, and hands, and was easily acquired on investigators' hands. Skin contamination often persisted on patients' chest and abdomen after resolution of diarrhea. Thus, skin contact of the patient by a health-care worker is a means of *C. difficile* transmission^[169].

It is important to emphasize that asymptomatic fecal excretion of *C. difficile* is transient in most patients, and treatment with metronidazole is not effective. Although treatment with vancomycin is temporarily effective in asymptomatic carriers, it is also associated with a significantly higher rate of *C. difficile* carriage 2 mo after treatment and, therefore, is not recommended^[63].

An increase in hospital-acquired *C. difficile* infection rate was found at the University of Pittsburgh Medical Center. A comprehensive *C. difficile* infection control 'bundle' was implemented by hospital personnel to control the outbreak of *C. difficile* infection. This *C. difficile* infection control bundle consisted of education, increased and early case-finding, expanded infection-control measures, the development of a *C. difficile* infection management team, and antimicrobial management. Process measures, antimicrobial usage, and hospital-acquired *C. difficile* infection rates were analyzed, and *C. difficile* infection isolates were typed. The rates of compliance with hand hygiene and isolation were 75% and 68%, respectively.

The *C. difficile* infection management team evaluated a mean 31 patients per month (11% were evaluated for moderate or severe disease). The use of antimicrobial

therapy associated with increased *C. difficile* infection risk decreased by 41% during the period 2003-2005. The aggregate rate of *C. difficile* infection during the period 2001-2006 decreased to 4.8 infections per 1000 HDs; and, by 2006, it had decreased to 3.0 infections per 1000 HDs, a rate reduction of 71%. During the period 2000-2001, the proportion of severe *C. difficile* infection cases peaked at 9.4% (37 of 393 *C. difficile* infections were severe); this rate decreased to 3.1% in 2002 and further decreased to 1.0% in 2006, a 78% overall reduction. In 2005, 13% of *C. difficile* isolates were type BI (20% were hospital acquired), which represented a significant reduction from 2001. These authors concluded that the outbreak of *C. difficile* infection with the BI strain in hospital was controlled after implementing this infection control 'bundle'. Thus, early identification, coupled with appropriate control measures, reduces the rate of *C. difficile* infection and the frequency of adverse events. However, it requires a multipronged approach.

Methods of contact precautions and control: (1) Place patients with *C. difficile* in private rooms. (2) If private rooms are not available, place these patients in rooms with other patients who have *C. difficile*-associated disease. (3) Perform hand hygiene procedures preferably using soap and water-not alcohol. To reduce the transmission of *C. difficile* spores, environmental disinfection with 10% sodium hypochlorite and hand-washing with soap and water can be effective at removing the spores from hands and surfaces.

Strict antiseptic procedures should be followed by health care workers in contact with the patient, and these procedures should include the use of disposable gloves, and a mask and gown. Because alcohol is ineffective at killing *C. difficile* spores, health care workers must frequently wash their hands with soap and water, rather than with alcohol-based waterless hand sanitizers, especially when caring for CDAD patients. Patient-care equipment (e.g. blood-pressure cuffs, stethoscopes and thermometers) should either be used only for the infected patient or cleaned well before they are used with another patient.

Enhanced environmental cleaning following a regular schedule with dilute bleach should be used to eliminate *C. difficile* spores from all patient contact surface areas. These spores may remain on infected surface areas for months or even years.

In addition, note the ability of the vegetative form of *C. difficile* to survive on moist surfaces. On dry surfaces, vegetative *C. difficile* cells die rapidly, whereas they remained viable for up to 6 h on moist surfaces in room air. This illustrates the importance of washing and drying room surfaces when cleaning contaminated rooms.

A very important method of controlling outbreaks of *C. difficile*-associated disease should be restricting the use of antimicrobial agents that have been implicated as risk factors for the disease, as recommended by Gerding *et al.*^[116]. Davey *et al.*^[170] documented that interventions to improve antibiotic prescribing practices to hospital inpatients can be successful, and that they can reduce antimicrobial resistance and the rates of hospital-acquired infections.

Control of fluoroquinolone use

Effective surveillance of antibiotic-resistant bacteria and CDAD must be intensified in every healthcare setting, but especially in long-term care and rehabilitation facilities. All these facilities must have easy laboratory access for prompt and active surveillance culturing and *C. difficile* cyto-toxin testing, for both A and B, at the earliest indication of any infection or CDAD. In addition, Furuno *et al*^[171] advise that those patients at higher risk for carriage of antibiotic-resistant bacteria should be identified early for active surveillance targeting-culturing for methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE); e.g. patients who report having had antibiotics or prior hospital admissions within the past year. The authors found that this was very cost-effective, saving a projected \$19 000-\$26 000 relative to non-directed hospital wide screening for resistant organisms (MRSA and VRE), during the 8-mo study period at their tertiary care facility. They also found that there often is a significant delay between the onset of CDAD symptoms and the full implementation of CDC contact precautions.

ADDENDUM II

Note that current Proper Hand Hygiene techniques for *C. difficile* differ from previous 2002 CDC Guidelines for hand hygiene in health-care settings, which were as follows.

IV.A.1. During the delivery of healthcare, avoid unnecessary touching of surfaces in close proximity to the patient to prevent both contamination of clean hands from environmental surfaces and transmission of pathogens from contaminated hands to surfaces.

IV.A.2. When hands are visibly dirty, contaminated with proteinaceous material, or visibly soiled with blood or body fluids, wash hands with either a non-antimicrobial soap and water or an antimicrobial soap and water.

IV.A.4. Wash hands with non-antimicrobial soap and water or with antimicrobial soap and water if contact with spores (e.g. *C. difficile* or *Bacillus anthracis*) is likely to have occurred. The physical action of washing and rinsing hands under such circumstances is recommended, because alcohols, chlorhexidine, iodophors, and other antiseptic agents have poor activity against spores.

Alcohol-containing hand disinfection products were recommended over soap and water in the control of most organisms of epidemiological importance^[172]. Alcohol, however, does not eradicate *C. difficile* spores, maintain both Bettin *et al*^[173] and Boyce *et al*^[174]. Thus, what hand cleaning method to use in the presence of *C. difficile* infection is controversial. Papers conflict on the subject of alcohol eradicating *C. difficile* spores.

There even has been concern that the widespread use of alcohol-based hand sanitizers (instead of hand washing) has played a role in recent *C. difficile* outbreaks. Furthermore, because soap and water hand hygiene requires more time than alcohol-based hand hygiene, there is concern that alcohol-based hand hygiene may decrease overall effective hand hygiene compliance.

These concerns remain unproven. Overall CDAD rates have tended to decrease or remain the same after the introduction and increased use of alcohol-based sanitizers as the primary mode of hand hygiene^[175,176]. There is a lack of rigorous evidence, however, linking specific hand hygiene interventions with the prevention of health care associated infections (HCAIs). The varied nature of the interventions used and the diverse factors affecting the acquisition of HCAIs make it difficult to show any specific effect of hand hygiene alone. The most frequent methodologies currently used in this research area have been before-and-after observational studies without a control comparison group^[177]. However, the CDC recommends soap and water hand hygiene when caring for patients with CDAD.

In summary, if a facility is experiencing a *C. difficile* outbreak, it is prudent to emphasize that health care workers should frequently wash their hands with soap and water, in addition to using an alcohol-based hand sanitizer^[178].

The 2008 recommendations have been ambivalent, as seen below.

If your institution experiences an outbreak of *C. difficile*, consider using only soap and water for hand hygiene when caring for patients with *C. difficile*-associated disease; alcohol-based hand rubs are not as effective against spore-forming bacteria.

Current (Reviewed 3/08) CDC hand hygiene guidelines, available at <http://www.cdc.gov/handhygiene/> [7A] Accredited organizations are required to provide health care workers with a readily accessible alcohol-based hand rub product (CDC recommendations 8 C&D). However, use of an alcohol-based hand rub cleaner by any individual health care worker is not required. If you choose not to use it, then soap and water should be used instead.

In addition, use gloves when entering patients' rooms and during patient care; use gowns if soiling of clothes is likely; dedicate equipment, whenever possible.

Implement an environmental cleaning and disinfection strategy. Ensure adequate cleaning and disinfection of environmental surfaces and reusable devices, especially items likely to be contaminated with feces and surfaces that are touched frequently. Use an Environmental Protection Agency (EPA)-registered hypochlorite-based disinfectant for environmental surface disinfection after cleaning, in accordance with label instructions; generic sources of hypochlorite (e.g. household chlorine bleach) also may be appropriately diluted and used. Follow the manufacturer's instructions for the disinfection of endoscopes and other devices. Infection control practices in long-term care and home health settings are similar to those practices taken in traditional health-care settings.

How to clean and disinfect surfaces and devices according to the CDC's evidence-based guidelines for the prevention of CDAD (as reported at http://www.cdc.gov/ncidod/dhqp/id_CdiffFAQ_HCP.html). (1) Surfaces should be kept clean, and body substance spills should be managed promptly, as outlined in the CDC's 'Guidelines for Environmental Infection Control in Health-Care Facilities'. (2) Hospital cleaning products can be used for

routine cleaning. (3) Hypochlorite-based disinfectants have been used with some success for environmental surface disinfection in those patient-care areas where surveillance and epidemiology indicate ongoing transmission of *C. difficile*. (4) Consult the aforementioned guidelines for the use conditions for generic sources of hypochlorite-based products (e.g. household chlorine bleach) for disinfection of environmental surfaces. Note: EPA-registered hospital disinfectants are recommended for general use, whenever possible, in patient-care areas. At present, there are no EPA-registered products with specific claims for inactivating *C. difficile* spores, but there are a number of registered products that contain hypochlorite.

If an EPA-registered proprietary hypochlorite product is used, consult the label instructions for proper and safe use conditions. The literature supports the role of environmental disinfection with unbuffered hypochlorite solutions (diluted 1:10)^[179].

Fawley *et al.*^[180] studied the differences between the activity of various cleaning agents and germicides against *C. difficile* spores and the potential for some of these products to promote sporulation. When used at recommended working concentrations, only chlorine-based germicides were able to deactivate *C. difficile* spores. *C. difficile* epidemic strains had a greater sporulation rate than non-epidemic strains. The mean sporulation rate, expressed as the proportion of a cell population that is in spore form, was 13% for all strains not exposed to any cleaning agent or germicide, and it was significantly increased by exposure to cleaning agents or germicides containing detergent alone (34%), a combination of detergent and hypochlorite (24%), or hydrogen peroxide (33%). By contrast, the mean sporulation rate did not change substantially after exposure to germicides that contain either a combination of detergent and dichloroisocyanurate (9%) or dichloroisocyanurate alone (15%).

A study by White *et al.*^[181] revealed that all floor cleaning methods reduce the overall microbial load, though high counts and bacterial pathogens occasionally persist despite cleaning. Spray cleaning yielded marginally better results than traditional mopping and vacuuming. Wet scrubbing significantly reduced levels of coagulase-positive staphylococci ($P = 0.03$), which, in combination with routine methods, produced an effect that persisted for at least a week. Any sudden change in CDAD incidence in any medical institution should be reported immediately to public health officials.

The use of copper surfaces within the clinical environment and the application of a germination solution in infection control procedures may offer a novel way by which to eliminate *C. difficile* from contaminated surfaces and reducing CDAD^[182].

Three novel copper-based biocidal formulations, but not their components (copper sulfate and inorganic binders), were found by Gant *et al.*^[183] to have potent activity against organisms highly relevant to healthcare-associated infections, and all were active against *C. difficile* spores. This biocidal activity was not achieved by copper sulfate or the inorganic binders used in the formulations.

Table 9 Strength of recommendation and quality of evidence

Category/grade	Definition
Strength of recommendation	
A	Good evidence to support a recommendation for use
B	Moderate evidence to support a recommendation for use
C	Poor evidence to support a recommendation
Quality of evidence	
I	Evidence from ≥ 1 properly randomized, controlled trial
II	Evidence from ≥ 1 well-designed clinical trial, without randomization; from cohort or case-control analytic studies (preferably from > 1 center); from multiple time series; or from dramatic results from uncontrolled experiments
III	Evidence from opinions of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees

Adapted from Canadian Task Force on the Periodic Health Examination. *Can Med Assoc J* 1979; 121(9): 1193-1254.

All three copper-based formulations completely decontaminated UMF cloths containing MRSA, ACCB or *C. difficile* spores, suggesting that any of these copper-based formulations could be highly beneficial in the healthcare environment. None of the three copper-based formulations or copper sulfate was cytotoxic to human epithelial cells, up to concentrations of 100-200 ppm.

ADDENDUM III

C. difficile infection rates are rising (Figures 3 and 4). The most recent guidelines on *C. difficile* infection based on the strength of recommendations and quality of evidence were issued on 9 October 2008. This was issued as part of the latest updated guidelines for hospital acquired infections by the American Hospital Association, the Joint Commission on Accreditation of Health Organizations, the Infectious Diseases Society of America, the Society for Healthcare Epidemiology of America, and the Association for Professionals in Infection Control and Epidemiology in-“A Compendium of Strategies to Prevent Healthcare-Associated Infections in Acute Care Hospitals.” *Infect Control Hosp Epidemiol* 2008; 29: S12-S21 (Authors-Yokoe, Mermel, Anderson, Arias, Burstin, Calfee, Coffin, Dubberke, Fraser, Gerding, Griffin, Gross, Kaye, Klompas, Lo, Marschall, Nicolle, Pegues, Perl).

(1) Basic practices for prevention and monitoring of *C. difficile* infection

Recommended for all acute care hospitals using strength of recommendations and quality of evidence from Table 9.

A. Components of a *C. difficile* infection prevention program: (1) Use contact precautions for infected patients, with a single-patient room preferred (A-II for hand hygiene, A-I for gloves, B-III for gowns, and B-III for single-patient room). (2) Ensure cleaning and disinfection of equipment and the environment (B-III

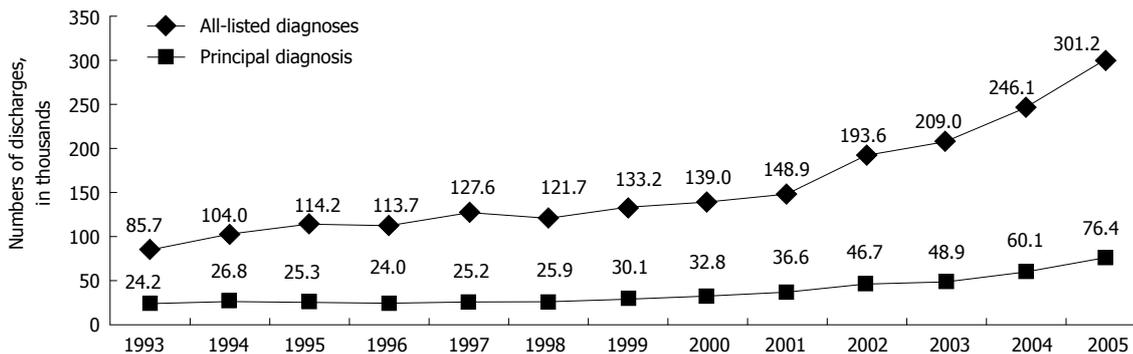


Figure 3 Trends in hospital stays associated with *C. difficile*-associated disease, 1993-2005. (From Elixhauser and Jhung^[82]) shows the trend in CDAD from 1993 through 2005. During the 8-year period from 1993 until 2001, the total number of hospital discharges with CDAD increased from approximately 85 700 to 148 900 per year, 74% increase. However, during the following 4-year period, from 2001 to 2005, the rate of increase for CDAD escalated, when the numbers of cases more than doubled to 301 200 (a 102 percent increase in 4 years). There were a total of 2 037 900 hospital discharges with CDAD over this 12-year period.

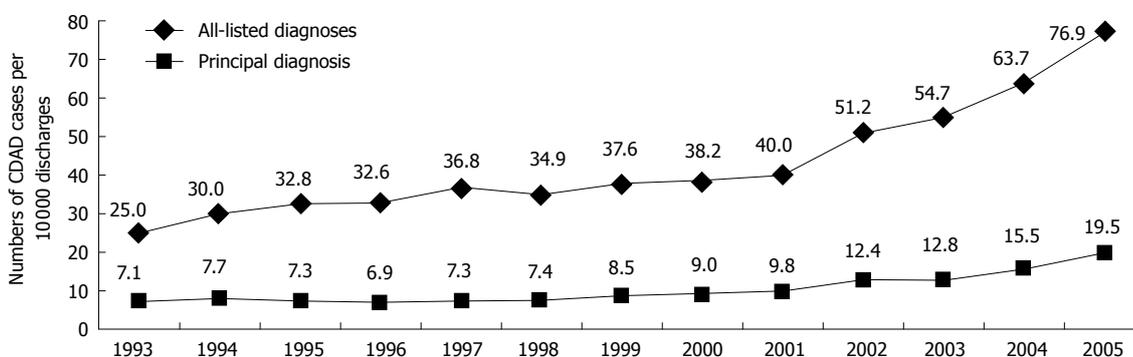


Figure 4 Discharge rate for *C. difficile*-associated disease, per 10 000 hospital discharges, 1993-2005. (From Elixhauser and Jhung^[82]) shows the number of CDAD discharges per 10 000 hospital discharges from 1993 through 2005. The findings are similar to those of the previous figure. From 1993 to 2001, the rate of CDAD per 10 000 discharges increased by 60% while the rate of increase from 2001 to 2005 was considerably steeper, 92%. Thus, the recent sharp rise in CDAD was not attributable solely to an increase in the number of hospital discharges.

for equipment and B-II for the environment). (3) Implement a laboratory-based alert system to provide immediate notification to infection prevention and control personnel and clinical personnel about patients with newly diagnosed *C. difficile* infection (B-III). (4) Conduct *C. difficile* infection surveillance and analyze and report *C. difficile* infection data (B-III). (5) Educate healthcare personnel, housekeeping personnel, and hospital administration about *C. difficile* infection (B-III). (6) Educate patients and their families about *C. difficile* infection, as appropriate (B-III). (7) Measure compliance with CDC or World Health Organization hand-hygiene and contact precaution recommendations (B-III).

(II) Special approaches for the prevention of *C. difficile* infection

Perform a *C. difficile* infection risk assessment. These special approaches are recommended for use in locations and/or populations within the hospital for which outcome data and/or risk assessment suggest lack of effective control despite implementation of basic practices.

A. Approaches to minimize *C. difficile* transmission by healthcare personnel: (1) Intensify the assessment of compliance with process measures (B-III). (2) Perform hand hygiene with soap and water as the preferred method

before exiting the room of a patient with *C. difficile* infection (B-III). (3) Place patients with diarrhea under contact precautions while *C. difficile* test results are pending (B-III). (4) Prolong the duration of contact precautions after the patient becomes asymptomatic until hospital discharge (B-III).

B. Approaches to minimize *C. difficile* infection transmission from the environment: (1) Assess the adequacy of room cleaning (B-III). (2) Use sodium hypochlorite (bleach)-containing cleaning agents for environmental cleaning. Implement a system to coordinate with the housekeeping department if it is determined that sodium hypochlorite is needed for environmental disinfection (B-II).

C. Approaches to reduce the risk of *C. difficile* infection acquisition: Initiate an antimicrobial stewardship program (A-II).

(III) Approaches that should not be considered a routine part of *C. difficile* infection prevention

(1) Do not test patients without signs or symptoms of *C. difficile* infection for *C. difficile* (B-II). (2) Do not repeat *C. difficile* testing at the end of successful therapy for a patient recently treated for *C. difficile* infection (B-III).

ADDENDUM IV

***C. difficile* infection as a unique infectious problem**

In the US, The Deficit Reduction Act of 2005 (P.L. 109-171) requires the Centers for Medicare & Medicaid Services (CMS), the US federal agency which administers Medicare, Medicaid, and the State Children's Health Insurance Program to deny the assignment of a case to a higher DRG (payment to a health care facility) based on the occurrence of one of a selected number of hospital-acquired conditions, if that condition was acquired during the hospitalization. This rule is named CMS-1390-P: Medicare Program; Proposed Changes to the Hospital Inpatient Prospective Payment Systems and Fiscal Year 2009 Rate - Provisions on Preventable Hospital-Acquired Conditions Including Infections. The US Congress requires CMS to select conditions that are high cost, high volume, or both; assigned to a higher paying DRG when present as a secondary diagnosis; and reasonably preventable through the application of evidence-based guidelines.

In its original ruling, CMS had proposed adding nine hospital-acquired conditions, including *C. difficile* to the list of hospital acquired infections for which it proposed to deny payment. This would have taken effect on 1 October 2008. In July 2008, however, in response to an April 2008 letter sent by all three of the US major gastroenterology organizations, CMS, in a final rule setting policies and payment rates for the hospital setting, decided not to add *C. difficile* to the list of 'Hospital-Acquired Conditions for Which It Will Deny Payment'.

The gastroenterology organizations in its April 2008 letter to CMS focused on the last criterion necessary for 'non-reimbursement'-reasonably preventable through the application of evidence-based guidelines.

US gastroenterology organizations, in the letter to CMS, made much about the alleged fact that alcohol-based products are effective against the majority of microorganisms other than (i.e. not for) *C. difficile*, with the statement that "Alcohol-based products, in compliance with CDC guidelines, have played a significant role in potentially complicating efforts to avoid the spread of CDAD."

"Indeed", stated the letter, quoting Shen *et al*^[184] "the proportion of all hand hygiene episodes performed with soap and water dropped from 90% to 15%, three years after the introduction of alcohol hand gels in one U.S. teaching hospital". The letter continued by stating "that the trade-off of higher overall compliance against more focused use of soap and water is one that CMS must consider given that 'CDC guidelines have played a significant role in potentially complicating efforts to avoid the spread of CDAD'. "In conclusion", ended the letter to CMS, "the ACG, AGA and ASGE urge CMS not to add *C. difficile* to its list of hospital-acquired conditions for which additional payment as a complicating condition would not be available. We strongly believe that the disease is not reasonably preventable. Adding it to the list would create a very expensive and unworkable situation for CMS, hospitals,

physicians and patients."

However, as we have seen in our above review of *C. difficile* infection, the fact is that the hand washing issue is controversial and basically not objectively ascertained with good RCTs. Nevertheless, one can agree that hand washing at least may be superior secondary to the mechanical shedding of *C. difficile* spores with vigorous hand washing.

One can agree with the arguments against adding *C. difficile* infection to the list of non-reimbursable hospital services because of its variable incubation period. Complicating the accurate diagnosis of CDAD is that, while symptoms typically occur within 48 h of infection, patients infected in the hospital with *C. difficile* usually become infected within 3 wk of admission. However, the onset of symptoms can be delayed by 2-3 mo^[30]. Also, although most cases of CDAD occur on days 4-9 of antibiotic therapy^[15], the subsequent diagnosis of CDAD is not always possible upon admission to the hospital, due to a variable incubation period. Therefore, one can agree that it is not reasonable to hold an inpatient hospital liable for a condition acquired in a different setting, one which is not even always detectable upon the patient's admission into their setting.

The CMS accepted these arguments against including *C. difficile* infection in those nosocomial infections that are not reimbursable. However, that still does not alleviate the responsibility of each healthcare facility to make aggressive attempts to counteract this problem. One can look to successful efforts made by others.

The University of Pittsburgh Medical Center developed a program, as mentioned in our review, consisting of education, increased early case finding, expanded infection-control measures, development of a *C. difficile* infection management team, and microbial management. The aggregate rate of *C. difficile* infection decreased from 7.2 infections per 1000 (9.4 during a peak) hospital discharges to 4.8 infections per 1000 hospital discharges, and later, was 3.0 infections per 1000 hospital discharges. The rates of compliance with hand hygiene and isolation were 75% and 68%, respectively^[185].

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Hugh James Freeman, MD, FRCPC, FACP, Series Editor

Malignancy in adult celiac disease

Hugh James Freeman

Hugh James Freeman, Department of Medicine (Gastroenterology), University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver V6T 1W5, Canada

Author contributions: Freeman HJ contributed all to this paper.
Correspondence to: Dr. Hugh James Freeman, MD, FRCPC, FACP, Department of Medicine (Gastroenterology), University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver V6T 1W5, Canada. hugfree@shaw.ca

Telephone: +1-604-8227216 Fax: +1-604-8227236

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Abstract

Prior studies have suggested that the incidence of some neoplastic disorders, particularly malignant lymphoma and small intestinal adenocarcinoma, are increased in celiac disease. Earlier studies from the United Kingdom have also suggested a link between celiac disease and esophageal carcinoma, although this has not been confirmed in North America. The risk of other gastrointestinal cancers seems to be limited. Gastric cancer does not appear to be detected more frequently, although direct endoscopic visualization of the upper gastrointestinal tract is now very common in patients with celiac disease. Colon cancer also appears to be limited in celiac disease, even in patients first diagnosed with celiac disease late in life. This has led to the hypothesis that untreated celiac disease may be protective, possibly owing to impaired absorption of fat or fat-soluble agents, including hydrocarbons and putative co-carcinogens implicated in the pathogenesis of colon cancer, which may be poorly absorbed and rapidly excreted.

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Key words: Adult; Celiac disease; Adenocarcinoma; Lymphoma; T cell enteropathy

Peer reviewer: Kazuichi Okazaki, Professor, Third Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka, 570-8506, Japan

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INTRODUCTION

Malignant disease is a serious concern in celiac disease^[1] and recently has been reviewed in detail^[2,3]. Some patients may even present with lymphoma^[4,5] or a small-intestinal adenocarcinoma^[6], and the celiac disease is only detected later. In others, malignancy, particularly lymphoma, complicates the clinical course of well established celiac disease, but may be especially difficult to diagnose^[7]. The precise risk of malignant disease in adult celiac disease is difficult to evaluate, but about 8%-10% with severe biopsy changes develop lymphoma^[8], and this figure has remained remarkably constant over several years^[9]. Age of first diagnosis of celiac disease seems to be a critical factor. In those first diagnosed late in life (and presumably, initiating a protective gluten-free diet much later), detection of lymphoma may be much higher^[8].

Mechanisms involved in development of malignant disease in celiac disease require elucidation. Significantly, however, the small-intestinal mucosa that is involved, with changes caused by celiac disease, may still pathologically respond to a gluten-free diet, even after lymphoma is detected^[4,5]. There are likely to be many potential confounding variables that alter the pathogenesis of lymphoma in a celiac population and influence risk measurements for various malignancies in different populations. These include genetic, geographic, infectious, and other epidemiological factors. Finally, the duration of gluten restriction and the degree of compliance with the gluten-free diet are specific factors that may be difficult to measure precisely, but seem crucial to malignant change in celiac disease.

DIAGNOSTIC DIFFICULTIES

Most lymphomas are detected in the small intestine, usually the jejunum, but ileal localization may also occur^[2]. Gastric or colonic lymphoma also occurs^[2]. Lymphomas are usually ulcerating lesions, or stenosing and obstructing tumors^[3]. Occasionally, the lymphoma may be multifocal or diffuse and localized only in the mucosa^[5]. Often, concomitant nodal involvement is present^[2]. The diagnosis may be especially difficult if small-intestinal (including duodenal) erosions and ulcers are present, as neoplastic cells may be more difficult to identify pathologically if significant superimposed inflammatory changes are present^[4]. In some, benign

ulcers may lead to a mistaken diagnosis of Crohn's disease or a label of "ulcerative jejunoileitis"^[4]. Some of these ulcers may contain frankly neoplastic lymphoma cells. Free perforation of the small intestine is a condition that should lead the clinician to a high level of suspicion of lymphoma in a patient with known or suspected celiac disease^[10]. Even if there is a very high degree of suspicion, lymphoma may be notoriously difficult to diagnose, despite multiple endoscopic or suction small-intestinal biopsies^[7]. In some patients that eventually prove to have lymphoma, even full thickness biopsies of the small intestine may not yield a definitive pathological diagnosis, especially if only mucosal disease is present. Additional tissue may be helpful for immunohistochemical labeling or PCR may be helpful in showing an altered binding pattern of antigen expression or a monoclonal cell population.

TYPES OF LYMPHOMA

Lymphoma may be classified based on pathological and immunophenotypical features. B-cell and T-cell lymphomas both occur in celiac disease. However, detection of a T-cell type more often leads to suspicion of underlying celiac disease. Primary intestinal T-cell lymphoma is recognized under the WHO classification as enteropathy-associated T-cell lymphoma (ETL or EATL). They are very uncommon and represent an estimated 5% of all gastrointestinal lymphomas^[3,11]. Previously, these were thought to be histiocytic in origin (and labeled malignant histiocytosis) but their origin now appears to be from T cells, specifically intra-epithelial lymphocytes^[3,11]. In celiac disease (without lymphoma), the intra-epithelial lymphocytes express the following antigens (among others): surface CD3 and CD8. In a subset of patients that seem clinically refractory to a gluten-free diet, intra-epithelial lymphocytes have a different form of T-cell phenotypic expression: CD3 shows intra-cytoplasmic expression while CD8 expression is absent. Some believe this may reflect a specific form of refractory celiac disease (type 2) with a poor prognosis and a possible precursor lesion for the development of lymphoma^[12-15].

Even lymphomas with T-cell immunophenotypic features have been detected in extra-intestinal sites, which complicates the clinical course of celiac disease. These may be very rare and include lymphoma diffusely involving the liver and spleen (i.e. hepatosplenic type T-cell lymphoma) without evidence of small-intestinal involvement^[16], or lymphoma in other embryologically related or gut-derived sites, such as the thyroid gland or broncho-pulmonary and pleural sites^[17].

Recent studies have also provided evidence for an increased risk of other lymphoma types. In a pathological review of tumor materials from celiac disease patients, there was an apparent aggregation of autoimmune disorders, female sex and B-cell lymphoma^[18]. More than double the risk for B-cell lymphoma was recorded, with

the most common type classified as a diffuse large B-cell lymphoma. In the same study, T-cell lymphomas had an approximately 50-fold risk along with a poorer prognosis (reflected in mean survival time after diagnosis and 5-year survival rate)^[18].

Recent studies have also evaluated risk of lymphoma in celiac disease. While the risk of lymphoma in celiac disease, especially of the T-cell type, is increased, the risk appears not to be as significant. The relative risk has been estimated to be close to 3 and likely is lower in clinically silent disease^[19].

OTHER GASTROINTESTINAL CANCER

Also intriguing are studies related to malignant disease elsewhere in the gastrointestinal tract. Small-bowel adenocarcinoma is increased in celiac disease. Normally, this is a rare tumor. Some have suggested that this carcinoma may be related to an adenoma-carcinoma sequence^[2], but the risk of duodenal adenoma may not be increased in celiac disease^[20]. Most patients appear to present with proximal small-intestinal localization, usually with small-bowel obstruction or bleeding. If complete surgical resection of a small-intestinal adenocarcinoma can be accomplished, the prognosis is better than if lymphoma is present^[21].

Some European studies have shown that there may be an increased risk of esophageal and pharyngeal carcinoma^[1,22]. However, these findings have not been confirmed in America. In one report^[8], only a single terminal hypopharyngeal squamous cell carcinoma was detected in a celiac disease patient with lymphoma. No esophageal or gastric cancer was detected, despite repeated endoscopic studies during the course of diagnosis and treatment of celiac disease. Interestingly, however, Barrett's esophagus, a known precursor lesion of esophageal adenocarcinoma was frequently detected^[8]. It may be that exposure to different environmental factors in different geographic areas or other confounding variables are important in cancer etiology and pathogenesis in celiac disease.

In a population-based cohort of celiac disease patients, overall colorectal cancer risk was marginally increased, owing to an increased risk in the ascending and transverse colon^[23], but not in dermatitis herpetiformis^[23]. However, others have noted that colorectal cancer may not be increased^[9], especially in celiac disease patients with a diagnosis established late in life^[9]. Possibly, untreated celiac disease is protective; dietary fat or fat soluble agents, including hydrocarbons or other putative co-carcinogens, may be implicated in the pathogenesis of colon cancer if poorly absorbed and rapidly excreted. Alternatively, immunological changes (e.g. increased intraepithelial lymphocytosis) in celiac disease may inhibit the development of epithelial malignancies at other gastrointestinal sites. Additional studies are needed to further clarify this information for celiac disease patients.

TREATMENT

Treatment of lymphoma associated with celiac disease to date has not substantially differed from lymphoma in the absence of celiac disease, and generally involves a combination of surgical treatment, radiation and chemotherapy. Most believe that the best treatment results occur in those diagnosed early^[24]. Biological agents are also being evaluated.

In newly diagnosed lymphoma patients with chronic diarrhea and weight loss, underlying celiac disease should be excluded, preferably prior to lymphoma treatment (since both radiation and chemotherapy may structurally alter the small intestine), because concomitant recognition of celiac disease may have important nutritional implications.

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REVIEW

Capsule endoscopy

Miguel Muñoz-Navas

Miguel Muñoz-Navas, Gastroenterology Department, University Clinic of Navarra, Pamplona 31008, Spain

Author contributions: Muñoz-Navas M wrote the paper.

Correspondence to: Miguel Muñoz-Navas, MD, Gastroenterology Department, University Clinic of Navarra, Pio XII 36, Pamplona 31008, Spain. mmunoz@unav.es

Telephone: +34-948-296730 Fax: +34-948-296500

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Abstract

Capsule endoscopy (CE) is a simple, safe, non-invasive, reliable technique, well accepted and tolerated by the patients, which allows complete exploration of the small intestine. The advent of CE in 2000 has dramatically changed the diagnosis and management of many diseases of the small intestine, such as obscure gastrointestinal bleeding, Crohn's disease, small bowel tumors, polyposis syndromes, *etc.* CE has become the gold standard for the diagnosis of most diseases of the small bowel. Lately this technique has also been used for esophageal and colonic diseases.

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Key words: Capsule endoscopy; Small intestine; Gastrointestinal hemorrhage; Crohn's disease; Gastrointestinal neoplasms; Intestinal polyposis

Peer reviewer: Dr. Klaus R Herrlinger, Hepatology and Endocrinology, Robert-Bosch-Hospital, Auerbachstrasse, 110, D-70376 Stuttgart, Germany

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INTRODUCTION

A few years ago, the assessment of small bowel pathology was a major dilemma, especially when it came to the management of obscure gastrointestinal bleeding. Evaluation of the patients was frequently unsatisfactory because of the inability to completely visualize the small bowel mucosa with the available endoscopic and

radiological techniques. Capsule endoscopy (CE) was launched at the beginning of this millennium and since then has had a very important impact on managing obscure gastrointestinal bleeding and many other small bowel diseases.

CAPSULE ENDOSCOPY

Until a few years ago, the small bowel was an organ which was very difficult to explore with the available endoscopic, radiological and nuclear medicine techniques. In routine practice, only the last few centimeters of the ileum were accessible to retrograde visualization by ileocolonoscopy. Exploration from the proximal side by push, sonde or intraoperative enteroscopy were invasive procedures that did not always allow us to visualize the lesions in the small bowel^[1]. Sonde enteroscopy had been abandoned in the 90's because it was a tedious technique (long duration of the procedure) and it had several technical limitations. Push enteroscopy is limited by the depth of insertion of the scope and is poorly tolerated. Intraoperative enteroscopy is the most effective of these techniques, but it is the most invasive with a significant percentage of adverse side effects^[2].

With wireless CE we can provide a simple, safe, non-invasive, reliable procedure, well accepted and tolerated by the patient, which has revolutionized the study of the small bowel. This technique evaluates endoscopically, with high resolution images, the whole small bowel, avoiding any sedation, surgery or radiation exposure^[2].

Currently, CE is recommended as a third stage examination, after negative gastroscopy and colonoscopy in patients with obscure gastrointestinal bleeding. Also many studies have established, with a growing body of evidence, that this technique is cost-effective in other clinical situations, such as detection of small bowel lesions in Crohn's disease in patients in which other methods have failed to provide a diagnosis, non-steroidal anti-inflammatory drug enteropathies, celiac disease, small bowel polyposis syndromes and small bowel tumors^[2]. Other possible indications are HIV patients with gastrointestinal symptoms^[3], malabsorptive syndromes other than celiac disease^[4], Henoch-Schonlein purpura^[5], patients with small bowel transplants^[6] and with intestinal graft *versus* host disease, particularly in monitoring the response to immunosuppressive therapy^[7].

The acquired knowledge of the wide range of lesions that can be found in the small bowel, encouraged the

implementation of some diagnostic and therapeutic techniques, such as double balloon enteroscopy, MRI-enteroclysis and CT-enteroclysis^[2].

The capsule endoscope is a disposable, small, swallowable, wireless, miniature camera which allows us to get a direct visualization of the gastrointestinal mucosa^[8]. The initial capsule endoscope was developed by Given Imaging (Yoqneam, Israel) and approved in Europe by the European Medicines Agency and in the United States by the Food and Drug Administration in 2001^[8]. This technique is available in over 4500 gastrointestinal centers throughout the world.

The capsule which measures only 11 mm × 26 mm and weighs 3.7 g, holds a metal oxide semiconductor imaging-chip video camera, 6 white light-emitting diode illumination sources, 2 silver-oxide batteries and a radio telemetry transmitter. The image field is 140 degrees, magnification is × 8 and the depth of view is 1 to 30 mm^[9,10].

Before the capsule is swallowed, 8 skin antennas are taped to the patient's anterior abdominal wall and connected to the hard drive. After an overnight fast, the patient swallows the capsule with a few sips of water, then the capsule is passively moved along by peristalsis. Two hours after ingestion, the patient is allowed to drink, while eating is allowed after 4 h. During the procedure the patient may carry on with his daily activities^[11].

The camera is activated by removal of the capsule from its magnetic holder and takes 2 images per second and transmits these by means of radio frequency to a sensor array placed on the patient's abdomen and from here to a recording device in a belt that the patient wears for the duration of the battery life (8 h). The use of the real time viewer may shorten procedures, as the patient can be disconnected once the cecum is visualized^[11].

After those 8 h, the sensor array and recorded data are removed and the recorded images are downloaded to the computer. It takes on average 40-60 min to read these images^[3,12] and since it is very time-consuming, one possible cost-effective strategy could be the use of expert nurse endoscopists to select images. Some studies have shown that highly motivated nurses and gastrointestinal residents trained to read CE can detect clinically significant lesions at a similar rate to physicians^[13-15]. Since its development, additional support systems have been added to the software to assist the reader, such as localization capability, suspected blood indicator, a multi-viewing feature and quick view modality^[3].

The capsule is excreted with the feces, usually within 24 to 48 h^[16]. CE is usually performed as an outpatient procedure. The presence of intestinal contents or a motility disorder may cause the incomplete visualization of the intestinal mucosa. Several studies have examined the possibilities of improving bowel cleanliness and shortening transit time by means of different medications and different fasting periods. Nevertheless, small bowel preparation is still a controversial issue^[6]. We have participated in a prospective multicenter randomized trial which has shown that bowel preparation with different laxatives does not improve the visualization of the small intestine^[17].

The main contraindication to performing CE is the suspicion or knowledge of an obstruction in the gastrointestinal tract.

The retention of the device is the main complication of the procedure and is defined when CE remains in the digestive tract for a minimum of 2 wk^[18]. The frequency of this problem varies, depending mostly on the clinical indication for CE, and ranges from 0% in healthy subjects, to 1.5% in patients with obscure gastrointestinal bleeding, to 5% in patients with suspected Crohn's disease^[6] and to 21% in patients with intestinal obstruction^[3]. At present CE has some technical limitations: it cannot be used to obtain biopsy specimens or for endoscopic treatment and it cannot be controlled remotely^[8]. CE has also some clinical limitations which are problems in sizing and locating small bowel lesions^[2], a possible false-negative CE result, due to the fact that the global miss rate is about 11%, ranging from 0.5% for ulcerative lesions to 18.9% for neoplastic disease and the fact that sometimes we can get findings of uncertain relevance in healthy subjects^[8]. Another drawback is that in almost 20% of procedures the capsule does not reach the cecum while it is active^[11].

Since its development, more than 650 000 capsules have been swallowed worldwide^[19] and more than 1000 peer-reviewed publications have appeared in the medical literature. The most important gastrointestinal societies have published guidelines about its use (ASGE^[20], ESGE^[21], BSG^[22]).

In latter years, breakthrough developments in CE technology have enabled the direct visualization of the upper^[23,24] and lower segments^[25,26] of the gut using specifically designed capsules.

In recent issues of this journal we have coordinated the publication of several papers, some covering the latest advances in this field, presenting the use of CE in diagnosis of gastrointestinal bleeding^[27], inflammatory bowel disease^[28], celiac disease^[29], neoplastic disease^[30], non-steroidal anti-inflammatory drugs-enteropathy and rare intestinal diseases^[31], and also its use in pediatric patients^[32]. Others studied the value of intestinal preparation before CE^[33], CE use in the colon^[34] and esophagus^[35], and the Patency and Agile^[36] capsules, and finally another paper was about the future of the CE^[37].

I wish to emphasize here that we have been very successful in convincing some of the most important groups working in this area to write the above-mentioned papers.

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Endoscopic ultrasound elastography for evaluation of lymph nodes and pancreatic masses: A multicenter study

Marc Giovannini, Thomas Botelberge, Erwan Bories, Christian Pesenti, Fabrice Caillol, Benjamin Esterni, Geneviève Monges, Paolo Arcidiacono, Pierre Deprez, Robert Yeung, Walter Schimdt, Hanz Schrader, Carl Szymanski, Christoph Dietrich, Pierre Eisendrath, Jean-Luc Van Laethem, Jacques Devière, Peter Vilmann, Adrian Saftoiu

Marc Giovannini, Thomas Botelberge, Erwan Bories, Christian Pesenti, Fabrice Caillol, Benjamin Esterni, Endoscopy Unit, Paoli-Calmettes Institute, Marseille 13400, France

Geneviève Monges, Department of Pathology, Paoli-Calmettes Institute, Marseille 13400, France

Paolo Arcidiacono, Endosonography Unit, Gastroenterology and Gastrointestinal Endoscopy Unit, San Raffaele Hospital, Milan, 68052, Italy

Pierre Deprez, Robert Yeung, Division of Gastroenterology, University Hospital Saint-Luc, B-1200 Brussels, Belgium

Walter Schimdt, Hanz Schrader, Carl Szymanski, Department of Medicine I, University Hospital, D-44801 Bochum, Germany

Christoph Dietrich, Department of Internal Medicine 2, Caritas-Krankenhaus, Bad Mergentheim, D-97980, Germany

Pierre Eisendrath, Jean-Luc Van Laethem, Jacques Devière, Division of Gastroenterology, Erasme Hospital, B-1070 Anderlecht, Brussels, Belgium

Peter Vilmann, Adrian Saftoiu, Department of Surgical Gastroenterology, Gentofte University Hospital, DK-2400 Hellerup, Denmark

Author contributions: Giovannini M and Botelberge T Analyzed the data and performed the statistical study; Pesenti C and Caillol F performed the EUS + Elastography; Monges G was the pathologist; Arcidiacono P, Schimdt W, Schrader H, Szymanski C, Dietrich C, Van Laethem JL, Devière J and Vilmann P included and performed EUS + Elastography; Bories E, Esterni B, Deprez P, Eisendrath P, Yeung R and Saftoiu A performed the interobserver study.

Correspondence to: Marc Giovannini, Endoscopy Unit, Paoli-Calmettes Institute, Marseille 13400,

France. giovanninim@wanadoo.fr

Telephone: +33-4-91223568 Fax: +33-4-91223658

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Abstract

AIM: To evaluate the ability of endoscopic ultrasound (EUS) elastography to distinguish benign from malignant pancreatic masses and lymph nodes.

METHODS: A multicenter study was conducted and included 222 patients who underwent EUS examination with assessment of a pancreatic mass ($n = 121$) or lymph node ($n = 101$). The classification as benign

or malignant, based on the real time elastography pattern, was compared with the classification based on the B-mode EUS images and with the final diagnosis obtained by EUS-guided fine needle aspiration (EUS-FNA) and/or by surgical pathology. An interobserver study was performed.

RESULTS: The sensitivity and specificity of EUS elastography to differentiate benign from malignant pancreatic lesions are 92.3% and 80.0%, respectively, compared to 92.3% and 68.9%, respectively, for the conventional B-mode images. The sensitivity and specificity of EUS elastography to differentiate benign from malignant lymph nodes was 91.8% and 82.5%, respectively, compared to 78.6% and 50.0%, respectively, for the B-mode images. The kappa coefficient was 0.785 for the pancreatic masses and 0.657 for the lymph nodes.

CONCLUSION: EUS elastography is superior compared to conventional B-mode imaging and appears to be able to distinguish benign from malignant pancreatic masses and lymph nodes with a high sensitivity, specificity and accuracy. It might be reserved as a second line examination to help characterise pancreatic masses after negative EUS-FNA and might increase the yield of EUS-FNA for lymph nodes.

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Key words: Endoscopic ultrasound; Elasticity coefficient; Elastography; Pancreatic mass; Lymph node

Peer reviewer: Marko Duvnjak, MD, Department of Gastroenterology and Hepatology, Sestre milosrdnice University Hospital, Vinogradska cesta 29, 10 000 Zagreb, Croatia

Giovannini M, Botelberge T, Bories E, Pesenti C, Caillol F, Esterni B, Monges G, Arcidiacono P, Deprez P, Yeung R, Schimdt W, Schrader H, Szymanski C, Dietrich C, Eisendrath P, Van Laethem JL, Devière J, Vilmann P, Saftoiu A. Endoscopic ultrasound elastography for evaluation of lymph nodes and pancreatic masses: A multicenter study. *World J Gastroenterol* 2009; 15(13): 1587-1593 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1587.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1587>

INTRODUCTION

A major limitation of endoscopic ultrasound (EUS) examination is its limited capacity to determine the exact nature of a lesion. Differential diagnosis between benign and malignant lymph nodes and focal pancreatic masses based on the EUS appearance is difficult and frequently requires EUS-guided fine needle aspiration (EUS-FNA) for confirmation of malignancy^[1-4].

Elastography has recently been presented as a novel technique that can be applied during ultrasound (US) examination to assess and measure tissue elasticity. Knowing that malignant tissues are generally harder than normal surrounding tissue, elastography might provide interesting clinical information to help distinguish benign from malignant tissue based on their specific tissue consistency. Clinical research has shown promising results in differentiating between benign and malignant tissue in the thyroid gland^[5], breast^[6-8], prostate^[9,10] and to assess liver fibrosis^[11-14]. Recently, elastography has also been introduced during EUS examination^[15-19]. The current study is a continuation of previous research^[15] to validate the potential role of elastography in distinguishing benign from malignant lymph nodes and focal pancreatic lesions in a large retrospective trial. The aim of this multicenter study was to classify lymph nodes and pancreatic masses during EUS examination as benign or malignant based on the real time (qualitative) elastography patterns and to compare the results with a classification based on the conventional B-mode EUS images and with the final diagnosis obtained by EUS-FNA and/or by surgical pathology.

MATERIALS AND METHODS

Patients, procedure and examination technique

Every patient ($n = 222$) who underwent EUS examination with evaluation of a pancreatic mass ($n = 121$) or lymph nodes ($n = 101$), between October 2006 and February 2007, was included. The study was conducted in seven different centers throughout Europe. Only one lesion per patient was examined and by one single endoscopist per center. Each center started the study after six months experience of EUS elastography. The EUS examinations were performed with conventional linear EUS probes (Pentax EG38-UT and EG38-70UTK, Hamburg, Germany). The examined lesion was first classified as benign or malignant based on the conventional B-mode images. Subsequently, elastography was carried out in real time using a commercially available module incorporated into the Hitachi EUB-8500 system (Hitachi Medical Systems Europe, Zug, Switzerland). The technology measures the degree of tissue deformation after compression as an indicator for the stiffness of tissue. This compression during EUS examination is naturally obtained by arterial pulsations and respiratory movements. Detailed reviews on the technical aspects of elastography have been previously published^[15,20,21]. The sample area was adjusted to the region of interest and the suitability of the elastographic signal was indicated

by a numeric scale within the image. Tissue elasticity was shown superimposed on the conventional B-mode EUS image by colors reflective of stiffness. Hard tissue areas were marked with blue, intermediate areas with green, medium soft areas with yellow and soft areas with red. Elastographic and B-mode images were displayed simultaneously side by side. The complete spectrum from blue to red was applied to each elastographic image and represented the graduation of relative elasticity within the sample area. Elastographic images were interpreted during the examination and a 60 s video loop was recorded for an interobserver study. After assessing the elastographic images, EUS-FNA was performed in all cases for clinical reasons using a 22-gauge needle (Wilson-Cook Medical, Winston-Salem, North Carolina). The technique of EUS-FNA is described elsewhere^[22]. In all participating centers, the specimen were examined using the monolayer cytology technique^[23]. An on-site pathologist was present in only four centers during the examination. In the remaining centers, the endoscopist assessed the sample to ensure sufficient tissue was obtained based on the presence of tissue filaments in the conservation solution and repeat punctures were performed if necessary. The pathologist was blinded to the elastography results. The final diagnosis was based on histology obtained by EUS-FNA and surgical specimen when available. If EUS-FNA was found to be negative (after at least one repeat examination), a 12 mo clinical and imaging follow-up was carried out in the absence of surgical specimens. The following parameters were recorded in a protocol: the classification as benign or malignant based on the B-mode images, the elastography score based on the elastographic pattern and the classification as benign or malignant based on this pattern and the final result based on histology.

Scoring system

The elastographic images were scored according to elastographic patterns based on previous research^[15]: a score equal to 1 was assigned when the image showed a homogenous soft tissue area (green) corresponding to normal tissue (Figure 1), a score equal to 2 when the image indicated heterogenous soft tissue (green, yellow, and red) corresponding to fibrosis or inflammatory tissue (Figure 2A and B), a score equal to 3 when the image displayed mixed colors or a honeycombed elastography pattern indicative of mixed hard and soft tissue making the interpretation difficult (Figure 3), a score equal to 4 when the image displayed a small soft (green) central area surrounded by mainly hard (blue) tissue corresponding to a malignant hypervascularized lesion (Figure 4) and a score equal to 5 was assigned to lesions representing mainly hard (blue) tissue with areas of heterogeneous soft tissue (green, red) representing zones of necrosis in an advanced malignant lesions (Figure 5). For the study purpose and to facilitate the use in clinical practice, we subsequently classified an elastography score equal to 1 and 2 as: (A) representing normal tissue or a benign

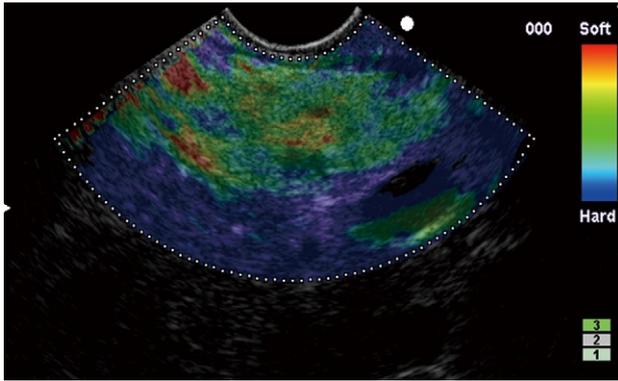


Figure 1 Elastographic image showing homogenous soft tissue corresponding to normal tissue.

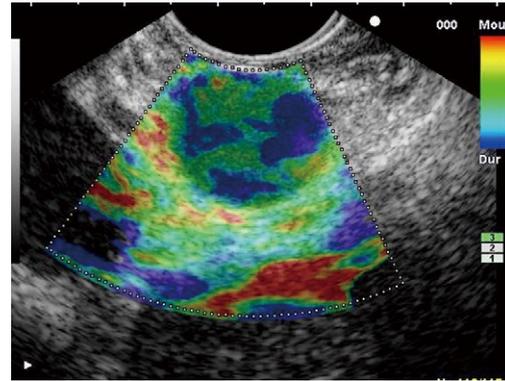


Figure 3 Elastographic image showing mixed hard and soft tissue ("honeycombed pattern") making the interpretation difficult.

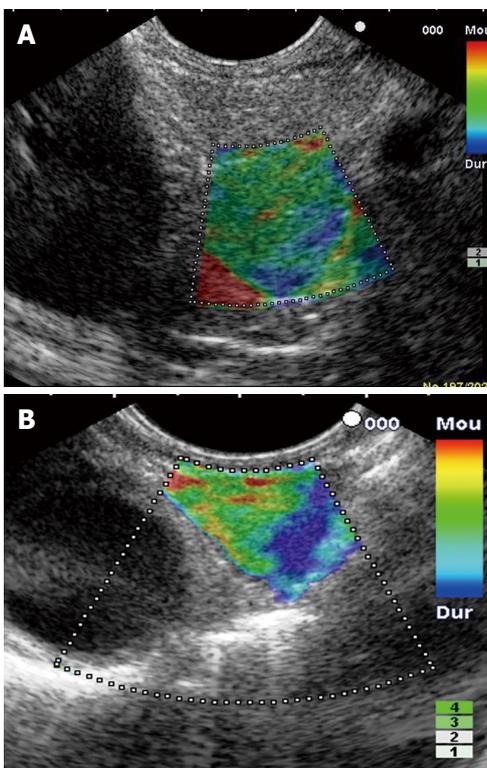


Figure 2 Elastographic image. A: Heterogenous soft tissue corresponding to fibrosis (benign nodule in patient who had an acute pancreatitis 2 mo before); B: Heterogenous soft tissue corresponding to inflammatory tissue (benign lymph node).

tumor; a score equal to 4 and 5 was classified as (C) representing a malignant lesion; and a score equal to 3 was classified as (B) which represented tissue difficult to classify as benign or malignant based on the elastographic pattern. However a score equal to (B) was considered as malignant for statistical analysis (Figure 6).

Statistical analysis

The results are presented as means plus or minus standard deviation or as medians with ranges, depending on the data distribution. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated as appropriate.

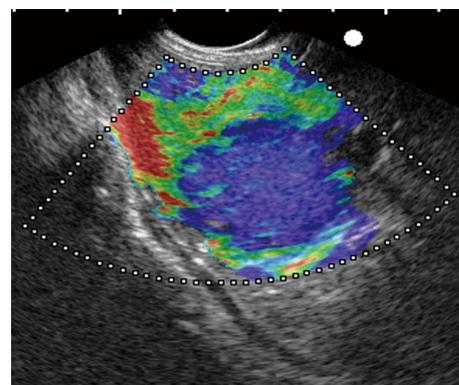


Figure 4 Elastographic image showing mainly hard tissue with a small soft central area corresponding to a malignant hypervascularized lesion (pancreatic neuroendocrine tumor).

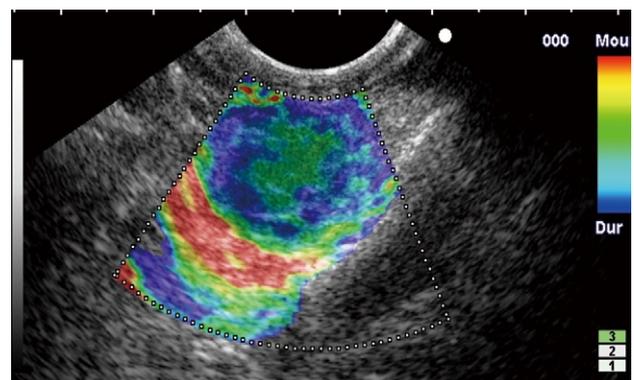


Figure 5 Elastographic image showing mainly hard tissue with areas of heterogenous soft tissue corresponding to an advanced malignant lesion with necrotic areas (pancreatic adenocarcinoma).

An interobserver study was performed on a statistically representative and blinded selection of 30 videos (15 of a pancreatic mass and 15 of a lymph node). These videos were each evaluated by five endoscopists experienced in EUS and elastography. The elastographic images were scored with a whole number from 1 to 5 using the previous described criteria, constituting an ordered variable. The agreement between two different examiners was measured by an adapted kappa coefficient. The

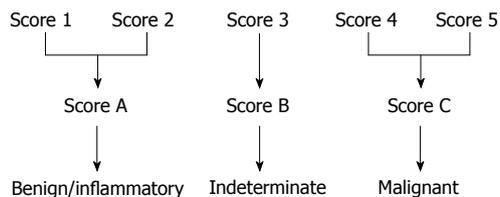


Figure 6 Elastography score.

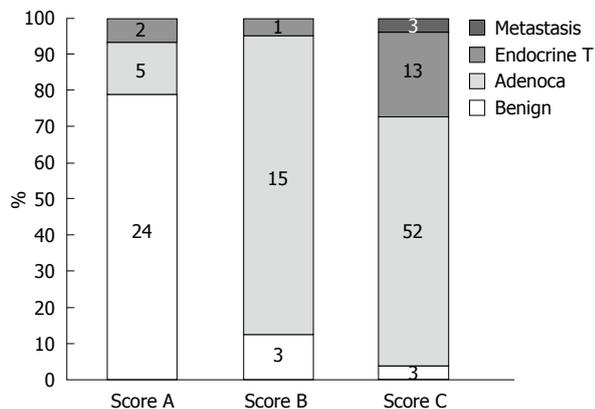


Figure 7 Pancreatic masses: elastography score and final histology.

interobserver study also evaluated the agreement between two examiners to classify the examined tissue as benign or malignant. The result is a binary variable (“malignant” or “benign”). The results being “inconclusive” were considered as missing data for the calculation of the agreement. This agreement was measured by the Cohen kappa coefficient.

RESULTS

Pancreatic masses

One hundred and twenty-one patients (77 M and 44 F, mean age 63 years) underwent EUS examination with elastography for evaluation of a pancreatic mass (mean diameter 29.5 mm, range 7-80 mm). The masses were located in the pancreatic head ($n = 48$), isthmus ($n = 17$), body ($n = 29$), tail ($n = 13$) and uncinata process ($n = 14$). No complications occurred during the study. The final histological assessment was based on the FNA results in 82 cases and on surgical pathology in 39 cases. The final diagnosis of the pancreatic masses included pancreatic adenocarcinoma ($n = 72$), malignant endocrine tumor ($n = 16$), benign endocrine tumor ($n = 2$), benign chronic pancreatitis related nodules ($n = 28$) and pancreatic metastasis ($n = 3$) (Figure 7). The elastographic images were interpreted as benign (score 1 + 2 = A) in 31 cases, indeterminate (score 3 = B) in 19 cases and malignant (score 4 + 5 = C) in 71 cases. Considering the “indeterminate” result equal to score (B) as malignant, the calculated sensitivity, specificity, positive and negative predictive values of EUS elastography to differentiate benign from malignant pancreatic masses were, respectively, 92.3%, 80.0%, 93.3% and 77.4% with a global accuracy of this new technology of 89.2%. The

Table 1 Pancreatic masses: classification as benign or malignant based on EUS elastography, conventional B-mode imaging and the final diagnosis based on histology

		Histology	
		Malignant (n)	Benign (n)
Elastography/ conventional B-mode	Malignant	84/85	6/27
	Benign	7/7	24/2

Elastography: sensitivity = $84/91 = 92.3\%$, specificity = $24/30 = 80\%$, accuracy = $108/121 = 89.2\%$; Conventional B-mode: sensitivity = $85/92 = 92.3\%$, specificity = $2/29 = 68.9\%$, accuracy = $87/121 = 71.9\%$.

calculated sensitivity, specificity, positive and negative predictive values of conventional B-mode images to differentiate benign from malignant pancreatic masses were, respectively, 92.3%, 68.9%, 75.8% and 22.2% with an accuracy of 71.9% (Table 1).

Lymph nodes

One hundred and one patients (56 M and 45 F, mean age 61.1 years) underwent EUS examination of a lymph node for staging of lung cancer ($n = 25$), oesophageal carcinoma ($n = 25$), gastric cancer ($n = 13$), pancreatic cancer ($n = 13$), for suspicion of lymph node relapse of kidney cancer ($n = 2$) and of breast cancer ($n = 8$). EUS examination was also performed for evaluation of isolated lymph nodes ($n = 15$). Lymph nodes (mean diameter 20.1 mm, range 7-50 mm) were located in the mediastinum ($n = 51$), in the cervical area ($n = 4$), in the celiac or mesenteric area ($n = 44$), and in the perirectal space ($n = 2$). No complications occurred during the study. The final histological assessment was based on FNA and classified the lymph nodes as malignant in 57 cases, including metastasis of an adenocarcinoma ($n = 35$), metastasis of a squamous cell carcinoma ($n = 13$), metastasis of an endocrine tumor ($n = 3$), metastasis of a melanoma ($n = 1$), lymphomas ($n = 5$), and benign in 44 cases (including three cases of sarcoidosis) (Figure 8). The elastographic images were interpreted as benign (score 1 + 2 = A) in 38 cases, indeterminate (score 3 = B) in 10 cases and malignant (score 4 + 5 = C) in 53 cases. Considering the “indeterminate” result equal to score (B) as malignant, the calculated sensitivity, specificity, positive and negative predictive values were, respectively, 91.8%, 82.5%, 88.8% and 86.8% with a global accuracy of this new technology of 88.1%. The calculated sensitivity, specificity, positive and negative predictive values for the conventional B-mode images were respectively 78.6%, 50.0%, 70.5% and 60.6% with an accuracy of 67.3% (Table 2).

Inter-observer study

The kappa coefficient of the sonoelastography score for pancreatic masses was 0.524, for the lymph nodes 0.519, and 0.520 for all cases confound.

The kappa coefficient for the differentiation between benign and malignant tissue was 0.785 for the pancreatic masses, 0.657 for the lymph nodes and 0.725 for all cases confound.

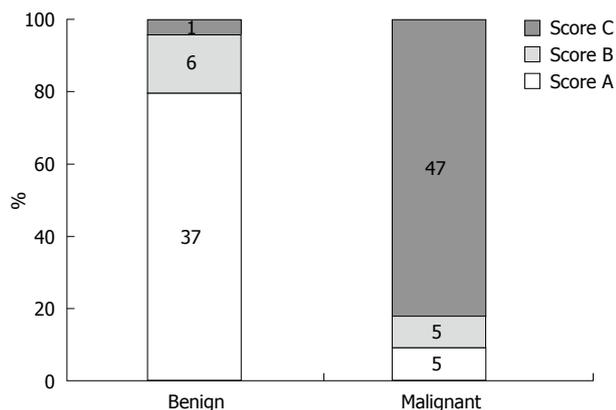


Figure 8 Lymph nodes: elastography score and final histology.

Table 2 Lymph nodes: classification as benign or malignant based on EUS elastography, conventional B-mode imaging and the final diagnosis based on histology

		Histology	
		Malignant (n)	Benign (n)
Elastography/ conventional B-mode	Malignant	51/48	2/20
	Benign	10/13	38/20

Elastography: sensitivity = 51/61 = 83.6%, specificity = 38/40 = 95%, accuracy = 89/101 = 88.1%; Conventional B-mode: sensitivity = 48/61 = 78.6%, specificity = 20/40 = 50%, accuracy = 68/101 = 67.3%.

DISCUSSION

The aim of this multicenter study was to evaluate the ability of EUS elastography to distinguish benign from malignant focal pancreatic masses and lymph nodes and to compare the results with the conventional B-mode images and final histology.

Our study shows that EUS elastography has high sensitivity, specificity and accuracy and a much higher specificity than conventional B-mode images to differentiate between benign and malignant focal pancreatic lesions. Using our current scoring system, 15.7% of the cases still obtain an elastography score equal to 3 indicating tissue difficult to classify as benign or malignant. However, 84% of these cases with an elastography score equal to 3 turned out to be malignant and we believe that the soft tissue parts of these focal lesions on elastography represent necrotic areas in an adenocarcinoma ($n = 15$) or a hypervascularised area in an endocrine tumor ($n = 1$). Hence, an elastography score equal to 3 should be considered as malignant, in our opinion.

There were seven false negative cases (five adenocarcinoma and two neuroendocrine tumors) that may be explained in a similar way: the presence of abundant necrotic or vascular tissue resulted in an elastographic pattern mainly consisting of soft tissue. By contrast, the false positive cases in our study ($n = 6$) might represent patients with (early) chronic pancreatitis having areas of hard fibrotic nodules. Unfortunately, lack of surgical specimens in these patients cannot confirm

this hypothesis. However, in a recent publication by Janssen *et al*^[16], the elastographic patterns of the normal pancreas and the pancreas affected by inflammatory or focal disease were studied. They concluded that elastography does not distinguish between chronic pancreatitis and tumors because of their similar fibrous structure. This implies that EUS elastography will not be able to help target suspicious lesions and improve the rather low accuracy of EUS-FNA in patients with chronic pancreatitis.

In distinguishing benign from malignant focal pancreatic lesions, EUS elastography does not replace tissue confirmation and we believe that EUS elastography should not be used as a first line examination in the evaluation of focal pancreatic lesions. However, when facing (repeated) negative EUS-FNA or technical problems in performing EUS-FNA, the interpretation of the EUS elastographic images could help orientate the diagnosis and influence the decision making for surgery when the lesion is suspicious on elastography, or justify a follow-up when the elastographic images are in favour of a benign lesion.

Our data also shows that EUS elastography has high sensitivity, specificity and accuracy in distinguishing benign from malignant lymph nodes and seems to be superior to conventional B-mode images. Whether the false negative and false positive cases in this study are due to the presence of necrotic and fibrotic areas in lymph nodes, respectively, is less certain. Our results confirm comparable results obtained by Săftoiu *et al*^[18] using similar elastography pattern criteria to differentiate benign from malignant lymph nodes in 42 patients with a reported sensitivity, specificity and accuracy of, respectively, 91.7%, 94.4% and 92.86%. The role of EUS elastography to distinguish benign from malignant lymph nodes should be considered as complementary to other imaging techniques rather than a replacement for tissue confirmation. Based on a high PPV, EUS elastography might help in selecting more suspicious lymph nodes for tissue sampling, especially in patients presenting multiple lymph nodes, such as in oesophageal or lung cancer. Based on a high NPV, it might be used to reduce the number of unnecessary biopsies. As for focal pancreatic lesions, EUS elastography might offer an alternative for differential diagnosis in the case of negative EUS-FNA of a lymph node, as well as in situations where EUS-FNA is not possible (technical problems, interposed malignant tissue or interposed vascular structure).

The current results are different from the results obtained during our previous research^[15]. In this previous study, EUS elastography was shown to have a sensitivity of 100% and specificities of 67% and 50% for diagnosing malignant pancreatic masses and lymph nodes, respectively. Although false positive results in both study groups were reported, it should be recalled that the number of benign lesions in the previous study was relatively small.

For both pancreatic masses and lymph nodes, EUS elastography might also help in guiding the puncture in a non necrotic part of the suspicious lesion when necrotic

tissue is present, as in advanced cancer.

One of the main criticisms of EUS elastography is the variability of the elastographic images and the difficulty of interpretation^[19]. However, our interobserver study showed a satisfying interobserver concordance for the differentiation between benign and malignant pancreatic masses and lymph nodes ($\kappa = 0.725$).

In the absence of pathologic assessment of surgical specimens, we considered the EUS-FNA result as a gold standard. Although the specificity of EUS-FNA is close to 100%^[24-28], it has the potential to miss micro-invasion of malignancy into lymph nodes or to give false negative results for a necrotic pancreatic lesion. However, we consider it as representative of daily practice, particularly when it is combined with an adequate clinical and imaging follow-up period.

To overcome the difficulty in classifying the EUS elastography score equal to 3 or (B) as benign or malignant, we are currently evaluating the next generation of elastography software. This new software provides a quantitative histogram analysis of the elastographic images and has already proven to be useful in the evaluation of lymph nodes^[18].

The potential role of EUS elastography to help detect and differentiate submucosal tumors as well as any other solid masses situated nearby the gastrointestinal tract has still to be evaluated. The exact role of EUS elastography in patients manifesting symptoms suggestive of chronic pancreatitis with equivocal EUS (3 features or fewer) has still to be validated^[29].

EUS elastography is a new application in the field of the endosonography and seems to be able to differentiate benign from malignant lymph nodes and pancreatic lesions with a high sensitivity, specificity and accuracy. EUS elastography is superior compared to conventional B-mode imaging and the interobserver reproducibility is satisfying. The goal is not to replace tissue confirmation. Instead, the information obtained by EUS elastography should be considered as complementary to the conventional EUS imaging. It should be reserved as a second line examination to orientate further decision making after repeat negative EUS-FNA for pancreatic lesions. It may increase the yield of FNA and reduce the number of unnecessary biopsies when assessing lymph nodes. However, further research is necessary to improve our current elastography scoring system. The second generation of elastography software providing quantitative analysis of tissue elasticity might be able to increase the accuracy of this technique.

COMMENTS

Background

Elastography has recently been presented as a novel technique that can be applied during ultrasound examination to assess and measure tissue elasticity. Clinical research has shown promising results in differentiating between benign and malignant tissue in the thyroid gland, breast, prostate and to assess liver fibrosis.

Research frontiers

endoscopic ultrasound (EUS) elastography is a new application in the field of the endosonography and seems to be able to differentiate benign from

malignant lymph nodes and pancreatic lesions with a high sensitivity, specificity and accuracy. EUS elastography is superior compared to conventional B-mode imaging and the interobserver reproducibility is satisfying.

Innovations and breakthroughs

In distinguishing benign from malignant focal pancreatic lesions, EUS elastography does not replace tissue confirmation and should not be used as a first line examination in the evaluation of focal pancreatic lesions. However, when facing (repeated) negative EUS-guided fine needle aspiration (EUS-FNA) or technical problems to perform EUS-FNA, the interpretation of the EUS elastographic images could help orientate the diagnosis and influence the decision making for surgery when the lesion is suspicious on elastography, or justify a follow-up when the elastographic images are in favour of a benign lesion.

Applications

The goal is not to replace tissue confirmation. Instead, the information obtained by EUS elastography should be considered as complementary to the conventional EUS imaging. It should be reserved as a second line examination to orientate further decision making after repeat negative EUS-FNA for pancreatic lesions. It might increase the yield of FNA and reduce the number of unnecessary biopsies when assessing lymph nodes.

Peer review

The importance of the research and the significance of the research contents are high. Presentation and readability of the manuscript is highly acceptable.

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BRIEF ARTICLES

Use of mycophenolate mofetil in inflammatory bowel disease

Terrence Tan, Ian Craig Lawrance

Terrence Tan, Ian Craig Lawrance, Department of Gastroenterology, Fremantle Hospital, 6059 WA, Australia

Ian Craig Lawrance, Centre for Inflammatory Bowel Diseases, Fremantle Hospital, 6059 WA, Australia; Department of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, 6059 WA, Australia

Author contributions: Tan T and Lawrance IC contributed equally to this work.

Correspondence to: Ian Craig Lawrance, Department of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, 6059 WA, Australia. ian.lawrance@uwa.edu.au

Telephone: +61-8-94316347 Fax: +61-8-94313160

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therapy, without the need for dose escalation. Further evaluation of MMF comparing it to conventional immunosuppressants is required.

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Peer reviewer: Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, University Hospital Heraklion, Department of Gastroenterology, PO Box 1352, 71110 Heraklion, Crete, Greece

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Abstract

AIM: To assess the efficacy and safety of mycophenolate mofetil (MMF) prospectively in inflammatory bowel disease (IBD) patients intolerant or refractory to conventional medical therapy.

METHODS: Crohn's disease (CD) or ulcerative colitis/IBD unclassified (UC/IBDU) patients intolerant or refractory to conventional medical therapy received MMF (500-2000 mg *bid*). Clinical response was assessed by the Harvey Bradshaw index (HBI) or colitis activity index (CAI) after 2, 6 and 12 mo of therapy, as were steroid usage and adverse effects.

RESULTS: Fourteen patients (9 CD/5 UC/IBDU; 8M/6F; mean age 50.4 years, range 28-67 years) were treated and prospectively assessed for their response to oral MMF. Of the 11 patients who were not in remission on commencing MMF, 7/11 (63.6%) achieved remission by 8 wk. All 3 patients in remission on commencing MMF maintained their remission. Ten patients were still on MMF at 6 mo with 9/14 (64.3%) in remission, while of 12 patients followed for 12 mo, 8 were in remission without dose escalation (66.7%). Three patients were withdrawn from the MMF due to drug intolerance. There were no serious adverse events attributed due to the medication.

CONCLUSION: MMF demonstrated efficacy in the management of difficult IBD. MMF appeared safe, well tolerated and efficacious for both short and long-term

INTRODUCTION

The natural history of both forms of the inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC), is characterized by a lifelong course of remissions and relapses and a proportion of these patients are steroid refractory or develop steroid dependence requiring maintenance immunosuppression. The most commonly used immunomodulatory medications are azathioprine (AZA), or its metabolite 6-mercaptopurine (6MP). Approximately 10% of patients, however, will be intolerant of these drugs, resulting in their withdrawal and the need for an alternative immunomodulator^[1]. Up to 50% of CD and 20% of UC patients will also develop a severe acute episode of their disease requiring hospitalization^[2] and almost half of these patients will require rescue therapy or surgery^[2,3]. In severe steroid refractory UC, remission may be achieved through the use of cyclosporine or infliximab, but despite continued maintenance therapy for these patients with AZA/6MP, over 65% will relapse by 12 mo and 30% will require colectomy^[4]. Thus, despite the advent of new biological agents, used in combination with AZA/6MP, efficacy is not universal so the need for other immunomodulatory medications remains imperative.

Mycophenolate mofetil (MMF) is a powerful

immunosuppressant primarily indicated for prevention of solid organ transplantation rejection. It is an anti-metabolite with pharmacodynamic properties similar to AZA. MMF appears to be very safe and efficacious for this indication and is used as a first-line anti-rejection drug in many transplant centers. More recently, however, this immunosuppressant has been employed in the management of difficult IBD cases^[5,6]. Its efficacy has primarily been assessed in small, uncontrolled case series with only a few small randomized trials^[7-9]. They indicate that MMF may be effective in IBD, but its role is controversial. The problem arises primarily from observations that despite clinical remission and response achieved early in the course of treatment, a large proportion of patients ultimately flare and require biological agents or surgery. There is also a suggestion that the MMF dose needs to be increased over time in order to maintain an effect and some studies also suggest non-superiority of MMF to conventional immunosuppressants such as AZA^[10,11].

Most of the early studies on MMF were undertaken in patients with chronic active CD who failed, or were intolerant to, AZA, and demonstrated good efficacy^[7,9]. These findings, however, were not supported by later studies, with either a low response or high relapse rate^[5,8,12]. The rate of treatment discontinuation due to side effects was also high^[6,12,13] and studies comparing MMF to AZA yielded conflicting and inconsistent results^[7,10]. In one study MMF was identified to be more likely to be effective in AZA intolerant, rather than refractory patients, and was not inferior to AZA in the management of UC for the induction or maintenance of remission at 6 mo^[11]. Another study with longer term outcomes evaluating MMF in a cohort of AZA resistant/intolerant patients, however, observed that although MMF was initially effective, relapses were common^[14].

This study presents our experience in the use of MMF in the treatment of IBD patients in the short and long term. We prospectively assessed the efficacy of MMF in both the induction and maintenance of remission in patients who were intolerant of AZA/6MP and had previously failed courses of either methotrexate (MTX), antibiotics and/or infliximab. We particularly examined the need for dose escalation over time in patients who initially responded to the MMF as this has been a criticism of its long-term efficacy.

MATERIALS AND METHODS

All subjects were patients at the Centre for Inflammatory Bowel Diseases, Fremantle Hospital, which is a specialist IBD unit in a 450 bed tertiary institution that services the southern metropolitan region of Perth, Australia. Patients with IBD were classified as CD or ulcerative colitis/IBD unclassified (UC/IBDU) according to the "Montreal Classification" (a modification of the Vienna Classification). The diagnosis of IBD had to be definite, and was made in accordance with previously established criteria based upon clinical, endoscopic, histopathological

and radiological findings. The diagnoses of CD or UC/IBDU were exclusive of infective enterocolitis (excluded by stool microscopy and culture, bacterial and amoebic serology, acid-fast staining of biopsies and mycobacterial cultures), Behcet's disease and microscopic colitis. Patient demographics, disease status, infusion number, response and remission rates and adverse effects were recorded.

All patients were treated between Jan 2003 and July 2008. Patients treated with MMF received between 500 mg and 2000 mg twice a day with the dose optimized to maintain the white cell count (WCC) between 4 and $6 \times 10^9/L$, neutrophil count $> 2.0 \times 10^9/L$ and lymphocyte count at or just below the normal range of $1.1 \times 10^9/L$ without side effects. A clinical response to the MMF in CD patients was determined by a reduction in the Harvey Bradshaw index (HBI) of greater than or equal to 3, with a remission defined as a HBI less than 5 off steroids. A clinical response to the MMF in the UC/IBDU patients was defined as a reduction of 4 or more points in the colitis activity index (CAI) and remission was considered to be CAI of less than or equal to 4 off steroids. The response and remission rates after 8 wk of therapy and long-term response to treatment with MMF were assessed.

Serious adverse effects (SAE) were analyzed. Serious adverse effects are defined as any adverse drug experience occurring that results in death, life-threatening adverse event, persistent or significant disability/incapacity, required in-patient hospitalization, or prolonged hospitalization or congenital anomaly or birth defect.

RESULTS

The primary indications for treatment were either steroid refractoriness, or dependence, and allergy, or intolerance, to AZA/6MP therapy. Patients were steroid dependant if they were unable to be withdrawn from steroids without a disease flare and patients were steroid refractory if they continued to suffer active inflammation whilst on steroids of 20 mg or greater per day. All patients with active disease were considered for treatment with MMF only after demonstrating failure of disease control or steroid dependency. Two CD and 1 UC/IBDU patients were in clinical remission at the time of commencing the MMF. One of these patients suffered from severe psoriasis in addition to her CD and was changed to MMF in consultation with the dermatologists in an attempt to control both the psoriasis and the CD. The second patient had undergone 3 terminal ileal resections with recurrent severe ileal inflammation occurring within 1 to 2 years after each surgery, but was allergic to AZA/6MP, while MTX and infliximab were ineffective. The third patient required 6MP to maintain remission, but was intolerant of this medication due to severe alopecia.

Patient demographics

Fourteen patients (9 male, 5 female) were treated with MMF during the study period (Table 1). The ages at time of commencing the MMF ranged from 28-67 years (mean age 50.4 ± 12.9 years). Nine patients suffered from CD

Table 1 Demographics of the IBD patients using the Montreal classification

	CD patients <i>n</i> = 9	UC/IBDU patients <i>n</i> = 5
Gender: male	57.1% (8/14)	57.1% (8/14)
Age at diagnosis		
Mean ± SE (range)	38.6 ± 13.3 yr (19-54)	44.0 ± 12.7 yr (30-63)
A1- ≤ 16	0% (0/9)	0% (0/5)
A2-17-40	44.4% (4/9)	40% (2/5)
A3- > 40	55.6% (5/9)	60% (3/5)
Disease duration		
Mean (range)	10.4 yr (1-26)	9.8 yr (1-28)
Crohn's disease		
L1-terminal ileum	22.2% (2/9)	
L2-colon	33.3% (3/9)	
L3-ileocolonic	44.4% (4/9)	
L4-upper GI	11.1% (1/9)	
P-perianal	22.2% (2/9)	
B1-inflammatory	44.4% (4/9)	
B2-stricturing	33.3% (3/9)	
B3-perforating	22.2% (2/9)	
Ulcerative colitis/IBDU		
E1-proctitis		20% (1/5)
E2-left sided		40% (2/5)
E3-extensive		40% (2/5)
Raised CRP	55.6% (5/9)	80.0% (4/5)

and 5 had UC/IBDU. Of the CD patients, 77.8% (7/9) suffered from colonic inflammation, 22.2% (2/9) had ileal involvement alone, 11.1% (1/9) had jejunal CD, and 22.2% (2/9) suffered from perianal disease. Of the UC/IBDU patients, 2 had extensive colitis, while 2 suffered left-sided colitis and 1 patient had proctitis. The age of diagnosis was lower in the CD patients (mean 38.6 ± 13.3 years, range 19-54 years) compared to the UC/IBDU patients (mean 44.0 ± 12.7 years, range 30-63 years), but this was not statistically significant. Both the CD and UC/IBDU patient groups had similar disease duration at the time of the MMF therapy (mean 10.4 years and 9.8 years respectively). Four (44.5%) of the CD patients had previously undergone at least one surgery (1 subtotal colectomy, 2 small bowel resections and 1 total colectomy and ileal surgery). C-reactive protein (CRP) levels were also elevated in 5 of the 9 CD patients and 4 of 5 UC/IBDU patients prior to commencement of the MMF.

Current and previous medical therapy

Conventional therapies had been tried in all patients (Table 2). Surgical options had been discussed in detail and were considered to be either medically inappropriate at that stage, or were declined by the patient. Of the 9 CD patients, 88.9% (8/9) were on 5-aminosalicylic acid (5ASA) and 77.8% (7/9) were dependent on, or intolerant to, oral steroids. Antibiotic therapy with metronidazole and ciprofloxacin had been tried and was unsuccessful in 44.4% (4/9) of CD patients. All but 1 (88.9%) of the CD patients were allergic or intolerant to the use of AZA/6MP (drug fevers, severe vomiting requiring hospitalization, severe alopecia and hepatotoxicity). MTX was ineffective in 33.3% (3/9) with 44.4% (4/9) intolerant to, or refusing to take, this medication. Infiximab was ineffective and not continued

Table 2 Medications taken by study patients at time of the commencement of MMF therapy

	CD patients <i>n</i> = 9	UC/IBDU patients <i>n</i> = 5
5-ASA		
Current	88.9% (8/9)	100% (5/5)
Steroids		
Current	55.5% (5/9)	100% (5/5)
Intolerant	22.2% (2/9)	0%
AZA/6MP		
Intolerant	88.8% (8/9)	100% (5/5)
Methotrexate		
Ineffective	33.3% (3/9)	N/A
Intolerant	11.1% (1/9)	
Refused	33.3% (3/9)	
Antibiotics		
Ineffective	33.3% (3/9)	N/A
Intolerant	11.1% (1/9)	
Infiximab		
Current	0%	0%
Ineffective	44.4% (4/9)	40% (2/5)
Intolerant	22.2% (2/9)	0%

N/A: Not applicable.

Table 3 Response and remission rates at 8 wk and CRP levels with MMF therapy

	CD patients <i>n</i> = 9	UC/IBDU patients <i>n</i> = 5
Remission	66.7% (6/9)	80% (4/5)
Response	66.7% (6/9)	80% (4/5)
Intolerant	22.2% (2/9)	20% (1/5)
Ineffective	11.1% (1/9)	0% (0/5)
Raised CRP		
In responders	0% (0/6)	0% (4/5)
In non responders	33.3% (1/3)	100% (1/1)

in 44.4% (4/9) and 22.2% (2/9) were intolerant to its use (anaphylaxis and serum sickness).

Of the UC/IBDU patients, all were currently on both 5ASA and oral steroid therapy. All of these patients were intolerant to AZA/6MP therapy, while infiximab had been tried and was ineffective in 2 patients.

Efficacy at 8 wk

After 8 wk of MMF therapy, 63.6% (7/11) of patients (3 CD and 4 UC/IBDU) who were not in remission at commencement of MMF responded and went into remission, while 71.4% (10/14) went into remission or maintained clinical remission (Tables 3 and 4) as determined either by the HBI or CAI. The 2 patients who had AZA/6MP ceased and MMF commenced due to concurrent severe psoriasis and severe alopecia, maintained their disease remission. The other CD patient who was placed on MMF to prevent post-surgical recurrence was still in remission. All patients in remission at 8 wk also had normal CRP levels. Three of the 14 patients (2 CD and 1 UC/IBDU) were intolerant to MMF and took the medication for 1 mo or less. In only 1 patient was MMF ineffective after 8 wk of therapy, with the patient undergoing surgery 6 mo after commencing the MMF. The surgical pathology

Table 4 Individual patient data of disease extent, age at treatment, duration of MMF therapy and response

	Sex	Diagnosis	Age at diagnosis	Age at MMF	Indication for MMF	Disease extent	Duration of MMF (mo)	Response after 8 wk	Steroids continued at 8 wk
1	M	CD	27	28	Steroid dependant	Pancolitis	30	Remission	Ceased
2	M	CD	54	55	Steroid dependant	L Sided colitis/fistula	12	Remission	Ceased
3	F	CD	50	67	Severe Psoriasis	Ileocolonic disease	48	Remission	N/A
4	M	CD	41	41	Steroid dependant	Ileocolonic disease	12	Remission	Ceased
5	M	CD	53	63	Recurrent TI resections	Recurrent ileal disease	15	Remission	N/A
6	F	CD	32	58	Steroid intolerant	Colectomy/2x TI resection/ recurrent ileal disease	< 1	Intolerant	Continued
7	F	CD	19	28	Steroid dependant	Subtotal colectomy/recurrent ileal disease/fistula	< 1	Intolerant	Continued
8	M	CD	21	48	Steroid dependant	Ileal disease	6	Ineffective	Surgery
9	F	UC/IBDU	43	50	Steroid dependant	Pancolitis	12	Remission	Ceased
10	M	UC/IBDU	31	33	Steroid dependant	L Sided colitis	9	Remission	Ceased
11	M	UC/IBDU	53	64	Steroid dependant	L Sided colitis	22	Remission	Ceased
12	F	CD	50	50	Recurrent flares	Pancolitis	12	Remission	Ceased
13	M	UC/IBDU	63	63	Steroid dependant	Pancolitis	8	Remission	Ceased
14	F	UC/IBDU	30	58	Steroid dependant	Subtotal colectomy/proctitis	< 1	Intolerant	Continued

demonstrated chronic active inflammation and fibrosis of the previous ileocolonic anastomosis.

Efficacy at 6 mo

Of the 10 patients on MMF who responded or were in remission at 8 wk, all were still on MMF at 6 mo. Only one patient suffered a disease flare in that 6-mo period. This patient flared 10 wk after commencing the MMF and required further steroids and a single dose of infliximab to induce remission, but continued on the MMF with subsequent successful withdrawal of the steroids and no further need for infliximab therapy. None of the other patients required an increase in their dose of MMF over the 6-mo period in order to maintain their remission. The patient who was on the MMF for recurrent inflammation following previous terminal ileal resections for uncontrolled CD inflammation underwent a colonoscopy at 6 mo, which demonstrated no ileal or colonic CD inflammation.

Efficacy at 12 mo

Ten patients (Table 4) had been on MMF for more than 6 mo (mean 18.1 ± 12.1 mo, max 48 mo) with 8 patients taking MMF for 12 mo or more. Of the 10 patients, 1 flared at 8 mo and was withdrawn from MMF due to lack of efficacy. One of the patients died 12 mo after commencing the MMF from an unrelated cause while in remission from his CD. One patient who flared after 30 mo of MMF was withdrawn and commenced on adalimumab with good effect. A total of 12 patients were followed for 12 mo or more and of these 8 were in remission (66.7%). All the 8 patients on MMF maintained their remission without the need for dose escalation.

Adverse effects

There was one serious adverse event (SAE) in this patient cohort. This patient died from decompensated alcoholic liver disease. He had previously denied any significant alcohol consumption on numerous occasions

and had been on a stable dose of MMF for over 10 mo. The patient presented to hospital jaundiced with ascites and blood results consistent with an acute hepatitis. The MMF was ceased and the patient was subsequently diagnosed with acute severe alcohol-induced hepatitis. His condition deteriorated over a 2-wk period and he died from liver failure. This SAE was considered to be 'unlikely related' to the MMF use. Adverse events that resulted in cessation of the medication occurred in 3 (21.4%) patients (2 CD and 1 UC/IBDU). These were GI disturbances (nausea and vomiting) and severe headaches. There were no other adverse events that required modification of the MMF dose.

DISCUSSION

The treatment of refractory IBD has always been one of the most challenging aspects in the clinical practice of luminal gastroenterology. MTX has been the primary alternative therapy for CD patients who are treatment refractory or intolerant to AZA/6MP. Although MTX has demonstrated efficacy in CD, the rate of adverse events at the higher doses often required to achieve clinical response/remissions has limited its use^[15]. At low doses, however, MTX is often ineffective^[15] and definitely less effective than AZA/6MP^[16] with longer-term studies demonstrating a frequent loss of efficacy over time^[17]. A systematic review of 5 trials identified only one large randomized trial that recommended high dose parenteral MTX to induce clinical remission^[18]. The remaining studies using oral forms have disappointing results^[15] and because of its route of administration MTX is not acceptable to many patients. Despite some evidence justifying the use of MTX in UC^[19], and fistulising CD^[20], data remains limited and confined to retrospective chart reviews. AZA/6MP, therefore, has been the mainstay of immunosuppressive maintenance therapy in IBD. The use of MMF has, therefore, been proposed as an alternative immunosuppressive therapy for patients who either are refractory or intolerant to AZA/6MP.

The aim of our study was to prospectively evaluate the short and long-term efficacy and safety of MMF in patients who were either steroid refractory, or dependent, as well as intolerant or allergic to AZA/6MP therapy. We also wanted to examine the need for dose escalation of MMF over time as this has been suggested as a problem with the use of MMF by some studies^[13]. As with many of the other published data on MMF, ours was a small cohort of IBD patients with open-label use of MMF. Our patients, however, were assessed at numerous time points and were followed for over a year. The patients in our cohort were also medication resistant, with two thirds failing anti-TNF- α therapy, suggesting a more difficult-to-treat population of patients compared to some other studies. Despite this the results were encouraging. Overall the response rate observed was 71% of patients achieving or maintaining a complete clinical remission after 8 wk of therapy. Excluding the 3 patients who were in remission and off steroids at the time of commencing the MMF, the response/remission rates were still 63.6% at 8 wk. These findings are in contrast with current literature, which reports short-term response rates of only between 25%-40%^[8,13,14].

A proportion of MMF-treatment failures in previous studies have been attributed to discontinuation secondary to significant adverse effects. In our study MMF was generally well tolerated, but discontinuation of the MMF secondary to adverse effects was still 21.4%, similar to the 30% observed in other studies. This does not explain the difference, however, in the overall response rates and the reasons behind the difference remains unclear. Relapses over time have also been previously reported as common^[8,13,14]. Early relapse in our cohort, however, was not commonly observed and even after 12 mo of MMF therapy, 57.1% (8/14) of our IBD patients were still in remission. Of particular note is the lack of dose escalation required over time in our patients responding to MMF. None of the 8 patients on MMF in remission at 12 mo had their dose of MMF increased in the previous 6 mo.

In our experience, the efficacy of MMF appears to differ in some aspects to the published data. Our data demonstrate that MMF can be efficacious and well tolerated in treating refractory IBD patients who are intolerant to AZA/6MP. Problems of lack of long-term efficacy and early disease flare as well as the need for dose escalation over time did not eventuate. Our findings support the use of MMF in the management algorithm of resistant IBD, but its role needs further clarification in larger randomized, double-blind studies comparing it to conventional immunosuppressants. Long-term efficacy would appear to be demonstrated in our study and our current experience suggests that MMF can and should be considered in patients who have failed conventional immunosuppressive therapy.

COMMENTS

Background

Treatment for patients with inflammatory bowel disease (IBD) refractory, or intolerant, to conventional immunosuppressive therapy such as azathioprine/6-

mercaptopurine and methotrexate is difficult. The advent of biological therapies has alleviated this problem to a certain degree but there are still a proportion of patients who fail to respond to them or develop drug reactions. The need for alternative effective immunosuppressive agents in the management of IBD are thus required.

Research frontiers

This study aimed to further define the role of mycophenolate mofetil (MMF) in the treatment of inflammatory bowel disease. The use of this immunosuppressant has been studied in patients refractory, or intolerant, to conventional treatments and results have varied with some studies showing a lack of efficacy or high rates of adverse events. The authors describe a single center experience in the use of MMF for difficult-to-treat IBD patients.

Innovations and breakthroughs

In contrast to previous reports the study identified MMF to be a safe and efficacious choice in the treatment of difficult IBD and found that the agent to be well tolerated and the response to be sustained. The reported clinical remission rates also seem to be higher than those in previous studies.

Applications

The findings of study supported the use of MMF in the treatment of patients with IBD who are refractory or intolerant to conventional therapies such as azathioprine/6-MP or methotrexate.

Terminology

Inflammatory bowel disease is a group of chronic diseases involving the gastrointestinal tract particularly in the small and large bowel. It is divided into 2 groups: Crohn's disease and ulcerative colitis. Crohn's disease is characterized by transmural rather than superficial mucosal inflammation and often presents as a discontinuous disease involving the small or large intestine, or both. Ulcerative colitis/IBD unclassified is the Montreal classification of patients with IBD but without the features needed to diagnose Crohn's disease.

Peer review

The authors examined the use of mycophenolate mofetil in the treatment of inflammatory bowel disease and found it to be a good alternative immunomodulator in those with IBD who have either failed or become intolerant to conventional therapy. The presence of a good response in those who previously failed biological agents suggests a possible role of MMF in the management of this subgroup of patients as well.

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Oral allopurinol to prevent hyperamylasemia and acute pancreatitis after endoscopic retrograde cholangiopancreatography

Hector Martinez-Torres, Xochilt Rodriguez-Lomeli, Carlos Davalos-Cobian, Jesus Garcia-Correa, Juan Manuel Maldonado-Martinez, Fabiola Medrano-Muñoz, Clotilde Fuentes-Orozco, Alejandro Gonzalez-Ojeda

Hector Martinez-Torres, Xochilt Rodriguez-Lomeli, Carlos Davalos-Cobian, Jesus Garcia-Correa, Juan Manuel Maldonado-Martinez, Department of Gastroenterology and Endoscopy, UMAE-Hospital de Especialidades del Centro Medico Nacional de Occidente-IMSS, Avenida Belisario Dominguez # 1000, Guadalajara, Jalisco 44340, Mexico
Fabiola Medrano-Muñoz, Clotilde Fuentes-Orozco, Alejandro Gonzalez-Ojeda, Research Unit in Clinical Epidemiology, UMAE-Hospital de Especialidades del Centro Medico Nacional de Occidente-IMSS, Avenida Belisario Dominguez # 1000, Guadalajara, Jalisco 44340, Mexico

Author contributions: Medrano-Muñoz F, Fuentes-Orozco C and Gonzalez-Ojeda A Participated in the protocol design, collection of clinical information, statistical analysis and were involved in editing the manuscript; Martinez-Torres H, Rodriguez-Lomeli X, Davalos-Cobian C, Garcia-Correa J and Maldonado-Martinez JM were involved in the protocol design, identification and inclusion of candidates and performed the endoscopic procedures.

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Correspondence to: Alejandro Gonzalez-Ojeda, MD, PhD, Research Unit in Clinical Epidemiology, UMAE-Hospital de Especialidades del Centro Medico Nacional de Occidente-IMSS, Avenida Belisario Dominguez # 1000, Guadalajara, Jalisco 44340, Mexico. avygail5@yahoo.com.mx

Telephone: +52-33-36189538 Fax: +52-33-36189538

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Abstract

AIM: To assess the efficacy of allopurinol to prevent hyperamylasemia and pancreatitis after endoscopic retrograde cholangiopancreatography (PEP).

METHODS: One hundred and seventy patients were enrolled and randomized to two groups: a study group ($n = 85$) who received 300 mg of oral allopurinol at 15 h and 3 h before endoscopic retrograde cholangiopancreatography (ERCP) and a control group ($n = 85$) receiving an oral placebo at the same times. Main Outcome Measurements included serum amylase levels and the number severity of the episodes of

pancreatitis. Serum amylase levels were classified as normal (< 150 IU/L) or hyperamylasemia (> 151 IU/L). Episodes of PEP were classified following Ranson's criteria and CT severity index.

RESULTS: Gender distribution was similar between groups. Mean age was 53.5 ± 18.9 years for study group and 52.8 ± 19.8 years for controls. Also, the distribution of benign pathology was similar between groups. Hyperamylasemia was more common in the control group ($P = 0.003$). Mild PEP developed in two patients from the study group (2.3%) and eight (9.4%) from control group ($P = 0.04$), seven episodes were observed in high-risk patients of the control group (25%) and one in the allopurinol group (3.3%, $P = 0.02$). Risk factors for PEP were precut sphincterotomy ($P = 0.02$), pancreatic duct manipulation ($P = 0.002$) and multiple procedures ($P = 0.000$). There were no deaths or side effects.

CONCLUSION: Oral allopurinol before ERCP decreased the incidences of hyperamylasemia and pancreatitis in patients submitted to high-risk procedures.

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Key words: Endoscopic retrograde cholangiopancreatography; Hyperamylasemia; Acute pancreatitis; Oral allopurinol; Risk factors

Peer reviewers: Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan; Stefano Bellentani, Professor, Fondo Studio Malattie Fegato-ONLUS, Sezione di Campogalliano, Via R. Luxemburg, 29/N, 41011 Campogalliano (MO), Italy

Martinez-Torres H, Rodriguez-Lomeli X, Davalos-Cobian C, Garcia-Correa J, Maldonado-Martinez JM, Medrano-Muñoz F, Fuentes-Orozco C, Gonzalez-Ojeda A. Oral allopurinol to prevent hyperamylasemia and acute pancreatitis after endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2009; 15(13): 1600-1606 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1600.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1600>

INTRODUCTION

Pancreatitis is the most common complication of endoscopic retrograde cholangiopancreatography (ERCP)^[1-4], with the reported incidence ranging from 1.8% to 7.2% in most prospective series^[5-9]. However, the reported incidence can be up to 30%, depending on the criteria used to diagnose pancreatitis, the type and duration of patient follow-up and the type of case mix^[10]. More commonly, hyperamylasemia occurs in up to 30% of patients undergoing ERCP^[11].

The generally accepted criteria for the diagnosis of post-ERCP pancreatitis (PEP) were proposed in 1991 during a consensus workshop. These criteria include the new onset of pancreatic-type abdominal pain associated with at least a threefold increase in serum amylase or lipase occurring within 24 h after an ERCP. The pain symptoms need to be severe enough to require admission to a hospital or to extend the length of stay of patients who are already hospitalized^[12]. Most of the episodes of acute pancreatitis are catalogued as mild. However, based on the presence of organ failure or local complications, acute severe pancreatitis occurs after 0.3% to 0.6% of ERCP procedures^[10,13,14].

Numerous attempts have been made to find a pharmacologic agent that could be used to reduce the incidence and severity of PEP. An ideal agent should be highly effective in reducing PEP, safe for the patient, well tolerated, relatively affordable and not require a prolonged administration time. Unfortunately, nearly all of the agents investigated have fallen short of these goals, but several agents have shown some promise^[15,16]. An early step in the pathogenesis of acute pancreatitis is capillary endothelial injury manifested by an increase in capillary permeability^[17,18]. Subsequent research has suggested that this capillary injury might be mediated by oxygen-derived free radicals^[19-21]. Xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine, which generates an oxygen-derived free radical. This catalyst is commonly derived from a ubiquitous inactive precursor, xanthine dehydrogenase, which is present in the pancreas and the intestinal mucosa. Xanthine dehydrogenase is converted to xanthine oxidase by the proteolytic cleavage of a peptide fragment. These findings have prompted attempts at the prevention of pancreatitis by treatment with free radical scavengers (e.g. superoxide dismutase, dimethyl sulfoxide or catalase), protease inhibitors (e.g. gabexate) or xanthine oxidase inhibitors (e.g. allopurinol)^[22-25].

The efficacy of oral allopurinol to reduce PEP has been investigated in an *in vivo* animal model^[26]. Pretreatment was not only associated with a significant (sixfold) reduction in the incidence of pancreatitis, but when pancreatitis did occur it was less severe. Other animal models using pretreatment with allopurinol have demonstrated a significant reduction in the progression of histological pancreatic injury and in the severity of experimental pancreatitis in dog and rat models^[27-29]. These findings in animals supported the need for human studies on the utility of allopurinol pretreatment to reduce the incidence of hyperamylasemia and PEP.

One randomized clinical trial has reported positive clinical results^[30], whereas three have reported negative outcomes^[31-33]. Given the findings of these published clinical results, the beneficial animal data and the practical benefits of allopurinol's potential use for prevention of hyperamylasemia and PEP, we designed a randomized clinical trial to compare the rates of these symptoms seen with treatment using either allopurinol or a placebo.

MATERIALS AND METHODS

Trial design and patient selection

This was a randomized clinical trial carried out in patients who underwent ERCP within a six-month period (July through December 2007) at the Endoscopy and Gastroenterology Departments of the High Specialty Medical Unit, Specialties Hospital of the Western National Medical Center of the Mexican Institute of Social Security. From the 300 candidates for ERCP, only 170 met the trial criteria. Patients needed to be over 18 years old and undergoing ERCP due to suspected bile duct obstruction with intact papilla of Vater. No patients were enrolled in the study if they had clinically evident acute pancreatitis or hyperamylasemia (> 150 IU/L) before the procedure or if they had ingested nonsteroid anti-inflammatory drugs (NSAIDs) within a week prior to assessment. Patients submitted to diagnostic, therapeutic or failed ERCP 12 mo before the inclusion in the study were not admitted nor were those who had undergone previous endoscopic or surgical sphincterotomy. Patients were also excluded if they were being treated with anticoagulants or platelet antiagregants, such as acetyl salicylic acid and platicaxel or with a prothrombin time with a difference of > 5 s against the blind sample taken no earlier than 72 h before the study. We also eliminated patients who were allergic or hypersensitive to allopurinol or hydrosoluble contrast solutions or those with active hemorrhages of peptic origin. Additional exclusion criteria included a hemoglobin level of less than 8 g/dL; a platelet count of less than 60×10^9 /L; relative neutropenia (absolute neutrophil count $< 2.0 \times 10^9$ /L); significant renal dysfunction (serum creatinine level, > 200 μ mol/L); decompensated cirrhosis; a known or suspected pregnancy or presence of lactation; current or recent use of allopurinol (within 48 h); current use of drugs with a known interaction with allopurinol, including cyclophosphamide, chlorpropamide, azathioprine/mercaptopurines, or probenecid and an inability to swallow or absorb oral medication.

Main outcome measurements

We included 170 patients in this study. Randomization was performed at the Department of Gastrointestinal Endoscopy by using computer-generated random numbers. Allopurinol and the placebo were similar in presentation and packed in appropriate containers with the identification code. The drug or placebo was only administered after informed consent was obtained. Eighty-five patients were randomly assigned to the study

group receiving 600 mg of allopurinol divided in two oral doses before the procedure (300 mg at 15 h and 300 mg at 3 h before ERCP) and 85 patients were assigned to the control group receiving two doses of an oral placebo at the same time. Blood samples were drawn from all patients to determine serum amylase levels before the procedure and 2 h later and classified as normal level (< 150 IU/L); or hyperamylasemia (> 151 IU/L). If the amylase serum level was > 151 IU/L and there was no evidence of acute pancreatitis (abdominal pain, nausea or vomiting), patients were started on a liquid diet and discharged 8 h to 24 h after the endoscopic procedure. If the serum amylase was above 600 UI/L or three times above the normal value and the patient had a sharp pain irradiating to the back and nausea or vomiting, the diagnosis of PEP was established in the absence of radiological evidence of a pneumoperitoneum or emphysema in the retroperitoneal space through a plain radiologic examination of the abdomen or CT scan. These patients were managed in the hospital with fasting, hydration with crystalloid solutions, antiemetics (metoclopramide) and analgesics. Pancreatitis episodes were classified according to Ranson's prognostic criteria and CT severity index^[34].

Details concerning the endoscopic procedure, specifying the difficulty for cannulation, number of pancreatic duct injections, sphincterotomies, characteristics of the bile duct, presence of choledocolithiasis, as well as defining whether the procedure was diagnostic or therapeutic (endoprosthesis placement or stone extraction). Patients were classified as low-risk for the development PEP or those male and older than 50 years old, when the procedure was diagnostic or therapeutic with sphincterotomy, biliary or pancreatic stenting and stone extraction or presence of chronic pancreatitis. Otherwise, patients were considered as high-risk for the development of PEP in the case of female gender and younger than 50 years old, those submitted to pancreatic duct manipulation or precut sphincterotomy, multiple endoscopic procedures, difficult or failed cannulation and patients with suspected sphincter of Oddi dysfunction^[10,16]. Other complications such as perforation, bleeding and infection, were recorded.

Statistical analysis

The results are shown as percentages and as means with standard deviations. Statistical inference was tested using chi-squared or Fisher's exact test for qualitative variables, while Student's *t* test was used for quantitative variables. To explore the risk factors, the relative risks and 95% confidence intervals were estimated. $P < 0.05$ was considered statistically significant. Finally, the reduction in absolute risk (ARR), the reduction in relative risk (RRR) and number needed to treat (NNT) were analyzed to estimate factors needed to prevent an episode of pancreatitis.

Ethical considerations

The research protocol was reviewed and approved by the Research and Ethics Committees of our Institution. All patients signed informed consent forms before

Table 1 Demographics and ERCP data

	Allopurinol <i>n</i> = 85	Placebo <i>n</i> = 85	<i>P</i>
Age	53.5 ± 18.9	52.8 ± 19.8	0.82
Gender M/F	36/49	34/51	0.86
Diagnosis			
Benign			
Choledocholithiasis	35	35	0.51
Iatrogenic injury of the biliary tract	11	14	0.48
Chronic pancreatitis	3	1	0.31
Chronic hepatopathy	2	2	0.60
Sphincter of oddi dysfunction	2	2	0.60
Mirizzi's syndrome	1	0	0.50
Malignant			
Pancreatic adenocarcinoma	11	12	0.82
Cholangiocarcinoma	4	5	0.50
Periampullary carcinoma	2	2	0.60
Gallbladder cancer	0	1	0.50
Normal cholangiography	8	5	0.48
Failed procedure	6	6	0.61
Total	85	85	

taking part in the study. The study was financed with funds from the Department of Gastroenterology, Gastrointestinal Endoscopy and the Medical Research Unit in Clinical Epidemiology of the Medical Center.

RESULTS

The patients participating in the trial comprised 70 men (41.2%) and 100 women (58.8%). The study group had 36 men and 49 women; in the control group there were 34 men and 51 women. The average age for the study group was 53.5 ± 18.9 years and for the control group it was 52.8 ± 19.8 years. Basal amylase levels were 50.8 ± 19.3 U/dL for the study group and 46.9 ± 16.1 U/dL for the control group.

A benign diagnosis for both groups was reported in 108 patients (63.5%): 54 in the study group and 54 in the control group. Malignant diseases were diagnosed in 17 and 20 cases respectively (21.8%). Normal cholangiography was determined in eight and five cases respectively (7.6%) and difficult or failed ERCP occurred in six patients in each group (7%). The diagnoses reached are shown in Table 1. No significant statistical differences were found between groups, and there were no differences in the procedural details described in Table 2. Twenty-three patients developed hyperamylasemia (> 151 IU/L), five (5.8%) from the study group and eighteen (21.1%) from the control group ($P = 0.003$). Ten patients developed pancreatitis, two from the study group (2.3%) and eight from the control group (9.4%; $P = 0.04$). In all cases amylase levels were above 600 IU/L (range 771 to 8886 IU/L). These patients were classified according to Ranson's criteria at admission and at 48 h by CT Severity index as having mild pancreatitis (less than 3 positive signs and Balthazar's A an B without necrosis, severity index of 0 to 1 points). They were handled conservatively and all did well. All patients were discharged within three days of starting the treatment.

Table 2 Procedural details, endpoints and post-ERCP morbidity *n* (%)

	Allopurinol group <i>n</i> = 85	Placebo group <i>n</i> = 85	<i>P</i>
Procedural details			
Total procedural time (min)	37.8 ± 11.9	38.2 ± 12.4	0.82
Cannulation time (min)	15.4 ± 5.5	15.6 ± 5.6	0.81
Pancreatic cannulation and injection	24 (24.7)	18 (21.1)	0.18
Number of injections	1.23 ± 0.42	1.27 ± 0.44	0.60
Acinarization	9 (10.5)	9 (10.5)	0.58
Invasive diagnostics			
Cytology	15 (17.6)	17 (20)	0.42
Intrabiliary biopsy	2 (2.3)	2 (2.3)	0.69
Therapeutics			
Any Therapeutics	71 (83.5)	74 (87)	0.51
Precut sphincterotomy	15 (17.6)	18 (21.1)	0.56
Biliary sphincterotomy	20 (23.5)	17 (20)	0.57
Stone extraction	29 (34.1)	27 (31.7)	0.74
Biliary stenting	32 (37.6)	37 (43.5)	0.43
Pancreatic stenting	2 (2.3)	3 (3.5)	0.64
End points			
Hyperamylasemia	5 (5.8)	18 (21.1)	0.003
Pancreatitis	2 (2.3)	8 (9.4)	0.049
PEP in low-risk procedures	1/55 (1.8)	1/57 (1.7)	0.70
PEP in high-risk procedures	1/30 (3.3)	7/28 (25)	0.02
ERCP morbidity			
Bleeding	2 (2.3)	2 (2.3)	0.69
Perforation	1 (1.1)	0	0.50

The analysis of the risk factors for the development of PEP revealed that Gender ($P = 0.52$, RR, 0.83, CI 95% 0.24-3.1), age [younger or older than 50 years old ($P = 0.31$, RR, 0.38, CI 95% 0.04-3.12)] and etiology ($P = 0.18$, RR, 0.77, CI 95% 0.46-1.29) were not statistically different between groups. When sphincterotomy or biliary stenting was performed, no risk of developing acute pancreatitis was observed ($P = 0.31$, RR, 0.38, CI 95% 0.04-3.12). Otherwise, we observed a marked tendency to favor the development of acute pancreatitis if precut sphincterotomy was performed ($P = 0.022$, RR, 4.9, CI 95% 1.3-18.19), if there was instrumentation of the pancreatic duct ($P = 0.002$, RR 9.3, CI 95% 1.91-45.4) or if multiple endoscopic procedures such as pre-cut sphincterotomy plus pancreatic duct manipulation plus biliary stenting during the same ERCP were performed, ($P = 0.000$, RR 14.8, CI 95% 3.0-73.06). PEP was observed in eight patients submitted to high-risk procedures (Table 2), one (3.3%) corresponded in the allopurinol group and seven (25%) patients for the control group ($P = 0.02$). In contrast, two episodes of PEP were observed in patients submitted to low-risk procedures, one (1.8%) from the allopurinol group and one (1.7%) from the control group ($P = 0.70$).

We found an ARR of 21.7%, with an RRR of 86.8% and an NNT of 4.6 patients submitted to high-risk ERCP procedures to avoid a clinically evident episode of pancreatitis. Major complications were observed in four patients (two from each group) consisting of mild to moderate bleeding which required blood transfusion and resolved without surgical intervention and one perforation was observed in a patient of the study group

treated surgically without complications or mortality. No adverse events were recorded with the use of allopurinol or the placebo.

DISCUSSION

Xanthine oxidase (XO) was first discovered in milk over a century ago and in rat serum nearly 70 years ago^[35,36]. This enzyme is now known to be present in many different tissues and in a wide range of species from bacteria to humans^[37,38]. It is a cytosolic metalloflavoprotein that is predominantly responsible for the oxidation of endogenous purines and exogenous ethanol^[39-41]. Granger and colleagues demonstrated that XO was an important source of the oxidative stress associated with ischemia and reperfusion^[38]. This enzyme has since been implicated in the pathogenesis of a wide spectrum of diseases^[42], and it is thought to be the most important source of oxygen-derived free radicals and cell damage during reoxygenation of hypoxic tissues and pancreatitis^[40-44]. A number of studies in animal models conducted during the past two decades have highlighted the potential benefit of XO inhibition in a range of clinical settings. Thus, clinical studies have shown that it is safe and effective for the treatment of gout and tumor-lysis syndrome (a life-threatening constellation of metabolic abnormalities resulting from spontaneous or treatment-related tumor necrosis or fulminant apoptosis) and to reduce complications such as postoperative arrhythmias, myocardial infarction and associated mortality after cardiovascular surgery^[39].

Allopurinol has high oral bioavailability (80%-90%), a rapid onset of action (peak circulating level reached in 0.5-2 h) and a 70% hepatic transformation to a long-lasting active metabolite (oxypurinol, with a half-life of 15 h)^[39]. These pharmacokinetic attributes mean a single oral dose of allopurinol before ERCP could conceivably prevent PEP, because the drug targets those changes that contribute to the initial triggering of pancreatitis^[42,43]. Allopurinol is also an inexpensive generic drug with an excellent safety record and is not included in the catalog of drugs inducing pancreatitis^[45].

Four randomized clinical trials have been published in full to date (Table 3): a negative study from Budzyńska *et al*^[31] ($n = 300$), a positive study from Greece^[30] ($n = 250$), a negative study from the USA^[32] ($n = 701$) and the most recent study published by Romagnuolo *et al*^[33], from Canada ($n = 586$) with negative results. In the present study, we demonstrated that the use of allopurinol led to a significant reduction in the incidence of hyperamylasemia (5.8% *vs* 21.1% in placebo-treated controls, $P = 0.003$) and acute pancreatitis (2.3% *vs* 9.4%, $P = 0.04$). According to the particular patient's conditions, type of endoscopic procedure or multiple procedures, patients were divided as low and high risk for the development of PEP. The incidence was similar between patients submitted to low-risk procedures. In contrast, the difference was statistically significant in high-risk procedures, favoring the use of allopurinol (incidence 3.3% in the study group *versus* 25%

Table 3 Summary of randomized trials using allopurinol to prevent post-ERCP pancreatitis

Study (year), SC vs MC, country	n	Dose, mg	Allopurinol vs placebo PEP rates	Percentage high risk ¹	Comment
Budzyńska <i>et al</i> ^[31] (2001) SC, Poland	300	400 ²	12.1% vs 7.9%; 12 vs 8	0	3-arm study, with third arm (n = 100) given prednisone
Kastinelos <i>et al</i> ^[30] (2005) SC, Greece	250	1200 ³	3.2% vs 17.8%; 4 vs 21	0	2 patients with suspected SOD
Mosler <i>et al</i> ^[32] (2005) MC, USA	701	900 ⁴	13.0% vs 12.1%; 46 vs 42	70.2	4% absolute benefit in high-risk patients; 4% absolute harm in average risk
Romagnuolo <i>et al</i> ^[33] (2008) MC, Canada	586	300 ⁵	5.5% vs 4.1%; 16 vs 12	11.3	Harm in average risk patients; benefit in high-risk patients
Current study (2009) SC, Mexico	170	600 ⁶	2.3% vs 9.4%; 2 vs 8	34.1	21.7% absolute benefit in patients with high-risk procedures favoring allopurinol, no difference in low-risk procedures
Raw pooled	2007 (1008 vs 999)	-	7.9% vs 9.1%; 80 vs 91	-	1.2% difference (95% CI, 3.2% to 2.0%)

¹As defined in this protocol, namely sphincter manometry and/or pancreatic therapy. Other higher-risk cases (e.g. precut sphincterotomy or suspected SOD) were not considered; ²200 mg 15 h before, 200 mg 3 h before; ³600 mg 15 h before, 600 mg 3 h before; ⁴600 mg 4 h before, 300 mg 1 h before; ⁵300 mg 1 h before; ⁶300 mg 15 h before, 300 mg 1 h before; SC: Single centre; MC: Multicenter.

in the control group, $P = 0.02$). Fortunately, all episodes of acute pancreatitis were catalogued as mild and there were no deaths.

There was variability in the doses used in the previous studies and in the baseline rates of PEP in the control (placebo) groups (some of which are out of the usual range reported), but these differences do not appear to completely explain the heterogeneity in the results. There remains a possibility for a threshold effect or a minimally effective dose for allopurinol, given that the positive study^[30] used the highest dose (1200 mg); however, there does not seem to be a clear dose-response relationship as the larger negative studies^[31-33] used different lower doses (300, 400 and 900 mg; Table 3). The four earlier studies all checked formally or informally for interactions, presenting the active treatment and placebo PEP rates in different subgroups. None found significant interactions between diagnostic and therapeutic procedures. The most detailed analyses of this type were found in the studies by Mosler *et al*^[32] and Romagnuolo *et al*^[33]. Both demonstrated a benefit in the reduction of the episodes of acute pancreatitis as well as the severity when analyzing high-risk patients or those requiring sphincter of Oddi manometry or planned pancreatic therapy. Mosler *et al*^[32] demonstrated that allopurinol reduced the incidence of PEP from 27% to 23% in the high-risk group (4% absolute risk reduction) and also reduced harm (8% versus 12% PEP) in the non-high-risk group (Table 3). Romagnuolo *et al*^[33] found that, for non-high-risk patients, the crude rate of PEP was 5.4% in the allopurinol group and 1.5% in the placebo group ($P = 0.017$ favoring the placebo, indicating harm associated with allopurinol), whereas in the high-risk group the PEP rates were 6.3% in the allopurinol group and 23% in the placebo group ($P = 0.050$ favoring allopurinol). It is also necessary to note that more patients in the allopurinol group (44% vs 34% $P = 0.02$) required pancreatic duct injection as well as more injections (two versus one, $P = 0.01$). However, confounding was not confirmed statistically, and correcting for pancreatic injection in a stratified model still showed a nonsignificant trend toward harm for allopurinol in the non-high-risk subgroup. If

allopurinol is truly harmful for non-high-risk patients undergoing ERCP (the adjusted subgroup OR was not significant), the mechanism responsible is unclear. It could be the result of an idiosyncratic reaction to the medicine itself; one study did suggest that medications with a history of inducing pancreatitis could increase the risk of PEP^[46].

Budzyńska *et al*^[31], also included primarily non-high-risk patients and showed a higher rate of PEP with allopurinol. In contrast, the patients in the study by Kastinelos *et al*^[30] were also primarily non-high-risk patients and yet the study showed a significant benefit for allopurinol. In our results, using 600 mg of allopurinol we observed a significant reduction in the episodes of mild acute pancreatitis (2.3% vs 9.4, $P = 0.04$), but the difference was attributed to a beneficial effect of allopurinol in patients submitted to high-risk procedures, since in low-risk procedures the difference was not statistically significant.

The debate still continues. In a recent meta-analysis just published in September, 2008, Bai *et al*^[47] concluded that allopurinol may not be useful to prevent PEP. However, they recognized the limitations of their meta-analysis since it was a study-level analysis and the authors denoted the difficulties in stratifying high-risk patients and high-risk procedures because this information was not available in reviewed trials^[30-33,47]. To overcome the limitations, they recommended the design of multicenter trials with appropriate numbers of high-risk patients and high-risk procedures.

In conclusion, extensive evidence supports a beneficial effect of allopurinol in the prevention and severity of experimental pancreatitis. Clinical evidence supports a favorable effect of oral allopurinol in the prevention of PEP in patients submitted to high-risk procedures. Our results establish a reduction of the incidence of asymptomatic hyperamylasemia and PEP, particularly in patients submitted to high-risk procedures. More clinical trials with different dosification and patient selection are required to definitively determine any positive or deleterious effect of oral allopurinol in the prevention of PEP.

COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) is a widely applied method for the diagnosis and treatment of pancreatobiliary disease. Post-ERCP pancreatitis is the most common postoperative complication of ERCP and its prevention has become an urgent clinical challenge.

Research frontiers

ERCP is an indispensable method for the diagnosis and treatment of pancreatobiliary disease, and pancreatitis is the most common postoperative complication of it. There are some studies on drugs for preventing post-ERCP pancreatitis, but their results remain debatable. Therefore, most endoscopy centers do not give patients a conventional chemoprophylaxis.

Innovations and breakthroughs

This trial revealed that oral allopurinol 300 mg 15 and 3 h (600 mg) before ERCP could reduce pancreatitis and hyperamylasemia.

Applications

Oral allopurinol 300 mg 15 and 3 h (600 mg) before ERCP can prevent post-ERCP pancreatitis. Compared with other drugs, oral allopurinol is inexpensive, convenient and has very few side-effects, and can be used as a protective drug for preventing post-ERCP pancreatitis.

Peer review

This paper is interesting since aiming to demonstrate the effect, and possible effectiveness, of allopurinol on the occurrence of post ERCP acute pancreatitis. The design is well organized and the conclusion is that this drug has a preventive effect on post ERCP - hyperamylasemia and pancreatitis, especially in high risk patients.

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An autoregressive integrated moving average model for short-term prediction of hepatitis C virus seropositivity among male volunteer blood donors in Karachi, Pakistan

Saeed Akhtar, Shafquat Rozi

Saeed Akhtar, Department of Community Medicine and Behavioral Sciences, Faculty of Medicine, Kuwait University, PO Box 24923, Safat 13110, Kuwait

Shafquat Rozi, Department of Community Health Sciences, Medical College, Aga Khan University, Stadium Road, Karachi 74800, Pakistan

Author contributions: Akhtar S designed the study, analyzed the data, and wrote the manuscript; Rozi S participated in HCV surveillance, helped in data collection, and data management.

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Correspondence to: Saeed Akhtar, PhD, Department of Community Medicine and Behavioral Sciences, Faculty of Medicine, Kuwait University, PO Box 24923, Safat 13110, Kuwait. saeed.akhtar@hsc.edu.kw

Telephone: +965-2498-6542 Fax: +965-2533-8948

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Abstract

AIM: To identify the stochastic autoregressive integrated moving average (ARIMA) model for short term forecasting of hepatitis C virus (HCV) seropositivity among volunteer blood donors in Karachi, Pakistan.

METHODS: Ninety-six months (1998-2005) data on HCV seropositive cases ($1000^{-1} \times \text{month}^{-1}$) among male volunteer blood donors tested at four major blood banks in Karachi, Pakistan were subjected to ARIMA modeling. Subsequently, a fitted ARIMA model was used to forecast HCV seropositive donors for 91-96 mo to contrast with observed series of the same months. To assess the forecast accuracy, the mean absolute error rate (%) between the observed and predicted HCV seroprevalence was calculated. Finally, a fitted ARIMA model was used for short-term forecasts beyond the observed series.

RESULTS: The goodness-of-fit test of the optimum ARIMA (2,1,7) model showed non-significant autocorrelations in the residuals of the model. The forecasts by ARIMA for 91-96 mo closely followed the pattern of observed series for the same months, with mean monthly absolute forecast errors (%) over 6 mo of 6.5%. The short-term forecasts beyond the observed

series adequately captured the pattern in the data and showed increasing tendency of HCV seropositivity with a mean \pm SD HCV seroprevalence ($1000^{-1} \times \text{month}^{-1}$) of 24.3 ± 1.4 over the forecast interval.

CONCLUSION: To curtail HCV spread, public health authorities need to educate communities and health care providers about HCV transmission routes based on known HCV epidemiology in Pakistan and its neighboring countries. Future research may focus on factors associated with hyperendemic levels of HCV infection.

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Key words: Hepatitis C virus; Blood donor; Ecological analysis; Autoregressive integrated moving average model; Pakistan

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INTRODUCTION

Hepatitis C virus (HCV) infection poses a major public health problem in developing countries, including Pakistan. However, the results of prevalence studies have shown variable estimates in select groups including 1.8% to 3.0% in volunteer blood donors^[1,2] and 16% to 20.5% in familial contacts of infected patients^[3,4]. A community-based study in Hafizabad, Punjab, found a 6.5% HCV seroprevalence^[5]. Using these estimates, Pakistan has been grouped into intermediate category with respect to burden of HCV infection^[6]. Several routes have been implicated for nosocomial and community acquired

HCV infection including unsafe injections, recycling of used syringes, inadequate sterilization of surgical and dental equipment, and facial shaving by barbers^[2,7].

Public health authorities in Pakistan intermittently run educational campaigns in electronic and print media to create awareness in the general population to halt HCV spread. However, in the absence of adequate HCV surveillance, the true impact of the HCV control efforts remains uncertain. Volunteer blood donors are generally considered to be the healthier segment of any community and the proportions of HCV seropositivity among them may be considered to mirror the situation in the general population^[8]. We have previously reported a significant increase in HCV seroprevalence among volunteer blood donors over the past several years using data from two blood banks^[2]. However, there is need to expand this HCV surveillance network countrywide to obtain more reliable and representative estimates.

Recently, mathematical models have been used to project the future HCV prevalence among intravenous drug users^[9], and its impact on the future development of HCV related morbidity and mortality^[10]. Modeling and forecasting HCV seropositivity among volunteer blood donors in Pakistan, and perhaps in other neighboring countries, might provide useful information for allocating resources, and re-shaping and planning future control activities^[11]. This study aimed to develop a univariate time series model for HCV seropositivity ($1000^{-1} \times \text{month}^{-1}$) among volunteer blood donors attending four large blood banks. Specifically, the objective of this study was to identify the stochastic autoregressive integrated moving average (ARIMA) model for short term forecasting of HCV seropositivity ($1000^{-1} \times \text{month}^{-1}$) among volunteer blood donors in Karachi, Pakistan.

MATERIALS AND METHODS

Setting

This study was conducted in Karachi-the largest cosmopolitan city and the hub of economic activity of Pakistan. It has an estimated population of 9.3 million, accounting for approximately 10% of the total population of the country. Forty three percent of the city's population is under the age of 15 years. The population of Karachi comprises several ethnic groups defined by mother tongue, including predominantly Urdu, Sindhi, Punjabi, Pushto, and Balochi. The healthcare facilities for the population include several small and tertiary care hospitals, both in the private and public sector.

Data

Eight-year (1998-2005) data on monthly aggregates of number of donors attending four large blood banks (blood bank I-IV) in Karachi were available for this study. These blood banks receive blood donations only from non-remunerated volunteer blood donors. Blood bank I is part of a tertiary care hospital in the private sector and receives blood donations as replacements

from friends and relatives of inpatients requiring blood transfusions. Blood banks II-IV belong to non-governmental organizations and cater for the needs of those in Karachi who need blood transfusions, including the patients with leukemia, hemophilia, thalassemia and other blood related diseases. Blood banks II-IV also receive blood donations from volunteers on an exchange basis. Prior to blood donation, each blood donor is subjected to screening for known risk factors for transfusion transmissible infections. All the blood banks follow similar criteria to receive blood donations and exclude potential donors who admit known risk factors of transfusion transmissible infections or any medical or non-medical condition associated with high risk (e.g. use of narcotic drugs, history of jaundice in the past 5 years and recent hospitalization). All four blood banks in the study use commercially available enzyme-linked immunosorbant assay kits and results are interpreted according to the manufacturer's instructions.

As noted earlier, blood donations between January 1998 and December 2005 by men aged 18-64 years were included in this evaluation. HCV serological results of consecutive blood donations from these blood banks were available from variable starting dates depending on the completed records, to assess the proportions of HCV seropositive donors.

Analytic approach

We used methods developed by Box and Jenkins to build an ARIMA time series model^[12]. This model-building process is designed to take advantage of associations in the sequentially lagged relationships that usually exist in data collected periodically. The general form of the ARIMA model was

$$\Delta^d z_t = \Phi_1 z_{t-1} + \dots + \Phi_p z_{t-p} + a_t - \theta_1 a_{t-1} - \dots - \theta_q a_{t-q}$$

where:

$\Delta^d z_t$ = differenced series i.e. $z_t - z_{t-1}$

z_t = set of possible observations on the time-sequenced random variable

a_t = random shock term at time t

$\Phi_1 \dots \Phi_p$ = autoregressive parameters of order p

$\theta_1 \dots \theta_q$ = moving average parameters of order q

The series was subjected to Box-Cox transformation^[13]. The transformed series was then differenced at the non-seasonal level and mean corrected to induce stationarity. Sample autocorrelation and partial autocorrelation functions were used to identify the ARIMA model of the appropriate order. Estimates of the model's parameters were obtained by the maximum likelihood method. Diagnostic checking included residual analysis and the Akaike Information Criterion was used to compare goodness-of-fit among ARIMA models. The final model was a result of several iterations of the identification, estimation, and checking process, and met the conventional criteria for the adequacy of the model^[14].

Assessment of forecast accuracy

The last 6 observations in the data set were used for validation of the forecast accuracy of the ARIMA

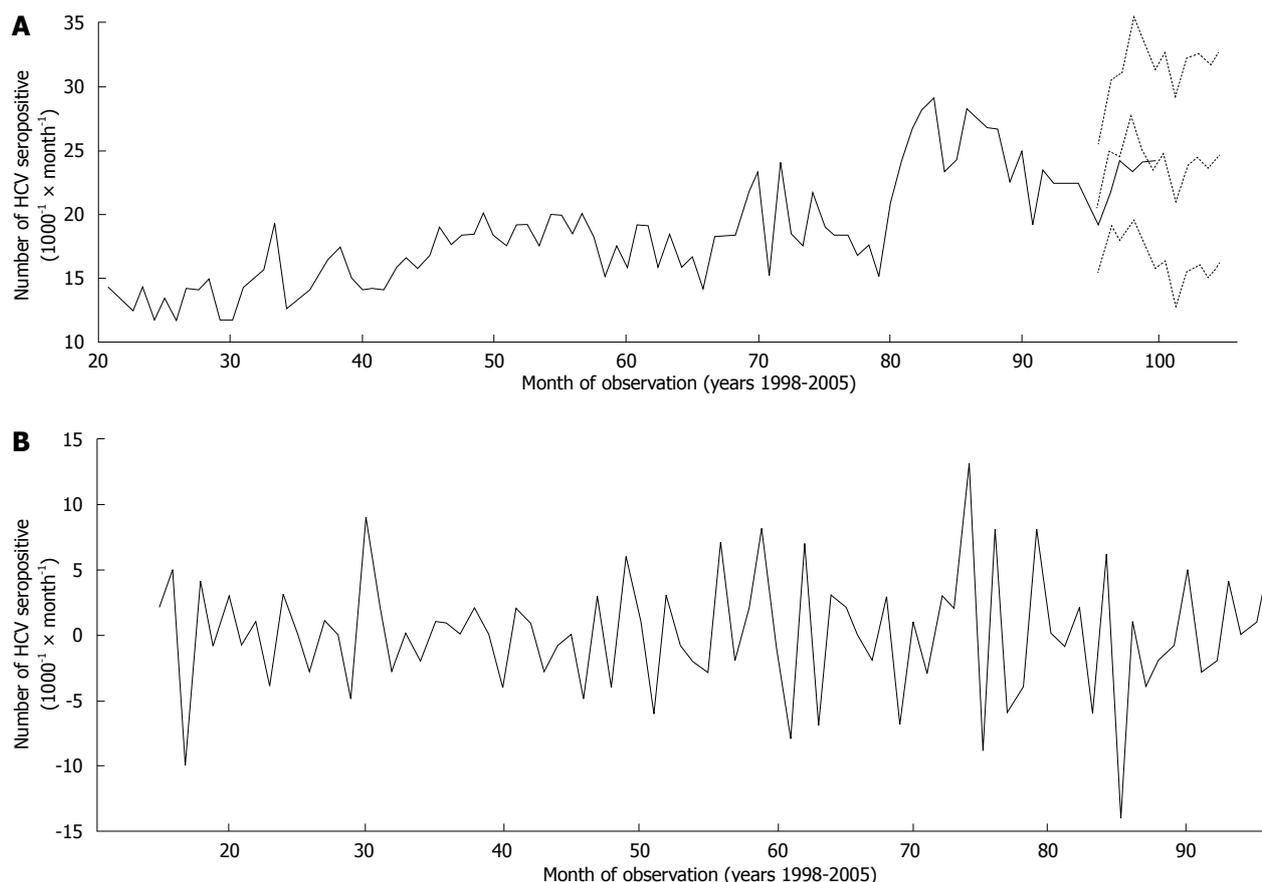


Figure 1 Hepatitis C virus seroprevalence (1000⁻¹ × month⁻¹) among volunteer male blood donors in Karachi, Pakistan 1998-2005. A: Observed data along with forecasts; B: Transformed series.

Table 1 Hepatitis C virus seroprevalence (1000⁻¹ × year⁻¹) among male volunteer blood donors at four large blood banks in Karachi (1998- 2005)

Yr	Mean	SD	95% CI for mean		Minimum	Maximum
			Lower limit	Upper limit		
1998	13.8	1.5	12.8	14.7	12	16
1999	16.3	2.2	14.8	17.7	13	21
2000	18.5	2.2	17.1	19.9	15	22
2001	19.8	2.0	18.6	21.1	16	22
2002	19.9	3.0	18.0	21.8	15	26
2003	20.3	3.2	18.2	22.3	16	27
2004	29.3	2.6	27.6	30.9	25	33
2005	24.8	2.1	23.4	26.1	21	27
Total	20.3	5.1	19.3	21.3	12	33

F = 47.9; df = 7, 88; P < 0.001.

model. The fitted ARIMA model was used to forecast the HCV seroprevalence (1000⁻¹ × month⁻¹) for 91-96 mo (June 2005 to December 2005) to contrast with the observed series of the same months. The average forecast error at prediction interval of *m* months ($\bar{\epsilon}_m$) was calculated as:

$$(\bar{\epsilon}_m) = \left[\sum_{m=1}^6 (y_{t=m} - \hat{y}_{t=m}) / 6 \right]^{1/2}$$

Where $y_{t=m}$ and $\hat{y}_{t=m}$ denote the observed and forecast values for month *t* + *m*. Finally, the fitted ARIMA model was used for short term (January 2006 to June 2006) forecasts along with their 95% confidence limits beyond the observed series.

RESULTS

Descriptive analysis

The crude HCV seropositivity (1000⁻¹) among the male volunteer blood donors during the study period was 20.3 (12792/630134). The mean prevalence (1000⁻¹ × month⁻¹) was 18.3 [95% confidence interval (CI): 16.8-19.9]. There was no statistically significant difference in HCV seropositivity (1000⁻¹) across various months of the years (*F* = 0.201; *P* = 0.997) (data not shown). However, a substantial variation in HCV seroprevalence (1000⁻¹) was observed across different calendar years (*F* = 47.895; *P* < 0.001) (Table 1). The observed and transformed series are presented in Figure 1.

ARIMA model

The parameters' estimates for the optimum ARIMA (2,1,7) model for the series of monthly HCV seropositive donors (1000⁻¹) are shown in Table 2. The autocorrelation and partial autocorrelation functions of the residuals showed good-fit (Figure 2). The residual plots showed small variations around the zero mean. None of these residuals had its magnitude larger than twice the standard deviation. Residuals' autocorrelations were not significantly different from zero as a set and had constant variance, thus confirming the adequacy of the model (Ljung-Box statistic = 20.4; *P* = 0.433).

The forecasts by the ARIMA (2,1,7) model for 91-96 mo (June 2005 to December 2005) using the

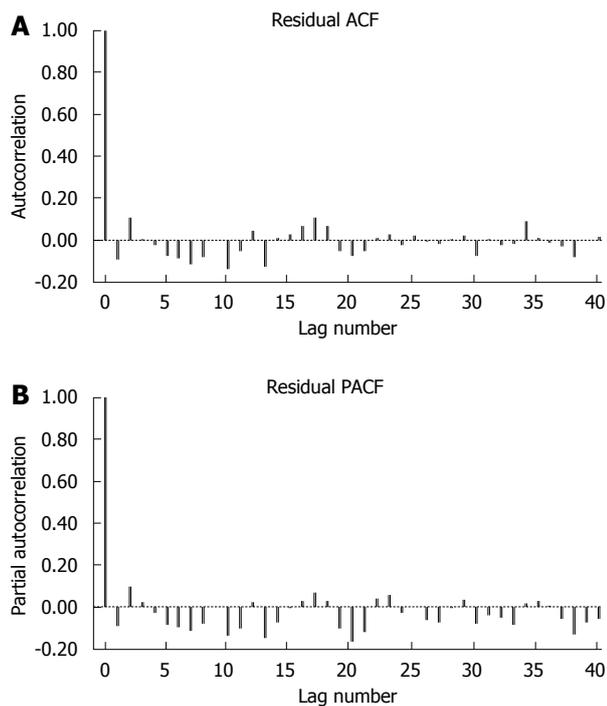


Figure 2 Residual plots for the final ARIMA (2,1,7) model of HCV seroprevalence (1000⁻¹ × month⁻¹) among male volunteer blood donors in Karachi, Pakistan 1998-2005. A: Autocorrelation function; B: Partial autocorrelation function.

observed series of months 1-90, closely followed the pattern of observed series for the same months (Figure 1), with mean ± SD and maximum monthly absolute forecast errors (%) over 6 mo interval being 6.5% ± 3.4% and 10%, respectively. Furthermore, the short term (January 2006 to June 2006) forecasts beyond the observed series adequately captured the pattern in the data (Figure 1) and showed evidence of increasing tendency of HCV seroprevalence (1000⁻¹ × month⁻¹) with the mean ± SD as 24.3 ± 1.4 over the forecast interval.

DISCUSSION

Epidemiological surveillance of communicable diseases is one of the more traditional public health activities. Time series analysis of surveillance data on prevalence and/or incidence of various infections may be helpful in developing hypotheses to explain and anticipate the dynamics of the observed phenomena and subsequently in the establishment of a quality control system and re-allocation of resources^[15,16]. This method is an ecologic approach and takes advantage of the strong association in the sequentially lagged relationship that usually exists in the data collected periodically^[17].

During the study period, the overall HCV seroprevalence (1000⁻¹) in volunteer blood donors was 20.3, which falls in the range of 14.9 to 38.9 known for first time blood donors in other developing countries. However, HCV seroprevalence (1000⁻¹) in this study was much higher than the 2.1 reported for developed countries^[18]. The low HCV seroprevalence in resource-

Table 2 Autoregressive integrated moving average model (2,1,7) of hepatitis C virus seroprevalence (1000⁻¹ × month⁻¹) among male volunteer donors in Karachi, Pakistan, (January 1998-December 2005)

Parameters	Estimate	Standard error	t-ratio
Autoregressive parameter (Φ)			
Φ ₂	0.67	0.15	4.5
Moving average parameter (θ)			
θ ₂	-0.59	0.18	3.3
θ ₃	0.49	0.15	3.3
θ ₄	-0.80	0.11	7.3
θ ₆	0.74	0.21	3.5
θ ₇	-0.37	0.17	2.2

White noise variance = 9.74; Ljung-Box Q statistic = 20.4 (P = 0.433).

rich countries is attributed to safe blood transfusion, whereas, in poor regions of the world, several million people acquire HCV infection each year as a result of contaminated transfusions and the re-use of infected medical devices^[18,19]. Therefore, public health practices adopted by the developed countries need to be strictly enforced in less developed countries to break the chain of transmission of HCV and other blood-borne pathogens.

Monitoring of HCV seropositivity among volunteer blood donors may provide clues about the effectiveness of control efforts of public health authorities and future trend of the proportion of HCV infected donors in Pakistan. In this paper, we used the ARIMA model on a time series of HCV seropositivity (1000⁻¹) collected monthly over a period of 96 mo on asymptomatic male volunteer blood donors from four major blood banks in Karachi. The forecasts made in a prospective manner over six months demonstrated increasing tendency of HCV seropositivity among the blood donors in this cosmopolitan city. Such a predicted increase in HCV seropositivity might result from inconsistent and naïve HCV control efforts on the part of public health officials in Pakistan. Therapeutic injections in a health-care setting have consistently been shown as a strong risk factor for HCV infection in Pakistan^[2,7,20,21], and if concerted efforts by the public health authorities are not made, might continue to contribute to the increasing load of HCV infection in this and similar settings in the region. An increasing trend among first time US blood donors of 50 to 59 years of age from 1995 to 2002 has been demonstrated^[22]. According to the authors, teenage children and young adults in 1960, and 1970s might have experimented with drug injection and were infected with HCV. These people entered into the 50 to 59 years age group during 1995 to 2002. However, in other age groups of donors in the same study and two other studies from US^[23,24], and from other developed countries (France^[25,26] and Spain^[27]) have shown a decreasing trend of residual risk of HCV infection in blood donors. According to these investigators different factors could have played a role in this reduction, for instance, increased awareness about the factors associated with increased risk of HCV infection, voluntary deferral by potential high risk

donors, improvement in donor recruitment, and /or an overall decrease in HCV infection level in the general population. Such factors need to be evaluated in our population in future studies.

Results from our previous study^[2], and those predicted by ARIMA model for 6 mo beyond the observed data exhibited a slightly increasing tendency of HCV seropositivity among male volunteer blood donors over the forecast period. This increasing pattern of HCV seroprevalence among these asymptomatic male volunteer donors merits further investigation of factors contributing to HCV seroprevalence in this population, which is thought to be a mirror image of the situation in the general population.

Some limitations of this study need to be taken into account when interpreting the results. Our HCV seroprevalence estimates are based on ELISA, which has sensitivity of more than 95%. However, these results do not reflect possible HCV infections that do not produce detectable seropositivity during the window period of HCV infection. The exact proportion of these HCV infected, but HCV seronegative, is not known, however, it has been argued that this figure must be very small given the use of current sero-assays^[28]. Our HCV seroprevalence estimates in male volunteer donor population were based on data from a limited number of blood banks; we do not know whether they reflect the national average. The blood banks that participated in this study however, account for a substantial proportion of donations made annually in Karachi. These centers are located in large metropolitan areas where the prevalence and/or incidence of HCV may be higher than the national figures. Therefore, we think we are justified in making generalizations from our data. In conclusion, in the absence of comprehensive HCV surveillance in the general population in Pakistan and perhaps in other neighboring countries, further monitoring of HCV seropositivity in blood donors and the investigation of factors associated with hyperendemic HCV infection using multivariate ARIMA models might further expand our understanding about HCV epidemiology in this region. Furthermore, effective screening of all blood donors for HCV infection at all blood banks should be seriously considered, because one single HCV infected regular blood donor could transmit the infection to several recipients.

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COMMENTS

Background

Less developed countries such as Pakistan generally lack effective surveillance systems for communicable diseases. In this study, the authors used the Box-Jenkins approach to fit an autoregressive integrated moving average (ARIMA)

model that might be used to monitor and predict trends in hepatitis C virus (HCV) seroprevalence in the general population using volunteer blood donors as a sentinel group. This information may be helpful to facilitate early public health responses to minimize HCV related morbidity and mortality.

Research frontiers

Developed countries have been able to control the HCV transmission in the general population by public health measures. However, such initiatives are practiced at sub-optimal level in resource-constrained countries. This problem is further compounded by the absence of effective surveillance of communicable diseases including blood-borne pathogens. Therefore, alternative methods to monitor and predict the burden of such infections are needed for rational allocation of resources.

Innovations and breakthroughs

This is the first application of an ARIMA model to monitor and predict the HCV seroprevalence in volunteer blood donors at multiple blood banks. Such data on infections with HCV and other blood-borne pathogens may mirror the situation in a setting that lacks an effective surveillance system for these infections.

Application

The fitted ARIMA model could be used for sentinel surveillance of blood-borne infections in volunteer blood donors. Therefore, the estimates for current and predicted future burden of these infections could be used by public health authorities for making rational policy decisions for control and prevention of HCV and other blood-borne pathogens in resource-constrained countries including Pakistan.

Peer review

The authors describe an effective model to predict HCV seropositivity in Pakistan.

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Pentoxifylline *versus* prednisolone for severe alcoholic hepatitis: A randomized controlled trial

Binay Krishna De, Subhabrata Gangopadhyay, Deep Dutta, Sumanta Das Baksi, Adyapad Pani, Pramit Ghosh

Binay Krishna De, Subhabrata Gangopadhyay, Deep Dutta, Sumanta Das Baksi, Adyapad Pani, Pramit Ghosh, Department of Medicine, Medical College & Hospitals, Calcutta, 88 College Street, Calcutta 700073, India
Pramit Ghosh, Department of Preventive and Social Medicine, Medical College & Hospitals, Calcutta, 88 College Street, Calcutta 700073, India

Author contributions: De BK conceptualized the study; De BK and Dutta D designed the research; Gangopadhyay S and Dutta D performed the research; Baksi SD randomized the patients; Pani A administered the drugs to the patients; Dutta D and Ghosh P analyzed the data; De BK and Dutta D wrote the manuscript.

Correspondence to: Binay Krishna De, Department of Medicine, Medical College & Hospitals, Calcutta, 88 College Street, Calcutta 700073, India. binaykde@hotmail.com
Telephone: +91-33-23731060 Fax: +91-33-25740733

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profile and renoprotective effects of pentoxifylline compared with prednisolone suggest that pentoxifylline is superior to prednisolone for treatment of severe alcoholic hepatitis.

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Key words: Alcoholic hepatitis; Pentoxifylline; Prednisolone; Maddrey discriminant function score; Model for end-stage liver disease score; Glasgow alcoholic hepatitis score

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Abstract

AIM: To compare the efficacy of pentoxifylline and prednisolone in the treatment of severe alcoholic hepatitis, and to evaluate the role of different liver function scores in predicting prognosis.

METHODS: Sixty-eight patients with severe alcoholic hepatitis (Maddrey score ≥ 32) received pentoxifylline ($n = 34$, group I) or prednisolone ($n = 34$, group II) for 28 d in a randomized double-blind controlled study, and subsequently in an open study (with a tapering dose of prednisolone) for a total of 3 mo, and were followed up over a period of 12 mo.

RESULTS: Twelve patients in group II died at the end of 3 mo in contrast to five patients in group I. The probability of dying at the end of 3 mo was higher in group II as compared to group I (35.29% *vs* 14.71%, $P = 0.04$; log rank test). Six patients in group II developed hepatorenal syndrome as compared to none in group I. Pentoxifylline was associated with a significantly lower model for end-stage liver disease (MELD) score at the end of 28 d of therapy (15.53 ± 3.63 *vs* 17.78 ± 4.56 , $P = 0.04$). Higher baseline Maddrey score was associated with increased mortality.

CONCLUSION: Reduced mortality, improved risk-benefit

INTRODUCTION

Severe alcoholic hepatitis is an acute or acute-on-chronic hepatic inflammatory response syndrome, which is part of the spectrum of diseases that result from alcohol-induced liver injury, ranging from the most common asymptomatic fatty liver to fulminant hepatitis and cirrhosis in the long term. However, it is difficult to predict the clinical response in an individual patient, as only a minority of individuals consuming large amounts of alcohol develop alcoholic hepatitis^[1,2]. The importance of acute alcoholic hepatitis lies in its significant morbidity and mortality, with a reported in-hospital mortality as high as 44%^[3]. Large amounts of alcohol with binge drinking, malnutrition, and female sex, are some of the factors associated with more severe disease^[4]. The presence of coexisting hepatitis C has been found to be associated with worse prognosis^[5]. Recent studies have shown that impaired immune response, endoplasmic reticulum stress, mitochondrial dysfunction, and free-radical injury induced by alcohol and its acetaldehyde adduct metabolites, Kupffer cell activation and cytokine production, have an important role in accentuating the hepatocyte injury and disease

precipitation^[6,7]. Serum level of cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6 and IL-8 are elevated in acute alcoholic hepatitis^[8]. Studies have shown a linear relationship between TNF- α receptors and mortality from acute alcoholic hepatitis^[9].

Maddrey discriminant function (DF) has commonly been used in estimating mortality among patients with acute alcoholic hepatitis, with an elevated DF (> 32) indicating an increased likelihood of death, and conversely, a low DF suggesting a generally favorable prognosis^[10,11]. Recently, model for end-stage liver disease (MELD) score and glasgow alcoholic hepatitis score (GAHS) have also gained interest as predictors of disease outcome in patients with severe acute alcoholic hepatitis^[12,13].

Although prednisolone is used widely and considered the standard treatment for severe acute alcoholic hepatitis with DF score ≥ 32 , it is not free of adverse effects and has had its share of controversies^[14]. Recently, pentoxifylline, a non-specific phosphodiesterase inhibitor, with combined anti-inflammatory (TNF- α inhibition) and antifibrogenic properties, has been found to be useful in patients with acute alcoholic hepatitis with DF ≥ 32 ^[15-17]. The beneficial effects are believed to occur through various mechanisms such as inhibition of phosphodiesterases, increased cAMP levels and down-regulation of TNF- α , IL-1, IL-6, transforming growth factor-beta (TGF- β), interferon-gamma (IFN- γ), stellate cell activation and procollagen- I mRNA expression^[18].

Although many individual studies are available on the efficacy of pentoxifylline and prednisolone in the treatment of severe alcoholic hepatitis, as far as we are aware, no study has compared the two drugs head to head in a randomized controlled study.

The present study compared the efficacy of pentoxifylline and prednisolone in the management of severe alcoholic hepatitis (DF ≥ 32), and their immediate and short-term outcomes. Also, we evaluated the GAHS and MELD score in patients with severe alcoholic hepatitis and compared them to traditional scores like DF and Child's score.

MATERIALS AND METHODS

One hundred and fifty-eight chronic alcoholic patients attending the liver clinic, outpatient department or the emergency medical services of the Medical College and Hospitals Calcutta were initially considered. The study was carried out from July 2006 to September 2008. The patients were initially examined clinically, evaluated, and subsequently were admitted for the duration of the study. The study protocol was approved by the institutional ethical committee. All the patients underwent investigations for liver chemistry (liver function tests, prothrombin time), complete hemogram, random blood sugar level, urea, creatinine, electrolytes, viral markers such as hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (HCV) antibody, hepatitis A virus IgM, hepatitis E virus IgM, serum ceruloplasmin, 24-h urinary copper (as and when required), and antinuclear antibody, upper gastrointestinal endoscopy, and Doppler abdominal

ultrasound, as and when required. Patients were included who had a history of chronic alcohol intake of more than 50 g/d^[19] with clinical and biochemical features of severe alcoholic hepatitis [Maddrey DF ≥ 32 and aspartate aminotransferase: alanine aminotransferase (AST: ALT) $> 2:1$, with absolute values of AST < 500 IU/L and ALT < 200 IU/L]. Patients with any other potential etiology of liver injury (acute or chronic viral hepatitis, autoimmune liver disease, Wilson's disease) even in the background of chronic alcohol intake were excluded from the study. Also, patients with a history of abstinence from alcohol in the last month, or who were positive for human immunodeficiency virus antibodies were excluded. Patients with infection, sepsis or spontaneous bacterial peritonitis, gastrointestinal bleeding, hepatorenal syndrome, acute pancreatitis or any other severe associated disease (uncontrolled diabetes, hypertension, heart failure, pulmonary disease or malignancy) at the time of inclusion or in the previous 3 mo were also excluded.

MELD score, GAHS and Child's score were calculated for all the patients who were included in the study. Only those patients were considered for final study who gave a prior informed written consent for pharmacotherapy.

The included patients were then divided into two groups by a computer-generated randomization table: group I, patients receiving pentoxifylline, and group II, patients receiving prednisolone. The pharmacotherapy (pentoxifylline or prednisolone) was started within a week of admission.

Patients in group I received pentoxifylline (Trental tablets, Sanofi Aventis, Mumbai, India) at a dose of 400 mg thrice daily orally and a placebo tablet in the place of prednisolone for the first 4 wk. Patients in group II received prednisolone tablet (Wysolone, Wreath, Mumbai, India) at a dose of 40 mg once daily for 4 wk and a placebo tablet taken thrice daily in place of pentoxifylline for the same duration. During the study, concomitant treatments with salicylates, nonsteroidal anti-inflammatory drugs, budesonide, anti-TNF α agents, vitamin E, s-adenosyl methionine or ursodeoxycholic acid were not allowed. The investigators who allocated the patients to the groups, administered the drugs and collected the clinical and laboratory data, as well the statisticians, were all blinded regarding the nature of the pharmacotherapy. All the patients were admitted in the wards of the Department of Medicine, Medical College and Hospitals, Calcutta for the initial period of 4 wk. All investigations such as liver function tests, prothrombin time, electrolytes, renal profile and abdominal ultrasound were repeated after the initial 4 wk of pharmacotherapy. After the initial 4 wk, the study was opened and the patients allocated to the different groups were revealed. After 4 wk of initial therapy, the dose of prednisolone in group II was tapered by 5 mg/wk over a period of 7 wk and then stopped. Patients in group I (pentoxifylline) who tolerated the drug well, continued to receive the medication at the same dose for the next 8 wk, and then stopped.

Only those patients who were clinically stable at the end of 4 wk were discharged and later followed-up in the

liver clinic. All the patients were counseled for strict alcohol abstinence at the time of discharge from the hospital.

The patients were reviewed at least once a month in the liver clinic. During follow-up, all the patients were examined clinically, and asked about drug compliance, intake of alcohol or potential drug adverse effects. Liver function tests, prothrombin time, renal function test, electrolytes, and abdominal ultrasound were performed as and when required. Maddrey DF, MELD, GAHS and Child's scores were calculated for all the patients during follow-up. Patients who had any alcohol intake in the follow-up period were excluded thereafter from the study.

Statistical analysis

Student's *t* test was used for analysis of continuous variables, Fisher's exact test for binary variables, and the χ^2 test was used for categorical variables. All results of continuous variables are expressed as mean \pm SD. Survival curves were estimated according to the Kaplan-Meier method and were compared using the log-rank test. Survival comparisons between groups were performed on an intent-to-treat basis. Results were considered statistically significant at $P < 0.05$.

RESULTS

Of the 158 patients initially evaluated, 74 who fulfilled the inclusion criteria without any other potential etiology of liver injury or severe co-morbid states were considered. Two patients refused consent for the study and another two patients refused to be admitted for the duration of the study. Seventy patients who fulfilled the inclusion and exclusion criteria and who gave informed written consent were randomized and divided into two groups: group I (pentoxifylline) had 34 patients, and group II (prednisolone) had 36 patients. The total duration of follow-up was 12 mo, with the patients being examined and evaluated in the liver clinic on a monthly basis. Two patients in group II withdrew voluntarily from the study and were excluded.

A total of 68 patients, 34 in each group, were considered for the final analysis. The baseline clinical and biochemical parameters of the patients receiving pentoxifylline or prednisolone are elaborated in Table 1, and were found to be comparable.

In group I, pentoxifylline therapy had to be stopped prematurely (within 3 mo) in five patients because of the development of life-threatening complications, all of whom unfortunately succumbed to the disease. Two patients expired following massive gastrointestinal bleeding. Two patients were lost to progressive hepatic encephalopathy and one patient died of sepsis, not responding to conservative management. Out of the five patients lost, two patients succumbed in the first 4 wk and three expired between 4 wk and 3 mo of therapy.

In group II, prednisolone therapy was stopped prematurely (within 3 mo) in 13 patients because of development of life-threatening complications. Two patients developed sepsis and both of them died of septic

Table 1 Comparison of baseline parameters of patients receiving pentoxifylline (group I) vs those receiving prednisolone (group II) in the treatment of severe alcoholic hepatitis (mean \pm SD)

Parameter	Group I (pentoxifylline) (n = 34)	Group II (prednisolone) (n = 34)	P value
Age (yr)	47.53 \pm 11.16	46.47 \pm 9.67	0.68
Male:female	34:0	33:1	-
Ascites	31	33	0.37
Encephalopathy	20	23	0.61
Varices	23	22	0.80
Maddrey DF score	54.25 \pm 16.24	57.78 \pm 17.08	0.39
MELD score	23.14 \pm 3.97	22.65 \pm 3.33	0.58
GAHS	8.23 \pm 1.07	7.94 \pm 0.95	0.24
Child's score	11.85 \pm 1.62	12.15 \pm 1.28	0.41
Mean TLC (/cm ³)	13926.47 \pm 3068.15	15225 \pm 11836.18	0.5379
Serum Na (mEq/L)	135.26 \pm 8.26	132.80 \pm 6.90	0.1908
Serum K (mEq/L)	4.18 \pm 0.72	4.293 \pm 0.98	0.6207
Urea (mg/dL)	31.68 \pm 27.63	25.74 \pm 16.92	0.2889
Creatinine (mg/dL)	1.42 \pm 0.61	1.19 \pm 0.32	0.057
Bilirubin (mg/dL)	5.40 \pm 2.50	6.604 \pm 3.90	0.1345
Albumin (gm/dL)	3.19 \pm 0.67	3.040 \pm 0.75	0.3870
ALT (IU/L)	54.88 \pm 23.25	57.38 \pm 20.50	0.6397
INR	1.97 \pm 0.34	2.04 \pm 0.31	0.3493

$P < 0.05$ considered statistically significant. TLC: Total leucocyte count.

Table 2 Causes of death in patients receiving pentoxifylline or prednisolone in the treatment of severe alcoholic hepatitis (n = 34)

Cause of death	Group I (pentoxifylline)	Group II (prednisolone)
Hepatorenal syndrome	0	6
Sepsis	1	2
Gastrointestinal bleed	2	2
Encephalopathy	2	1
Unknown	0	1
Total	5	12

shock. Two patients had upper gastrointestinal bleed and succumbed to hemodynamic failure. One patient developed acute pancreatitis 26 d after inclusion; prednisolone was stopped and the patient responded to conservative management who has been doing well till the end of this study. Six patients died of hepatorenal Syndrome, not responding to conservative management. This is in sharp contrast to Group- I where none of the included patients developed hepatorenal Syndrome. One patient died of progressive hepatic encephalopathy and the cause of death could not be determined in one of the patients. Out of the total of 12 patients who expired in group II, seven succumbed in the first 4 wk and five more were lost between 4 wk and 3 mo of therapy. The cause of death and the complication profile are shown in Tables 2 and 3. The mortality was significantly higher among patients receiving prednisolone (35.29%) as compared to 14.71% among those receiving pentoxifylline, as elaborated by Kaplan-Meier analysis shown in Figure 1 ($P = 0.04$).

Thirty-two patients in group I and 27 in group II were evaluated in the liver clinic at the end of 4 wk. The study was opened at this point in time and the allotment

Table 3 Morbidity/complication profile of patients receiving pentoxifylline (group I) or prednisolone (group II) in the treatment of severe alcoholic hepatitis

Complications	Duration of follow-up			
	0-3 mo		3 mo to 1 yr	
	Group I (n = 34)	Group II (n = 34)	Group I (n = 29)	Group II (n = 22)
Nausea	24	19	14	4
Vomiting	12	8	4	1
Dyspepsia	3	7	1	1
GI bleed	-	2	2	4
Oral thrush	-	6	-	-
Sepsis	2	2	-	3
Recurrent encephalopathy	2	-	5	-
Worsening ascites	-	2	-	2
Impaired glucose tolerance	-	2	-	2
Delayed wound healing	-	2	-	1
Deep vein thrombosis	-	1	-	-
Pancreatitis	-	1	-	-
Hepatorenal syndrome	-	6	-	-

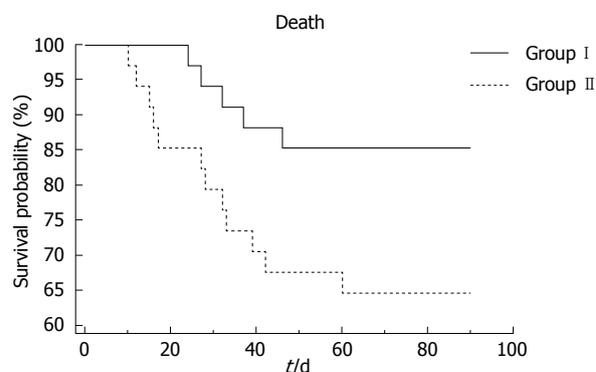
Table 4 Comparison of baseline parameters of patients succumbing to various complications to those surviving at the end of the study (12 mo)

Parameter	Patients succumbing to complications (n = 17)	Surviving patients (n = 51)	P value
Age (yr)	44.53 ± 11.19	47.82 ± 10.07	0.26
Male:female	17:0	50:1	-
Ascites	17	47	0.23
Encephalopathy	8	35	0.19
Maddrey DF score	63.22 ± 18.58	53.61 ± 15.39	0.038
MELD score	23 ± 4.15	22.86 ± 3.50	0.89
GAHS	8.35 ± 0.99	8 ± 1.02	0.21
Child's score	12 ± 1.06	12 ± 1.57	1.00
Mean TLC (/cm ³)	14008.82 ± 2804.14	14764.71 ± 9827.88	0.77
Serum Na (mEq/L)	131.76 ± 4.51	134.80 ± 8.35	0.16
Serum K (mEq/L)	4.22 ± 1.12	4.25 ± 0.76	0.90
Urea (mg/dL)	31.94 ± 27.95	27.63 ± 21.22	0.51
Creatinine (mg/dL)	1.19 ± 0.32	1.33 ± 0.55	0.34
Bilirubin (mg/dL)	6.88 ± 4.92	5.71 ± 2.56	0.21
Albumin (gm/dL)	2.97 ± 0.74	3.16 ± 0.70	0.32
ALT (IU/L)	52 ± 20.66	57.51 ± 22.18	0.37
INR	2.14 ± 0.32	1.96 ± 0.31	0.049

All values are expressed as mean ± SD. $P < 0.05$ considered statistically significant.

of patients to the different groups was revealed. The investigations done at the baseline were repeated, and the patients were re-admitted if deemed necessary. The patients were followed-up on a monthly basis and the investigations were repeated at the end of 3 mo, 6 mo and 1 year. The patients did relatively well beyond 3 mo of follow-up, and no more patients succumbed to the disease. In group I (pentoxifylline), one patient resumed alcohol consumption and another was lost to follow-up after 5 mo, and they were excluded from further analysis. In group II (prednisolone), two patients resumed alcohol consumption, after 8 and 10 mo of follow-up, respectively, and both were excluded from further analysis.

The morbidity/complication profiles among the two groups were comparable (Table 3). Nausea followed by vomiting and dyspepsia were the most common adverse

**Figure 1** Survival curves (Kaplan-Meier life table analysis) of patients receiving pentoxifylline (group I) as compared to patients receiving prednisolone (group II), at the end of 3 mo of therapy.

effects encountered in both groups. Patients receiving pentoxifylline more frequently complained of nausea and vomiting, whereas dyspepsia was more common among those receiving prednisolone. Recurrent hepatic encephalopathy was only seen in group I (pentoxifylline), while oral thrush, worsening of ascites, impaired glucose tolerance, delayed wound healing and deep vein thrombosis were seen only in group II (Table 3). On follow-up, recurrent encephalopathy was observed among five patients in group- I (pentoxifylline) in contrast to none in group II. The summary of the trial and its design is shown in Figure 2.

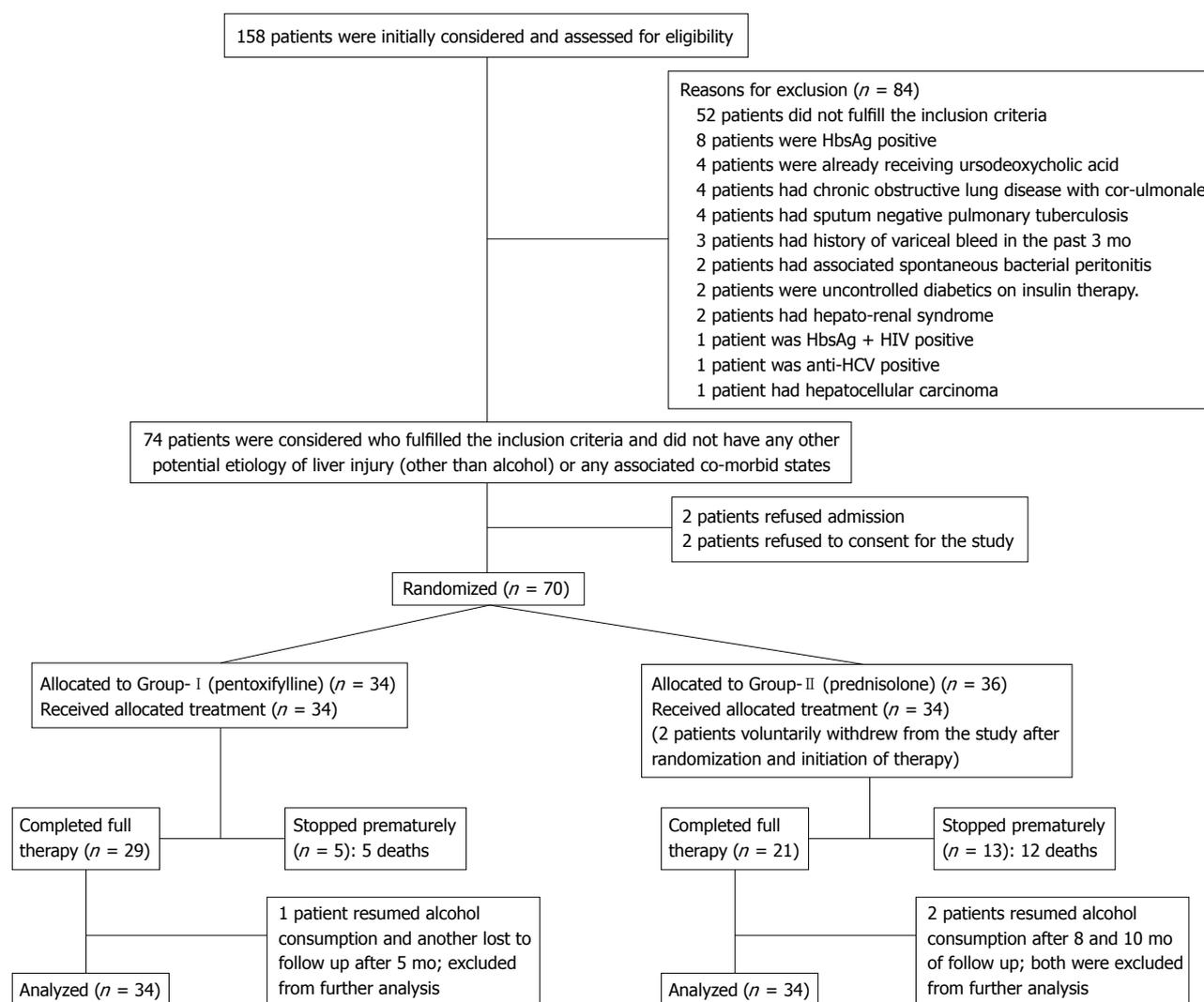
Table 4 shows the baseline profile of patients who succumbed to various complications as compared to those surviving at the end of the study. It shows that baseline Maddrey DF score and international normalized ratio (INR) was significantly higher among patients who succumbed to the disease as compared to those who survived ($P = 0.038$ and 0.049 respectively; Table 4). The baseline MELD score, GAHS and Child's score was not significantly different among the patients who expired as compared to those who survived (Table 4).

Table 5 shows the progression of GAHS, MELD, Child's and Maddrey DF score in the patients over 12 mo. The fall in Maddrey DF score and GAHS was comparable among the patients receiving pentoxifylline

Table 5 Progression of scores evaluating the severity of liver disease of patients receiving pentoxifylline (group I) as compared to those receiving prednisolone (group II) in the treatment of severe alcoholic hepatitis (mean \pm SD)

Liver disease score	Baseline	Duration of follow-up			
		4 wk	3 mo	6 mo	1 yr
Maddrey DF score					
Group I ¹	54.25 \pm 16.24	23.29 \pm 12.07	14.3 \pm 4.53	10.24 \pm 4.27	7.79 \pm 3.2
Group II ²	57.78 \pm 17.08	27.82 \pm 11.73	15.60 \pm 6.21	11.16 \pm 3.70	7.27 \pm 2.67
<i>P</i> value	0.39	0.15	0.39	0.43	0.94
MELD Score					
Group I ¹	23.14 \pm 3.97	15.53 \pm 3.63	12.41 \pm 2.88	10.37 \pm 2.32	9.18 \pm 1.59
Group II ²	22.65 \pm 3.33	17.78 \pm 4.56	13.45 \pm 2.77	11.14 \pm 1.83	9.4 \pm 1.88
<i>P</i> value	0.58	0.04	0.20	0.21	0.67
GAHS					
Group I ¹	8.23 \pm 1.07	6.37 \pm 0.79	6.10 \pm 0.77	5.96 \pm 0.90	5.74 \pm 0.66
Group II ²	7.94 \pm 0.95	6.52 \pm 1.09	5.91 \pm 0.61	5.91 \pm 0.61	5.7 \pm 0.57
<i>P</i> value	0.24	0.56	0.34	0.81	0.83
Child's score					
Group I ¹	11.85 \pm 1.62	9.69 \pm 2.57	7.14 \pm 1.60	5.96 \pm 1.09	5.78 \pm 0.89
Group II ²	12.15 \pm 1.28	9.81 \pm 2.08	7.59 \pm 1.68	6.23 \pm 0.97	5.9 \pm 0.79
<i>P</i> value	0.41	0.84	0.33	0.38	0.63

P < 0.05 considered statistically significant. ¹In group I: *n* = 34 at baseline, *n* = 32 at 4 wk and *n* = 29 at 3 mo, *n* = 27 at 6 mo and 1 year. ²In group II: *n* = 34 at baseline, *n* = 27 at 4 wk and *n* = 22 at 3 mo, 6 mo and *n* = 20 at 1 year.

**Figure 2** Summary of trial design and follow-up.

or prednisolone. MELD score was observed to be significantly lower among the patients receiving

pentoxifylline at the end of 4 wk, as compared to those receiving prednisolone.

DISCUSSION

The pathogenesis of alcohol-induced liver injury has not yet been clearly elucidated. Oxidative and nitrosative stress are believed to have a key role in the pathogenesis of alcoholic liver disease, and greater emphasis has been given to the role of cytochrome P450 2E1 in mitochondrial stress and disruption^[20]. Altered signaling pathways and involvement of extrahepatic mediators such as adiponectin may also have a key role^[20]. Augmented TNF- α production by macrophages and Kupffer cells and signaling *via* the p55 TNF receptor have been shown to be critical in the development of steatosis and hepatitis following chronic alcohol intake^[21]. Pentoxifylline, a non-specific phosphodiesterase inhibitor, with combined anti-inflammatory and antifibrogenic properties, has been shown to block the activation of hepatic stellate cells in culture^[22]. It also has inhibitory effects on basic mechanisms of fibrogenesis such as cell proliferation and extracellular matrix synthesis^[23]. Pentoxifylline has an added advantage of fewer adverse effects, such as gastrointestinal bleeding and renal shutdown, as compared to steroids. In the present study, none of the patients developed hepatorenal syndrome in the pentoxifylline group as compared to six in the prednisolone group. The MELD score in the pentoxifylline group was found to be significantly lower at the end of 4 wk of therapy (Table 5), as compared to the prednisolone group, confirming the renoprotective effects of pentoxifylline (as serum creatinine is a component of MELD score). Also gastrointestinal bleeding occurred more frequently in the prednisolone group as compared to the pentoxifylline group (Table 3).

The most important observation was the significantly reduced mortality among patients in the pentoxifylline group (14.71%) as compared to those receiving prednisolone (35.29%, $P = 0.04$, Figure 1). This reduced mortality in the pentoxifylline group was observed in spite of the increased occurrence of recurrent encephalopathy among patients in the pentoxifylline group. The patients with recurrent attacks of encephalopathy responded well to conservative management. This reduced mortality among patients in the pentoxifylline group can at least in part be explained by the renoprotective effects of pentoxifylline and the lower occurrence of gastrointestinal bleeding. In spite of the increased occurrence of nausea, and to a lesser extent vomiting, among patients in the pentoxifylline group, they were not severe enough to warrant stoppage of therapy. Also, with time, the occurrence of these complications was reduced (Table 3). Oral thrush, impaired glucose tolerance, poor wound healing, deep venous thrombosis and pancreatitis were some of the significant problems faced by the patients in the prednisolone group (Table 3).

Retrospectively, on analyzing the different liver function scores at the time of inclusion, only a higher Maddrey DF score was associated with the occurrence of increased mortality among patients with severe alcoholic hepatitis (Table 4). Thus Maddrey DF score remains the score of choice in determining prognosis

of patients with severe alcoholic hepatitis, even after the advent of newer scores like MELD and GAHS.

One of the limitations of this study is the absence of evidence of histological improvement and survival among patients receiving pentoxifylline or prednisolone, because of the lack of availability of transjugular liver biopsy. Also the assessment of immunological and inflammatory status (e.g. TNF- α) of the patients was not possible. Nevertheless, a reduced mortality and more advantageous risk-benefit profile of pentoxifylline compared with prednisolone in patients with severe alcoholic hepatitis suggest that pentoxifylline is at least equivalent to prednisolone in the treatment of severe alcoholic hepatitis. However, further studies with a larger cohort of patients is warranted to decide if pentoxifylline is actually superior to the traditional drug prednisolone in the treatment of severe alcoholic hepatitis.

COMMENTS

Background

Severe alcoholic hepatitis is an acute, potential life-threatening manifestation of alcohol-induced liver injury, and forms part of the spectrum of liver disease, ranging from asymptomatic fatty liver to cirrhosis. The importance of severe alcoholic hepatitis lies in its significant morbidity and mortality, with a reported in-hospital mortality as high as 44%. Prednisolone has been used widely and is considered the standard treatment for severe acute alcoholic hepatitis with maddrey discriminant function (DF) score ≥ 32 . However, it is not free of adverse effects and has had its share of controversies.

Research frontiers

Various other drugs have been tried in the treatment of alcoholic hepatitis, such as antioxidants, colchicines, calcium channel inhibitors, propylthiouracil and d-penicillamine, without much success. Augmented tumor necrosis factor (TNF)- α production by macrophages and Kupffer cells plays an important role in the pathogenesis of severe alcoholic hepatitis. However, infliximab, a human-mouse chimeric antibody to TNF- α , when used with prednisolone, has been found to be associated with severe infections, and is thus potentially harmful. The challenge is to find a drug whose efficacy is not only comparable to that of the standard drug prednisolone, but also safe and easy to administer over long periods of time.

Innovations and breakthroughs

Recently, pentoxifylline, a non-specific phosphodiesterase inhibitor, with anti-inflammatory (TNF- α inhibition) and antifibrogenic properties has been found to be useful in patients with severe alcoholic hepatitis. The idea was to evaluate the efficacy of pentoxifylline and compare it to the standard drug prednisolone in the treatment of severe alcoholic hepatitis in a randomized controlled study, and to study the immediate and short term outcomes. Significantly reduced mortality, a more advantageous risk-benefit profile, and renoprotective effects of pentoxifylline compared with prednisolone in patients with severe alcoholic hepatitis may be considered as a breakthrough.

Applications

The authors found that pentoxifylline was tolerated well in the treatment of severe alcoholic hepatitis, and was associated with significantly lower mortality, significantly lower model for end-stage liver disease (MELD) score at the end of 4 wk, and absence of hepatorenal syndrome. This should encourage the use of pentoxifylline in the treatment of severe alcoholic hepatitis. However, long-term prospective studies with a larger cohort of patients are needed to decide if pentoxifylline is actually superior to the traditional drug prednisolone in the treatment of severe alcoholic hepatitis.

Terminology

MELD score is a measure of the severity of liver dysfunction and has been recently used in the assessment of patients with severe alcoholic hepatitis. However Maddrey DF score remains the standard for the assessment of patients with severe alcoholic hepatitis.

Peer review

The experiments were planned and executed well and the manuscript is well written.

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BRIEF ARTICLES

Altered spontaneous contractions of the ileum by anesthetic agents in rats exposed to peritonitis

Cengiz Aydin, Ihsan Bagcivan, Sinan Gursoy, Ahmet Altun, Omer Topcu, Ayhan Koyuncu

Cengiz Aydin, Omer Topcu, Ayhan Koyuncu, Department of General Surgery, Cumhuriyet University School of Medicine, 58140 Sivas, Turkey

Ihsan Bagcivan, Ahmet Altun, Department of Pharmacology, Cumhuriyet University School of Medicine, 58140 Sivas, Turkey

Sinan Gursoy, Department of Anesthesiology, Cumhuriyet University School of Medicine, 58140 Sivas, Turkey

Author contributions: Aydin C, Bagcivan I, Gursoy S and Koyuncu A contributed equally to this work; Aydin C, Bagcivan I and Gursoy S designed the research; Bagcivan I, Altun A performed the research; Bagcivan I, Altun A analyzed the data; Aydin C, Bagcivan I and Topcu O wrote the paper.

Correspondence to: Dr. Cengiz Aydin, Associate Professor, Department of General Surgery, Cumhuriyet University School of Medicine, 58140 Sivas, Turkey. caydin2@yahoo.com

Telephone: +90-346-2580493 Fax: +90-346-2581305

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Abstract

AIM: To investigate *in vitro* effects of propofol, midazolam and dexmedetomidine, which are commonly used anaesthetic or sedatives, on spontaneous contractions of the ileum both in normal rats and those exposed to hyperdynamic peritonitis.

METHODS: Spontaneous contractions of isolated ileum muscle segments from sham operated rats and those exposed to peritonitis, were studied *in vitro*. The amplitude and the frequency of spontaneous contractions of ileum muscle segments were studied after adding dexmedetomidine, propofol, and midazolam to the organ bath in a cumulative manner.

RESULTS: Both amplitude (85.2 ± 6.6 vs 47.4 ± 7.1) and frequency (32.8 ± 4.6 vs 20.2 ± 3.9) of spontaneous contractions in ileum smooth muscle segments were decreased significantly in the peritonitis group compared to the control group ($P < 0.05$). Dexmedetomidine significantly increased the amplitude of spontaneous contractions (85.2 ± 6.6 vs 152.0 ± 5.4 , $P < 0.05$) whereas, propofol (85.2 ± 6.6 vs 49.6 ± 4.8 , $P < 0.05$) and midazolam (85.2 ± 6.6 vs 39.2 ± 4.5 , $P < 0.05$) decreased it in both control and peritonitis groups. The frequency of spontaneous contractions were significantly decreased by propofol

in both control (32.8 ± 4.6 vs 18.2 ± 3.4 , $P < 0.05$) and peritonitis groups (20.2 ± 3.9 vs 11.6 ± 3.2 , $P < 0.05$). Dexmedetomidine and midazolam did not cause significant changes in the number of spontaneous contractions in both control and the peritonitis groups ($P > 0.05$).

CONCLUSION: Propofol, midazolam and dexmedetomidine have various *in vitro* effects on spontaneous contractions of the rat ileum. While dexmedetomidine augments the spontaneous contraction of the rat ileum, propofol attenuates it. However, the effects of these compounds were parallel in both control and peritonitis groups.

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Key words: Ileum; Propofol; Midazolam; Dexmedetomidine; Peritonitis

Peer reviewer: Inge I Depoortere, PhD, Centre for Gastroenterological Research, Gasthuisberg OandN, bus 701, Leuven 3000, Belgium.

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INTRODUCTION

Dysmotility of the gastrointestinal tract is a major complication in critically ill patients in intensive care units. Most of the time, this dysmotility manifests itself as inhibition of gastrointestinal motility, and rarely as hypermotility^[1]. Hypomotility can cause a functional, nonmechanical obstruction, most commonly an adynamic ileus. Impaired motility in critically ill patients can be caused by intestinal ischemia, electrolyte imbalances, peritoneal injury, abdominal surgery, lower-lobe pneumonia, pancreatitis, cholecystitis, intraabdominal abscesses, and medications (opiates, dopamine, diltiazem, verapamil, and anticholinergics)^[2]. Sedatives such as propofol and midazolam further inhibit delayed intestinal transit in critically ill patients

in a dose dependent manner. The delay may cause complications such as intolerance to enteral feeding and overgrowth of bacteria in the gastrointestinal tract resulting in increase the incidence of aspiration pneumonitis^[3].

Abdominal sepsis or peritonitis is also a major cause of morbidity and mortality in surgical intensive care units. Gastrointestinal dysmotility commonly accompany peritonitis and those patients suffering peritonitis are also exposed to the additive effects of sedatives or anesthetics in surgical intensive care units. Koyluoglu *et al*^[4] recently demonstrated that peritonitis induced a decrease in the amplitude and frequency of spontaneous contractions of ileum and jejunum segments from rats. Therefore, it is preferable to use a sedative or an anaesthetic that has few inhibitory effects on gastrointestinal transit in patients with peritonitis. However, there has been little study on this topic in the literature. In the present study, we aimed to investigate *in vitro* effects of propofol, midazolam and dexmedetomidine on spontaneous contractions of the ileum both in normal rats and those are exposed to hyperdynamic peritonitis.

MATERIALS AND METHODS

Animal preparation

Sixteen male Wistar albino rats each weighing approximately 280 g were used in this study. The study was approved by the ethics committee of Cumhuriyet University School of Medicine. Cecal ligation and puncture were used as the peritonitis model^[5]. Animals were divided into two groups. The first group consisted of sham surgical controls that underwent the same procedure as the peritonitis group, such that laparotomy was performed under anesthesia, with manipulation of the cecum, but cecum ligation and puncture were not performed. Rats in the second group underwent cecal puncture and ligation as previously described by Martin *et al*^[5]. Animals were anesthetized with intramuscular injections of 3 mg/kg xylazine (Rompun[®], Bayer, Istanbul, Turkey) and 90 mg/kg ketamine (Ketalar[®], Pfizer, Istanbul, Turkey), following which, laparotomy was performed *via* a 2 cm midline incision and the cecum was exposed. The cecum was ligated using 4/0 silk suture material just below the ileocecal valve, so that intestinal continuity was maintained. Then, the cecum was punctured using an 18-gauge needle in three locations, 1 cm apart, on the antimesenteric surface of the cecum, and cecum was gently compressed until feces were extruded. The cecum was replaced into the peritoneal cavity and the abdomen was then closed. When the animals were alert, they were transferred to single housing cages where they were left with ad libitum food and water. We then observed the rats in a recovery cage for 24 h.

A summary of the experimental treatments is presented below: Groups: Group I ($n = 8$): Sham surgical controls; Group II ($n = 8$): Peritonitis group.

At the second laparotomy, 24 h later, the rats were killed by cervical dislocation. The abdomen was opened with a midline incision and the ileum was removed and placed in previously aerated (95% O₂ and 5% CO₂) Krebs-bicarbonate solution (composition in mmol/L: NaCl, 120; KCl, 4.6; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 22; NaH₂PO₄ and glucose 11.5). Whole full-thickness segments of ileum were placed in circular direction in a 10 mL tissue baths, filled with pre-aerated Krebs-bicarbonate solution (KBS) at 37°C. The upper end of the preparation was tied to an isometric transducer (Grass FT 03, Quincy, MA, USA) and preloaded with 1-1.5 g. Tissues were allowed to equilibrate for 30 min.

In vitro muscle contractility studies

Muscle segments from each group were contracted with 80 mmol/L KCl to ensure that they worked properly at the beginning and end of each experiment.

At the beginning of each experiment, 80 mmol/L KCl was added to the organ bath, and the contraction was considered as reference response. Subsequently, the amplitude of spontaneous contractions of the isolated ileum muscle segments were calculated as a percentage of the contraction induced by KCl (80 mmol/L) from both control and peritonitis groups. Changes in the frequency (number/min.) of spontaneous contractions were expressed as the number of contractions for 10 min intervals.

Following the KCl response, smooth muscle segments were allowed to equilibrate for 30 min before addition of cumulative doses of propofol (10⁻⁸-10⁻⁴ mol/L), midazolam (10⁻⁸-10⁻⁴ mol/L) and dexmedetomidine (10⁻⁸-10⁻⁴ mol/L). Amplitudes of the contractions induced by these compounds from both control and peritonitis groups were calculated as the percentage of the initial spontaneous contractions. Changes in the frequency of spontaneous contractions were expressed as the number of spontaneous contractions for 10 min after drug application. Isometric tensions were recorded on a Grass model 79 E polygraph. All experiments were performed in duplicate.

Drugs

The following compounds were used: Propofol (2,6diisopropylphenol[®], Aldrich Chemicals Co., USA), Midazolam (Midazolam hydrochloride[®], Sigma, St Louis, USA), Dexmedetomidine (Abbot Laboratories, Abbot Park, IL, USA). All drugs were dissolved in distilled water. All drugs were freshly prepared on the day of the experiment.

Data analysis

All data are expressed as mean \pm SD. Statistical comparisons between groups were performed using general linear models of analysis of variance (ANOVA) followed by the Newman-Keuls test and a *t* test when appropriate and *P*-values of less than 0.05 were considered to be statistically significant.

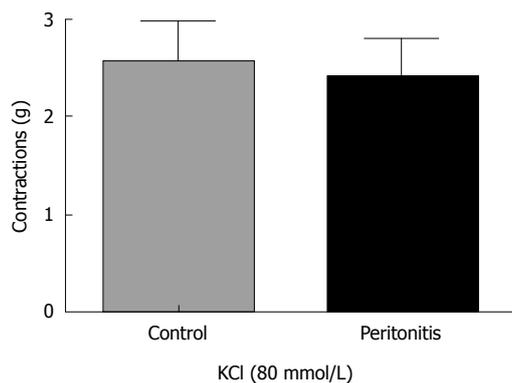


Figure 1 KCl (80 mmol/L) induced contractions of isolated ileum muscle segments in control and peritonitis groups. No statistical difference was observed between groups ($P > 0.05$).

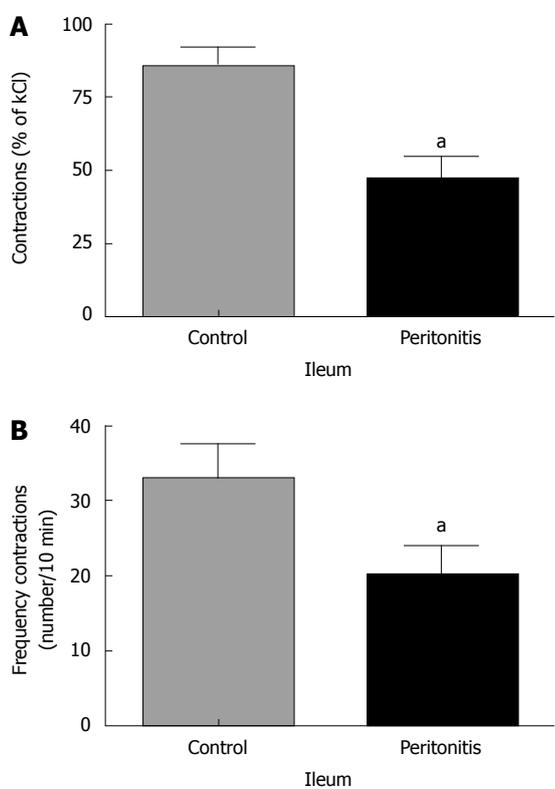


Figure 2 Changes in the spontaneous contractions of the isolated ileum muscle segments. A: Amplitudes were calculated as a percentage of the contraction induced by KCl (80 mmol/L) from both control and peritonitis groups; B: Frequencies were expressed as the number of contractions for 10 min from both control and peritonitis groups. (^a $P < 0.05$ vs control group; analysis of variance followed by Newman-Keuls test).

RESULTS

Contractions induced by 80 mmol/L KCl were not significantly different between the peritonitis group and the control group in isolated ileum smooth muscle segments which indicated that muscle segments from both groups worked properly (Figure 1).

The mean amplitude of the spontaneous contractions was 85.2 ± 6.6 in the control and 47.4 ± 7.1 in the peritonitis group, respectively. The number of spontaneous contractions obtained in 10 min in the

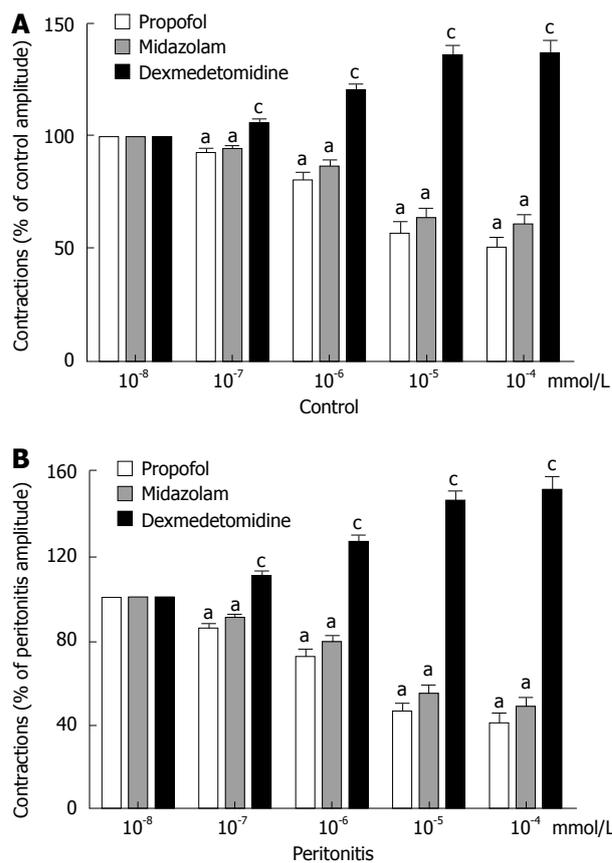


Figure 3 Amplitudes of the contractions induced by anaesthetic agents. A: Control group; B: Peritonitis group; both were calculated as the percentage of the initial contractions. (^a $P < 0.05$ vs initial contractions, ^c $P < 0.05$ vs propofol and midazolam; analysis of variance followed by Newman-Keuls test).

peritonitis group was 32.8 ± 4.6 and 20.2 ± 3.9 in the control group. Both the amplitude and the frequency of spontaneous contractions of ileum smooth muscle segments were decreased significantly in the peritonitis group compared to the control group ($P < 0.05$), (Figure 2A and B).

The amplitudes of spontaneous contractions of ileum muscle segments were studied after adding dexmedetomidine, propofol, and midazolam to the organ bath. Dexmedetomidine (10^{-8} - 10^{-4} mol/L) significantly increased the amplitude of spontaneous contractions starting from 10^{-7} mol/L in isolated ileum muscle segments, in both the control and peritonitis groups, in a concentration-dependent manner. Propofol (10^{-8} - 10^{-4} mol/L) and midazolam (10^{-8} - 10^{-4} mol/L) decreased the amplitude of spontaneous contractions starting from 10^{-7} mol/L as the molar concentrations of these drugs were increased ($P < 0.05$). However, there was no statistical difference between the decreasing effects of propofol and midazolam ($P > 0.05$), (Figure 3A and B), (Tables 1 and 2).

The frequency of spontaneous contractions of the ileum segments were significantly decreased by cumulative doses of propofol (10^{-8} - 10^{-4} mol/L) in both the control and peritonitis groups ($P < 0.05$). Dexmedetomidine (10^{-8} - 10^{-4} mol/L) and midazolam (10^{-8} - 10^{-4} mol/L) did not cause a significant change in the number of spontaneous

Table 1 Effects of Propofol, midazolam and dexmedetomidine on spontaneous contractions in experiment groups

	Control	Peritonitis
Amplitude (%) ¹	85.2 ± 6.6 (100)	47.4 ± 7.1 ^a (100)
Propofol	49.6 ± 4.8 ^b	59.4 ± 5.2 ^b
Midazolam	39.2 ± 4.5 ^b	51.8 ± 5.0 ^b
Dexmedetomidine	136.6 ± 5.8 ^{b,c}	152.0 ± 5.4 ^{b,c}
Frequency (#/10 min)	32.8 ± 4.6	20.2 ± 3.9 ^a
Propofol	18.2 ± 3.4 ^b	11.6 ± 3.2 ^b
Midazolam	28.2 ± 3.8	17.4 ± 3.0
Dexmedetomidine	36.2 ± 4.0	22.2 ± 3.1

¹Amplitudes of the contractions induced by anesthetic agents from both control and peritonitis groups were calculated as the percentage of the initial spontaneous contractions; ^a*P* < 0.05 *vs* control; ^b*P* < 0.05 *vs* initial spontaneous contractions; ^c*P* < 0.05 *vs* propofol and midazolam.

Table 2 Effects of Propofol, midazolam and dexmedetomidine on amplitude and frequency of the spontaneous contractions

	Amplitude	Frequency
Propofol	Decreased	Decreased
Midazolam	Decreased	No significant change
Dexmedetomidine	Increased	No significant change

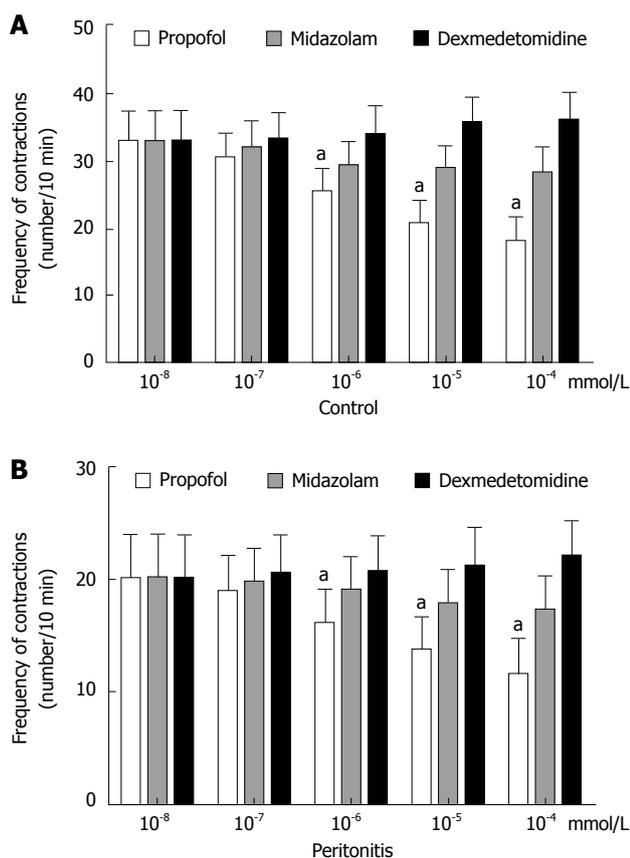


Figure 4 Changes in the frequency of spontaneous contractions. A: Control group; B: Peritonitis group. Both were expressed as the number of contractions for 10 min. (^a*P* < 0.05 *vs* initial contractions; analysis of variance followed by Newman-Keuls test).

contractions of the isolated ileum muscle segments in both the control and peritonitis groups (*P* > 0.05) (Figure 4A and B) (Tables 1 and 2).

DISCUSSION

The first finding of our study is that peritonitis altered the spontaneous activity of the rat ileum by decreasing both the amplitude and the frequency of the contractions in accordance with the previous study reported recently by Koyluoglu *et al.*⁴¹. The main findings are that dexmedetomidine, a selective α_2 -adrenergic agonist, increased the amplitude, midazolam decreased the amplitude and propofol decreased both the amplitude and frequency of the spontaneous contractions in rat ileum in a concentration-dependent manner in both the control and peritonitis groups. Liu *et al.*⁶¹ reported that the α_2 -adrenoceptors inhibited neurogenic contractions of the rat ileum. Dexmedetomidine inhibited peristalsis in the guinea pig small intestine *in vitro* in a concentration dependent manner⁷¹. Dexmedetomidine strongly inhibited intestinal transit in the rat, however this inhibition was less potent than morphine⁸¹. However, in our study, dexmedetomidine, when applied in a concentration dependent manner significantly increased the amplitude but did not change the frequency of spontaneous contractions in the rat ileum *in vitro* both in the control and peritonitis groups. Our result seems to contradict previous reports. However, similarly to our results, Karaman *et al.*⁹¹ showed that dexmedetomidine *in vitro* caused a significant increase in the amplitude and frequency of spontaneous contractions in rat myometrium in a dose-dependent manner. The augmenting effect of this agent on spontaneous contractions needs to be investigated further.

Lee *et al.*¹⁰¹ demonstrated that propofol has an *in vitro* inhibitory effect on spontaneous contractile activity and causes acetylcholine induced contractions of human gastric and colonic smooth muscles at clinically relevant concentrations. It was demonstrated in a previous study that propofol, at concentrations of 10^{-7} and 10^{-6} mol/L, potentiated the guinea pig ileum contractile responses to γ -aminobutyric acid (GABA), but only at the lower dose range of applied GABA; at a concentration of 10^{-5} mol/L, it inhibited the contractile effect over the entire dose range of applied GABA¹¹¹. In the present study, propofol decreased both amplitude and frequency of contractions of rat ileum muscle segments in the control and peritonitis groups. Previous studies have reported the relaxant effects of propofol on other smooth muscle tissues such as vascular¹²¹ and uterine smooth muscles¹³¹. The action of propofol involves a positive modulation of the inhibitory function of the neurotransmitter GABA through GABA A receptors¹⁴¹. Jensen *et al.*¹⁵¹ evaluated the influences of propofol, nitrous oxide and isoflurane and found that recovery and postoperative bowel function were not influenced by the anaesthetic technique after major gastrointestinal surgery. However, it might be different in case of a critically ill patient with peritonitis in an intensive care unit. The combined inhibitory effects of peritonitis and propofol might further slow intestinal transit and lead to an adynamic ileus.

Like other benzodiazepines, midazolam acts on the benzodiazepine binding site of GABA A receptors. When bound, it enhances the binding of GABA to the GABA A receptor, resulting in inhibitory effects on the central nervous system^[16]. Castedal *et al*^[17] reported in their manometric study that midazolam had relatively few effects on small bowel motility. In the present study, midazolam decreased the amplitude but caused very little change in the frequency of ileum contractions. Midazolam is often used for intravenous conscious sedation in endoscopic retrograde cholangiopancreatography (ERCP). Midazolam significantly altered the mobility of the sphincter of Oddi and caused a significant reduction in basal pressure of the sphincter of Oddi but did not affect the phasic frequency^[18].

In conclusion, dexmedetomidine, midazolam and propofol have various *in vitro* effects on spontaneous contractions of the rat ileum. While dexmedetomidine augments the spontaneous contraction of the rat ileum, propofol attenuates it. The effects of these agents were parallel in both control and peritonitis groups. The clinical implications of these findings need to be tested in surgical intensive care units, which might help in choosing the most appropriate drug for the sedation of patients with peritonitis.

COMMENTS

Background

Gastrointestinal dysmotility commonly accompanies peritonitis and those patients suffering peritonitis are also exposed to the additive effects of sedatives or anaesthetics in surgical intensive care units. Therefore, it is preferable to use a sedative or an anaesthetic that has few inhibitory effects on gastrointestinal transit in the patients with peritonitis. The *in vitro* effects of propofol, midazolam and dexmedetomidine, which are commonly used anaesthetic or sedatives, are worth investigating for that purpose.

Research frontiers

The first finding of the study is that peritonitis altered the spontaneous activity of the rat ileum by decreasing both the amplitude and the frequency of the contractions. The main findings are that dexmedetomidine increased the amplitude, midazolam decreased the amplitude and propofol decreased both the amplitude and frequency of the spontaneous contractions of the rat ileum in a concentration-dependent manner in both the control and peritonitis groups.

Innovations and breakthroughs

In this study, the *in vitro* effects of dexmedetomidine, midazolam and propofol on spontaneous contractions of ileum muscle in rats exposed to peritonitis were demonstrated.

Applications

These findings will aid the selective use of sedatives or anaesthetics that have fewer inhibitory effects on gastrointestinal transit in patients with peritonitis.

Peer review

The authors of the present study have compared the acute effects of different sedatives or anaesthetics on the spontaneous contractions in the ileum of sham-operated rats and rats with peritonitis. The amplitude and the frequency of the spontaneous contractions were affected to a different extent by the respective sedatives. This study was well performed.

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Treatment of massive pancreaticojejunal anastomotic hemorrhage after pancreatoduodenectomy

Chen Liu, Ying-He Qiu, Xiang-Ji Luo, Bin Yi, Xiao-Qing Jiang, Wei-Feng Tan, Yong Yu, Meng-Chao Wu

Chen Liu, Ying-He Qiu, Xiang-Ji Luo, Bin Yi, Xiao-Qing Jiang, Wei-Feng Tan, Yong Yu, Meng-Chao Wu, Billiary I Unit of Eastern Hepatobiliary Surgery Hospital, Shanghai 200438, China

Author contributions: Liu C and Qiu YH contributed equally to this work; Qiu YH wrote the manuscript; Liu C designed and revised the manuscript; Jiang XQ, Yi B, Yu Y and Tan WF provided the collection of all the subjects material in addition to providing financial support for this work.

Correspondence to: Xiao-Qing Jiang, Billiary I Unit of Eastern Hepatobiliary Surgery Hospital, Shanghai 200438, China. jxq1225@sina.com

Telephone: +86-21-81875388 Fax: +86-21-81870788

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Abstract

AIM: To compare the treatment modalities for patients with massive pancreaticojejunal anastomotic hemorrhage after pancreatoduodenectomy (PDT).

METHODS: A retrospective study was undertaken to compare the outcomes of two major treatment modalities: transcatheter arterial embolization (TAE) and open surgical hemostasis. Seventeen patients with acute massive hemorrhage after PDT were recruited in this study. A comparison of two treatment modalities was based upon the clinicopathological characteristics and hospitalization stay, complications, and patient prognosis of the patients after surgery.

RESULTS: Of the 11 patients with massive hemorrhage after PDT treated with TAE, one died after discontinuing treatment, the other 10 stopped bleeding completely without recurrence of hemorrhage. All the 10 patients recovered well and were discharged, with a mean hospital stay of 10.45 d after hemostasis. The patients who underwent TAE had a re-operation rate of 18.2% and a mortality rate of 9.1%. Among the six patients who received open surgical hemostasis, two underwent another round of open surgical hemostasis. The mortality was 50%, and the recurrence of hemorrhage was 16.67%, with a mean hospital stay of 39.5 d.

CONCLUSION: TAE is a safe and effective treatment

modality for patients with acute hemorrhage after PDT. Vasography should be performed to locate the bleeding site.

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Key words: Pancreatoduodenectomy; Massive hemorrhage; Transcatheter artery embolization; Complication; Treatment

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INTRODUCTION

Massive pancreaticojejunal anastomotic hemorrhage is the second most common complication of pancreatoduodenectomy (PDT). Blind open surgical hemostasis, however, poses additional risks and complications, which can be prevented by transcatheter arterial embolization (TAE). The present study was to evaluate the effectiveness of TAE and open surgical hemostasis. Between June 2005 and August 2008, a total of 308 patients underwent PDT in our hospital. Of these patients, 17 had massive pancreaticojejunal anastomotic hemorrhage following PDT. In this retrospective study, we summarized our clinical experiences with these patients in order to compare the safety and efficacy of TAE and open surgical hemostasis. The results may help determine the therapeutic approaches to massive pancreaticojejunal anastomotic hemorrhage after PDT.

MATERIALS AND METHODS

Patient information

A total of 17 subjects were enrolled in our study, including 13 men and five women, aged 42-68 years (mean 60 ± 2.45 years). There were 10 cases of lower

common bile duct carcinoma, three cases of pancreatic head carcinoma and four cases of ampullary carcinoma. Obstructive jaundice was found in 12 patients. A catheter was inserted into the pancreatic duct in four cases, pancreatic duct exterior drainage was placed in two cases, and T-tube external drainage was placed in six cases, respectively. No T-tube or pancreatic duct drainage was placed in the remaining five patients. Post-surgical hemorrhage occurred in 10 cases from the gastroduodenal artery, from the posterior edge of the pancreatic stump in one case, and from the inferior pancreaticoduodenal artery in one case. Patients with upper gastrointestinal hemorrhage after PDT could be divided into early- and late-stage groups depending on the occurrence of hemorrhage within 5 d after PDT^[1]. In our study, there were two cases of early-stage hemorrhage and 15 cases of late-stage hemorrhage. Pancreatic leakage was confirmed in five cases. One patient withdrew from the study because his family gave up the treatment. All patients enrolled in this study signed the informed consent.

Inclusion criteria

Patients who were diagnosed as lower common bile duct cancer, pancreatic head cancer, or digestive tract tumors such as ampullary carcinoma, with the need of pancreatoduodenectomy. Modified Child procedures were adopted in each patient, were included in this study. Digestive tract reconstruction was performed in order of pancreas-intestine, bile duct-intestine and stomach-intestine. An internal support tube was placed in the pancreatic duct and a cross-section of the pancreas was sutured to stop bleeding. Patients with massive pancreaticojejunal anastomotic hemorrhage after PDT and specific criteria are listed below.

Exclusion criteria

Patients not meeting the above diagnostic criteria or with accompanying mental disorders or severe primary diseases in cardiovascular, liver, kidney and hematopoietic systems, or those giving up treatment and withdrawing from the study were excluded, except for women in pregnancy or breast-feeding, or those going to be pregnant.

Diagnosis of massive pancreaticojejunal anastomotic hemorrhage after PDT

In our study, a modified Child's procedure was adopted for all subjects, namely digestive tract reconstruction was performed in order of pancreas-intestine, bile duct-intestine and stomach-intestine. An internal support tube was placed in the pancreatic duct and a cross-section of the pancreas was sutured to stop bleeding as previously described^[2-4]. The diagnosis of massive pancreaticojejunal anastomotic hemorrhage after PDT was based on the literature^[3]. The main diagnostic criteria for patients in our study were as follows. (1) Hemorrhage occurred within 1 mo of PDT (the last hemorrhage in our study occurred on day 23 post-surgery) and the presence of a

massive hemorrhage impacting vital signs was confirmed by angiography or gastroscopy (subjects whose origin of hemorrhage could not be found by angiography or those with acute ulcer hemorrhage were confirmed by gastroscopy). (2) Massive hemorrhage manifested as fresh blood effusing suddenly from the abdominal drainage tube or T-tube, or massive hematemesis or blood drainage from the gastric tube (> 200 mL). (3) Patients experienced hypovolemic shock accompanying a simultaneous decrease in hemoglobin (hemoglobin decreased more than 30 g/L in 24 h). Massive pancreaticojejunal anastomotic hemorrhage after PDT was diagnosed when the patients had hemorrhage and any other manifestations.

Treatment of massive hemorrhage

TAE: Of the 11 patients treated with TAE, eight underwent hepatic artery embolization, two underwent embolization of hepatic artery proper, and one refused any treatment upon initiating TAE. Hemorrhage was stopped in six cases after a single embolization procedure. Two patients underwent TAE twice for hemostasis (Figure 1). Emergency arteriography showed that the two patients had pancreatic stump artery bleeding into the jejunum. Hepatic artery was embolized during the first TAE, but recurrence of bleeding was found 4 h later. Arteriography showed that the pancreatic stump artery bled again, and microcoils embolizing the hepatic artery were displaced into the right hepatic artery. TAE was performed again with the common hepatic artery embolized using five microcoils, which stopped postoperative hemorrhage. Arteriography showed no obvious origin of hemorrhage in patient 3 during the first TAE. However, 7 h later, the patient received another arteriogram because of recurrence of bleeding. Duodenal stump bleeding was detected, and the common hepatic artery was then embolized to stop the hemorrhage. Arteriography revealed a crude edge on hepatic artery in patient 5, and the bleeding was stopped after embolization of the hepatic artery. A "vascular pool" image was observed in the inferior pancreaticoduodenal artery of patient 6, which was considered the origin of bleeding stopped by embolizing the common hepatic artery.

Complications such as hepatophyma and spleen necrosis may occur after TAE. In this study, patient 2 suffered from fever 2 mo after operation, with its peak at 39°C. Hepatophyma larger than 5 cm in diameter was seen in the right liver lobe. The patient recovered after paracentesis and anti-infective therapy. No such complications were found in the other patients.

Open surgery: A total of six patients received direct open surgical hemostasis for their hemorrhage.

RESULTS

Efficacy of TAE

A total of 11 cases of massive hemorrhage after PDT

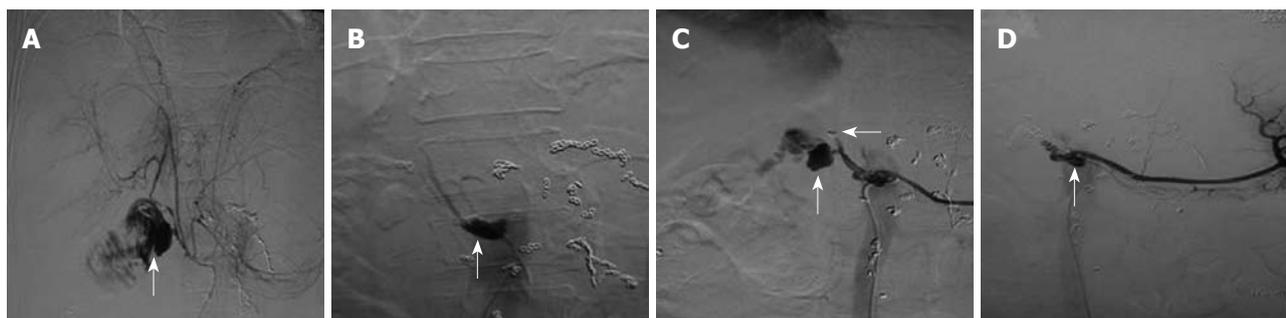


Figure 1 Images from patient 2 (treated using TAE). A: Angiogram of the gastroduodenal stump bleeding before the first embolization. Arrow: Angiogram of the gastroduodenal stump bleeding; B: Angiogram of the gastroduodenal stump, which stopped bleeding after the first TAE. Arrow: Angiogram of the gastroduodenal stump); C: Angiogram of the stump prior to the second embolization. Angiography indicates that microcoils have entered the hepatic artery, causing renewed bleeding from the stump. Arrows: The second angiogram of the gastroduodenal stump bleeding; D: After the second embolization, angiography confirmed that the bleeding was stopped. Arrow: Angiogram of the gastroduodenal stump.

Table 1 Overview of 11 patients with massive hemorrhage after PDT treated with TAE

Case	Time until bleeding	Symptom	Bleeding site	Embolization site	TAE	Hospital stay	Complications after TAE	Prognosis
1	21	T-tube bleeding	Pancreatic stump artery	Proper hepatic artery	1	9	High TB	Good
2	23	T-tube bleeding, hematemesis	Gastroduodenal stump	Common hepatic artery	2	14	High TB	Good
3	12	Double catheterization cannula, T-tube bleeding	Gastroduodenal stump	Common hepatic artery	2	14	High TB	Good
4	14	Hematemesis	Gastroduodenal stump	Common hepatic artery	1	5	Hyperpyrexia, pyemia	Dead
5	3	Double catheterization cannula bleeding	Proper hepatic artery	Proper hepatic artery	1	11	None	Good
6	5	Double catheterization cannula bleeding	Inferior pancreaticoduodenal artery	Common hepatic artery	1	8	High TB	Good
7	7	Single lumen cannula bleeding	Gastroduodenal Stump	Common hepatic artery	1	7	None	Good
8	9	Hematemesis	Gastroduodenal stump	Common hepatic artery	1	11	High TB	Good
9	10	Hematemesis	Gastroduodenal stump	Common hepatic artery	1	13	High TB	Good
10	7	Double catheterization cannula bleeding	Gastroduodenal stump	Common hepatic artery	1	13	High TB	Good
11	8	Double catheterization cannula bleeding	Gastroduodenal stump	Common hepatic artery	1	10	None	Good

TB: Total bilirubin.

underwent TAE which completely stopped their bleeding, except for one patient who died after refusing further treatment. Bleeding was stopped in eight patients after a single TAE procedure and in two patients after two treatments with TAE. All 10 patients recovered well and were discharged.

Total bilirubin (TB) exceeded the ULN level in seven out of the 10 patients and was as high as 178 $\mu\text{mol/L}$ in patient 2 after TAE, who was discharged with a full recovery of liver function after treatment. Patient 4 experienced a fever at 39.5°C after TAE and his TB level was 140 $\mu\text{mol/L}$. Blood bacterial culture showed pyemia, and this patient abandoned treatment 5 d after TAE, and died 7 d after discharge. The other patients had a good prognosis after anti-infective and supportive treatment. Recurrence of hemorrhage was not found during a follow-up period of 2 mo and all 10 patients reported having an acceptable quality of life. The mean hospital stay was 10.45 d after hemostasis. Patients requiring a second operation accounted for 18.2%, and the overall mortality rate was 9.1% (Table 1).

Efficacy of open surgery

Six patients underwent a second open surgical hemostasis for hemorrhage. Patients 1, 3 and 6 recovered and were discharged. However, patient 6 received an additional open surgical hemostasis, and patient 2 received emergency open surgical hemostasis again because of rebleeding 5 and 9 d after surgery, respectively. Patient 2 died of multiple organ failure. Liver and renal failure was observed in patients 4 and 5 after surgery. Both patients abandoned treatment, and one of them died 1 wk after discharge. The other patients had a good prognosis after anti-infective and supportive treatment. The mortality rate for patients undergoing open surgery was 50%, the recurrence rate of hemorrhage was 16.67%, and the mean hospital stay was 39.5 d (Table 2).

DISCUSSION

Pancreatojejunal and choledochojejunal anastomotic internal drainage can effectively prevent biliary and pancreatic leakage. PDT is a common abdominal

Table 2 Overview of six patients with massive hemorrhage after PDT treated with open surgical hemostasis

Case	Time until bleeding	Symptom	Bleeding site	Hemostasis surgeries	Duration of hospital stay after hemostasis	Complications after surgery	Prognosis
1	17	T-tube bleeding	Gastroduodenal stump	1	59	None	Good
2	9	T-tube bleeding, hematemesis	Gastroduodenal stump	2	32	Multiple organ failure	Dead
3	10	T-tube bleeding	Gastroduodenal stump	1	63	None	Good
4	11	Hematemesis	Gastroduodenal stump	1	16	Liver failure	Dead
5	8	T-tube bleeding	Gastroduodenal stump	1	13	Renal failure	Dead
6	8	T-tube, pancreatic duct bleeding	Gastroduodenal stump	2	54	None	Good

operation. However, it is associated with the most common complications of hemorrhage and pancreatic leakage, with a relatively high risk^[5]. The incidence of postoperative massive hemorrhage is 7.5%-12.4%. From June 2005 to August 2008, we performed 308 PDT, and massive pancreaticojejunal anastomotic hemorrhage occurred in 16 patients, accounting for 5.2% of its overall incidence rate, which is slightly lower than the reported rate^[6]. Massive hemorrhage after PDT is difficult to stop, and if it is not stopped immediately, the mortality rate can be as high as 30%-58%^[7,8]. Massive pancreaticojejunal anastomotic hemorrhage after PDT, mainly from the gastroduodenal stump, is often caused by transudatory digestive juices or corrosion of peripheral tissues by local fluid infection. Corrosion of the anastomotic vicinal vessels is especially common. In the present study, pancreatic leakage was confirmed in five of the 16 patients, and the number of leukocytes was increased before bleeding in six of them, and the number of WBC was $20.5 \times 10^9/L$ in one patient. In addition, four of the six patients had a fever. Gastroduodenal arterial bleeding was detected by DSA in five of our patients, suggesting that it is particularly important to prevent intra-abdominal infection after PDT. Abdominal CT and B-ultrasound should be regularly performed to monitor the fever or blood pressure of such patients, to ensure that seroperitoneum is not compromised. If there is any indication of infection, puncture and drainage should be carried out to prevent further infection. During the surgery, arteries should be ligated twice or transfixed, with longer suturing ends as appropriate^[9].

Pancreaticojejunal anastomotic hemorrhage after PDT is difficult to treat. TAE with or without surgery might be a more effective procedure to stop bleeding. Since PDT may produce significant surgical trauma, and the site of hemorrhage is often difficult to locate, blind open surgical exploration often ends in failure. In addition, postoperative complications such as pancreatic leakage and intra-abdominal infection can result in more severe consequences than those caused by hemorrhage. Thus, better treatment modalities are needed for pancreaticojejunal hemorrhage after PDT. In our study, six patients underwent open surgical hemostasis again after PDT, and two of them received additional open surgical hemostasis because of recurrence of bleeding, which can be explained as follows. Namely, their overall poor condition following two major operations may have hindered recovery; the exact site of bleeding could

not be accurately located during the first operation for hemorrhage; intra-abdominal organ edema such as pancreatic edema was common and pancreatic leakage and intra-abdominal infections were not completely controlled. One patient with an intra-abdominal infection died of multiple organ failure. Liver or renal failure was also found in two patients after surgery. Both patients abandoned treatment, and one of them died 1 wk after discharge. Overall, the mortality rate for open surgery was 50%, the recurrence rate of bleeding was 16.67%, and the mean hospital stay was 39.5 d, suggesting that the prognosis of patients undergoing TAE is rather poor.

However, TAE can prevent re-operation risk and complications. In our study, 18.2% patients had a second TAE with a mortality of only 0.9%. In addition, the mean hospital stay after TAE was 10.45 d. Angiography should be performed as soon as hemorrhage is diagnosed to locate the site of bleeding. Then, embolization can be performed to stop hemorrhage^[4,5].

Angiography can diagnose hemorrhage and evaluate the efficacy of its treatment.

Angiography can locate the site of bleeding. TAE after PDT is needed for successful treatment of hemorrhage, and cooperation between surgeons is necessary to maximize the therapeutic efficacy and minimize the risk of TAE.

Since patients will be transported and allergy testing of contrast medium will be carried out during TAE, preoperative and intraoperative anti-hemorrhagic shock should be prevented. Any change in vital signs should be monitored while performing TAE. Larger microcoils should be selected for TAE because the common hepatic artery is large with a high-pressure blood flow.

After TAE, patients should be closely observed, and the outcome of hemostasis should be confirmed by arteriography. Patients without recurrence of bleeding can be sent to the intensive care unit for observation. The site of bleeding was found in eight of our 10 patients who underwent TAE. No recurrence of bleeding was observed at first in the other two patients who required additional surgery, which may have resulted from their poor condition (unresolved shock and low blood pressure), leading to reduced bleeding despite. Spastic contraction of blood vessels and/or obstruction of vessels or blood clots may have prevented accurate angiography. It was reported that gastroduodenal arterial stump bleeding is one of the main sources of pancreaticojejunal anastomotic hemorrhage after PDT^[10]. If pancreaticojejunal

anastomotic hemorrhage is suspected but not dealt with, massive hemorrhage may occur. Common hepatic arterial embolization may be a better choice of treatment.

Nevertheless, we found that common hepatic artery embolization with TAE was likely to result in liver injury, which was manifested as elevated TB and aminotransferase level. In our study, TB level was higher in five patients than in normal controls after TAE, and the aminotransferase level was three-fold higher in four patients than in normal controls. However, if not accompanied with liver disease, liver failure rarely occurs after TAE. We provided supportive treatment for patients with liver injury by increasing the oxygen flow and concentration, extending oxygen absorption time, maintaining a high oxygen pressure and 100% oxygen saturation, and improving liver support. We also provided low-dose hormone therapy with prostaglandin E, growth hormone, and hepatocyte growth factor to increase the liver blood flow and promote liver cell regeneration. The patients received additional supportive treatment with plasma and human serum albumin when necessary.

In summary, TAE, which can avoid reoperation and complications of surgery, is an effective and safe treatment modality for acute postoperative hemorrhage after PDT. However, TAE may damage liver function due to hepatic artery embolization. Angiography can locate the site of bleeding, and if there are indications for surgery, TAE should be performed in time.

COMMENTS

Background

Massive pancreaticojejunal anastomotic hemorrhage after pancreaticoduodenectomy (PDT) is the second most common severe complication, only next to pancreaticojejunal anastomotic dehiscence. Blind open surgical hemostasis has more surgical risks and complications. However, transcatheter arterial embolization (TAE) can prevent reoperation risk and complications.

Research frontiers

An internal support tube was placed in the pancreatic duct and a cross-section of pancreas was sutured to stop bleeding. If the bleeding site was located by selective angiography, embolization could be performed to stop bleeding.

Applications

TAE is an effective and safe treatment modality for acute hemorrhage after PDT. Vasography should be performed in time to locate the site of bleeding. TAE can also prevent reoperation risk and complications.

Peer review

The authors showed that TAE was an effective and safe treatment modality for acute hemorrhage after PDT. The initial observations are interesting.

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BRIEF ARTICLES

Passage of bone-marrow-derived liver stem cells in a proliferating culture system

Yun-Feng Cai, Ji-Sheng Chen, Shu-Ying Su, Zuo-Jun Zhen, Huan-Wei Chen

Yun-Feng Cai, Shu-Ying Su, Zuo-Jun Zhen, Huan-Wei Chen, Department of Hepatobiliary Surgery, the Affiliated Foshan Hospital of Sun Yat-sen University, Foshan 528000, Guangdong Province, China

Ji-Sheng Chen, Department of Hepatobiliary Surgery, the Second Affiliated Hospital of Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China

Author contributions: Cai YF and Chen JS contributed equally to this work; Cai YF, Chen JS, Su SY, Zhen ZJ and Chen HW designed the research; Cai YF, Su SY, Zhen ZJ and Chen HW performed the research; Chen HW contributed new reagents/analytic tools; Cai YF, Chen JS and Zhen ZJ analyzed the data; Cai YF, Chen JS, Chen HW wrote the paper.

Correspondence to: Yun-Feng Cai, MD, Department of Hepatobiliary Surgery, the affiliated Foshan Hospital of Sun Yat-sen University, Foshan 528000, Guangdong Province, China. yfc70@yahoo.com.cn

Telephone: +86-757-83161119 Fax: +86-757-83812566

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Abstract

AIM: To explore the feasibility of passage of bone-marrow-derived liver stem cells (BDLSCs) in culture systems that contain cholestatic serum.

METHODS: Whole bone marrow cells of rats were purified with conditioning selection media that contained 50 mL/L cholestatic serum. The selected BDLSCs were grown in a proliferating culture system and a differentiating culture system. The culture systems contained factors that stimulated the proliferation and differentiation of BDLSCs. Each passage of the proliferated stem cells was subjected to flow cytometry to detect stem cell markers. The morphology and phenotypic markers of BDLSCs were characterized using immunohistochemistry, reverse transcription polymerase chain reaction (RT-PCR) and electron microscopy. The metabolic functions of differentiated cells were also determined by glycogen staining and urea assay.

RESULTS: The conditioning selection medium isolated BDLSCs directly from cultured bone marrow cells. The selected BDLSCs could be proliferated for six passages and maintained stable markers in our proliferating system. When the culture system was changed to a differentiating system, hepatocyte-like colony-forming

units (H-CFUs) were formed. H-CFUs expressed markers of embryonic hepatocytes (alpha-fetoprotein, albumin and cytokeratin 8/18), biliary cells (cytokeratin 19), hepatocyte functional proteins (transthyretin and cytochrome P450-2b1), and hepatocyte nuclear factors 1 α and -3 β). They also had glycogen storage and urea synthesis functions, two of the critical features of hepatocytes.

CONCLUSION: BDLSCs can be selected directly from bone marrow cells, and pure BDLSCs can be proliferated for six passages. The differentiated cells have hepatocyte-like phenotypes and functions. BDLSCs represent a new method to provide a readily available alternate source of cells for clinical hepatocyte therapy.

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Key words: Liver stem cells; Bone marrow; Cell separation; Cell proliferation; Cell differentiation

Peer reviewer: Masaki Nagaya, MD, PhD, Islet Transplantation and Cell Biology, Joslin Diabetes Center, One Joslin Place, Joslin Diabetes Center, Boston 02215, United States

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INTRODUCTION

About a decade ago, many reports highlighted the broad developmental potential of bone-marrow-derived stem cells^[1]. This gave new hope for cell therapy using autologous bone marrow cells, which has few ethical problems and has been applied to severe liver diseases^[2]. However, little progress has been made in recent years because of the difficulties of selection and proliferation of this specific cell population^[3]. It is necessary to find a new way to isolate and purify bone-marrow-derived liver stem cells (BDLSCs). Following the principle that cells in culture can survive only when they become accommodated to the surrounding environment, we developed a culture system to isolate BDLSCs from

bone marrow cells. Within this system, only BDLSCs could survive, while the other bone marrow cells could not^[4]. This method could provide pure BDLSCs, but still could not harvest large numbers of cells, and the efficiency of differentiation was insufficient for therapeutic application. Here, we developed a proliferating culture system to passage BDLSCs and a differentiating system to yield hepatocyte-like cells, by modified culture environments.

MATERIALS AND METHODS

Selection, proliferation and differentiation of BDLSCs

Preparation of conditioning selection medium:

Common bile duct ligation and transection were carried out under general (ether) anesthesia in Sprague-Dawley rats weighing 200-250 g to induce cholestasis. After 10 d, whole blood was collected from each rat, and serum was separated. Cholestatic serum (50 mL/L) was added to Dulbecco's Modified Eagle's Medium (DMEM; Gibco) that contained 20 mmol/L HEPES (Sigma), 10^{-7} mol/L dexamethasone (Sigma), and antibiotics, to act as the conditioning selection medium (See our previous study)^[4].

Culture of bone marrow cells: Rat bone marrow cells were obtained by flushing the femurs. About 3×10^8 bone marrow cells were retrieved from one rat. Bone marrow cells were suspended in DMEM and plated at a density of 1×10^9 cells/L onto culture dishes. DMEM enriched with 100 mL/L fetal bovine serum (FBS; Hyclone), 20 mmol/L HEPES, 10^{-7} mol/L dexamethasone, and antibiotics was used. Dishes were placed in a humidified incubator containing 50 mL/L CO₂ and 950 mL/L O₂ at 37°C.

Selection and passage of liver stem cells: Three days after culture, the medium and suspended cells were discarded and replaced by conditioning selection medium. Pure liver stem cells were selected by this medium. The cells were then proliferated with a new proliferating culture system, which contained 5% cholestatic serum, 10% FBS (Gibco), 10 mmol/L nicotinamide (Sigma), 1 mmol/L 2-phosphate ascorbic (Sigma), 1 mg/mL galactose (Sigma), 30 µg/mL praline (Sigma), insulin/transferrin/selenite mixture (Gibco), 10 ng/mL epidermal growth factor (EGF; Pepro Tech) and 10 ng/mL hepatocyte growth factor (HGF; Pepro Tech). When the cells developed into the proliferating stage, 10 ng/mL leukocyte inhibitory factor (LIF; Cytolab) was added to inhibit the differentiation of the cells. Passaging was carried out when the cells overlapped in the plate.

Differentiation of liver stem cells: Each passage of the stem cells was cultured in the differentiating system (i.e. the above culture system containing 20 ng/mL EGF, 25 ng/mL HGF, 1% DMSO (Sigma) and 20 ng/mL interleukin (IL)-3 (Pepro Tech), and LIF was discarded at this time. Differentiated cells were harvested.

Growth curve of passaged BDLSCs

The passaged BDLSCs were digested and re-cultured on 24-well dishes at a concentration of 1×10^7 /L. The mean number of cells in every three wells were counted daily for 8 d. The cell growth curve was then drawn according to these numbers.

Flow cytometry detection of the stability of stem cell markers of cell passages

After each passage, the stem cells and the differentiated cells were digested to prepare single-cell suspensions. The cell surface markers beta-2 microglobulin (β_2m), Thy-1, CD34, Flt-3, c-kit and IL-3R were detected with cytometry. Each marker was detected six times in every passage, and mean values were determined. The stability of stem cell surface marker expression was determined, and the expression before and after differentiation was compared.

Morphological and phenotypic markers of differentiated cells

Immunohistochemistry: Stem cells of each passage were cultured in the differentiating system in six-well dishes with cover glasses. When hepatocyte-like cells came into being, cover glasses and the differentiated BDLSCs were removed and fixed for immunohistochemistry. The primary antibodies were goat anti-rat albumin, alpha-fetoprotein (AFP), and cytokeratin -8/18 (CK8/18) polyclonal antibodies. (see our previous study)^[4].

Electron microscopy: Culture dishes of differentiated cells were washed with PBS, and fixed in glutaraldehyde for 48 h. After fixation, cells were curretted and centrifuged to form aggregates. After post-fixation in osmium tetroxide, cells were dehydrated in graded alcohols and embedded in low-viscosity epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed under an electron microscope (see our previous study)^[4].

Reverse transcription polymerase chain reaction (RT-PCR): Total RNA was extracted from the differentiated cells and mRNA transcription of hepatocyte nuclear factor (HNF)-1 α , HNF-3 β , CK18, CK19, albumin, AFP, transthyretin (TTR) and cytochrome P450-2b1 (CYP2b1) were detected by RT-PCR (see our previous study)^[4].

Function tests of differentiated cells

Periodic acid-Schiff (PAS) staining for glycogen and urea assay of the differentiated cells were also conducted to confirm their hepatocyte-like function (see our previous study)^[4].

Statistical analysis

Data were presented as mean \pm SD. All the data were analyzed using the SPSS statistical package 13.0. Differences in means were tested by the unpaired Student's *t* test. All tests were considered statistically significant at $P < 0.05$.

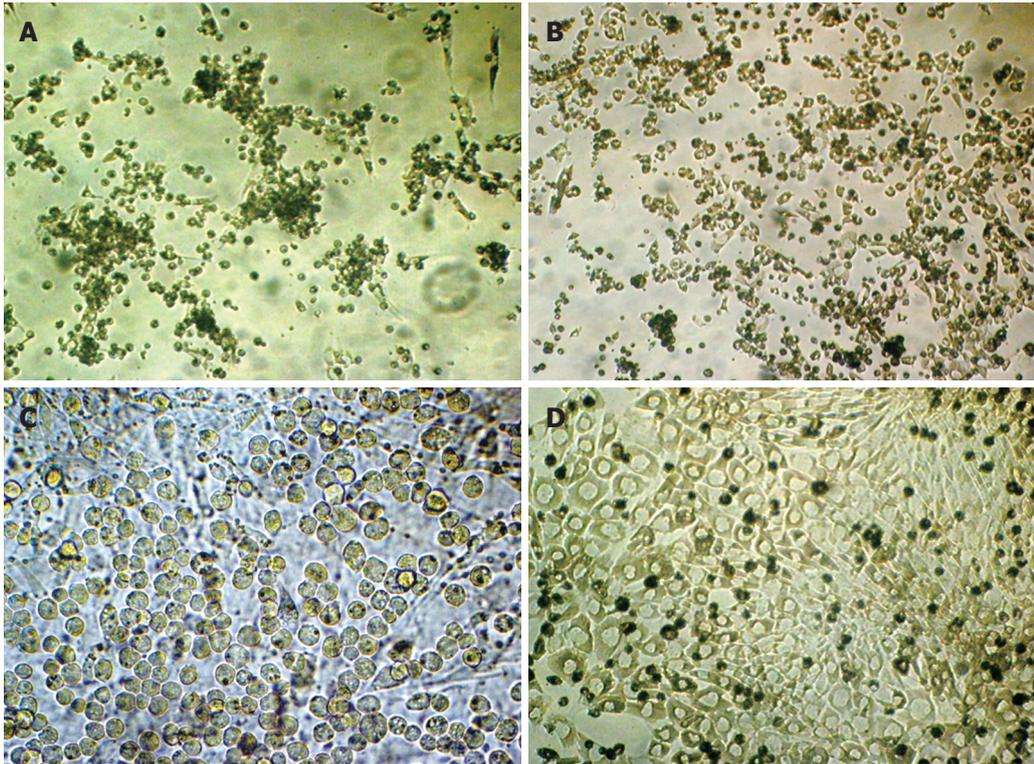


Figure 1 Morphological evidence of BDLSC differentiation. A: BDLSC clone selected from bone marrow cells, phase-contrast microscope (200 ×); B: Proliferation of liver stem cell clone-phase-contrast microscope (200 ×); C: Differentiation-prohibited passage stem cells, phase-contrast microscope (400 ×); D: Hepatocyte-like cells after differentiation, phase-contrast microscope (400 ×).

RESULTS

Morphological evidence of BDLSC differentiation

During the first 3 d, many colonies appeared in the conditioning cholestatic serum. These colonies were composed of small, undifferentiated cells in the center, and epithelioid cells at the periphery (Figure 1A). After replacing with the proliferating system, the colonies enlarged, and the cells proliferated rapidly in about 4 d (Figure 1B). With the addition of LIF, mature differentiation was inhibited, and colonies of small round cells with large nuclei, little endochylema and high nuclear-to-cytoplasmic ratio appeared (Figure 1C). The colonies maintained the ability of proliferation and passage was required in 5-7 d. The original aim of six passages could be achieved. After six passages, however, the proliferation was difficult to maintain and fibroblast-like cells appeared. After replacing with the differentiating system, hepatocyte-like colony-forming units (H-CFUs) started to appear. The H-CFUs were composed of small, undifferentiated cells in the center, and large cells with regular multilateral contours, low nuclear-to-cytoplasmic ratio, and single round nuclei at the periphery. The differentiated cells formed cords or trabeculae that resembled the hepatocyte cords in hepatic lobules (Figure 1D).

Growth curve of passaged BDLSCs

The curve showed that the number of cells increased as the culture time passed, and rapid proliferation appeared from day 2 to day 5 (Figure 2).

Flow cytometry detecting stability of stem cell markers of cell passage

Flow cytometry detecting the cell surface markers of

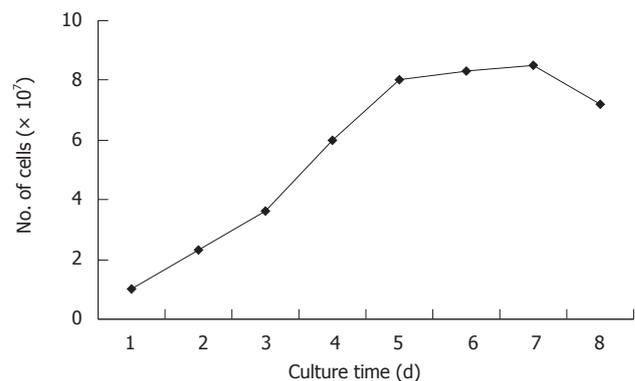


Figure 2 Cell growth curve of passaged BDLSC.

each passage showed that the undifferentiating cells were relatively stable $\beta_2m^{low}/Thy-1^+/CD34^{low}/c-kit^+$ cells. The markers changed after differentiation, with significant differences ($P < 0.05$) in all of the detected markers except CD34 (Figures 3 and 4).

Phenotypic markers of differentiated cells

The differentiated cells from each passage were analyzed for biochemical evidence of hepatocytic differentiation, in order to confirm their characteristics. Just as in our previous study^[4], immunohistochemistry was performed on differentiated cells grown on cover glasses to determine the presence of albumin, AFP and CK8/18, the characteristic proteins expressed during hepatocyte development, which revealed diffuse cytoplasmic staining for these proteins. RT-PCR further confirmed the hepatocytic characteristics of differentiated cells as the results showed that there were mRNA transcripts of HNF-1 α , HNF-3 β , albumin, AFP, CK-18, CK-19, TTR,

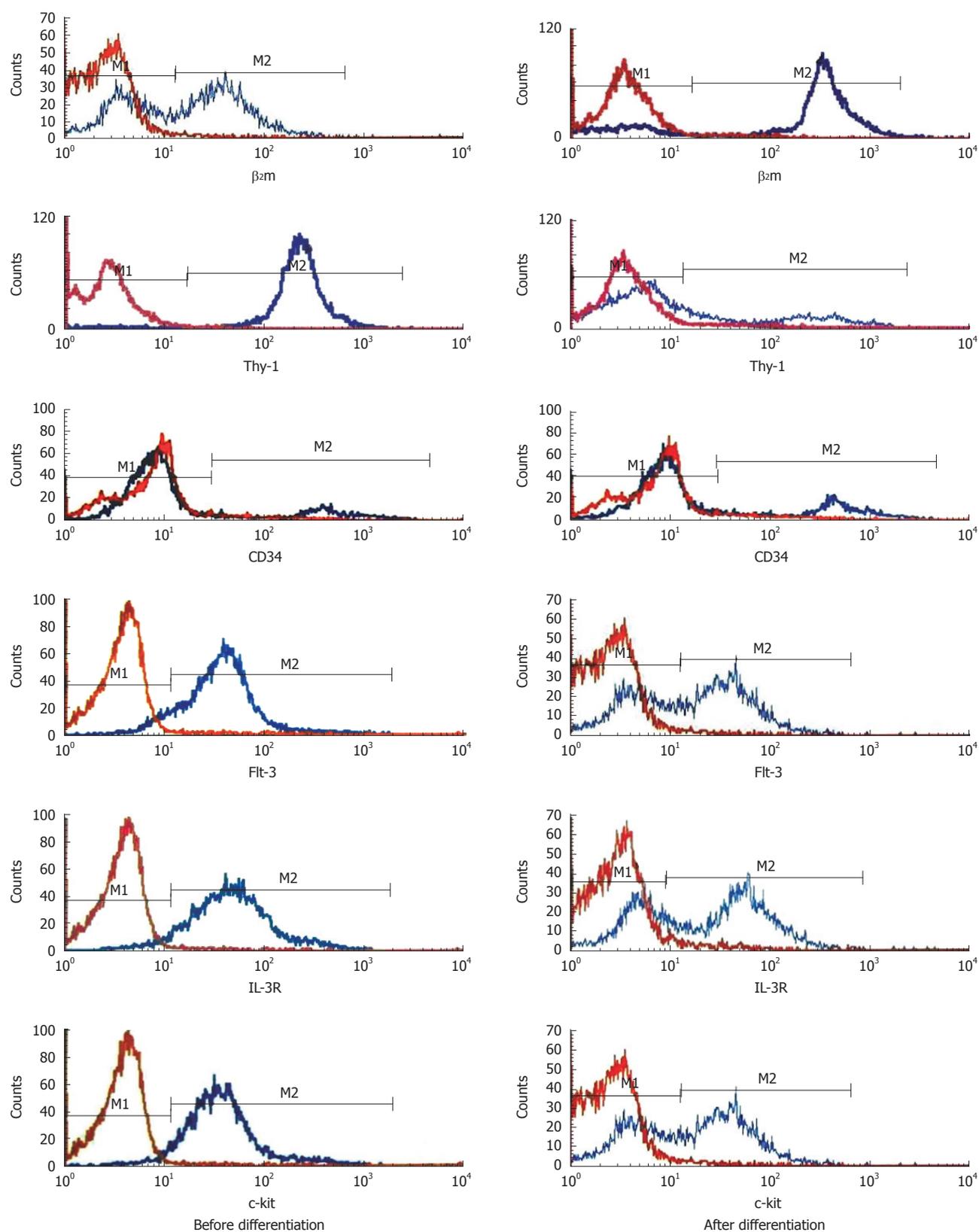


Figure 3 Differences of stem cell markers before and after differentiation by flow cytometry. Red curves: Negative control, M1: Negative part, M2: Positive part.

and CYP2b1, all of which were hepatocyte specific.

Ultrastructurally, the differentiated cells were rich in endoplasmic reticulum and ribosomes and contained abundant ellipsoid mitochondria, which were typical features of adult hepatocytes.

Function tests of differentiated cells

We also found glycogen storage in the cytoplasm of hepatocyte-like differentiated cells by PAS staining, and urea production and secretion by urea assay of the culture medium^[4].

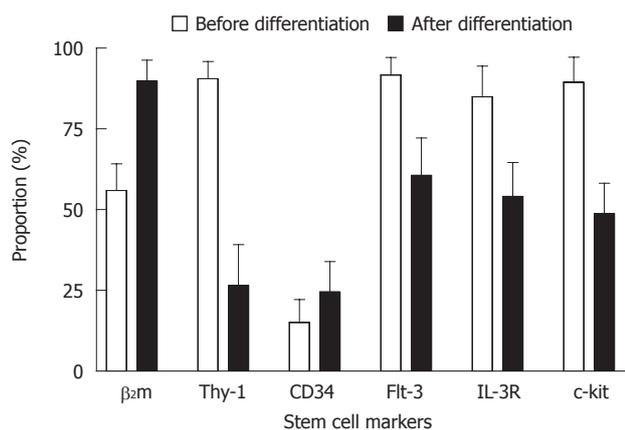


Figure 4 Expression of stem cell markers before and after differentiation.

DISCUSSION

Bone-marrow-derived stem cells are able to transdifferentiate into hepatic cells as shown in cross-sex and cross-strain bone marrow and whole liver transplantation experiments^[5]. Great interest has been aroused in the identification and isolation of BDLSCs^[6]. However, the characteristic surface markers of BDLSCs and their pedigrees in the derivation of bone marrow stem cells remain obscure^[7]. It is difficult to identify and sort particular cells by immunological methods, such as fluorescence-activated and magnetic-activated cell sorting^[8,9]. Furthermore, the sorting of stem cells with complicated surface markers is difficult^[10]. We developed a conditioning culture system to solve this problem. Within such a system, only BDLSCs could survive, while other cells could not, therefore, it was possible to purify these specific stem cells^[4].

However, the passage of BDLSCs is still a challenge, which has hindered the proliferation of the cells. No report of successful passage has been published. In our experiments, we found that the key to passage of the stem cells was to isolate purified stem cells. We were able to harvest pure BDLSC colonies with our selection system. After that, a proliferating system that contained all the nutrients required for the proliferation of liver stem cells was introduced to culture the cells^[11,12]. This system contained all the known conditions required for the proliferation of oval cells^[13], with low concentrations of HGF and EGF and a mixture of FBS and cholestatic serum, so as to maintain the characteristics of the liver stem cells. At the same time, for stem cell proliferation, differentiation must be prevented. We therefore provided LIF, a factor known as a strong inhibitor of stem cell differentiation^[14]. When replaced with the proliferating system on day 4, rapid proliferation occurred and the cells maintained their undifferentiated state, under the action of LIF. To harvest the differentiated cells, LIF must be discarded, and the concentration of EGF and HGF must be higher. Under the conditions that contained the differentiating factors, hepatocyte-like cells appeared from each passage of the stem cells. The morphology and phenotypic markers manifested in the differentiated cells were similar to those of liver stem cells. These cells

expressed markers of embryonic hepatocytes (AFP, albumin and CK18), biliary cells (CK19), hepatocyte functional proteins (ITR and CYP2b1), and hepatocyte nuclear factors (HNF-1 α and HNF-3 β). To confirm that the differentiated cells had functional characteristics of hepatocytes, we tested the glycogen and urea synthesis functions of the cells, and demonstrated that the cells possessed hepatocyte-like functions. These all proved that each passage of the stem cells can differentiate into hepatocyte-like cells. We also detected the stability of each passage of the stem cells with flow cytometry, and the results showed that the proliferation was stable. In addition, the increase in β_2m expression on the differentiated cells demonstrated their maturation because β_2m is only expressed on mature cells. However, we had difficulty in maintaining the proliferation after six passages. The emergence of fibroblast-like cells became inevitable. The explanation might be that the proliferation of adult stem cells, unlike tumor cells, was limited, and that new factors or a network similar to the liver tissue were required for further proliferation.

In conclusion, we demonstrated that BDLSCs could be selected from whole bone marrow cells using conditioning medium. The stem cells could also be proliferated for six passages and differentiated into hepatocyte-like cells. These methods not only provide a new and effective method for the isolation and purification of extrahepatic liver stem cells, but also provide a readily available alternate source of cells for clinical hepatocyte therapy.

COMMENTS

Background

Bone-marrow-derived liver stem cells (BDLSCs) were once a hot topic in the field of stem cell research because of their important therapeutic implications, but little progress has been made in recent years because of the difficulty of isolation and proliferation of this special cell population. The authors developed a culture system to isolate BDLSCs from bone marrow cells, from which they could culture pure BDLSCs *in vitro*. However, the passage of BDLSCs is still a challenge, which has hindered the proliferation of the cells.

Research frontiers

Great interests has been aroused in the identification and isolation of liver stem cells from bone marrow cells. Several subsets of bone marrow cells have been found to have the potential to differentiate into hepatocytes, but no report of successful passage has been published.

Innovations and breakthroughs

This study provided a new method for BDLSC isolation and proliferation. With a careful designed culture system, BDLSCs can be purified *in vitro* and be passaged, which brings new hope to the clinical use of bone-marrow-derived stem cells.

Applications

BDLSCs can be selected directly from bone marrow cells, and pure BDLSCs can also be proliferated for six passages. The differentiated cells have hepatocyte-like phenotypes and functions. BDLSCs represent a new method to provide a readily available alternate source of cells for clinical hepatocyte therapy.

Peer review

In this study, the authors used their original method to retrieve cells that are possibly BDLSCs. Then, they used fluorescence-activated cell sorting to determine the cells' characteristics before and after differentiation. This is an interesting and potentially important study, which suggests that bone-marrow-derived cells can be stimulated to expand and then differentiate into hepatocyte-like cells, which may possibly be used to treat liver disease.

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BRIEF ARTICLES

B-cell clonality in the liver of hepatitis C virus-infected patients

He-Bin Fan, You-Fu Zhu, An-Shen Chen, Mu-Xiu Zhou, Fu-Ming Yan, Xiao-Ju Ma, Hao Zhou

He-Bin Fan, An-Shen Chen, Fu-Ming Yan, Xiao-Ju Ma, Hao Zhou, Department of Infectious Disease, The People's Liberation Army 161 Hospital, Wuhan 430010, Hubei Province, China

You-Fu Zhu, Department of Infectious Disease, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Mu-Xiu Zhou, Department of Pathology, The People's Liberation Army 161 Hospital, Wuhan 430010, Hubei Province, China

Author contributions: Fan HB, Zhu YF, Chen AS and Zhou MX performed the majority of experiments; Zhu YF provided vital reagents and analytical tools and was also involved in the collecting of all the human material; Yan FM, Ma XJ, Zhou H edited the manuscript.

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Correspondence to: Dr. He-Bin Fan, Department of Infectious Disease, The People's Liberation Army 161 Hospital, Wuhan 430010, Hubei Province, China. fan_hebin@126.com
Telephone: +86-27-50169074 Fax: +86-27-50169007

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Abstract

AIM: The association of hepatitis C virus (HCV) infection with type II mixed cryoglobulinemia is well established, but the role of HCV in B-cell lymphoma remains controversial. In patients with HCV infection, B-cell clonal expansions have been detected in peripheral blood and bone marrow, and a high prevalence of B-cell non-Hodgkin's lymphomas has been documented. Liver biopsies in chronic HCV infection frequently show portal lymphoid infiltrates with features of B follicles, whose clonality has not yet been investigated. The object of this study was to determine the frequency of liver-infiltrating monoclonal B-cells in 40 patients with HCV infection.

METHODS: Eight hundred and forty-eight patients were studied prospectively, including 40 HCV-positive patients and 808 patients with chronic hepatitis B virus (HBV) infection. Immunohistochemical study for B- and T-cell markers was performed on the paraffin-embedded liver tissue sections. The clonality of lymphoid B-cells was tested using a

polymerase chain reaction (PCR) approach designed to identify immunoglobulin heavy chain gene (*IgH*) rearrangements.

RESULTS: Liver-infiltrating monoclonal B-cells were detected in the liver for 4 (10%) of 40 HCV-positive patients but were present in only 3 (0.37%) of 808 liver biopsy specimens with chronic HBV infection. Chi-square testing showed that the monoclonal B-cells infiltration in the liver was more frequent in the HCV-infected patients ($P = 0.000$). A clonal *IgH* rearrangement was detected in 5 (71.4%) of 7 liver biopsy specimens with monoclonal B-cells infiltration. In 2 of 5 patients with both a clonal B-cell expansion and monoclonal B-cells infiltration in the liver, a definite B-cell malignancy was finally diagnosed.

CONCLUSION: Liver-infiltrating monoclonal B-cells are detected in the liver of patients with chronic HCV and HBV infection. A high percentage of patients with monoclonal B-cells infiltration and B-cell clonality in the liver were finally diagnosed as having a definite B-cell malignancy.

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Key words: Hepatitis; Hepatitis C virus; B-lymphocytes; Polymerase chain reaction; Gene rearrangement; Clonality

Peer reviewer: Vasilii I Reshetnyak, MD, PhD, Professor, Scientist Secretary of the Scientific Research Institute of General Reanimatology, 25-2, Petrovka str., 107031, Moscow, Russia

Fan HB, Zhu YF, Chen AS, Zhou MX, Yan FM, Ma XJ, Zhou H. B-cell clonality in the liver of hepatitis C virus-infected patients. *World J Gastroenterol* 2009; 15(13): 1636-1640 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1636.asp>
DOI: <http://dx.doi.org/10.3748/wjg.15.1636>

INTRODUCTION

The relationship between lymphoproliferative disorders and infectious agents has been recognised and studied for many decades. A causative association between

hepatitis C virus (HCV) and non-Hodgkin's lymphoma (NHL) was postulated relatively recently and has been the subject both of intense investigation and of some debate^[1-5]. On the strength of epidemiological data, emerging biological investigations and clinical observations, HCV appears to be involved in the pathogenesis of at least a proportion of patients with NHL^[6-7]. This hypothesis is supported by the evidence that HCV is not only hepatotropic, but also a lymphotropic virus^[8]. *In vitro*, HCV is able to replicate in human T-cell lines^[9] and in normal peripheral blood mononuclear cells from healthy subjects^[10]. Moreover, viral genomic sequences have been found in T- and B-cell populations, as well as in monocyte-derived cells from peripheral blood and liver tissue in patients with HCV-related chronic hepatitis^[10-12]. Most studies on HCV-associated B-cell proliferations have focused on peripheral blood and bone marrow lymphocytes. During HCV infection, liver tissue is frequently characterized by prominent lymphoid aggregates in portal tracts that show histological and immunophenotypical features of B follicles^[13-14]. The nature of these aggregates has not yet been investigated in detail; in particular, the clonality of B-cells within lymphoid infiltrates in the liver of HCV-infected patients has not been analyzed at the molecular level. In the present study, we analyzed the frequency of liver-infiltrating monoclonal B-cells from the paraffin-embedded liver biopsies of 40 patients with HCV infection. In 7 patients with monoclonal B-cells infiltration in the liver who were followed up, B-cell clonality was tested using a polymerase chain reaction (PCR) approach designed to identify immunoglobulin heavy chain gene (*IgH*) rearrangements.

MATERIALS AND METHODS

Ethics

All patients were notified of the risk of the liver puncturation and provided informed written consent.

Selection of cases

From June 2003 to December 2005, 848 patients were enrolled in a prospective study and were followed up as outpatients at the Department of Infectious Disease at our NanFang Hospital. In 40 HCV-positive patients (anti-HCV antibody and HCV RNA positive), anti-hepatitis B virus (HBV) and anti-human immunodeficiency virus antibodies were negative. The patients had not received anti-HCV therapy for at least 6 mo. For each patient, a sample of liver biopsy was performed. A control group, consisting of 808 HBV-infected patients with other non-immune chronic liver diseases, was followed up at the same department. All the patients were diagnosed as having chronic hepatitis according to the criteria formulated by the Chinese Society of Infectious disease and Parasitology and Chinese Society of Hepatology, Chinese Medical Association.

Immunohistochemical characterization of lymphoid aggregates

The 848 liver biopsies contained 7 monoclonal B-cell infiltrations. The composition of the 7 infiltration cell specimens was investigated by immunohistochemistry, using antibodies against B-cell (L26/CD20) markers, following the streptavidin–biotin–complex immunoperoxidase technique.

Polymerase chain reaction of *IgH* rearrangements

The DNA from the 7 monoclonal B-cells infiltration liver specimens successfully amplified for the β -actin gene was then subjected to clonality assessment by PCR amplification of rearranged *IgH* genes. After heating for 10 min at 95°C, 2 μ L from each sample was added to the PCR mixture containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 25 pmol/L of each primer, 200 μ mol/L of each dNTP, 1.25 units of *Taq* polymerase, and 4.5 mmol/L MgCl₂ in a final volume of 50 μ L.

We amplified the hypervariable complementary region (CDR-II and CDR-III), included between the third and joining regions (FR-III and JH) of *IgH* genes, with a 5'-primer homologous to the FR-III region, and JH as 3'-primers, using a seminested protocol. The primers were FR3, CTGTCGACACGGCCGTGTATTACTG; JH, AACTGCAGAGGAGACGGTGACC. The first PCR cycle consisted of denaturation of the sample DNA at 93°C for five minutes, annealing of the primers at 55°C for one minute, then extension of the DNA at 73°C for two minutes. Each experiment was duplicated and accompanied by a negative control containing no template DNA. Ten microlitres of the PCR products were analyzed by electrophoresis on 3% agarose gels, stained by ethidium bromide, and viewed under UV light.

The histologic features of liver biopsy specimens were analyzed

Liver biopsy specimens (> 10 mm in length) were fixed, paraffin-embedded, and stained with hematoxylin–eosin–safron, and Warthin–Starry stained for collagen. For each liver biopsy specimen, a stage of fibrosis and a grade of activity were established according to the criteria formulated by the Chinese Society of Infectious Disease and Parasitology and Chinese Society of Hepatology, Chinese Medical Association.

Data analysis

Categorical variables were compared by chi-square testing, and continuous variables were compared by the two-sided Student *t* test.

RESULTS

Among the 848 patients prospectively included in this study, 40 were HCV-positive (anti-HCV antibody

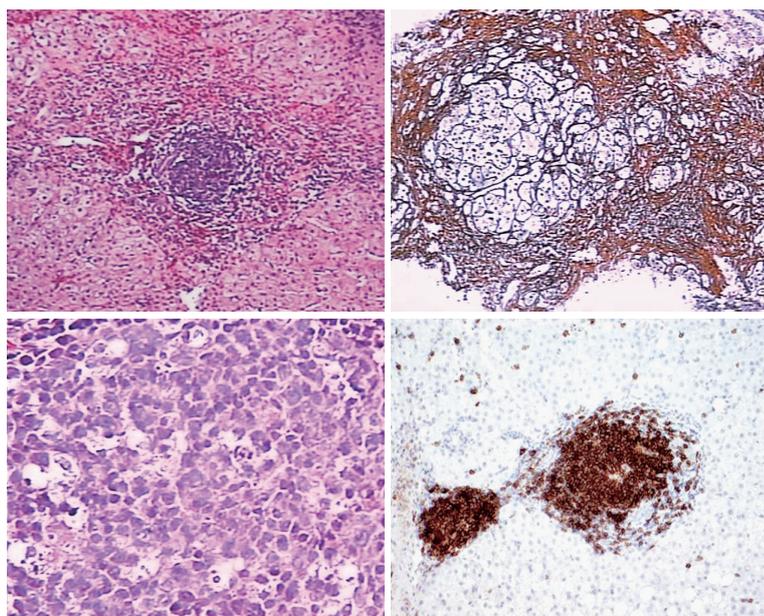


Figure 1 Histological appearances of a B-cell lymphoma, best classified as a marginal zone lymphoma involving the liver. A, B: Low power view of liver core biopsy shows chronic hepatitis and marked portal lymphoid infiltrates. C: High power view of liver core biopsy shows a monotonous portal lymphoid infiltrate composed of small lymphocytes with moderate amount of clear cytoplasm (so-called monocytoid appearance). Note that the infiltrate does not involve the biliary epithelium. D: CD20 immunostain shows that virtually all of the lymphoid cells are B-cells.

Table 1 Baseline characteristics of patients with, and those without, chronic HCV infection

Characteristic	HCV positive (n = 40)	HCV negative (n = 808)	P
Age, (mean ± SD, yr)	50 ± 14	51 ± 15	> 0.05
Male	28/40	550/808	> 0.05
ALT, (mean ± SD, upper limit of normal value)	136 ± 7.8	132 ± 6.9	> 0.05
Liver histologic activity			
None or mild (G0-G1)	7/40	142/808	> 0.05
Moderate or severe (G2-G4)	33/40	666/808	> 0.05
Liver histologic fibrosis			
None or portal fibrosis (S0-S1)	8/40	17/808	> 0.05
Few or many septa or cirrhosis (S2-S4)	32/40	791/808	> 0.05
Liver lymphoid infiltrate			
None	0/40	0/808	> 0.05
Mild	32/40	646/808	> 0.05
Severe	8/40 (65)	162/808	> 0.05
Liver lymphoid aggregate			
None	36/40	805/808	
Yes	4/40	3/808	0.000

and HCV RNA positive), and 808 were HBV-infected patients. The main characteristics of patients with, and those without, chronic HCV infection are detailed in Table 1.

The histological appearance of a B-cell lymphoma is shown in Figure 1.

A clonal B-cell expansion was detected in 5 (71.4%) of 7 of the livers with monoclonal B-cells infiltration. In 2 of 5 patients with both a clonal B-cell expansion and monoclonal B-cells infiltration in the liver, a definite B-cell malignancy was finally diagnosed.

DISCUSSION

In this study, we addressed the question of whether

lymphoid aggregates in the liver of patients with chronic hepatitis C are clonal B-cell proliferations.

Seven of the 848 patients have monoclonal B-cells infiltrating in the livers, including 4 of 40 chronic HCV-infected patients and 3 of 808 chronic HBV-infected patients. Five of the 7 patients with monoclonal B-cells infiltration showed a single band, suggesting that they were formed by a single B-cell clone. We were able to observe progression in patients who continued to be followed up after the end of this study. Lymphoma developed in 2 HCV-infected patients out of 5 patients who had monoclonal B-cells infiltration and B-cell proliferation in the liver.

Although epidemiological data link HCV infection and NHL, the pathobiological processes leading to clonal B-cell expansion and subsequent malignant transformation are only recently becoming better understood. CD81 has emerged as a potentially key mediator of B-cell/HCV interaction, in light of the finding that CD81 can bind to at least two sites on the HCV envelope protein, E2. CD81/E2 interaction does not apparently promote viral entry into B-cells; however, B-cells with specific anti-HCV surface immunoglobulins can simultaneously interact with viral E2 protein *via* CD81, resulting in dual activation signals leading to B-cell proliferation. Furthermore, clonal immunoglobulin gene rearrangements from HCV-positive lymphomas often share a similar restricted gene segment usage pattern, as seen in B-cells from patients with mixed cryoglobulinemia, and also show somatic hypermutation, emphasising the link between chronic viral antigenic stimulation and NHL pathogenesis^[15-20].

A multistep process has also been documented in a lymphoproliferative disorder in an HCV-infected patient, in whom the bcl-2 translocation was followed by myc translocation during the clinical progression of the disease^[21]. However, the wide spectrum of lymphomas that have been described in patients with HCV infection, ranging from lymphoplasmacytoid^[2], to MALT-type^[22], to

follicle-centre cell lymphomas^[23], seem to indicate that more heterogeneous and complex processes are probably involved in the lymphomagenesis associated with HCV.

In conclusion, in patients with chronic HCV infection, the presence of a B-cell clonality and monoclonal B-cells infiltration in the liver may be useful for detecting patients at high risk for developing malignant lymphoproliferative disease. The importance of B-cell clonality analysis in the course of chronic HCV disease needs to be further evaluated, as do the indications for, and the efficacy of, antiviral treatment in patients at risk for B-cell malignancy.

COMMENTS

Background

The association of hepatitis C virus (HCV) infection with type II mixed cryoglobulinemia is well established, but the role of HCV in B-cell lymphoma remains controversial. The incidence of B-cell lymphoma is currently rising in line with the progression of hepatitis C though the cause of this increase is largely unknown.

Research frontiers

Investigating the clonality of B-cells in the liver by analyzing the *IgH* gene rearrangement has been shown to correlate the development of B-cell lymphoma with HCV-infected patients. Liver biopsies in chronic hepatitis C frequently show portal lymphoid infiltrates with features of B follicles, whose clonality has not yet been investigated. In this study, the authors demonstrate that the clonality of B-cells in the liver may represent a low-grade lymphoma.

Innovations and breakthroughs

Recent reports have highlighted the importance of antiviral treatment in the HCV-infected patient with B-cell clonality in the liver. This is the first study to analyze the association of monoclonal B-cells infiltration in the liver with the B-cell clonality. Furthermore, our follow up study showed that the lymphoma developed more frequently in the patients who had monoclonal B-cells infiltration and B-cell proliferation in liver.

Applications

The presence of a B-cell clonality and monoclonal B-cells infiltration in the liver may be useful for detecting patients at high risk for developing malignant lymphoproliferative disease. The study results suggest a strategy for antiviral treatment in patients at risk for B-cell malignancy.

Terminology

Polymerase chain reaction (PCR) amplification is a method currently in widespread use for detection of clonal *IgH* rearrangements. In PCR, rearranged DNA is amplified with a series of consensus primers that are complementary to sequences of variable regions; framework 1, framework 2, and framework 3 and to joining regions of the *IgH* gene.

Peer review

The authors investigate the association of HCV infection and liver B-cell clonality in a prospective clinical trial. Liver biopsy specimens from 40 HCV-positive patients were analyzed, and specimens from hepatitis B virus (HBV)-positive patients served as a control. This is the first study to describe B-cell clonality in the liver of HCV-infected patients, and the results of their study are of interest.

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Liver transplantation for severe hepatic trauma: Experience from a single center

Spiros G Delis, Andreas Bakoyiannis, Gennaro Selvaggi, Debbie Wepler, David Levi, Andreas G Tzakis

Spiros G Delis, Andreas Bakoyiannis, Gennaro Selvaggi, Debbie Wepler, David Levi, Andreas G Tzakis, Department of Surgery, Division of Transplantation, University of Miami Miller School of Medicine, PO Box 012440 (R440), Miami, Florida 33101, United States

Author contributions: Delis SG, Selvaggi G, Wepler D and Levi D designed and performed the research; Delis SG, Selvaggi G, Wepler D and Tzakis AG analyzed the data; Delis SG wrote the paper; Bakoyiannis A reviewed the literature; Tzakis AG supervised the work.

Correspondence to: Spiros G Delis, MD, PhD, Department of Surgery, Division of Transplantation, University of Miami Miller School of Medicine, PO Box 012440 (R440), Miami, Florida 33101, United States. sdelis55@hotmail.com

Telephone: +1-305-3555111 Fax: +1-305-3555134

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Abstract

Liver transplantation has been reported in the literature as an extreme intervention in cases of severe and complicated hepatic trauma. The main indications for liver transplant in such cases were uncontrollable bleeding and postoperative hepatic insufficiency. We here describe four cases of orthotopic liver transplantation after penetrating or blunt liver trauma. The indications were liver failure, extended liver necrosis, liver gangrene and multiple episodes of gastrointestinal bleeding related to portal hypertension, respectively. One patient died due to postoperative cerebral edema. The other three patients recovered well and remain on immunosuppression. Liver transplantation should be considered as a saving procedure in severe hepatic trauma, when all other treatment modalities fail.

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Key words: Liver injury; Orthotopic liver transplantation; Severe liver trauma; Hepatic coma; Hepatic trauma

Peer reviewers: Justin H Nguyen, MD, Division of Transplant Surgery, Mayo Clinic, 4205 Belfort Road, Suite 1100, Jacksonville, Florida 32256, United States; Carla W Brady, MD, MHS, Duke University Medical Center, Division of Gastroenterology, DUMC Box 3913, Durham, NC 27705, United States

Delis SG, Bakoyiannis A, Selvaggi G, Wepler D, Levi D,

Tzakis AG. Liver transplantation for severe hepatic trauma: Experience from a single center. *World J Gastroenterol* 2009; 15(13): 1641-1644 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1641.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1641>

INTRODUCTION

The liver is the most commonly injured abdominal organ, despite its protected location under the rib cage. The therapeutic options for the management of both blunt and penetrating hepatic trauma include a range of operative and non-operative treatment modalities^[1-3]. Currently available methods for the management of hepatic trauma include observation, laparotomy with direct suturing, perihepatic gauze packing, application of fibrin tissue glue, mesh hepatorrhaphy, limited debridement resection and partial lobectomy. Extensive surgical techniques, such as formal hepatectomy or total hepatectomy with liver replacement, have been documented only in selected patients^[4,5]. The surgical aim is control of hemorrhage, preservation of sufficient hepatic function and prevention of secondary complications. Liver transplantation has a limited, though very important, role in specific life threatening cases, when all the above mentioned methods fail to control bleeding or when liver failure ensues. We here describe our experience over the course of 11 years (1996 through 2007) with four cases of severe hepatic trauma requiring liver transplantation.

CASE REPORT

Case 1

A 25-year-old Caucasian male presented with hypovolemic shock to the Trauma Center due to a gunshot wound to the abdomen. The patient was severely acidotic, requiring intense fluid resuscitation. His Glasgow Coma Scale (GCS) score was 9/15 upon admission. The patient was initially managed according the "advanced trauma life support" (ATLS) guidelines and very shortly thereafter was transferred to the operating theatre, due to signs of active bleeding. During an exploratory laparotomy, a trajectory wound affecting segments VII and VIII of the liver was documented, with active bleeding. A Pringle manoeuvre was initially used

along with repair of liver injury. The abdomen was then packed. On the first postoperative day the patient remained unstable and acidotic with further bleeding from the liver surface requiring re-exploration. Right hepatic artery ligation and packing were performed and the patient was transferred to the intensive care unit with a plan for a possible right hepatectomy. Liver and renal functions, however, deteriorated progressively, with persistent acidosis, prolonged prothrombin time, low fibrinogen level and acute renal failure. The patient was placed on the transplant list and two days later underwent an orthotopic liver transplant. A portal and systemic veno-venous bypass was utilized. During re-exploration of the abdomen, the native liver appeared necrotic; mass clamping of the hilum following by supra and infra-hepatic vena cava clamping was performed. The donor liver was implanted using a conventional method for the inferior vena cava. Postoperatively, the patient remained unstable, with progressive lactic acidosis, liver dysfunction and cerebral edema. Cerebral edema was managed with direct monitoring of intracranial pressure (ICP) and drainage of cerebrospinal fluid when decompression was necessary. Despite the above treatment and the complete support in the intensive care unit (ICU), with elevation of the patient's head by 25 degrees and maintenance of cerebral perfusion pressure by supporting systemic arterial pressure, reducing central venous pressure and avoiding agitation, the patient's condition gradually deteriorated and he died on the eleventh post-operative day.

Case 2

A 68-year-old white female developed a subcapsular hematoma of the right lobe of the liver due to blunt abdominal trauma. A right liver lobectomy was performed in another institution because of hematoma expansion. Liver function, however, continued to deteriorate after surgery. The patient was referred for further evaluation. GCS was 15/15 upon admission. Doppler ultrasound revealed main portal vein thrombosis. An exploratory laparotomy was performed to attempt portal vein thrombectomy through the right portal vein stump, but this was unsuccessful. The common bile duct was also found to be partially necrotic and external bile drain placement was performed. Due to postoperative liver failure, the patient was listed as a status 1 candidate for liver transplant. Transplantation was performed two days later using a veno-venous bypass, with caval reconstruction in a piggyback fashion. The patient recovered after prolonged hospitalization and remains on immunosuppression with tacrolimus and mycophenolate mophetil ten years after transplantation.

Case 3

A 58-year-old white female suffered a gunshot wound to the abdomen which resulted in a penetrating right lobe liver injury and a through-and-through injury of the duodenum. Suture ligation with packing and duodenal repair performed in another institution, were adequate to control initial bleeding. However, over the course of the

following two years she experienced multiple episodes of cholangitis due to biliary strictures and she required a choledoco-duodenostomy. Additionally, she went on to develop an arterio-venous fistula between the right hepatic artery and the right portal vein, which resulted in the development of significant portal hypertension. She experienced multiple episodes of gastrointestinal bleeding related to secondary biliary cirrhosis and the portal hypertension. An attempt to embolize the arterio-venous fistula failed and orthotopic liver transplantation was then considered. Her GCS score was 15/15. The native liver was cirrhotic with partial main portal vein thrombosis and a dilated hepatic artery. Under veno-venous bypass, a piggyback technique was used for the caval dissection and the recipient portal vein was thrombectomized. The spleno-portal junction was used for venous reconstruction. Due to intra-operative injury to the duodenum during the dissection, a Billroth II gastrojejunostomy was performed and a Roux-en-Y hepatico-jejunostomy was created for bile duct reconstruction. The patient recovered after an uneventful postoperative course. Explant pathology revealed cirrhotic liver with periportal abscess formation. Six months later, the patient developed cholestasis and hepatic artery thrombosis. He underwent re-transplantation and is alive and well 11 years later.

Case 4

A 35-year-old female was admitted to the casualty department with a gunshot injury. She presented in hypovolemic shock. Her GCS score upon admission was 9/15. After initial management according to ATLS guidelines she underwent exploratory laparotomy and segment II and III penetrating liver injuries with concomitant portal vein laceration were discovered. Longitudinal venorrhaphy of the portal vein, along with liver packing was performed without liver resection. She was then taken to angiography for embolization of the left hepatic artery. Two months later she developed liver gangrene with hepatic artery pseudo-aneurysm. Although septic, the patient was not excluded from evaluation for liver transplant due to the fact that the liver was primarily the source of infection. After removal of the native liver, the patient's hemodynamic status markedly improved. During transplant, the liver was fragile and the hilar structures were impossible to identify. The hilum was mass clamped and the structures isolated in a serial fashion after hepatectomy. The portal vein was dissected free to the confluence with the splenic vein because of the associated fibrosis and the native hepatic artery was suture ligated after removal of the pseudo-aneurysm. Transplant was performed in a piggyback fashion using a supra-celiac jump graft for the arterial inflow. The patient was discharged on postoperative trauma day 85 and is currently doing well at home nine years after transplantation.

DISCUSSION

The overall mortality of hepatic trauma has declined

Table 1 Liver injury scale (AAST)

Grade	Description	
I	Hematoma	Subcapsular, < 10% surface area
	Laceration	Capsular tear, < 1 cm parenchymal depth
II	Hematoma	Subcapsular, 10%-50% surface area: intraparenchymal, < 10 cm in diameter
	Laceration	1-3 cm parenchymal depth, < 10 cm in length
III	Hematoma	Subcapsular, > 50% surface area or expanding; ruptured subcapsular or intraparenchymal hematoma > 10 cm or expanding
	Laceration	> 3 cm parenchymal depth
IV	Laceration	Parenchymal disruption involving 25%-75% of hepatic lobe or 1-3 Couinaud's segments within a single lobe
V	Laceration	Parenchymal disruption involving > 75% of hepatic lobe or > 3 Couinaud's segments within a single lobe
	Vascular	Juxtahepatic venous injuries; i.e. retrohepatic vena cava/central major hepatic veins
VI	Vascular	Hepatic avulsion

Table 2 Type of injury, operations performed and patient outcome

Patient	Age	Injury	Primary operation	Indication for OLT	Re-transplant	Outcome
1	25	Gun shot wound right lobe	Packing, hepatic artery ligation	Acute liver failure	No	Died (cerebral edema)
2	68	Blunt trauma subcapsular hematoma right lobe	R lobectomy, failed portal vein thrombectomy	Portal thrombosis progressive liver failure	No	Discharged POD 45
3	58	Gun-shot wound right lobe, A-V fistula	Hepatorraphy, duodenal repair, embolization	Portal hypertension (A-V fistula), left portal vein thrombosis	Yes	Alive at 11 yr
4	35	Gun-shot wound left lateral lobe, hepatic artery pseudoaneurysm	Packing, embolization	Liver gangrene	No	Discharged POD 85

from 60% in the first half of the last century to approximately 6% today^{6l}. As many as 90% of patients with liver trauma are non-surgically managed with a remarkably high success rate, with only 10% requiring surgical intervention. The American Association for the Surgery of Trauma classified liver trauma degree and reported a liver injury scale (Table 1)^{7l}. The need for orthotopic liver transplantation (OLT) after liver trauma is clearly restricted. However, since the mortality rate of severe and complicated hepatic injuries remains significantly high, reaching 46% for grade IV and 80% for rate V hepatic injury^{8,9l}, OLT must be taken under consideration when all other methods to achieve hemostasis have failed or cannot be applied.

The indications for liver transplantation in the setting of severe and complicated liver trauma, reported in the literature are: (1) uncontrollable bleeding despite repeated previous surgical interventions; (2) postoperative evolution towards hepatic insufficiency (acute or progressive); (3) injuries of the portal vein that cannot be reconstructed^{4,5,9-13l}. In our series, the indications for OLT were the following: portal hypertension due to portal thrombosis and arterio-venous shunt; liver failure from massive injury; and portal thrombosis and liver gangrene with pseudo-aneurysm formation (Table 2). Sepsis was not an absolute contraindication in our study provided that the source of infection was limited to the liver. The above indications, such as fulminate liver failure without irreversible brain injury or extra hepatic sepsis, can also be used as criteria for prompt referral.

Esquivel^{12l} first reported the use of liver transplantation in two patients with progressive hepatic failure and uncontrollable bleeding. Ringe *et al*^{4l} proposed a two-stage procedure (total hepatectomy and subsequent liver transplantation) in cases of severe hepatic trauma,

when all other conventional methods failed to control bleeding. In reviewing the literature between 1987 and 2005, we found 13 reported cases of patients who underwent OLT for the management of severe and life threatening hepatic trauma^{4,5,10-14l}. All of them had severe (grade IV or V) hepatic trauma according to the organ injury scale of the American Association for the Surgery of Trauma^{7l}, and were hemodynamically unstable upon admission.

Furthermore, all patients in these studies had undergone a primary or even secondary operation to control bleeding, before they were finally referred to a transplant center. All our patients had also been managed with more conservative surgical procedures to control bleeding prior to referral for OLT. In our cohort OLT was partly planned due to complications related to the initial surgical management in addition to the severity of the initial liver injury.

To our knowledge, this is the largest series from a single center reported so far. The postoperative mortality rate was 25% and involves a patient with significant hemodynamic instability. In agreement with previous reports, we feel that OLT might be contraindicated when patients do not show any signs of hemodynamic stabilization despite intensive medical support. In such cases, rapid clinical deterioration follows the transplant surgery, leading to multi organ failure and death^{15l}.

Although liver transplantation can be life saving in selective cases with severe liver injury, the lack of immediately available liver grafts combined with the inability to keep a patient in an anhepatic state, are the main causes of why such a few cases have been reported. Patients have to be listed as status 1 and donors with expanded criteria may also be accepted (size mismatch or steatotic livers). Reduced liver grafts have also been used

in the literature but primary non-function is possible^[4].

Preexisting sepsis and associated organ injuries are usual contraindications of liver transplantation for the management of severe hepatic trauma^[16]. Bowel perforation with peritonitis, severe pancreatic trauma and loss of a large portion of the abdominal wall increase the mortality rate and preclude liver transplantation. A severe closed head injury with associated cerebral edema is also an absolute contraindication for orthotopic liver transplantation^[17]. However, localized sepsis in the liver is a relative contraindication, since the septic focus can be eradicated by the transplant itself^[10].

It is worth noting that from a technical point of view: (1) veno-venous bypass is favored due to absence of portal hypertension; (2) mass clamping of the hilum is advocated in situations of difficult dissection or need for rapid liver removal and (3) a piggyback technique is facilitated by the absence of pre-existing portal hypertension. *Ex situ* liver surgery with subsequent auto-transplantation has been reported for the management of otherwise unresectable hepatobiliary malignancies, with good results^[18,19]. It could be a viable alternative option for severe liver trauma, especially if a liver graft is not immediately available. In our series *ex vivo* liver repair was not performed. Patients with lethal injuries to the liver can survive only if they are referred to a transplantation center promptly as documented by our experience.

Liver transplantation is an acceptable surgical method for management of patients with severe traumatic liver injury, under the previously mentioned life-threatening conditions. Further reports are awaited, in order to support and expand the application of OLT in such devastating cases.

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Guillain-Barré syndrome following hepatitis E

Jean Philippe Loly, Estelle Rikir, Maxime Seivert, Emile Legros, Pierre Defrance, Jacques Belaiche, Gustave Moonen, Jean Delwaide

Jean Philippe Loly, Maxime Seivert, Jacques Belaiche, Jean Delwaide, Department of Hepato-Gastroenterology, CHU Sart Tilman, Université de Liège, 4000 Liège, Belgium
Estelle Rikir, Gustave Moonen, Department of Neurology, CHU Sart Tilman, Université de Liège, 4000 Liège, Belgium
Emile Legros, General Practitioner, rue Neuve, 144, 4860 Pepinster, Verviers, Belgium

Pierre Defrance, Department of Hepato-Gastroenterology, CHPLT La Tourelle, 4800 Verviers, Belgium

Author contributions: Loly JP, Rikir E, Seivert M, Moonen G, Delwaide J wrote the manuscript; Legros E and Defrance P provided the relevant clinical data; Belaiche J contributed to the discussion and reviewed the manuscript.

Correspondence to: Jean Delwaide, Department of Hepato-Gastroenterology, CHU Sart Tilman, Université de Liège, 4000 Liège, Belgium. jean.delwaide@chu.ulg.ac.be

Telephone: +32-4-3667256 Fax: +32-4-3667889

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Abstract

Guillain-Barré syndrome (GBS) is often triggered by a preceding bacterial or viral infection. Occasionally, it has been observed in association with acute hepatitis A, B and C, and three cases have been previously described in India in which GBS was associated with acute hepatitis E. A molecular mimicry mechanism is supposed to be involved in the pathogenesis of GBS triggered by infectious agents, although the nature of the shared epitopes has not been characterized in most instances, including that in the case of hepatotropic viruses. We report a case of GBS following acute hepatitis E in a European individual. The presence of antiganglioside GM2 antibodies in this patient suggested molecular mimicry involving ganglioside GM2 in the pathogenesis of GBS associated with hepatitis E.

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Key words: Gangliosides; Guillain-Barré syndrome; Hepatitis E; Molecular mimicry; Viral hepatitis

Peer reviewers: Rakesh Aggarwal, Additional Professor, Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India; Liang-Ping Hu, Professor, Consulting Center of Biomedical Statistics, Academy of Military Medical Sciences, Beijing 100850, China

Loly JP, Rikir E, Seivert M, Legros E, Defrance P, Belaiche J, Moonen G, Delwaide J. Guillain-Barré syndrome following hepatitis E. *World J Gastroenterol* 2009; 15(13): 1645-1647 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1645.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1645>

INTRODUCTION

Guillain-Barré syndrome (GBS) is an acute polyradiculoneuropathy presenting, in its classical form, as a rapidly evolving symmetric and ascending motor paralysis with hypotonia and areflexia accompanied by an leukocytic cerebrospinal fluid with elevated protein level. In over two-third of cases, an infection precedes the onset of neuropathy by 1 to 3 wk. Cytomegalovirus and Epstein-Barr virus account for a large proportion of virus-triggered cases. There are also many reports linking acute hepatitis A, B, and C, with GBS. Hepatitis E is a frequent cause of acute hepatitis in Asia, the Middle East, North Africa and South or Central America. Locally acquired hepatitis E in individuals who have not travelled to endemic areas is, however, becoming an emerging problem in European countries^[1,2]. We report a case of Guillain-Barré syndrome in a patient sporadically contaminated in a Western country. This is the third report of GBS in a patient with hepatitis E^[3,4], and the first occurring in a patient sporadically contaminated in a Western country. This is the first time, to our knowledge, that ganglioside molecular mimicry is suggested in the pathogenesis of GBS-associated with a hepatotropic virus.

CASE REPORT

A 66-year-old male general practitioner who worked in an urban area consulted due to an acute elevation of liver function tests (AST: 1062 IU/L, ALT: 1813 IU/L, γ -GT: 90 IU/L). Serum bilirubin and alkaline phosphatases were normal. The liver tests had been carried out during a routine check-up and the patient was completely asymptomatic at presentation. Three months prior to consultation his blood tests were normal. The patient had not recently received any hepatotoxic or neurotoxic drugs or vaccinations, and had not travelled abroad during the last year.

A few days later, the patient developed neurological symptoms, beginning with progressive loss of strength in both legs and paraesthesia of the lower limbs, mainly in the evening. Ataxia and neuropathic pain appeared a few days later. These symptoms led the patient to be hospitalized in the neurology department of our institution.

On physical examination, the patient was afebrile. Blood pressure was normal. Examination of heart, lungs and abdomen was unremarkable. There were no features of chronic liver disease and no signs of encephalopathy. The neurological examination showed a stance and gait ataxia with Romberg's sign and distal hypopallesthesia. Symmetric hyporeflexia in the upper limbs and areflexia in the lower limbs were associated with a severe proximal weakness prominent in the lower limbs.

Routine blood examination showed an AST of 68 IU/L, ALT of 443 IU/L and γ -GT of 94 IU/L. Renal function, electrolytes, glucose, and haematologic values were normal.

Cerebrospinal fluid analysis showed a major increase in protein concentration at 1722 mg/L, associated with a high level of immunoglobulin G, without an increased number of leucocytes. Electrophysiological examinations of the lower limbs demonstrated an acute demyelinating polyradiculoneuropathy.

These findings were consistent with a diagnosis of GBS. Of note, serum antiganglioside antibodies GM2 IgM were positive (Dotzen ganglio profile Ab IgG and IgM by Zentech[®]). Other antiganglioside antibodies were negative (GM1, GM3, GD1A and GD1B, GD3, GQ1B, GT1A and GT1B). Antibodies to Purkinje cells, to neurons and to myelin were negative. Sulfatide antibodies were negative.

A serological study showed IgM antibodies to hepatitis E (two assays were used: HEV IgM ELISA by Genelabs, with a sensitivity of 93% and a specificity of 99%; and Recomblot HEV IgM by Mikrogen, with a sensitivity of 85.7% and a specificity of 100% in non endemic regions^[5]).

Hepatitis B surface antigen, antibodies to hepatitis C, IgM anti-HAV were absent. The following serological tests were also negative: antibodies to HIV, Varicella-Zoster virus and cytomegalovirus. Serology for campylobacter was negative. IgG were positive, with negative IgM, for Epstein-Barré virus, adenovirus and herpesvirus.

A diagnosis of GBS associated with acute hepatitis E was made. Intravenous immunoglobulins were given at a dose of 0.4 g/kg per day for five days. This treatment significantly improved the patient's neurological condition with progressive recovery of walking perimeter and a reduction in neuropathic pain. Liver enzymes completely normalized. Four months later, a near-complete neurological recovery was noted.

DISCUSSION

Hepatitis E has become an emerging cause of acute hepatitis in western countries^[1,2]. In most cases, acute

hepatitis E in these regions is of autochthonous origin^[6]. The most frequent risk factors for hepatitis E, reported in a French survey, were water consumption from a personal water supply, uncooked shellfish consumption, and the recent acquisition of a pet pig^[7]. None of these risk factors were present in our patient. It is possible that the contamination was related to the patient's profession as a general practitioner. It has been shown that the clinical evolution of hepatitis E can be different in patients infected sporadically compared with patients infected in endemic areas. In autochthonous cases, the mean age is higher and the prognosis is more severe with a higher rate of fulminant liver failure^[8].

Guillain-Barré syndrome is clinically defined as an acute inflammatory demyelinating polyradiculoneuropathy causing limb weakness^[9]. Paralysis of muscles develops acutely over a period of days, but can take up to 4-6 wk. In most patients, after a brief plateau, improvement begins with a gradual resolution of paralysis which lasts from weeks to months. The syndrome is considered to be an autoimmune disease triggered by a preceding bacterial or viral infection. The most commonly identified triggering agents are *Campylobacter jejuni*, followed by cytomegalovirus, Epstein-Barr virus, and mycoplasma pneumonia. HIV, shigella, clostridium, haemophilus influenza, as well as hepatitis A, B and C were also identified as triggering agents^[10]. In our case, the temporal association between acute hepatitis E and GBS strongly suggested a relation between both disorders.

The mechanism by which infection can trigger GBS is not completely understood. It is thought that the immune system mistakenly attacks myelin or axons by a molecular mimicry mechanism (in which the host generates an immune response against an infectious organism that shares epitopes with the host's peripheral nerves)^[11]. The nature of the epitope, although still uncertain, is likely to be a glycolipid. The most attractive candidate targets are gangliosides, which are present in nodal and internodal membranes of nerve fibres^[12]. Ganglioside antibodies may perturb nerve conduction and, in a complement-dependant fashion, disrupt the molecular topography of nodal and paranodal proteins and induce motor axonal degeneration^[13]. It is postulated that infected cells can produce ganglioside-like epitopes that trigger the immune response. This mechanism of molecular mimicry has been observed for *Campylobacter jejuni* with the implication of several gangliosides (GM1, GD1B, and GQ1B)^[14]. The implication of antiganglioside GM2 antibodies in the pathogenesis of GBS related to CMV has been described^[15]. It has been demonstrated that CMV-infected fibroblasts express ganglioside-like epitopes that specifically recognize anti-GM2 antibodies^[16]. These results suggest that, in CMV-infected GBS patients, infected cells with CMV can express epitopes inducing an immune response against gangliosides.

For GBS related to hepatitis A, B, C, or E, no homologous epitopes to a component of the peripheral nerves have been described to date. We report the first

description of the presence of antiganglioside GM2 antibodies in GBS associated with a hepatotropic virus, suggesting possible molecular mimicry involving gangliosides. This possible relationship should be further documented in the very rare cases of GBS associated with viral hepatitis to confirm the mechanism.

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CASE REPORT

Schistosomal appendicitis: Incidence in Japan and a case report

Tadashi Terada

Tadashi Terada, Department of Pathology, Shizuoka City Shimizu Hospital, Shizuoka 424-8636, Japan

Author contributions: Terada T contributed all to this paper.

Correspondence to: Tadashi Terada, MD, PhD, Department of Pathology, Shizuoka City Shimizu Hospital, Miyakami 1231 Shimizu-Ku, Shizuoka 424-8636,

Japan. piyo0111jp@yahoo.co.jp

Telephone: +81-54-3361111 Fax: +81-54-3361315

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Abstract

Schistosomal appendicitis is very rare in developed countries like the USA, Europe, and Japan. The author reviewed 311 pathologic archival specimens of vermiform appendix over the past 10 years. One case of schistosomal appendicitis was recognized. Therefore, the incidence of this disease was 0.32% in all appendices surgically resected in our hospital. The patient was a 41-year-old woman presenting with lower abdominal pain. She was a sailor traveling to many countries including endemic areas. Physical examination, laboratory data, and imaging modalities suggested an acute appendicitis, and appendectomy was performed under the diagnosis of ordinary appendicitis. Histologically, numerous schistosomal eggs were present in the vasculatures throughout the appendiceal walls. Some of the eggs were calcified. Stromal foreign body reaction was also recognized. The appendicitis was phlegmonous consisting of severe infiltrations of neutrophils and eosinophils. Acute serositis was also noted. Examination of feces revealed numerous eggs of *Schistosoma mansoni*. Clinicians should be aware of schistosomal appendicitis.

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Key words: Appendix; Histopathology; Pathologic archival specimens; Schistosomiasis; Acute appendicitis

Peer reviewer: Maurice A Cerulli, MD, FACP, FACG, Division of Gastroenterology and Hepatology, New York Methodist Hospital, Brooklyn, New York 11215, United States

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INTRODUCTION

Schistosomiasis is caused by *Schistosoma mansoni* or *Schistosoma japonica*, and is a disease of waterborne trematode infestation. Schistosomal appendicitis is very rare in developed countries like the USA, Europe, and Japan^[1-5]. However, it is prevalent in endemic areas such as Africa and South Asia^[1-5]. In Japan, Yamanashi and Fukuoka prefectures are endemic areas. However, the incidence and pathological features of schistosomal appendicitis are not known. Therefore, the author herein reports the incidence and pathological features of this disease in Japan.

CASE REPORT

The author re-examined 311 pathologic archival specimens of vermiform appendix over the past 10 years. Of these specimens, one case of schistosomal appendicitis was recognized. Therefore, the incidence of this disease was 0.32% of all appendices surgically resected in our hospital. The specimen was from a 41-year-old woman, who was a sailor traveling to many countries including endemic areas, who had complained of lower abdominal pain. Physical examination was suggestive of acute appendicitis. Blood laboratory data showed leukocytosis. Imaging modalities including CT indicated an appendiceal swelling. An appendectomy was performed under the clinical diagnosis of ordinary appendicitis.

Histologically, numerous schistosomal eggs were present in the vasculatures throughout the appendiceal walls (Figure 1A). This finding was unexpected. Some of the eggs were calcified (Figure 1B). Stromal foreign body reaction was also recognized (Figure 1B). Severe infiltrations of neutrophils and eosinophils were recognized (Figure 1C). Acute serositis was also noted (Figure 1A). Examination of feces revealed numerous eggs of *Schistosoma mansoni*. The involvement of other organs was unclear.

DISCUSSION

Schistosomiasis is a waterborne parasitic disease caused by *Schistosoma mansoni* and *Schistosoma japonica*. This

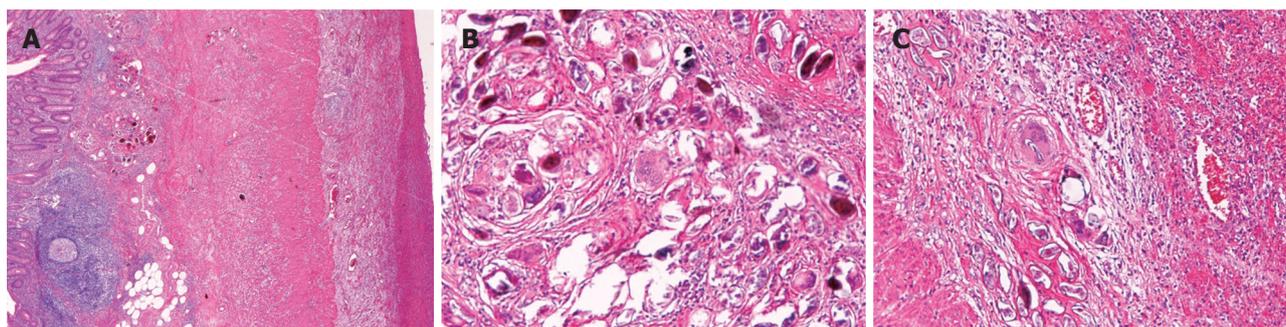


Figure 1 Histologic findings of schistosomal appendicitis. A: Low power view of the appendiceal walls in schistosomal appendicitis. Acute inflammation is recognized throughout the walls. Schistosomal eggs are seen in the mucosa and muscular layer. The serosa shows acute serositis (right). HE, $\times 40$; B: Schistosomal eggs in the mucosa. Foreign body granulomatous reaction is recognized. Some eggs show calcification. HE, $\times 200$; C: Schistosomal eggs in the subserosa. The eggs were located within vasculatures. Severe infiltrations of neutrophils and hemorrhage are recognized. HE, $\times 200$.

disease is endemic and particularly prevalent in Africa and South Asia^[1-5]. Our hospital is located in a non-endemic area. Schistosomiasis is a disease of intestine and liver, where the parasite resides and produces eggs in the vasculatures.

The incidence of schistosomal appendicitis is unclear in Japan. The present study revealed that the incidence was 0.32% of all vermiform appendices resected.

The present case is pathologically typical of schistosomal appendicitis, and feces examination strongly supported the diagnosis. Clinically, schistosomal appendicitis was not considered, and clinicians were first informed after pathologic examination. The present patient was a sailor traveling to many countries including endemic areas. Thus, clinicians should be aware of schistosomal appendicitis.

It is uncertain whether schistosomiasis of the vermiform appendix induces acute appendicitis^[1]. However, it is now thought that appendiceal schistosomiasis may cause acute appendicitis. This may be due to ischemic changes caused by egg emboli. This situation may diminish mucosal immunity, thus leading to bacterial infection.

In endemic areas like Nigeria, Badmos *et al*^[4] reported that appendices with schistosomiasis were present in 35/843 (4.2%) of surgically resected cases. Of these 35 positive cases, 23 (65.7%) were associated with acute appendicitis, while the remaining 12 cases (34.3%) were not associated with inflammation. Thus,

the presence of the parasite does not always give rise to acute appendicitis. In developed countries like the USA, Nandipati *et al*^[5] reported that schistosomal appendicitis was found in 3/1690 (0.2%) of surgically resected cases. All three cases were African Americans^[5]. Thus, in developed countries, schistosomal appendicitis is preferentially found in travelers or in an endemic area population.

In summary, the incidence of schistosomal appendicitis is 0.34% in Japan. The present patient with this disease was a sailor traveling to many countries. Thus, clinicians should be aware of schistosomal appendicitis.

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CASE REPORT

A combination treatment of entecavir and early-phase corticosteroid in severe exacerbation of chronic hepatitis B

Kazuyuki Matsumoto, Yasuhiro Miyake, Hirokazu Miyatake, Masahiro Takahara, Takayuki Imada, Satoru Yagi, Tatsuya Toyokawa, Morihito Nakatsu, Masaharu Ando, Mamoru Hirohata

Kazuyuki Matsumoto, Hirokazu Miyatake, Masahiro Takahara, Takayuki Imada, Satoru Yagi, Tatsuya Toyokawa, Morihito Nakatsu, Masaharu Ando, Mamoru Hirohata, Department of Internal Medicine, Mitoyo General Hospital, Kanonji 769-1695, Japan

Yasuhiro Miyake, Department of Molecular Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

Author contributions: Matsumoto K and Miyake Y analyzed the data and wrote the paper; Miyatake H, Takahara M, Imada T, Yagi S, Toyokawa T, Nakatsu M carried out patient care; Ando M and Hirohata M helped by supervising and approving the final manuscript.

Correspondence to: Yasuhiro Miyake, MD, Department of Molecular Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558,

Japan. miyake43@md.okayama-u.ac.jp

Telephone: +81-86-2357219 Fax: +81-86-2255991

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Peer reviewer: Sun-Lung Tsai, MD, PhD, Professor, Director, Hepatogastroenterology Section, Department of Internal Medicine and Liver Research Unit, Department of Medical Research, Chi Mei Medical Center, 901 Chung Hwa Road, Young-Kang City, Tainan County 710, Taiwan, China

Matsumoto K, Miyake Y, Miyatake H, Takahara M, Imada T, Yagi S, Toyokawa T, Nakatsu M, Ando M, Hirohata M. A combination treatment of entecavir and early-phase corticosteroid in severe exacerbation of chronic hepatitis B. *World J Gastroenterol* 2009; 15(13): 1650-1652 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1650.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1650>

Abstract

Of patients with severe exacerbation of chronic hepatitis B accompanied by jaundice and coagulopathy, 20%-30% have a fatal outcome. In this report, we describe 2 cases of severe exacerbation of chronic hepatitis B with jaundice and coagulopathy who were successfully treated with a combination of entecavir and corticosteroid. In both cases, rapid reductions in serum hepatitis B virus (HBV)-DNA levels were observed, and corticosteroid was stopped after serum HBV-DNA levels became undetectable. Entecavir treatment was continued. Generally, entecavir treatment reduced serum HBV-DNA levels rapidly, although the improvement in liver function was delayed by a few weeks. During this time lag, liver cell injury continued and the disease progressed. Corticosteroid suppressed the excessive host immune response and was useful for stopping progressive deterioration. A combination of entecavir and early-phase corticosteroid may be a useful treatment in severe exacerbation of chronic hepatitis B.

INTRODUCTION

An estimated 400 million people worldwide have chronic hepatitis B virus (HBV) infection, and more than 500 000 people die every year from complications of HBV-related chronic liver disease^[1]. In patients chronically infected with HBV, acute exacerbations are clinically important because they can have severe or even fatal consequences^[2]. An estimated 10%-30% of hepatitis B carriers experience acute exacerbation each year^[3]. Hepatitis B e antigen (HBeAg) seroconversion and mortality occur in 2.7% and 0.7% of patients with acute exacerbation, respectively^[4]. On the other hand, mortality occurs in 20%-30% of cases with severe exacerbation accompanied by jaundice and coagulopathy^[5,6].

In the past decade, lamivudine (LMV) has revolutionized the treatment of chronic hepatitis B. Treatment with LMV significantly decreases the rate of hepatic decompensation and prevents the development of hepatocellular carcinoma^[7]. On the other hand, LMV monotherapy confers no significant protection against rapid progression to hepatic failure in severe exacerbation of chronic hepatitis B, although LMV results in long-term benefits^[5]. Furthermore, viral resistance, which is usually followed by a loss of clinical response, a rise in aminotransferase levels and worsening of hepatic histology, is related to mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif which

occurs in 70%-80% of patients treated continuously for 4-5 years^[8]. Recently, the early introduction of high-dose corticosteroid was reported to improve the short-term prognosis of patients with severe exacerbation of chronic hepatitis B^[9].

In this report, we describe 2 cases with severe exacerbation of chronic hepatitis B successfully treated with a combination of entecavir (ETV) and corticosteroid.

CASE REPORT

Case 1

A 33-year-old Japanese man was admitted to our hospital with general fatigue. He had been diagnosed as a carrier of HBV but had never received treatment. His family history included chronic hepatitis B in his late mother, who had died of severe exacerbation of the disease. On physical examination at admission, he was conscious and the bulbar conjunctiva was not icteric. Neither ascites nor pretibial edema was noted. Laboratory data on admission were as follows: bilirubin, 19 mg/L; aspartate aminotransferase, 698 IU/L; alanine aminotransferase, 756 IU/L; albumin, 33 g/L; and prothrombin activity, 47.7%. He was positive for both hepatitis B surface antigen (HBsAg) and HBeAg, and his serum HBV-DNA level measured by real-time quantitative polymerase chain reaction (TaqMan PCR, Roche Diagnostics) was 2.6 LGE/mL. He was diagnosed with severe exacerbation of chronic hepatitis B, and oral ETV treatment (0.5 mg/d) was initiated. On his fifth day of hospitalization, prednisolone (30 mg/d) was added. At 6 wk, his serum transaminase level was normal, after which the dose of prednisolone was tapered. At 12 wk, his serum HBV-DNA level was 3.3 LGE/mL and prednisolone was stopped. Since then, his transaminase level has remained normal (Figure 1).

Case 2

A 44-year-old Japanese woman was admitted to our hospital with general fatigue and anorexia. She had been diagnosed as a carrier of HBV but had never received treatment. Her family history included a father who was a carrier of HBV. On physical examination at admission, she was conscious and her bulbar conjunctiva was icteric. Neither ascites nor pretibial edema was noted. Laboratory data on admission were as follows: bilirubin, 96 mg/L; aspartate aminotransferase, 1389 IU/L; alanine aminotransferase, 573 IU/L; albumin, 31 g/L; and prothrombin activity, 22.7%. She was positive for both HBsAg and HBeAg, and her serum HBV-DNA level measured by transcription-mediated amplification assay (Roche Diagnostics) was 5.3 LGE/mL. She was diagnosed with severe exacerbation of chronic hepatitis B. A combination of oral ETV (0.5 mg/d) and intravenous methylprednisolone (250 mg/d) was immediately started. One her sixth day of hospitalization, intravenous methylprednisolone was changed to oral prednisolone (50 mg/d). At 8 wk, her serum transaminase level was normal and her serum bilirubin levels and prothrombin activity were improved to

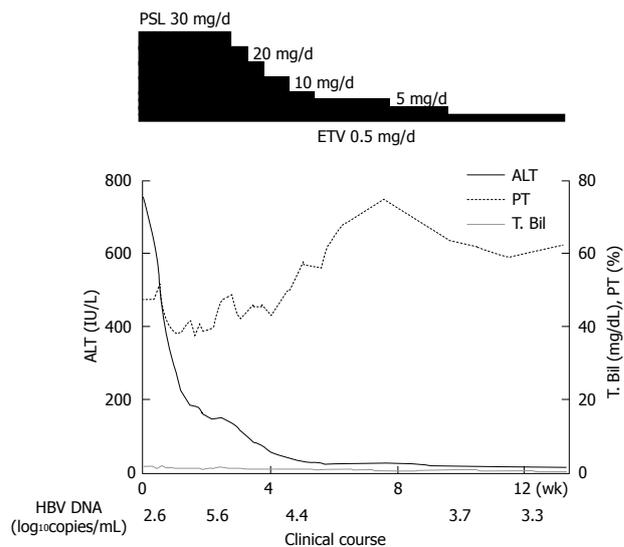


Figure 1 Patient clinical course. ALT: Alanine aminotransferase; PT: Prothrombin activity; T. Bil: Total bilirubin; HBV DNA: Hepatitis B virus DNA. At 6 wk, his serum transaminase level was normalized. At 12 wk, his serum HBV-DNA level became 3.3 LGE/mL and prednisolone was stopped.

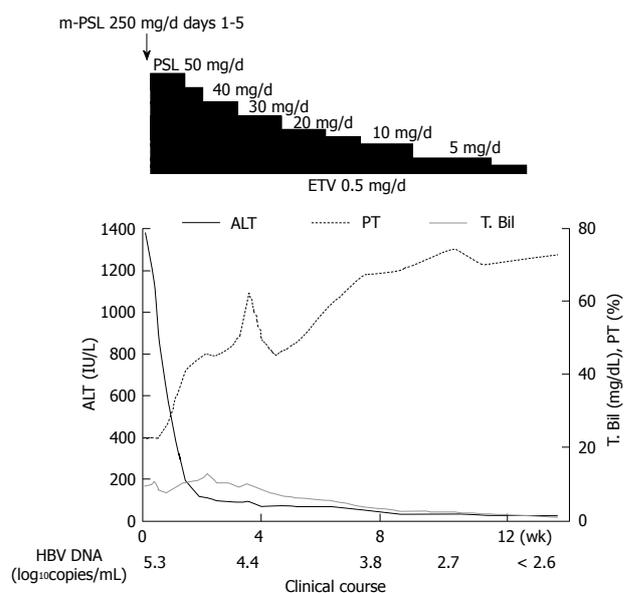


Figure 2 Patient clinical course. ALT: Alanine aminotransferase; PT: Prothrombin activity; T. Bil: Total bilirubin; HBV DNA: Hepatitis B virus DNA. At 8 wk, her serum transaminase level was normalized. At 15 wk, her serum HBV-DNA level became undetectable and the prednisolone was stopped.

68.8% and 29 mg/L, respectively. Subsequently, the dose of prednisolone was tapered. At 15 wk, her serum HBV-DNA level became undetectable and prednisolone was stopped. One year later, her ETV treatment has continued, and her serum HBV-DNA level has continued to be undetectable (Figure 2).

DISCUSSION

ETV suppresses HBV replication significantly better than LMV. The mean reduction in serum HBV-DNA levels from baseline to week 48 is reported to be 6.9 log in HBeAg-positive patients and 5.0 log in HBeAg-

negative patients^[10,11]. Furthermore, ETV shows a lower frequency of virologic rebound (2% in the first year of drug administration) compared with LMV. Thus, ETV is considered a first-choice therapy for patients with chronic hepatitis B not previously treated with a nucleoside analogue. However, for patients with severe exacerbation of chronic hepatitis B, treatment during the first 2 wk determines their prognosis^[9]. The liver cell injury caused by HBV infection is mediated mainly by the response of CD8+ cytotoxic T lymphocytes to small epitopes of HBV proteins, especially the hepatitis B core antigen, present on the surface of liver cells^[12]. ETV treatment reduces serum HBV-DNA levels rapidly, although the improvement in liver function is delayed by a few weeks. During this time lag, liver cell injury continues and the disease progresses. Corticosteroid suppresses the excessive host immune response and is useful for stopping progressive deterioration. On the other hand, patients not treated with any antiviral drugs show a subsequent rebound increase in serum transaminase levels 4 to 10 wk after withdrawal of corticosteroid^[13]. Thus, we consider that a combination of ETV and early-phase corticosteroid may be reasonable for improving prognosis in severe exacerbation of chronic hepatitis B.

Corticosteroid has been reported to directly stimulate HBV replication through specific glucocorticoid receptors in the HBV genome in cultured human hepatoma cells^[14]. Clinically, immunosuppressive treatment has been indicated to have a potentiating effect on HBV replication in patients with chronic active hepatitis B^[15]. Furthermore, corticosteroid has been reported to delay the normalization of serum transaminase levels^[16]. However, in this study, both our patients showed rapid reductions in serum HBV-DNA levels despite corticosteroid treatment. We consider that this may be attributable to the antiviral effect of ETV, which may be strong enough to overcome HBV replication activated by corticosteroid treatment.

In this study, 0.5 mg/kg or more of prednisolone was administered daily. When the patient showed a trend toward remission in serum transaminase levels, the dose of prednisolone was tapered. After serum HBV-DNA level became undetectable, prednisolone was stopped. In Japan, ETV is administered at a dose of 0.5 mg daily in order to treat chronic HBV infection in accordance with Japanese national health insurance rules. However, further study is required in order to confirm adequate doses of prednisolone and ETV in severe exacerbation of chronic hepatitis B.

In conclusion, the combination treatment of ETV and corticosteroid may improve the prognosis in severe exacerbation of chronic hepatitis B. However, this is a case report; prospective studies of large study populations are needed in order to confirm the effectiveness of this combination treatment.

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Needle track seeding: A real hazard after percutaneous radiofrequency ablation for colorectal liver metastasis

Shirley Yuk-Wah Liu, Kit-Fai Lee, Paul Bo-San Lai

Shirley Yuk-Wah Liu, Kit-Fai Lee, Paul Bo-San Lai, Division of Hepato-biliary and Pancreatic Surgery, Department of Surgery, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, China

Author contributions: Liu SYW, Lee KF and Lai PBS contributed equally to this work; Liu SYW wrote the draft of the manuscript; Lee KF and Lai PBS edited and prepared the manuscript.

Correspondence to: Paul Bo-San Lai, Professor, Chairman & Chief of Service, Division of Hepato-biliary and Pancreatic Surgery, Department of Surgery, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, China. paullai@cuhk.edu.hk

Telephone: +852-26321309 Fax: +852-26377974

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New Haven 06510, United States; Dr Takayuki Yamamoto, Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan; Professor Francis Seow-Choen, MBBS, FRCSEd, FAMS, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

Liu SYW, Lee KF, Lai PBS. Needle track seeding: A real hazard after percutaneous radiofrequency ablation for colorectal liver metastasis. *World J Gastroenterol* 2009; 15(13): 1653-1655 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1653.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1653>

Abstract

Neoplastic needle track seeding following percutaneous radiofrequency ablation (RFA) of secondary liver tumors is exceedingly rare. Reports on cutaneous tumor seeding after percutaneous RFA for colorectal liver metastasis are even rarer in the literature. Here we report a case of a 46-year-old female who developed an ulcerating skin lesion along the needle track of a previous percutaneous RFA site around 6 mo after the procedure. The previous RFA was performed by the LeVeen® needle for a secondary liver tumor from a primary rectal cancer. The diagnosis of secondary skin metastasis was confirmed by fine needle aspiration cytology. The lesion was successfully treated with wide local excision. We believe that tumor seeding after percutaneous RFA in our patient was possibly related to its unfavorable subcapsular location and the use of an expansion-type needle. Hence, prophylactic ablation of the needle track should be performed whenever possible. Otherwise, alternative routes of tumor ablation such as laparoscopic or open RFA should be considered.

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Key words: Radiofrequency catheter ablation; Needles; Neoplasm seeding; Liver neoplasms; Skin neoplasms; Neoplasm metastasis

Peer reviewers: Professor Walter E Longo, Department of Surgery, Yale University School of Medicine, 205 Cedar Street,

INTRODUCTION

Radiofrequency ablation (RFA) is a well established local ablative treatment for primary or secondary hepatic neoplasms. Despite a relatively low complication rate, the percutaneous application of RFA carries a potential risk of needle track seeding. As reported by a recent systematic review^[1], the seeding risk after percutaneous RFA for hepatocellular carcinoma (HCC) is around 0.6%. However, such a seeding risk in secondary liver tumors is less well defined. Reports on tumor seeding after percutaneous RFA for colorectal liver metastasis are rare. The following report illustrates a patient with cutaneous tumor seeding after percutaneous RFA for a small colorectal liver metastasis.

CASE REPORT

A 46-year-old female, who had no other major medical illness, was referred to our unit for stage IV rectal cancer with liver metastasis after receiving laparoscopic anterior resection of the primary rectal tumor in the private sector. Preoperative staging computed tomography (CT) of the abdomen showed no distant metastasis. The operation for the primary rectal tumor was uneventful. However, a suspicious liver lesion was incidentally found on the surface of the liver intra-operatively and was biopsied. Histopathology revealed a T3N2 well-differentiated adenocarcinoma of the rectum and a metastatic adenocarcinoma of the liver. A postoperative re-staging CT scan identified a 1.7 cm solitary subcapsular liver metastasis at segment 4 of the liver (Figure 1). A

Table 1 Literature review on neoplastic seeding after RFA for colorectal liver metastasis

Author (yr)	Age; gender	Size of liver metastasis (cm)	Subcapsular location of liver lesion	Number of RFA sessions	Size of seeding nodule (cm)	Time lag after RFA
Bonatti <i>et al</i> ^[4] (2003)	56; male	Not stated	Not stated	2	2	6 wk
Charalampopoulos <i>et al</i> ^[5] (2007)	64; male	3	Yes	2	Not stated	10 mo
Present case-2008	46; female	1.7	Yes	1	2	6 mo

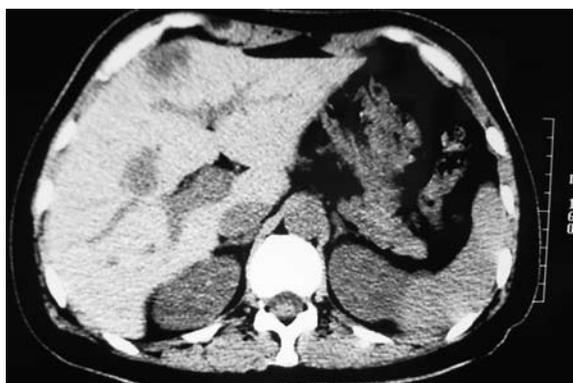


Figure 1 Subcapsular liver metastasis at segment 4 of liver.



Figure 2 Percutaneous radiofrequency ablation by LeVeen® needle.

subsequent positron emission tomography scan excluded additional distant metastasis. Unfortunately, the patient refused curative hepatic resection. Hence, a single session of CT-guided percutaneous RFA of the liver lesion using a 3 cm 17-gauge LeVeen® needle electrode (Super-slim, Boston Scientific, United States) with a single puncture was performed (Figure 2). Thermal ablation of the needle tract was not done in this case. She was subsequently put on a complete course of oxaliplatin-based chemotherapy.

Six months after RFA, she presented with a 2 cm ulcerating skin nodule at the previous RFA puncture site (Figure 3). Fine needle aspiration cytology of the nodule confirmed a metastatic adenocarcinoma of primary colorectal origin. At the same time, a CT scan revealed multiple simultaneous recurrences at the left liver. Left hepatectomy for the liver lesions and wide local excision of the cutaneous tumor were performed. Multiple suspicious peritoneal deposits were incidentally identified intra-operatively and they were all resected. Final histopathology revealed metastatic disease in the

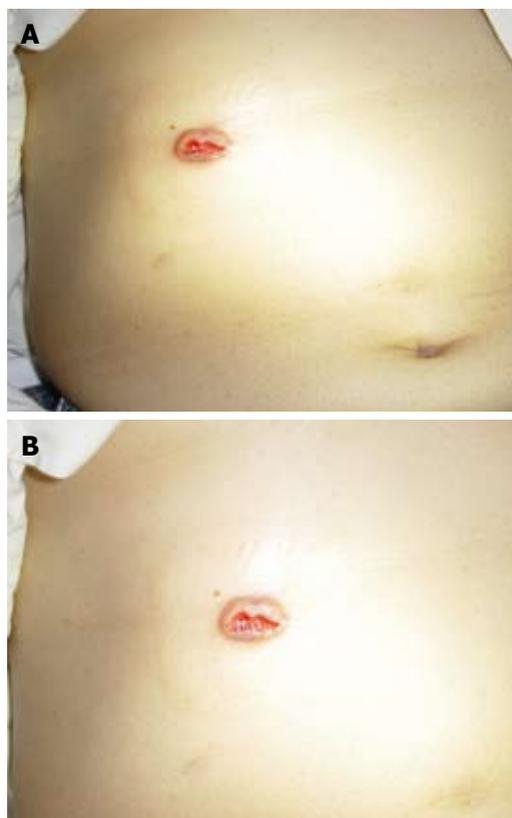


Figure 3 Ulcerating skin nodule. A: Cutaneous neoplastic seeding 6 mo after RFA. B: A closer view.

liver, skin and peritoneum. She then received further courses of palliative chemotherapy in view of likely recurrence. In the subsequent follow-up period, no cutaneous recurrence was identified at the RFA needle track. However, she developed progressive disease with lung metastasis and carcinomatosis. The patient finally died around 5 mo after the second operation.

DISCUSSION

Neoplastic seeding is an uncommon but well-recognized complication following percutaneous diagnostic and therapeutic procedures for primary liver cancer. For diagnostic percutaneous biopsy, the risk of neoplastic seeding was approximately 2.2%^[1]. As for therapeutic RFA of HCC, initial results from a small-scale Spanish study suggested an alarmingly high risk of 12.5%^[2]. A recent large-scale multicenter study by Livraghi *et al*^[3] in contrast identified a substantially lower risk of only 0.9%. As highlighted by a recent systematic review^[1], such a seeding risk for HCC was definitely lower with

an overall median risk of only 0.6%. For secondary liver tumors, objective evidence on the seeding risk following percutaneous RFA is lacking. In the English literature, there were only two related case reports identified^[4,5] (Table 1).

Several associated risk factors have been identified for neoplastic seeding following RFA for HCC, notably subcapsular tumor location^[2,6], poor tumor differentiation grade^[2], multiple RFA sessions^[6], multiple electrode placements^[6] and history of previous biopsy^[6]. In an Italian study by Latteri *et al*^[7], the risk of neoplastic seeding after open RFA was virtually zero but the risk was as high as 1.4% after percutaneous RFA. Remarkably, most of these identified factors were based on seeding risk for HCC. As for our patient, tumor seeding in the RFA needle track was possibly related to its unfavorable subcapsular location and the use of an expansion-type electrode. The previous use of laparoscopic biopsy in such a subcapsular tumor was probably a major detrimental factor related to its peritoneal dissemination. Nevertheless, potential risk factors of neoplastic seeding solely for secondary liver tumors were still undefined. To evaluate the seeding risk, a larger-scale prospective cohort study for secondary liver tumors is required. However, these sorts of studies are practically difficult to conduct because of the rare occurrence.

To prevent neoplastic seeding, some investigators advocated the application of thermocoagulative ablation along the needle track while withdrawing the RFA needle^[8]. However, this ablative technique may not be technically feasible in cases of subcapsular lesions as in our case. With regard to treatment, surgical excision with a wide margin seems to be the most justifiable option. Alternatively, different novel techniques had been described. Shibata *et al*^[9] successfully treated a chest wall neoplastic seeding from HCC by transarterial embolization of the feeding vessel. Espinoza *et al*^[10] suggested the use of RFA again for ablating the metastatic seeding tract as treatment.

To conclude, despite its rare occurrence, needle track seeding is a real hazard following percutaneous RFA for secondary liver tumors. Prophylactic ablation of the

needle track should be performed whenever possible for high risk patients. Otherwise, alternative routes of tumor ablation like laparoscopic or open RFA should be considered.

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CASE REPORT

A large congenital and solitary intrahepatic arterioportal fistula in an old woman

Zhen-Ya Lu, Jian-Yang Ao, Tian-An Jiang, Zhi-Yi Peng, Zhan-Kun Wang

Zhen-Ya Lu, Zhan-Kun Wang, Department of Internal Medicine, First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Jian-Yang Ao, Tian-An Jiang, Department of Ultrasound, First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Zhi-Yi Peng, Department of Radiology, First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Author contributions: Lu ZY wrote the article; Ao JY analyzed the images; Jiang TA, Peng ZY and Wang ZK provided their advices.

Correspondence to: Jian-Yang Ao, MD, Department of Ultrasound, First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China. aojysili@126.com

Telephone: +86-571-87236516 Fax: +86-571-87236514

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Abstract

Arterioportal fistula (APF) is a rare cause of portal hypertension and may lead to death. APF can be congenital, post-traumatic, iatrogenic (transhepatic intervention or biopsy) or related to ruptured hepatic artery aneurysms. Congenital APF is a rare condition even in children. In this case report, we describe a 73-year-old woman diagnosed as APF by ultrasonography, computed tomography, and hepatic artery selective arteriography. The fistula was embolized twice but failed, and she still suffered from alimentary tract hemorrhage. Then, selective arteriography of the hepatic artery was performed again and venae coronariae ventriculi and short gastric vein were embolized. During the 2-year follow-up, the patient remained asymptomatic. We therefore argue that embolization of venae coronariae ventriculi and short gastric vein may be an effective treatment modality for intrahepatic APF with severe upper gastrointestinal bleeding.

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Key words: Congenital intrahepatic arterioportal fistula; Liver; Embolization; Portal hypertension; Angiography

Peer reviewers: Dr. Paolo Del Poggio, Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza

Ospedale 1, Treviglio Bg 24047, Italy; David Cronin II, MD, PhD, FACS, Associate Professor, Department of Surgery, Yale University School of Medicine, 330 Cedar Street, FMB 116, PO Box 208062, New Haven, Connecticut 06520-8062, United States

Lu ZY, Ao JY, Jiang TA, Peng ZY, Wang ZK. A large congenital and solitary intrahepatic arterioportal fistula in an old woman. *World J Gastroenterol* 2009; 15(13): 1656-1659 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1656.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1656>

INTRODUCTION

Arterioportal fistula (APF) is a rare cause of portal hypertension and may lead to death. APF can be congenital, post-traumatic, iatrogenic (transhepatic intervention or biopsy) or related to ruptured hepatic artery aneurysms. Congenital APF is a rare condition in children. To date, only 18 cases of congenital intrahepatic APF have been reported^[1]. Most of them were found in their infancy, in which the oldest one was a 13-year-old boy^[1]. We report here the incidental findings of a large and solitary congenital APF in a 73-year-old woman. Digital subtraction angiography revealed a stubby fistular vessel between the left hepatic artery and portal vein of the patient. Transcatheter closure of APF was performed three times using multiple coils.

CASE REPORT

A 73-year-old woman was admitted to our hospital with complaints of ascites, splenomegaly, abdominal distension and pain. She had been asymptomatic before and denied any medication history as well as history of cirrhosis and hepatic neoplasms, blunt or penetrating trauma, percutaneous liver biopsy, transhepatic cholangiography, gastrectomy and biliary surgery. Her mother and aunt died of ascites and gastrointestinal bleeding in their thirties, about 60 years ago. There was no history of chronic hepatic disease in her family.

A recent physical examination revealed ascites, splenomegaly, and a subcutaneous varicose vein in the abdominal wall. Her laboratory results are listed in Table 1.

Values for other biochemical tests were within the normal ranges. All viral markers for hepatitis including

Table 1 Laboratory results

Item	Results
Hemoglobin	97 g/L
Hematocrit	40.30%
MCV	89.8 fL
Blood cell count	$4.0 \times 10^9/L$
Platelet count	$131 \times 10^9/L$
Prothrombin time	14 s (normal value: 9.8-15.0 s)
Serum ALT	37 IU/L (normal value: 3-40 IU/L)
Serum AST	62 IU/L (normal value: 3-40 IU/L)
Serum GGT	102 IU/L (normal value: 0-54 IU/L)
Serum ALP	141 IU/L (normal value: 30-115 IU/L)
Total bilirubin	10 $\mu\text{mol/L}$ (normal value < 22 $\mu\text{mol/L}$)
Direct bilirubin	4 $\mu\text{mol/L}$ (normal value < 7 $\mu\text{mol/L}$)
Albumin	39.7 g/L

hepatitis A-E viruses, autoantibodies (antinuclear, anti-mitochondrial, anti-smooth-muscle, anti-liver-kidney microsomal enzymes, anti-soluble liver antigen, and anti-mitochondrial antibody) were also negative. Serum alpha-fetoprotein level was normal. Upper gastrointestinal endoscopy revealed non-bleeding esophageal and fundus varices, but mild portal hypertensive gastropathy.

Real-time sonography of the liver demonstrated an anechoic lesion in continuity with a normal width of the left portal vein (LPV) branch, which was assumed to be an intrahepatic APF. Reverse flow in the left portal vein was observed on color Doppler sonography (Figure 1), and high-velocity flow at 1.31 m/s (Figure 2) was observed on spectral Doppler sonography. The distal portion of the LPV showed no enlargement but turbulent flow. The left hepatic artery branch was dilated, with a diameter of 5 mm. Color speckling (mosaic pattern), present in hepatic tissues adjacent to the aneurysmal lesion, was considered a typical sign of arteriovenous fistula.

Contrast-enhanced computed tomography (CT) also confirmed the diagnosis of intrahepatic APF. A high density of the LPV was observed with a CT value of 220.6 Hounsfield units (HU) (Figure 3A) at the arterial phase, and a CT value of 159.7 HU (Figure 3B) at the portal venous phase, close to the CT values of 226.2 HU (Figure 3A) and 168.0 HU (Figure 3B), respectively, for the abdominal aorta at the same phase.

After a comprehensive analysis, a diagnosis of portal hypertension secondary to hepatic portal fistula was established.

Selective digital subtraction angiography of the hepatic artery was performed, which revealed a large left hepatic artery-left portal vein fistula (Figure 4A). The aneurysmal lesion was embolized, and angiography CT and sonography showed no shunt after operation (Figure 4B).

Three months after operation, the patients had severe upper gastrointestinal bleeding from esophageal and fundus varices. She received another transcatheter closure, with no recurrence of bleeding. She underwent somatostatin infusion and conservative treatment but hemorrhage recurred 1 mo later. She refused any surgical intervention. Selective arteriography of the hepatic



Figure 1 Color Doppler sonography of the portal vein aneurysm showing increased blood flow in the LPV and turbulence (arrow) at the distal portion of the aneurysmal sac.

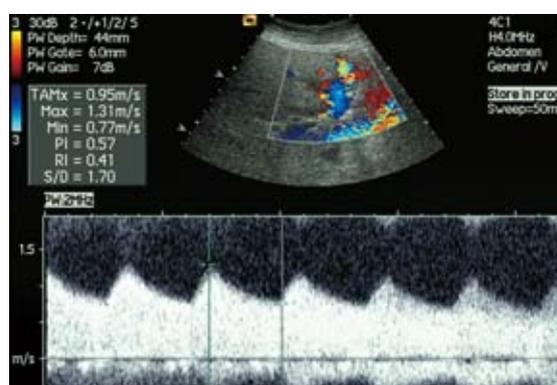


Figure 2 PW Doppler sonography of the portal vein aneurysm showing a high speed blood flow at 1.31 m/s in the aneurysm.

artery was performed again with her venae coronariae ventriculi and short gastric vein embolized (Figure 5A and B). During the 2-year follow-up, the patient remained asymptomatic.

DISCUSSION

Congenital APF is a rare cause of severe portal hypertension, with challenging diagnostic and therapeutic implications^[2,3]. Upon physical examination, a characteristic bruit can be heard over the right upper quadrant. Liver function tests are usually normal. Radiologic evaluation of APF is usually performed with color Doppler ultrasonography (US), helical CT, and magnetic resonance imaging. Arterial and direct portography as well as splenoportography may also be used. Doppler ultrasound is the best way in making the decisive diagnosis and helpful in the subsequent evaluation of these patients^[4,5]. Angiography is the most useful test because it can not only identify multiple APFs but also be therapeutic. These APFs are mostly intrahepatic and represent 12% of the cases developmental intrahepatic shunts^[3]. The presence of a patent ductus venosus is protective in the initial postnatal period. Symptoms usually develop after 1 mo of age, and these children are often investigated for generalized abdominal findings and are later noted to

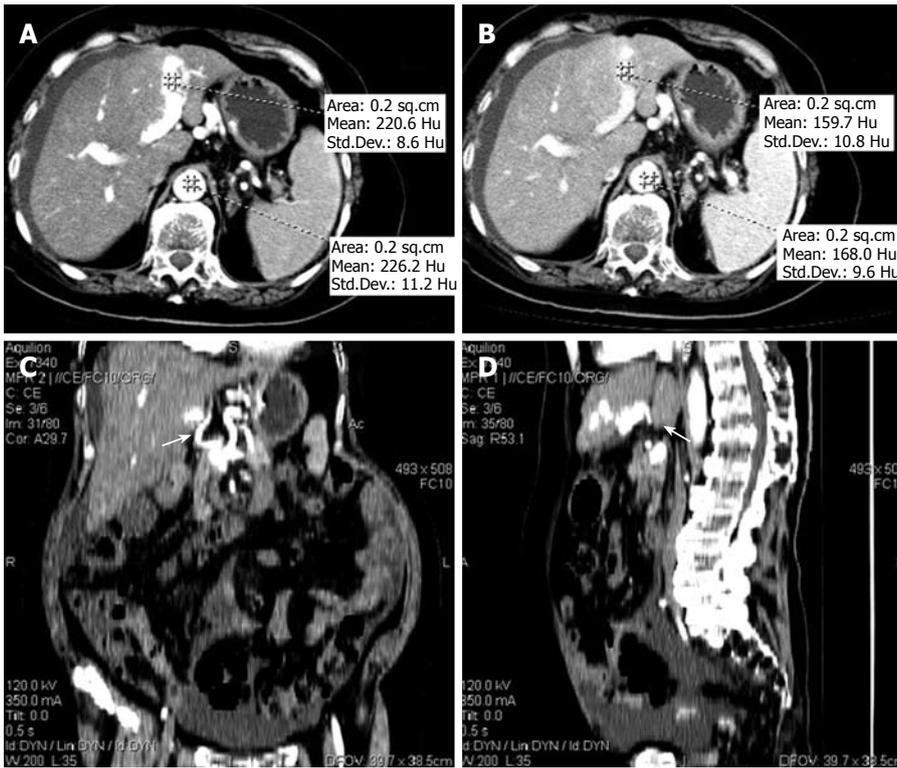


Figure 3 Contrast-enhanced CT showing a LPV density of 220.6 Hu and an abdominal aorta density of 226.2 Hu at artery phase (A), a LPV density of 159.7 Hu and an abdominal aorta density of 168.0 Hu at portal venous phase (B), and CT of coronal section (C) and sagittal section (D) showing the direct shunt of APF.

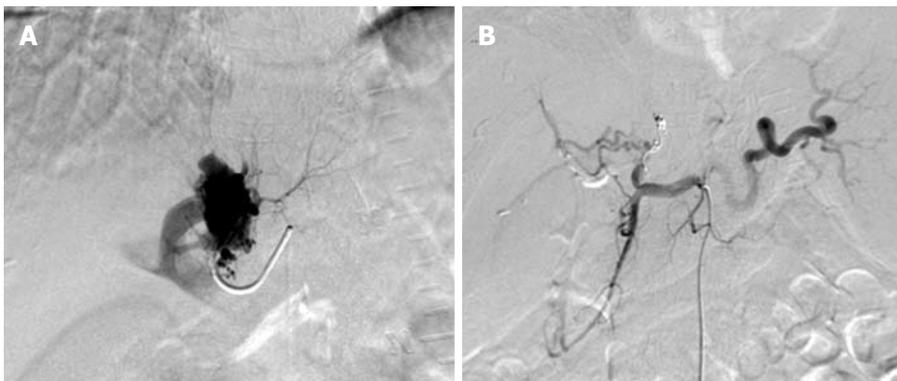


Figure 4 Selective digital subtraction angiography of the hepatic artery. A: A large left hepatic artery-left portal vein fistula; B: No more shunt after embolism.

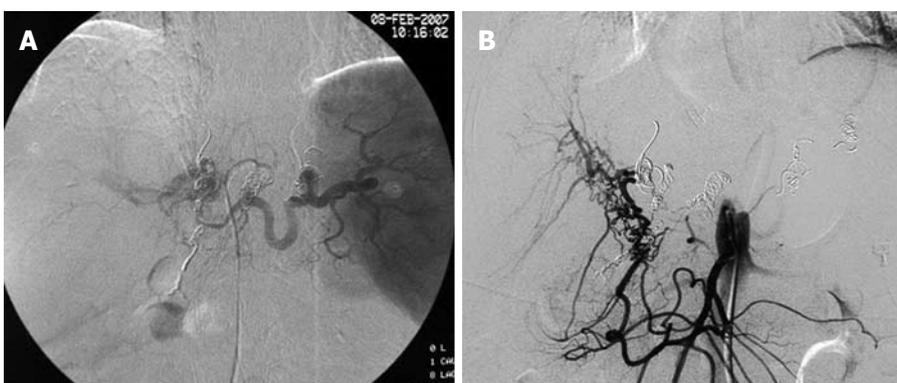


Figure 5 Angiography showing collateral circulation in fistula after embolism (A) and no more shunt after embolism (B).

develop symptoms related to portal hypertension^[2,3]. APF often presents in conjunction with major gastrointestinal tract bleeding and should be differentiated between small peripheral intrahepatic APF (type 1) and large central APF (type 2), whereas diffuse congenital intrahepatic APF that is difficult to manage is defined as typed 3^[6]. A few cases of congenital fistula from the hepatic artery to the

portal vein have been reported^[4], but this abnormality is not a common cause of portal hypertension. Increased blood flow in the portal system is considered to be the cause of hyperkinetic portal hypertension in patients with hepatoportal arteriovenous fistula. Arterioportal venous fistula can be treated with percutaneous arterial occlusion^[7]. Ligation of the hepatic artery has been proved

to be successful in reported cases^[8]. Recently, transcatheter arterial embolization has been attempted as the first choice, because of its low invasiveness and success in some cases. Recanalization of the embolized artery in some cases has also been reported^[9,10]. Therefore, some of the cases can undergo hepatic resection, including fistula.

To date, in the literature, the oldest congenital APF case was a 13-year-old child^[1]. We report here a 73-year-old woman. This patient had no history of cirrhosis and hepatic neoplasms, blunt or penetrating trauma, percutaneous liver biopsy, transhepatic cholangiography, gastrectomy, and biliary surgery, and was finally diagnosed having a congenital APF. Initially, the APF was demonstrated by contrast-enhanced CT and the fistula was subsequently identified by color Doppler imaging and angiography. The case was classified as type 3.

No more shunt was found after coil occlusion. Notably, esophageal varices and ascites disappeared after embolization. However, color Doppler sonography still displayed the shunt 3 mo after operation.

The aneurysmal lesion was embolized twice, but transcatheter closure did not work well. After undergoing embolization twice, she suffered alimentary tract hemorrhage again, suggesting that transcatheter closure is not so effective against a large APF. The reason why embolization failed was because of too much collateral circulation. The purpose of embolizing the venae coronariae ventriculi and short gastric vein is to cut off the collateral circulation, because it can effectively control hemostasis. Since it cannot cure the disease, the treatment modality is radical surgery.

In conclusion, this case suggests that interventional radiology plays an important role in the treatment of congenital APF with severe upper gastrointestinal bleeding caused by esophageal and fundus varices.

Liver function test, abdomen ultrasonography and gastroscopy should be performed regularly during the follow-up.

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Walter E Longo, Professor

Department of Surgery, Yale University School of Medicine, 205 Cedar Street, New Haven 06510, United States

Fanyin Meng, MD, PhD, Assistant Professor

Department of Internal Medicine, Ohio State University, Room 514A Medical Research Facility, 420 West 12th Avenue, Columbus, Ohio 43210, United States

Ibrahim A Al Mofleh, Professor

Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Dushyant V Sahani, MD, Director of CT

Department of Radiology, Associate Professor Radiology, Harvard Medical School, Division of Abdominal Imaging and Intervention, 55 Fruit Street, White 270, Boston, MA 02114, United States

Bruno Stieger, Professor

Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich 8091, Switzerland

Frank I Tovey, OBE, ChM, FRCS

Honorary Research Fellow, Department of Surgery, University College London, London, United Kingdom

Hitoshi Yoshiji, MD, PhD

Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan

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.apdwcgress.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

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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
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Gastro 2009 UEGW/World Congress of Gastroenterology
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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Za-zhi* 1999; **7**: 285-287

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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 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]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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^[3]Passed away on June 14, 2008



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EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
 Telephone: +86-10-59080039
 Fax: +86-10-85381893
 E-mail: wjg@wjgnet.com
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PUBLISHING

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MicroRNA signatures in liver diseases

Xian-Ming Chen

Xian-Ming Chen, Department of Medical Microbiology and Immunology, Creighton University Medical Center, Criss II, Room 529, 2500 California Plaza, Omaha, NE 68178, United States

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Correspondence to: Xian-Ming Chen, MD, Associate Professor, Department of Medical Microbiology and Immunology, Creighton University Medical Center, Criss II, Room 529, 2500 California Plaza, Omaha, NE 68178, United States. xianmingchen@creighton.edu

Telephone: +1-402-2803750 Fax: +1-402-2801875

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Abstract

MicroRNAs (miRNAs) are an emerging class of highly conserved non-coding small RNAs that regulate gene expression at the post-transcriptional level. It is now clear that miRNAs can potentially regulate every aspect of cellular activity, including differentiation and development, metabolism, proliferation, apoptotic cell death, viral infection and tumorigenesis. Recent studies provide clear evidence that miRNAs are abundant in the liver and modulate a diverse spectrum of liver functions. Deregulation of miRNA expression may be a key pathogenetic factor in many liver diseases including viral hepatitis, hepatocellular cancer and polycystic liver diseases. A clearer understanding of the mechanisms involved in miRNA deregulation will offer new diagnostic and therapeutic strategies to treat liver diseases. Moreover, better understanding of miRNA regulation and identification of tissue-specific miRNA targets employing transgenic/knockout models and/or modulating oligonucleotides will improve our knowledge of liver physiology and diseases.

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Key words: MicroRNAs; Non-coding RNAs; Liver; Tumorigenesis; Gene regulation

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INTRODUCTION

Genomic studies have demonstrated that many portions of the human genome do not encode conventional protein-coding genes but encode biologically active non-coding RNA species^[1]. With the development of small RNA interface techniques over the past decade, it becomes clear that many small RNA molecules could regulate gene and protein expression. One class of such small non-coding RNAs is microRNAs (miRNAs), a group of regulatory RNAs of 19-22 nucleotides involved in control of gene expression at the post-transcriptional level^[2]. Recent studies suggest that miRNAs are involved in regulating cell fate (cell death and proliferation), initiation and progression of human cancer, developmental timing, and inflammatory responses^[3-6]. Modulation of miRNA expression *in vitro* as well as *in vivo* has revealed an important role of miRNAs in liver functions. In this review, we appraise the recent findings on miRNAs in liver physiology and disease. We will also discuss the development and use of miRNA antagonists (antagomirs) to target miRNAs *in vivo*, which may translate into novel therapeutic strategies for liver disease in the future.

miRNAs ARE REGULATORY NON-CODING SMALL RNAs IMPORTANT TO POST-TRANSCRIPTIONAL GENE REGULATION

miRNAs are endogenous, single-stranded RNA molecules consisting of approximately 22 non-coding nucleotides that regulate target genes^[2,3]. miRNA molecules have been identified in over 80 species including those encoded by viral genomes. There are approximately 500-1000 different mammalian miRNA genes; a complete list and details about the nomenclature of the miRNAs can be viewed at Sanger mirBase 10.1 (<http://microrna.sanger.ac.uk/sequences/>)^[7]. Up to 600 miRNAs have been identified in humans^[2,3].

Most miRNAs are generated by RNA polymerase

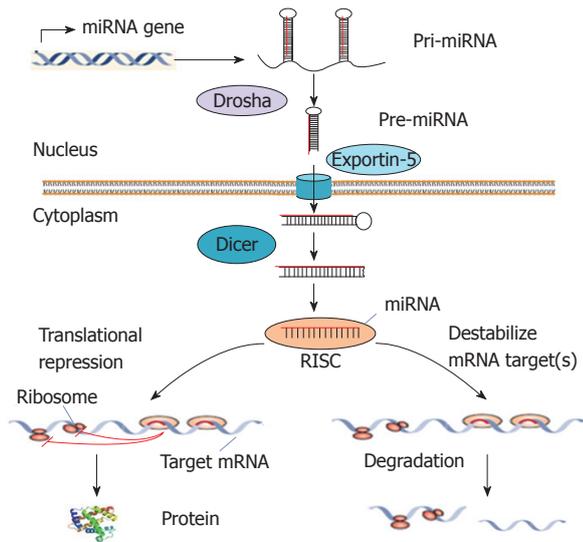


Figure 1 miRNA biogenesis and function in animal cells. Animal genomes have specific genes that encode miRNAs. Primary miRNA transcripts (pri-miRNAs) are processed into precursor miRNA (pre-miRNAs) stem-loops of approximately 60 nucleotides in length by the nuclear RNase III enzyme Droscha. These pre-miRNAs are transported to the cytoplasm via exportin-5 and are further processed by the ribonuclease Dicer. Mature miRNAs are then incorporated in the RNA-induced silencing complex (RISC) and interfere with the regulation of mRNA translation by targeting mRNAs resulting in mRNA degradation or translational repression.

as long primary transcripts (pri-miRNAs) that form a stem-loop structure^[8-11]. In the nucleus, pri-miRNAs are processed into 70-100 nucleotide-long hairpin pre-miRNAs by the RNase III Droscha. These pre-miRNAs are then exported into the cytoplasm by exportin-5^[11]. They are then further processed by another RNase III, Dicer. The resultant approximately 22-nucleotide RNA duplexes contain the mature miRNA and the passenger miRNA strand^[12,13]. The mature miRNA can be incorporated into the so-called RNA-induced silencing complex (RISC). This miRNA-mRNA interaction either blocks translation initiation, induces the endonucleolytic cleavage of the target mRNA, or both (Figure 1)^[12-14]. In mammal cells, the sequence of the miRNAs loaded in the complex targets the RISC to specific binding sites in the 3'-untranslated region (UTR) of mRNA transcripts. Each mRNA can be regulated by several miRNAs, and one miRNA can recognize several targets^[12,13,15]. Intriguingly, miRNAs may also lead to an upregulation of gene expression^[16]. However, the exact mechanism is currently unknown but may be the result of direct effects, such as chromatin remodeling, or indirect effects, e.g. suppression of transcriptional repressors. It is believed that expression of 30% of human genes may be regulated by miRNAs^[17].

miRNAs ARE ABUNDANT AND FINELY REGULATED IN THE LIVER

One of the first clues of the existence of miRNAs in mammals came from studies on genetic alterations in liver tumors. An unusual transcript, named hcr, was described

and characterized as liver-specific, essentially non-coding, specifically nuclear, and processed by endonucleases in one of woodchuck liver tumors investigated in 1989^[18]. Later on, the hcr transcript was found to encompass the so-called "pri-miRNA" for miR-122^[19]. miRNA-122 was later described as a liver specific miRNA and has been reported in mouse, woodchuck and human livers, in human primary hepatocytes, and in cultured liver derived cells^[19,20]. Besides miR-122, many other miRNAs, such as miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, and the *let-7* family, are also abundantly expressed in adult liver tissue. While miR-122 appears as the most highly expressed miRNA in adult liver, miR-92a and miR-483 seem to be more specifically expressed in the fetal liver (Table 1)^[21]. Thus, the liver displays a differential miRNA expression profile during its development.

The liver contains many cell types including parenchymal cells (i.e. hepatocytes) and "non-parenchymal cells" which include endothelial cells, stellate cells, lymphoid cells, and biliary epithelial cells (cholangiocytes). Each cell type may have completely distinct miRNA expression profiles. However, miRNA expression profile in those cell types, in particular from humans, is not fully tested yet. Current understanding is limited to available human cell lines. For example, a distinct expression profile of miRNAs has been identified in H69 cells, a cell line of SV40 transformed human cholangiocytes^[22,23].

The molecular mechanisms underlying transcriptional regulation of miRNA genes in the liver remain largely unknown. Hepatocyte nuclear factor 1 α (HNF-1 α) is a hepatocyte-enriched transcription factor. Manipulation of HNF-1 α function through RNA interference causes reciprocal changes in miR-107 expression and thus, may be involved in the regulation of miR-107 transcription in the liver^[24]. The myocyte enhancer factor-2 transcription factor can increase the expression of miR-1-2 and miR-133a-1^[25]. The transcription factor Myc can up-regulate the expression of miR17-92 cluster and down-regulate several other miRNAs in tumorigenesis^[26]. However, whether these transcription factors are also involved in the transcriptional regulation of those miRNAs in the liver is unclear. In addition, decrease of *let-7* family expression in human cholangiocytes in response to microbial stimulation appears to be nuclear factor (NF)- κ B-dependent^[23]. On the other hand, the functional expression of transcription factors can also be regulated by miRNAs, as it has recently been shown for signal transducer and activator of transcription 3 (STAT-3)^[22].

miRNAs MAY BE KEY PLAYERS IN THE REGULATION OF LIVER FUNCTIONS

For a comprehensive understanding of miRNA function and potential therapeutic use in liver physiology and disease, identification and validation of miRNA targets are of fundamental importance. Several bioinformatic methods have been developed to predict miRNA targets^[7]. Arora and Simpson adapted the 'ranked ratio

Table 1 Repertoire of miRNAs in human liver

	Atlas	Adult	Fetal		Atlas	Adult	Fetal
miR-122	+++	+++	++	miR-154	+	/	/
miR-126	+++	/	/	miR-193a	+	/	/
miR-16	++	++	/	miR-193b	+	/	/
let-7a	++	+++	/	miR-195	+	/	/
miR-22	++	+++	+	miR-199a	+	+++	+
miR-125b	++	+++	+	miR-21	+	+++	+
miR-143	++	+++	/	miR-223	+	/	/
let-7b	++	++	/	miR-25	+	++	/
miR-99a	++	++	/	miR-27a	+	++	/
let-7c	++	++	/	miR-29b	+	/	/
miR-181a	++	/	/	miR-29c	+	/	/
miR-194	++	+++	/	miR-3-c	+	+++	+
miR-451	++	/	/	miR-32-	+	/	/
miR-3-d	++	++	/	miR-377	+	/	/
miR-15b	++	/	/	miR-378	+	/	/
miR-193a-5p	++	/	/	miR-424	+	/	/
miR-24	++	+++	++	miR-425	+	/	/
miR 29a	++	/	/	miR-486-5p	+	/	+
miR-23b	++	+	+	miR-5-5	+	/	/
miR-26b	++	/	/	miR-874	+	/	/
miR-27b	++	++	/	miR-885-5p	+	/	/
miR-3-a	++	++	/	miR-1-7	Traces	/	/
miR-92a	++	++	+++	let-7e	Traces	/	/
miR-13-a	++	/	/	miR-98	Traces	/	/
miR-15a	++	++	/	miR-3-b	--	++	/
miR-186	++	/	/	miR-34	--	++	/
miR-191	++	++	/	miR-1-6a	--	++	/
miR-26a	++	/	++	miR-125a	--	++	/
miR-28	++	++	/	miR-148	--	++	/
miR-1- -	++	/	/	miR-149	--	++	/
let-7d	++	/	/	miR-189	--	++	/
miR-17	++	/	+	miR-199b	--	++	/
miR-185	++	/	/	miR-21-	--	++	/
miR-192	++	++	/	miR-321	--	+++	/
miR-3-e	++	/	/	miR-145	--	++	+
miR-381	++	/	/	miR-93	--	+	+
miR-99b	++	/	+	miR-483	--	/	+++
miR-1-3	++	+	/	miR-484	--	/	+
miR-23a	++	/	/	miR-485	--	/	+
let-7f	+	/	/	miR-487	--	/	++
let-7g	+	/	/	miR-2-	--	/	++
miR-139-5p	+	+	/	miR-18	--	/	+
miR-14-	+	/	/	miR-19b	--	/	+
miR-142	+	++	/	miR-1-6b	--	/	+
miR-144	+	/	/	miR-345	--	/	+
miR-151	+	/	+	miR-41-	--	/	+

The minus sign is used to indicate very low to undetectable levels and one plus to three pluses indicate a gradual expression from low levels to very high levels. The slash stands for the miRNAs that were not assayed. This figure is modified from Girard *et al*^[21] with the permission of the authors and the publisher.

(RR)' described by Yu *et al*^[27] and predicted the genes that are potentially targeted miRNAs in the liver^[28]. This RR value is an indicator of the distribution of miRNA target genes within a single mRNA population. A high RR indicates low expression in a greater proportion of target genes and is, therefore, indicative of miRNA activity in that tissue. The RR values for all miRNAs were calculated for the miRNAs in the liver^[28] and are shown in Figure 2. Clearly, many mRNAs could be the targets for miRNAs in the liver.

Validation of those potential targets for each miRNA requires experimental studies both *in vitro* and *in vivo*.

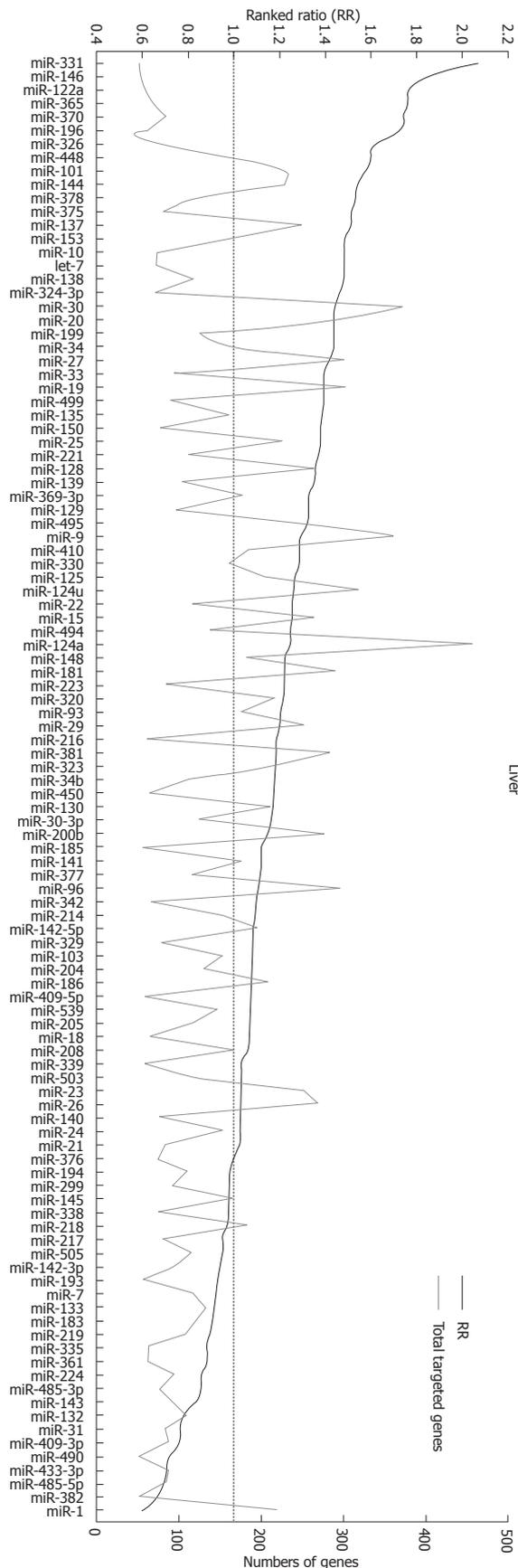


Figure 2 Prediction of targets by miRNAs in the liver. The miRNAs are ordered by RR values (upper-axis). The higher values reflect lower expression of predicted target genes and are, therefore, indicative of miRNA activity. The numbers of genes predicted to be targeted by each miRNA (lower-axis) are indicated by the grey line. This figure is reprinted from Arora and Simpson^[28] with the permission of the authors and the publisher.

Several groups have tested the overall importance of miR-122 in the regulation of metabolism in the liver^[29,30]. Through an antisense strategy to knock down miR-122, Esau *et al*^[30] observed that several genes which regulate lipid metabolism, specifically the key enzyme phosphomevalonate kinase, were down-regulated. Remarkably, silencing miR-122 in high-fat fed mice resulted in a significant reduction of hepatic steatosis, which was associated with reduced cholesterol synthesis rates and stimulation of hepatic fatty-acid oxidation^[30]. Functional inhibition of miR-122 by an “antagomir” resulted in increased expression of several hundred genes including those that are normally repressed in hepatocytes^[29]. In addition, Chang *et al*^[20] identified a binding site for miR-122 in the 3'-UTR of the cationic amino acid transporter (CAT-1) mRNA using the Lewis-based miRNA targets approach^[31]. Consistently, an inversed pattern of expression of CAT-1 and miR-122 was noted at all stages of liver development. Interestingly, miR-122-induced inhibition through the CAT-1 3'-UTR was efficiently relieved upon amino acid starvation, which validates CAT-1 as a target of miR-122^[32].

Several groups, including ourselves, have recently tested the role of miRNAs in the regulation of biliary epithelial immunity in the liver. Human cholangiocytes express *let-7* family members, miRNAs with complementarity to TLR4 mRNA. *let-7* regulates TLR4 expression *via* post-transcriptional suppression in cultured human cholangiocytes. Infection of cholangiocytes with *Cryptosporidium parvum* (*C. parvum*), a parasite that causes intestinal and biliary disease, results in decreased expression of primary *let-7i* and mature *let-7* in a MyD88/NF- κ B-dependent manner. The decreased *let-7* expression is associated with *C. parvum*-induced up-regulation of TLR4 in infected cells^[23]. miRNAs may also be involved in cholangiocyte responses to pro-inflammatory cytokines, such as interferon-gamma (IFN- γ), and actively participate in the regulation of biliary inflammatory response in the liver. Specifically, miR-513 regulates B7-H1 translation and mediates IFN- γ -induced B7-H1 expression in human cholangiocytes. B7-H1 (CD274, PD-L1) is a member of the B7 family of costimulatory molecules and plays a critical immunoregulatory role in cell-mediated immune responses. Resting human cholangiocytes express B7-H1 mRNA, but not B7-H1 protein. IFN- γ induces B7-H1 protein expression and alters miRNA expression profile in cholangiocytes. Of those IFN- γ -down-regulated miRNAs, miR-513 has complementarity to the 3'-UTR of B7-H1 and targeting of miR-513 to B7-H1 mRNA results in translational repression, but not B7-H1 mRNA degradation^[33].

miRNAS AND LIVER DISEASES

miRNAs and viral hepatitis

Genes encoding miRNAs have also been found in viruses and viral miRNAs have a regulatory effect on their protein-coding genes^[34]. This regulatory effect may be beneficiary to the virus toward maintaining its replication, latency and evading the host immune

system. Wu and colleagues analyzed the miRNA-encoding potential of the hepatitis B virus (HBV). Using computational approaches, the authors found that HBV putatively encodes only one candidate pre-miRNA. One viral mRNA was found to be targeted by the viral miRNA when they searched the target from viral mRNAs. Thus, HBV has evolved to use viral miRNAs as a means to regulate its own gene expression^[35].

miRNAs from the host cells may play a role in building up direct or indirect effect in regulating viral genes^[34,36,37]. Hepatitis C virus (HCV) is an enveloped RNA virus of the *Flavivirus* family, which is capable of causing both acute and chronic hepatitis in humans by infecting liver cells. miRNA-122 has been reported to facilitate the replication of HCV, targeting the viral 5' non-coding region^[38]. Indeed, HCV RNA can replicate in Huh 7 cells, which express miR-122, but not in HepG2 cells, which do not express miR-122. Silencing of miR-122 in hepatocytes resulted in a marked loss of replicating RNAs from HCV^[38]. A putative miR-122 binding site in the 50-end of HCV genome was identified, suggesting a direct role of miR-122 in HCV replication^[38]. An indirect effect of miR-122 inhibition on HCV regulation *via* the up-regulation of the cytoprotective enzyme heme-oxygenase 1 (HO-1) and the converse down-regulation of HO-1 repressor Bach1 was also characterized^[21]. In addition, HCV replication is associated with an increase in expression of cholesterol biosynthesis genes that are regulated by miR-122^[39].

On the other hand, Pedersen *et al*^[40] demonstrated IFN-mediated modulation of the expression of numerous cellular miRNAs in the treatment of hepatocytes infected with HCV. Expression of a total of 30 cellular miRNAs in hepatocytes was influenced by IFN- α/β or IFN- γ . Specifically, eight of the miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448), having nearly perfect complementarity in their seed sequences with HCV RNA genomes, were up-regulated. Importantly, these miRNAs are capable of inhibiting HCV replication and infection. This has opened the door to our understanding of novel host-defense mechanisms that exist in mammalian cells as well as the antiviral mechanisms employed by interferon.

miRNAs and human hepatocellular cancer (HCC)

Several studies have shown that specific miRNAs are aberrantly expressed in malignant HCC cells or tissues compared to non-malignant hepatocytes or tissue^[41-47]. Selected miRNAs such as miR-21, miR-224, miR-34a, miR-221/222, miR-106a, and miR-203 are up-regulated in HCC compared to benign hepatocellular tumors such as adenomas or focal nodular hyperplasia. Certain miRNAs have been noted to be decreased in HCC compared to non-tumoral tissue, such as miR-122a, miR-422b, miR-145, and miR-199a. Murakami *et al*^[48] showed a correlation between miR-222, miR-106a, miR-92, miR-17-5p, miR-20, and miR-18 and the degree of differentiation suggesting an involvement of specific miRNAs in the progression of the disease. Interestingly, the altered expression of some miRNAs was associated

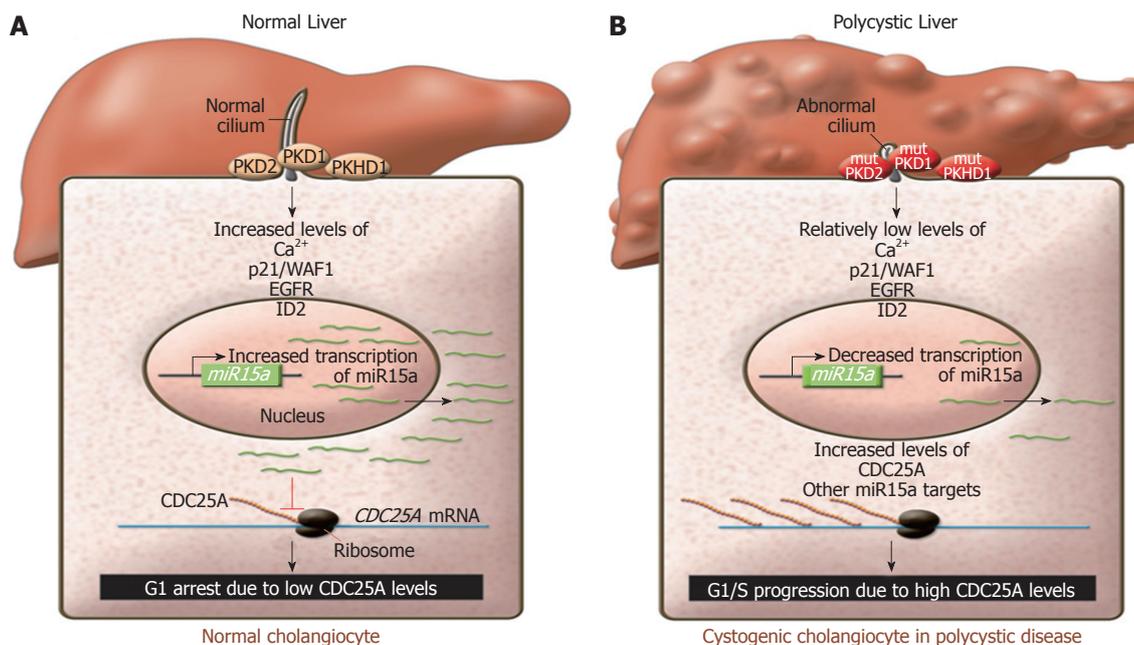


Figure 3 Model of miR15a function in hepatic cystogenesis. A: In normal cholangiocytes, ciliary signaling activates intracellular signaling pathways resulting in miR15a expression and CDC25A repression. Decreased levels of CDC25A then lead to G1 arrest, preventing cyst formation; B: In cholangiocytes of polycystic liver, miR15a level is reduced as a result of mutation of molecules important to the ciliary signaling. Reduction of miR15a results in elevated Cdc25A level, increased cell proliferation, and cyst growth. This figure is reprinted from Chu and Friedman^[49] with the permission of the authors and the publisher.

with distinctive risk factors, such as miR-96 with hepatitis B virus infection and miR-126* with alcohol use. Further investigations suggested the highly deregulated miR-223 and miR-222 could unequivocally distinguish HCC from adjacent non-tumoral liver, irrespective of viral association^[46]. In HCC patients with hepatitis C and liver cirrhosis, miR-122, miR-100, and miR-10a were overexpressed, whereas miR-198 and miR-145 were up to five-fold down-regulated in hepatic tumors compared to normal liver parenchyma^[47].

Alteration of expression profile in HCC for some miRNAs may be the consequence of malignant transformation. For other miRNAs, they may play a role in the transformation process. miRNA-122 was reported to be significantly and specifically down-regulated in HCC in humans as well as in rodents^[41,42]. Amongst the putative target genes of miR-122 that can be predicted using computational tools, at least three are of interest in tumorigenesis: the gene for N-Myc, which is frequently rearranged in woodchuck liver tumors by woodchuck hepatitis virus^[43], the gene referred to as “down-regulated in liver malignancy”^[44], and the gene for cyclin G1^[45]. In fact, miR-122 was shown to modulate cyclin G1 expression in HCC-derived cell lines, and an inverse correlation between miR-122a and cyclin G1 expression in primary liver carcinomas was further observed^[45]. These studies suggest an influence of the down-regulation of miR-122 and the converse expression of cyclin G1 in hepatocarcinogenesis^[21].

miRNAs and polycystic liver diseases

The polycystic liver and kidney diseases are a family of disorders with heterogeneous etiologies. Autosomal dominant polycystic kidney disease (ADPKD) is

associated with renal and liver cystogenesis that clinically manifests in adulthood, often leading to dialysis and renal transplantation. It is caused by mutations in either of two genes, *PKD1* and *PKD2*, which code for polycystin 1 and polycystin 2, respectively^[49]. Autosomal recessive polycystic kidney disease (ARPKD) can present in neonates with massive renal cysts, causing respiratory failure secondary to abdominal competition that subsequently leads to infant demise, although milder forms can present later in life. Proposed mechanisms of disease include ciliary dysfunction, excess cell proliferation, and altered cell-cell or cell-matrix interactions.

Lee and colleagues provide data to support a novel mechanism for cystogenesis involving miRNA. They demonstrate that levels of the miRNA miR15a are decreased in livers of patients with ARPKD and ADPKD, respectively, and congenital hepatic fibrosis as well as in the *PKC* rat model of ARPKD. This results in increased expression of the cell-cycle regulator Cdc25A, which is a direct target of miR15a, and increased cellular proliferation and cystogenesis *in vitro*. As a whole, the findings indicate that changes in miRNA expression contribute to the phenotypic changes found in cystic liver disease (Figure 3)^[49,50].

miRNAs FOR THE DIAGNOSIS OF LIVER DISEASES

miRNAs could be of diagnostic significance for many liver diseases but current literature has been focused on tumors in the liver. Hepatocellular tumors comprise diverse benign and malignant neoplasms. Although the phenotypes can be broadly distinguished histologically or immunologically, these tumors can vary widely

in their clinical behavior and prognosis. The use of miRNA-based classifications that correlate with etiology, pathogenetic changes, or malignant tendency will enhance molecular diagnosis and enable further definition of these phenotypes. In turn, this may yield clinically useful predictive markers of tumor behavior, as well as identify individual genetic and molecular contributors to tumorigenesis^[51,52]. Thus, miRNA profiling studies could be used for defining clinical phenotypes, as well as providing potentially useful molecular diagnostic markers.

Impairments in miRNA functioning seen in cancerogenesis can be used for determination of miRNA expression for diagnostics of tumor origin. Each type of cancer is characterized by a certain profile of miRNA expression^[52]. For example, the miRNA expression profiles in malignant hepatocytes differ from those of malignant cholangiocytes. Cluster analysis of miRNA expression profiles in tumors accurately determines not only type of the tissue (e.g. epithelium or hemopoietic system), but also discriminates tumors within the same type of tissue; this may reflect mechanism of transformation^[53]. Obviously, evaluation of miRNA profiles can be used for prognosis of the development of tumors^[54-56]. Such an approach for tumor diagnostics is very promising. However, it is not widely employed yet due to inadequately developed technology, lack of standards, requirements of very high purity of RNA samples, and not always reproducible results.

miRNAS AS THERAPEUTIC TARGETS FOR LIVER DISEASES

Development of miRNA/RNAi-based therapeutics requires several critical experimental steps, which include: (1) miRNA profiling of disease versus healthy tissue; (2) functional analysis of dysregulated miRNAs; and (3) *in vivo* studies with the use of different RNAi-based therapeutic methods to dysregulate miRNAs^[57]. The success of such strategies for gene therapy will provide clinicians with a larger repertoire that includes miRNA-therapeutic agents. For example, chemically engineered oligonucleotides, termed 'antagomirs', have recently been developed and proven to be efficient and specific silencers of endogenous miRNAs in mice^[29]. The silencing effect was considerably sustained over time probably because of a long half-life of endogenous miRNAs^[12]. In addition, induction of stable loss-of-function phenotypes for specific miRNAs by lentiviral-mediated antagomir expression has recently been described^[58].

Over the past several years, strategies based on targeting HBV, and to a lesser extent HCV, by both synthetic and expressed activators of the RNAi pathway have proved efficient to inhibit viral replication both *in vitro* and *in vivo*^[59,60]. The recent study by Pedersen *et al*^[40] provided great insights into validating sequence-predicted targets of cellular miRNAs within the HCV genome. miRNA-122 antagomir can down-regulate expression of several adult-liver genes^[29], providing

the potential to generate a new attractive expandable cell source for hepatocyte transplantation that would feature stem/progenitor cell phenotype. In addition, the effect of miR-122 antagomir in high-fat fed mice may be of therapeutic potential to reduce hepatic steatosis^[29].

The important breakthrough in the field of hepatocarcinogenesis came from the accurate correlation of alterations in miRNAs with tumor proliferation and differentiation. So far, there have been very limited insights into this characterization. Recent studies by Meng *et al*^[22,61] suggest a role for miRNAs in the influence of interleukin-6 in malignant cholangiocytes. MicroRNA-141, which showed strong overexpression in malignant cholangiocytes, was specifically localized in 12p, a region of known chromosomal aberration in biliary tract cancers^[42]. Inhibition of miR-21 sensitized the response of cholangiocarcinoma cell lines to chemotherapy^[42]. This observation gives rise to significant hope that miR-21 could serve as a biomarker for drug response in cholangiocarcinoma.

CONCLUSION

Study of miRNAs flourished during the decade after their discovery. It is now clear that miRNAs can potentially regulate every aspect of cellular activity, from differentiation and proliferation to apoptosis, and also modulate a large range of physiological processes from developmental timing to organogenesis^[1-6]. miRNAs also modulate a diverse spectrum of liver functions with developmental, (patho) physiological, and clinical implications. In the near future, the distinctive signature patterns of miRNA expression associated with liver cancer should allow classification of different stages in tumor progression^[21]. Further, creating artificial miRNAs with salutary effects by promoting the expression of beneficial gene products (e.g. tumor-suppressor proteins) or targeting viral genomes (e.g. molecules designed to specifically target HCV-genome sequences) may become part of our patient management and complement chemotherapy and antiviral treatments. Overall, unraveling the regulatory circuits of miRNAs in the liver is a great challenge, but may provide attractive targets for mechanism-based treatment of liver diseases.

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Chronic pancreatitis: Maldigestion, intestinal ecology and intestinal inflammation

Raffaele Pezzilli

Raffaele Pezzilli, Pancreas Unit, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 940138 Bologna, Italy

Author contribution: Pezzilli R wrote this paper.

Correspondence to: Raffaele Pezzilli, MD, Pancreas Unit, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 940138 Bologna, Italy. raffaele.pezzilli@aosp.bo.it

Telephone: +39-51-6364148 Fax: +39-51-6364148

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Abstract

Exocrine pancreatic insufficiency caused by chronic pancreatitis results from various factors which regulate digestion and absorption of nutrients. Pancreatic function has been extensively studied over the last 40 years, even if some aspects of secretion and gastrointestinal adaptation are not completely understood. The main clinical manifestations of exocrine pancreatic insufficiency are fat malabsorption, known as steatorrhea, which consists of fecal excretion of more than 6 g of fat per day, weight loss, abdominal discomfort and abdominal swelling sensation. Fat malabsorption also results in a deficit of fat-soluble vitamins (A, D, E and K) with consequent clinical manifestations. The relationships between pancreatic maldigestion, intestinal ecology and intestinal inflammation have not received particular attention, even if in clinical practice these mechanisms may be responsible for the low efficacy of pancreatic extracts in abolishing steatorrhea in some patients. The best treatments for pancreatic maldigestion should be re-evaluated, taking into account not only the correction of pancreatic insufficiency using pancreatic extracts and the best duodenal pH to permit optimal efficacy of these extracts, but we also need to consider other therapeutic approaches including the decontamination of intestinal lumen, supplementation of bile acids and, probably, the use of probiotics which may attenuate intestinal inflammation in chronic pancreatitis patients.

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Key words: Chronic pancreatitis; Exocrine pancreatic insufficiency; Leukocyte L1 antigen complex; Pancreatic elastase; Pancreatic extracts

INTRODUCTION

Exocrine pancreatic insufficiency caused by chronic pancreatitis results from various factors which regulate digestion and absorption of nutrients. Pancreatic function has been extensively studied over the last 40 years, even if some aspects of secretion and gastrointestinal adaptation are not completely understood. The pancreatic gland normally secretes more than 2 L of juice per day which is composed of water, bicarbonates and enzymes^[1]; protein secretion per gram of pancreatic tissue is elevated more than that of any other organ^[2], and more than 85% of the protein content is composed of enzymes which are able to digest lipids, proteins and carbohydrates^[3]. The pancreas normally produces more enzymes than are necessary for food digestion^[1], and normal digestion is guaranteed up to a loss of 95% of pancreatic secretory capacity^[4]. Recently, it has been demonstrated that gastric lipase can compensate pancreatic lipase even if it is not capable of complete lipolytic activity^[5]. Enzyme degradation in the intestinal lumen is the main factor controlling nutrient absorption. The activity of pancreatic enzymes progressively decreases during their progression in the intestinal lumen: 60% of active trypsin and chymotrypsin are present in the jejunum, whereas only 20% of these enzymes are present in the ileum; on the other hand, amylases and lipases are more stable^[6-8]. There are various explanations for the loss of enzymatic activity during progression in the intestinal lumen, including proteolytic degradation (chymotrypsin is the main lipase degradation factor)^[9], lipase acid inactivation (lipase is particularly sensitive to acid inactivation)^[10], and the brief half-life of some

enzymes, particularly lipase^[11]. This is the reason why, in patients with exocrine pancreatic insufficiency, fat maldigestion is more severe than that of carbohydrates and proteins. In addition to an optimal concentration of biliary acids and colipases in the intestinal lumen, good fat digestion requires an adequate blending of nutrients with the pancreatic juice and optimal intestinal motility. In pathological conditions, such as chronic pancreatitis, there is a deficit in bicarbonate production; a low duodenal pH determines biliary acid precipitation and the remaining lipase activity worsens. Finally, other causes of malabsorption may be an accelerated gastric emptying and a lower intestinal transit time^[12,13].

CLINICAL MANIFESTATIONS AND DIAGNOSIS OF EXOCRINE PANCREATIC INSUFFICIENCY

The main clinical manifestations of exocrine pancreatic insufficiency are fat malabsorption, known as steatorrhea, which consists of fecal excretion of more than 6 g of fat per day, weight loss, abdominal discomfort and abdominal swelling sensation. Fat malabsorption also results in a deficit of fat-soluble vitamins (A, D, E and K) with consequent clinical manifestations. The diagnosis of exocrine pancreatic insufficiency is based on these clinical symptoms and signs observed with direct and indirect tests. Some of these tests can be used to determine the degree of insufficiency which is usually classified as mild, moderate or severe. The most sensitive test is the secretin-cholecystokinin (CCK) or secretin-cerulein test; this test has a double-lumen tube capable of separately draining the gastric juice and the pancreatic juice. The test starts with pancreatic stimulation by secretin which produces the hydro-electrolyte pancreatic secretion and CCK or cerulein, which can stimulate enzymatic secretion. This test is highly sensitive and specific^[14] but is invasive, lengthy and expensive; moreover, it is only possible in patients with a normal gastrointestinal tract, and it is not useful in patients with an altered digestive anatomy. At present, fecal chymotrypsin and elastase 1 are more frequently used to diagnose exocrine pancreatic insufficiency^[15]. In particular, the determination of elastase 1 is more sensitive and specific than chymotrypsin determination. The advantage of these tests is that they can be used in patients who have undergone surgery involving the gastrointestinal tract, but they can not reveal a mild degree of exocrine pancreatic insufficiency^[15,16]. A cholesteryl-octanoate breath test is rarely used because of its high cost and possible interference with metabolic and pulmonary diseases^[17]. Fecal fat determination is useful in monitoring lipid malabsorption therapy. Pancreatic exocrine evaluation during magnetic resonance cholangiopancreatography with secretin administration is still under study and the results of the published studies seem to be promising^[18-20].

MALDIGESTION, INTESTINAL ECOLOGY AND INTESTINAL INFLAMMATION

The relationships between pancreatic maldigestion, intestinal ecology and intestinal inflammation have not received particular attention, even if in clinical practice these mechanisms may be responsible for the low efficacy of pancreatic extracts to abolish steatorrhea in some patients.

One mechanism which has been hypothesized between maldigestion and intestinal alterations relates to bacterial overgrowth in the small intestine; bacterial overgrowth is often seen in experimental models of exocrine pancreatic insufficiency^[21]. Furthermore, bacterial overgrowth has been observed in dogs with naturally occurring exocrine pancreatic insufficiency^[22]. The presence of bacterial overgrowth in human exocrine pancreatic insufficiency has been studied using non-invasive breath tests based on ¹⁴C-cholyglycine^[23], ¹⁴C-xylose^[24], glucose^[25,26] or by intubation followed by culture of intestinal aspirates^[27]. These studies indicated that bacterial overgrowth complicates 25%-50% of patients with exocrine pancreatic insufficiency, and it was suggested that bacterial overgrowth might either contribute to diarrhea or account for the persistence of diarrhea in patients with exocrine pancreatic insufficiency who receive adequate pancreatic enzyme supplementation. Furthermore, bacterial overgrowth might give rise to bile acid malabsorption and changes in intestinal permeability^[28].

However, Madsen *et al*^[29] found no bacterial overgrowth in any of their patients with exocrine pancreatic insufficiency, and these findings seem to conflict with previous observations seen in both humans and animals. In fact, in the study based on intestinal culture^[27], it was observed that bacterial overgrowth occurred in 50% of patients with severe pancreatic insufficiency who were not receiving enzyme replacement therapy, and studies in dogs indicated that bacterial overgrowth from ligation of the pancreatic duct can be reversed by bovine pancreatic extract replacement therapy; thus these results indicate that pancreatic enzymes might have an important influence on small-intestinal bacterial flora^[21]. Since all the patients studied by Madsen had oral enzyme supplementation, it is possible that enzyme substitution treatment normalized the luminal conditions of the small intestine, which otherwise would have facilitated bacterial overgrowth.

In a previous study, a wide range of bile salt malabsorption was observed in patients with exocrine pancreatic insufficiency secondary to alcoholic pancreatitis^[30]. Moreover, these data suggested that intraluminal factors, rather than a primary defect in the ileal mucosa, were responsible for bile salt malabsorption. The fecal loss of bile salts in their patients was markedly reduced by oral administration of pancreatic enzymes, indicating an important role for pancreatic enzymes in bile acid absorption. It has been postulated from studies performed *in vitro* that a lack

of pancreatic enzymes causes generalized maldigestion, which in turn may be responsible for persistent bile salt binding to maldigested protein, carbohydrate, or fiber in these patients^[31]. It is conceivable, therefore, that in patients with untreated pancreatic insufficiency, an exceedingly low concentration of intraluminal pancreatic enzymes during the postprandial period gives rise to persistent binding of bile acids to undigested dietary components, resulting in bile acid malabsorption.

Low intraluminal pH in the upper small intestine might be another important factor in the pathogenesis of fecal loss of bile acids in pancreatic insufficiency. Thus, bile acid malabsorption in patients with chronic pancreatitis and exocrine dysfunction does not occur until bicarbonate output is below a certain level^[32], and cimetidine has been shown to reduce pH-induced precipitation of bile acids, thereby improving the micelle concentration of bile salts in the duodenum^[33]. Moreover, food residues seem to absorb more bile salts at pH values less than 6.0^[34].

The study by Madsen^[29] also evaluated intestinal permeability in exocrine pancreatic insufficiency due to chronic pancreatitis in adult patients; these authors found that patients receiving enzyme replacement therapy had reduced urinary excretion of mannitol. The exact character of the underlying epithelial impairment was at that time not obvious, as the pathways for permeation of both smaller molecules such as mannitol and larger molecules are not yet known. It is possible that larger pores located in the crypts of intestinal epithelium permit absorption of larger molecules, while smaller molecules pass through both these pores and smaller pores located in the villi^[35,36]. According to this hypothesis, it is suggested that a defect localized in the villi of the intestinal epithelium may exist. It is possible, however, that more pronounced disturbances in intestinal permeability would have been revealed, if urine excretion of the test substances had been properly corrected for variations in the small-intestinal transit rate^[37]. Since bacterial overgrowth which often gives rise to defects in intestinal permeability was not found in any of our patients, it is suggested that pancreatic disease *per se* and not enzyme supplementation therapy caused the permeability defect. In a recent study, we evaluated fecal calprotectin in a total of 90 subjects; 22.2% with chronic pancreatitis, 16.7% with pancreatic cancer, 6.7% with chronic non-pathological pancreatic hyperenzymemia, 17.8% with non-pancreatic diseases and 25.6% with no detectable diseases^[38]. Calprotectin is a cytoplasmic antimicrobial component prominent in granulocytes, monocytes, and macrophages. It accounts for approximately 60% of the total protein in the cytosol. The release of calprotectin is most likely a consequence of cell disruption and death^[39] and is stable in stools for more than 7 d at varying temperatures, as well as being resistant to proteolysis even after transportation and storage^[40]. Calprotectin can inhibit bacterial proliferation both as a component of the innate immune response and through its iron-binding capacity^[41]. Fecal calprotectin determination has been demonstrated to

be useful in diagnosing various inflammatory diseases of the gastrointestinal tract^[42-46]. We found that patients with chronic pancreatitis had abnormally high fecal calprotectin concentrations in 55% of cases and most of these patients (40%) had pancreatic insufficiency^[38]. It is possible that, in these patients, pancreatic insufficiency may determine an alteration in intestinal ecology, and in intestinal inflammation. In the population studied, multivariate analysis showed that patients with abnormally low fecal elastase had more than a five-fold risk of increased fecal calprotectin suggesting that, in patients with pancreatic disease, the determination of fecal calprotectin may be useful in evaluating the possible presence of intestinal inflammation which may worsen the intestinal absorption of nutrients. Thus, our data further support the hypothesis that pancreatic insufficiency may cause intestinal inflammation probably due to a modification in intestinal ecology.

CONCLUSION

Pancreatic extracts are the basic treatment for pancreatic insufficiency. However, we need to explore the possibility that other drugs used to treat pancreatic insufficiency such as proton pump inhibitors or H2-blockers should be administered to our patients in order to modify the duodenal pH and to permit optimal efficacy of pancreatic extracts. Furthermore, we need to explore the possibility that other therapeutic approaches including the decontamination of intestinal lumen, supplementation of bile acids and the use of probiotics may attenuate intestinal inflammation permitting optimal efficacy of pancreatic extracts as well as the control of clinical signs and symptoms of pancreatic insufficiency.

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Jose JG Marin, Professor, Series Editor

Bile-acid-induced cell injury and protection

Maria J Perez, Oscar Briz

Maria J Perez, Oscar Briz, Research Unit, University Hospital of Salamanca; Laboratory of Experimental Hepatology and Drug Targeting, CIBERehd, University of Salamanca, E.D. 129 37007 Salamanca, Spain

Author contributions: Perez MJ and Briz O contributed equally to this work.

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Correspondence to: Maria J Perez, Department of Biochemistry and Molecular Biology Campus Miguel de Unamuno E.D. 129, 37007 Salamanca, Spain. mjperez@usal.es
Telephone: +34-923-294781 Fax: +34-923-294579

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Abstract

Several studies have characterized the cellular and molecular mechanisms of hepatocyte injury caused by the retention of hydrophobic bile acids (BAs) in cholestatic diseases. BAs may disrupt cell membranes through their detergent action on lipid components and can promote the generation of reactive oxygen species that, in turn, oxidatively modify lipids, proteins, and nucleic acids, and eventually cause hepatocyte necrosis and apoptosis. Several pathways are involved in triggering hepatocyte apoptosis. Toxic BAs can activate hepatocyte death receptors directly and induce oxidative damage, thereby causing mitochondrial dysfunction, and induce endoplasmic reticulum stress. When these compounds are taken up and accumulate inside biliary cells, they can also cause apoptosis. Regarding extrahepatic tissues, the accumulation of BAs in the systemic circulation may contribute to endothelial injury in the kidney and lungs. In gastrointestinal cells, BAs may behave as cancer promoters through an indirect mechanism involving

oxidative stress and DNA damage, as well as acting as selection agents for apoptosis-resistant cells. The accumulation of BAs may have also deleterious effects on placental and fetal cells. However, other BAs, such as ursodeoxycholic acid, have been shown to modulate BA-induced injury in hepatocytes. The major beneficial effects of treatment with ursodeoxycholic acid are protection against cytotoxicity due to more toxic BAs; the stimulation of hepatobiliary secretion; antioxidant activity, due in part to an enhancement in glutathione levels; and the inhibition of liver cell apoptosis. Other natural BAs or their derivatives, such as cholyl-N-methylglycine or cholylsarcosine, have also aroused pharmacological interest owing to their protective properties.

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Key words: Apoptosis; Cholestasis; Liver; Necrosis; Oxidative stress; Ursodeoxycholic acid

Peer reviewer: Yasushi Matsuzaki, Associated Professor, Division of Gastroenterology and Hepatology, Graduate School of Comprehensive Human Sciences and University Hospital, 1-1-1, Tennodai, Tsukuba 305-8575, Japan

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INTRODUCTION

Bile acids (BAs) are the major organic solutes in bile, and are involved in several important functions in the liver and the intestine. However, the retention of hydrophobic BAs in pathophysiological conditions, such as cholestatic diseases, is believed to play an important role in liver injury by inducing apoptosis or necrosis of hepatocytes^[1]. Primary BAs are directly synthesized from cholesterol by hepatocytes, by the addition of hydroxyl groups and the oxidation of its side chain to form a more water soluble end product. The hydroxylation is always on one side of the molecule resulting in an amphipathic molecule. In humans, the most abundant BA species are the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) (Figure 1), and the secondary BAs deoxycholic acid (DCA) and

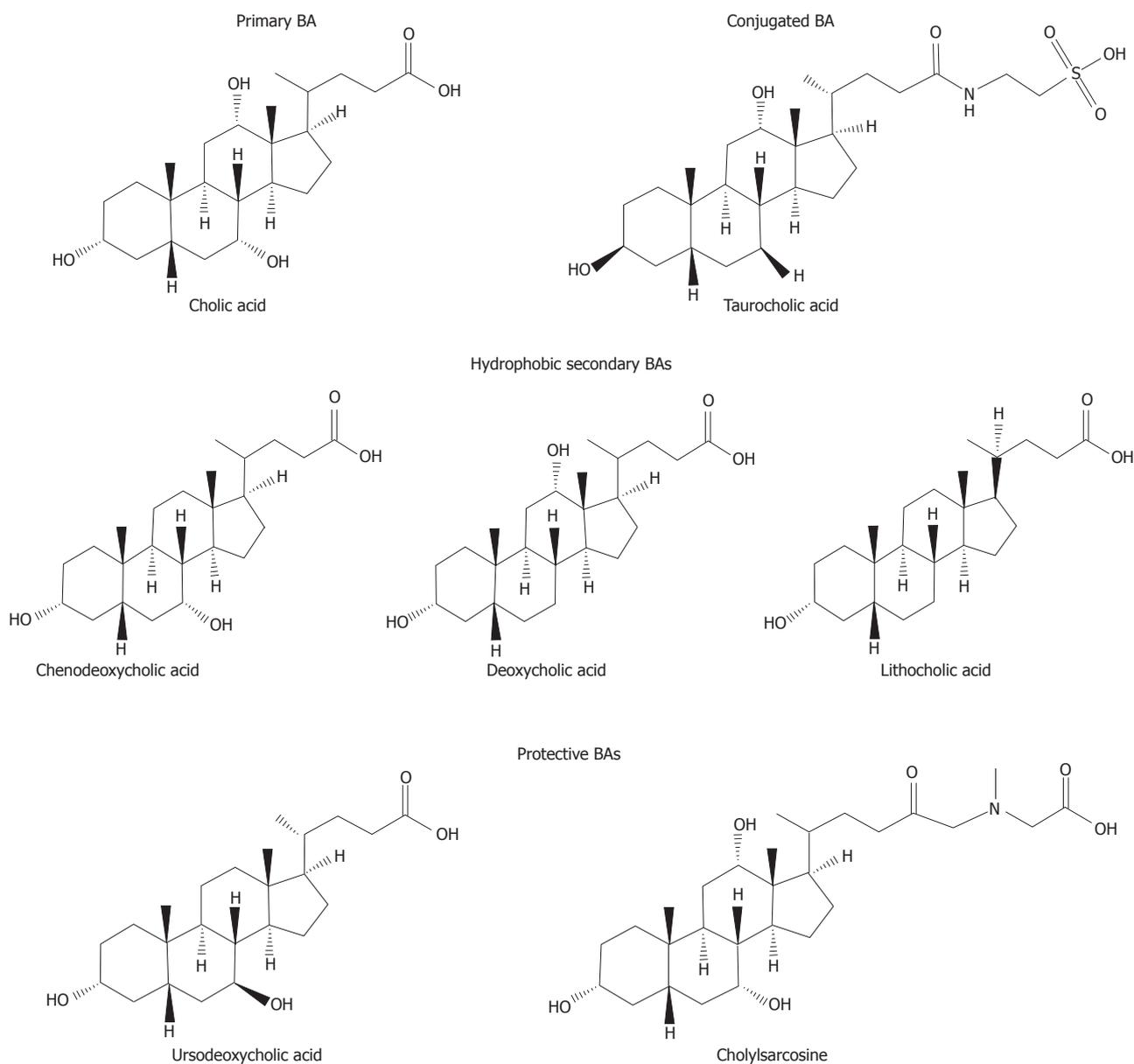


Figure 1 Molecular structures of different potentially toxic or protective natural bile acids and synthetic bile acid analogues.

lithocholic acid (LCA) (Figure 1), generated in the intestine by bacterial biotransformation of CA and CDCA, respectively. Small amounts of other secondary BAs such as ursodeoxycholic acid (UDCA; Figure 1), currently used in the treatment of cholestatic liver diseases, are also present in the human BA pool^[2,3].

BA hydrophobicity is an important determinant of the toxicity and protection of BAs, two biological properties of these compounds which will be reviewed in this manuscript. BA hydrophobicity depends on the number, position and orientation of the hydroxyl groups, as well as amidation at the C-24 position. Regarding the magnitude of hydrophobicity of BAs the order would be UDCA < CA < CDCA < DCA < LCA^[4]. Before being secreted into bile, most BA molecules are conjugated with glycine or taurine (Figure 1) within hepatocytes to more hydrophilic amidated forms. Under normal conditions, the amount of BAs undergoing further biotransformations to dianionic

glucuronidated or sulfated derivatives is negligible, although these may become important in cholestasis^[5].

BILE-ACID-INDUCED HEPATOCYTE INJURY

The retention and accumulation of hydrophobic BAs, such as CDCA and DCA, inside hepatocytes during cholestasis have long been implicated as a major cause of liver damage in this disease^[1]. Experimentally, hydrophobic BAs are known to induce injury to isolated hepatocytes^[6], cultured hepatocytes^[7], and whole liver^[8], but the mechanisms involved in this toxicity are not fully understood. In animal models^[9] and human cholestatic disorders^[10], hepatocyte swelling and the intracellular accumulation of bile pigments have been reported to occur. Moreover, swelling, pleomorphism and abnormal cristae have also been reported in

mitochondria from cholestatic hepatocytes^[11]. This injury to parenchymal cells, which occurs early on in the course of cholestasis, can be responsible for many of the subsequent inflammatory and fibrinogenic responses of non-parenchymal cells. Thus, altered hepatocytes may release molecules, including growth factors, cytokines, chemokines and lipid peroxide products, able to amplify the inflammatory response, stimulate fibrogenesis by hepatic stellate cells, or directly injure other nearby cells^[12].

Several mechanisms may account for the cytotoxicity associated with the most hydrophobic BAs in cholestatic liver diseases^[1]. BAs could disrupt cell membranes through their detergent action on lipid components^[13] and promote the generation of reactive oxygen species (ROS) that, in turn, oxidatively modify lipids, proteins, and nucleic acids, and eventually cause hepatocyte apoptosis^[14]. Additionally, they can activate Kupffer cells to generate ROS, which may further contribute to the liver cell insult^[15].

BILE-ACID-INDUCED OXIDATIVE STRESS

Several studies have suggested that oxidative stress may play an important role in the pathogenesis of hepatic injury during cholestasis in rats^[16] and humans^[17]. Thus, the antioxidant α -tocopherol is able to reduce both hydrophobic BA-induced ROS generation and injury to hepatocytes *in vitro*^[18] and *in vivo*^[8]. Hepatic mitochondria have been proposed as a major source of the oxidative stress induced by these BAs. In this respect, it has been demonstrated that hepatic mitochondria undergo lipid peroxidation during experimental cholestasis and BA toxicity in rats^[8,16]. Hydrophobic BAs impair respiration and electron transport in hepatic mitochondria. These compounds decrease the activities of several enzyme complexes involved in the electron transport chain, such as complexes I, III and IV, whereas complex II is not affected in isolated rat liver mitochondria^[19]. Moreover, toxic BAs decrease the mitochondrial membrane potential developed upon succinate energization^[20]. These compounds decrease state three and enhance state four respiration in mitochondria^[20]. The decrease in state three respiration is probably related to an inhibitory action of these compounds on the phosphorylation system, although by means of a mechanism that has yet to be defined. The reported stimulation of state four by hydrophobic BAs is associated with an enhanced permeability of mitochondria to protons^[20]. The observed uncoupling may be the consequence of several interactions of these compounds with the structure of mitochondria. These BAs induced membrane permeability to protons, either by acting as protonophores or by disruption of the structural organization of membrane components. Hydrophobic BAs can stimulate the generation of ROS in rat hepatic mitochondria and hepatocytes^[18,21] as well as in human hepatic mitochondria^[22]. Intracellular ROS generation by mitochondria appears to be an early event in hydrophobic BA-induced hepatocyte

toxicity^[21]. As occurs in cholestatic rats, this can lead to a depletion of antioxidant defences, including total hepatic and mitochondrial glutathione contents, and the concentrations of substances involved in electron transport, such as ubiquinone-9 and ubiquinone-10^[23].

Hydrophobic BAs can induce the mitochondrial permeability transition (MPT)^[22,24,25], a critical intracellular event that triggers both the apoptotic and necrotic forms of cell death in hepatocytes^[26]. MPT involves a rapid increase in the permeability of the mitochondrial membrane to low-molecular-weight solutes, caused by the opening of a channel composed of the adenine nucleotide translocator of the inner membrane, the voltage-dependent anion channel of the outer membrane, and mitochondrial cyclophilin D^[26]. Induction of MPT is associated with mitochondrial swelling, collapse of the mitochondrial membrane potential, reduced oxidative phosphorylation, rupture of the outer mitochondrial membrane, cytochrome c release, and generation of ROS^[26]. Hydrophobic BA-induced MPT has been associated with mitochondrial ROS generation in rat hepatocytes^[27] and in isolated mitochondria^[14]. An elevation of the cytosolic free calcium concentration induced by hydrophobic BAs^[28] may also be an important permissive factor that allows oxidant stress to open the permeability pore.

BILE-ACID-INDUCED CELL DEATH PATHWAYS

BA accumulation within the hepatocyte can result in cell injury and death through two mechanisms; lower BA concentrations induce hepatocellular apoptosis^[25,29-31], whereas higher concentrations induce necrosis^[7,21]. Both types of cell death seem to play a role in cholestatic liver injury, although the contribution of each is controversial. The role of apoptosis in cholestatic injury has been demonstrated^[32]. However, in certain models of BA toxicity in the mouse, it has been reported that hepatocyte necrosis is the predominant form of cell death^[33].

Interestingly, at the level of apoptosis induction, cytotoxicity of BAs does not always correlate with their hydrophobicity, which is not the case in necrosis. Apoptosis induction is indeed dependent on the BA, its concentration, or its conjugation state^[29].

Necrosis

In rat hepatocytes, cellular necrosis is characterized by cell swelling and disruption of the intracellular and plasma membranes. BA-induced lethal hepatocellular injury has been attributed to direct membrane damage due to the detergent-like properties of hydrophobic BAs^[13], as well as to depletion of ATP, ion dysregulation, mitochondrial and cellular swelling, plasma membrane failure, and cell lysis, releasing intracellular contents^[6]. In the liver of humans with cholestatic disorders, the histological features of hepatocyte necrosis, such as massive swelling of hepatocytes containing accumulated bile and elevated serum hepatocellular aminotransferase

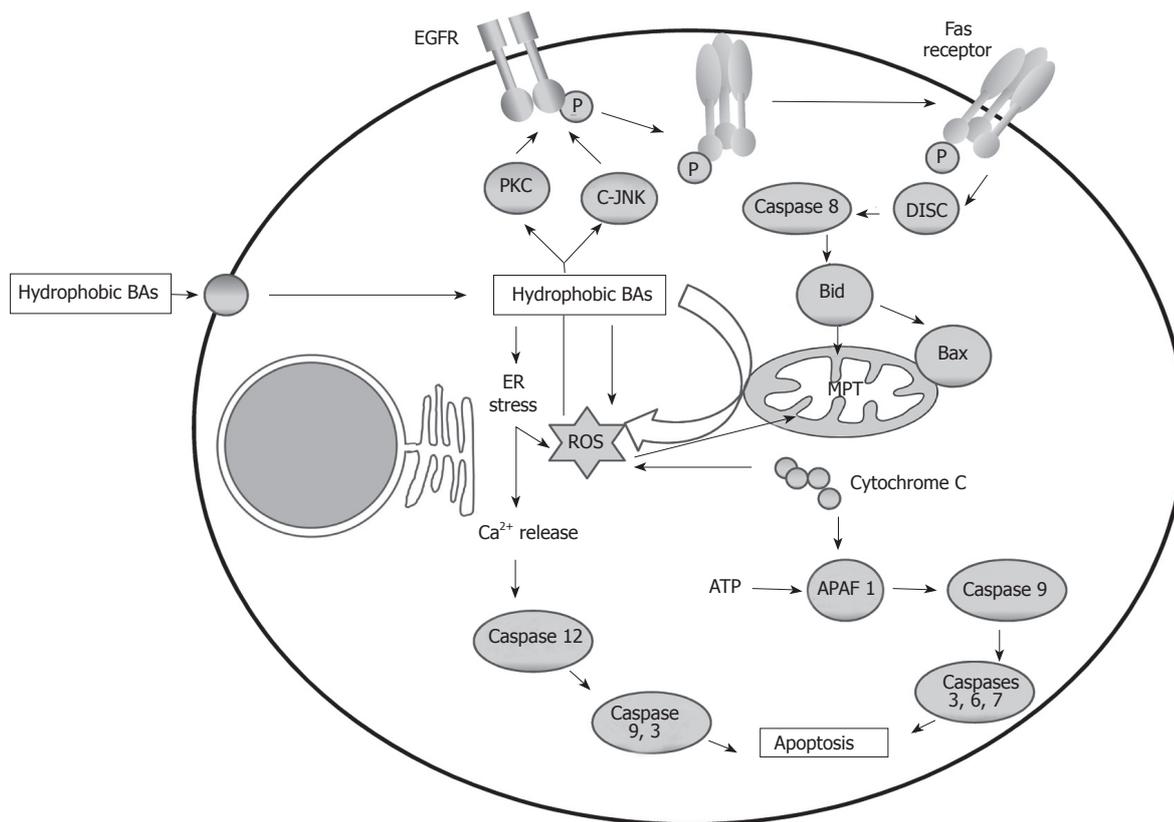


Figure 2 Intracellular mechanisms of bile acid-induced hepatocyte apoptosis. In this schema, further transduction after activation of death receptors and formation of the DISC, direct mitochondrial toxicity and ER stress are implicated.

enzymes, have been observed^[10]. Antioxidants have been shown to prevent hepatocellular necrosis and reduce oxidant stress in isolated hepatocytes exposed to hydrophobic BAs^[18,21] and to inhibit the dissipation of mitochondrial membrane potential^[14]. Thus, it has been proposed that MPT induction by ROS generated in hepatocyte mitochondria^[27] is a critical event promoting BA-induced hepatocyte necrosis. In contrast to BA-induced apoptosis, only high concentrations of BAs are able to induce hepatocyte necrosis^[7,21]. The generation of ROS can result from a direct detergent effect of BAs on membrane enzymes, such as phospholipase A₂, which upon activation release arachidonic acid. Mitochondrial damage due to BAs can arise from several causes, including the endogenous generation of arachidonic acid^[34].

Apoptosis

The impairment of apoptosis in hepatocytes and bile duct epithelial cells has been implicated in the pathogenesis of many liver diseases^[32]. It is well known that hydrophobic BAs can induce apoptosis by activating the death receptor or extrinsic pathway^[35,36], and through the mitochondrial or intrinsic pathway^[25,27,30] (Figure 2). More recently, it has been demonstrated that hydrophobic BAs can also induce apoptosis in hepatocytes by causing endoplasmic reticulum (ER) stress (Figure 2). In contrast to BA-induced cell necrosis, apoptosis is characterized by the maintenance of cellular ATP content^[29].

Extrinsic pathway of apoptosis: BA-associated hepatocyte apoptosis has been shown to occur through the death receptors Fas^[35] and TRAIL-R2. In contrast, BAs do not appear to enhance tumor necrosis factor (TNF)- α /TNF-R1 cytotoxicity^[36]. Toxic BAs cause cell death, partly due to the activation of a protease cascade. The proximal signaling protease caspase 8 appears to be activated by toxic BAs in a Fas-receptor-dependent manner. After caspase 8 activation, cathepsin B activity also increases. Inhibition of either protease attenuates apoptosis *in vitro*, suggesting that they both play a critical role in BA-induced apoptosis^[35]. Moreover, toxic BAs can induce Fas aggregation on the plasma membrane *via* a Fas-ligand-independent mechanism^[37]. These compounds appear to promote Fas activation by altering the cellular trafficking of this death receptor. The shuttling of Fas and the resulting apoptosis can be inhibited by Golgi-disrupting agents and microtubule poisons. By inference, a Golgi-associated and microtubule-dependent pathway appears to be involved in the trafficking of Fas to the cell surface during BA cytotoxicity^[38].

Oxidative stress has been implicated in the stimulation of Fas translocation induced by BAs. It has been demonstrated that BA-induced oxidative stress may trigger the activation of c-Jun-N-terminal kinases (JNKs) and protein kinase C (PKC). These are responsible for activating the epidermal growth factor receptor (EGFR), which associates with Fas in a JNK-dependent manner. The resulting phosphorylation of Fas induces its mobilization to the plasma membrane^[39].

The increased density of Fas on the cell surface also sensitizes hepatocytes to cell death induced by Fas agonists^[38]. Thus, toxic BAs also promote Fas-ligand-dependent hepatocyte apoptosis.

After death receptor activation and death-inducing signaling complex (DISC) formation, caspase 8 is activated and the pro-apoptotic protein Bid is cleaved and translocated to the mitochondria, which results in opening of the MPT pore, and the release of cytochrome c and other proapoptotic intermembrane space small molecules. Cytosolic cytochrome c leads to the binding of apoptosis activating factor-1 (APAF 1) with procaspase 9, resulting in the activation of caspase 9. A caspase cascade is then initiated and, finally, activation of the effector caspases, which leads to irreversible hepatocyte death^[40].

The death receptor TRAIL-R2 has been also suggested to be involved in the apoptosis induced by BA. Enhanced expression and oligomerization of this death receptor has been described in glycochenodeoxycholic acid (GCDCA)-induced apoptosis in Fas-deficient cell lines^[36].

Intrinsic pathway of apoptosis: BAs are able also to induce apoptosis through the mitochondrial or intrinsic pathway, in which intracellular stress causes mitochondrial dysfunction and the subsequent release of proapoptotic factors^[25,27,30]. As mentioned above, BAs can directly induce the generation of ROS by mitochondria^[18,21] and mitochondrial membrane potential depolarization^[27,30,31] in rat hepatocytes. MPT induction in apoptosis induced by several BAs has also been demonstrated^[24,25]. Antioxidants prevented GCDCA-induced apoptosis in rat hepatocytes through a mechanism involving the MPT^[25]. It has also been demonstrated that DCA-triggered apoptosis, involved in decreased mitochondrial membrane potential and alterations in Bax subcellular distribution, exhibits increased mitochondrial-associated Bax protein levels^[31]. These results suggest that BA-induced MPT is the initial event, and the initial cytochrome c release stimulates Bax translocation to the mitochondria, accomplishing further cytochrome c release^[31]. *In vivo* studies show that 3 d after bile-duct-ligation, Bax expression is increased but then decreases over time. Bax translocation to the mitochondria and cytochrome c release are also found in these conditions^[41].

Mitochondrial dysfunction can also occur in death-receptor-mediated apoptosis, especially the so called “type II cells”, such as hepatocytes. Mitochondrial cytochrome c release is (Fas-associated death domain) FADD/caspase 8-dependent during the death receptor-mediated apoptosis of type II cells^[42].

ER stress-induced apoptosis: More recently, it has been demonstrated that hydrophobic BAs can also induce apoptosis in hepatocytes through another intracellular pathway of cell death involving ER stress. Thus, GCDCA induces ER stress which, in turn, induces apoptotic signalling in a time-dependent manner in isolated rat hepatocytes. This BA caused Ca²⁺ release

from ER, which in turn induced extracellular Ca²⁺ influx followed by the activation of calpain and caspase-12. In this study it is suggested that ER stress induced by GCDCA may trigger the activation of both ER mediated apoptosis and mitochondria-mediated apoptosis in isolated rat hepatocytes by cross-talk between ER and mitochondria using calcium ions as signal substances^[43,44]. Recent studies have suggested that ER stress might be involved in hepatocyte cell death caused by cholestasis or BAs^[45,46]. It has been demonstrated that cholestasis induces ER stress mediated by CHOP, a key component in ER stress mediated apoptosis, and triggers hepatocyte cell death. Moreover, CHOP deficiency attenuates this cell death and subsequent liver fibrosis. The results demonstrate an essential role of CHOP in development of liver fibrosis due to cholestatic liver damage^[46].

BILE-ACID-INDUCED DAMAGE IN THE PLASMA MEMBRANE

In hepatocytes, high concentrations of BAs can cause damage to the basolateral membrane, cell organelle membranes, and because they are more concentrated in bile, these compounds might be particularly harmful to the outside layer of the canalicular membrane. Hydrophobic BA-induced structural and functional injury of hepatocyte membranes plays an important role in the pathogenesis of cholestatic liver diseases in humans^[47]. Conjugated-BAs are more hydrophilic and not usually cytotoxic until their concentrations approach their critical micellar concentration^[48]. The effect of BAs on cell membranes is induced by binding to the membrane components^[49]. The authors carried out experiments using large unilamellar vesicles and showed that at very low BA concentrations, BA/lipid aggregates are formed in the outer membrane monolayer, with a size and BA-binding strength that depended on the species of BA and lipids involved. As BA concentrations increased, binding to membranes also rose until a certain threshold was reached and the BA-membrane interaction then resulted in the formation of transient membrane holes^[49], which caused the disruption of plasma membrane integrity and subsequently lysis of hepatocytes^[1]. The efficiency of BAs to solubilise membrane lipids, such as phospholipids, cholesterol or fatty acids, is generally enhanced with increasing BA hydrophobicity^[1,13]. CDCA and DCA have critical micellar concentrations lower than that of CA; therefore at any given concentration they are more cytotoxic^[48]. In the hepatocyte, when the concentration of hydrophobic BAs exceeds the binding capacity of the cytosolic proteins, these compounds possibly damage organelle membranes, especially in the case of mitochondria, which leads to mitochondrial damage and ultimately to apoptosis or necrosis^[48].

The amount of BAs present in bile as monomers depends on their degree of association with phospholipids and cholesterol to form mixed micelles. Such mixed micelles are formed at concentration

values well below cytotoxic levels, which accounts for the lack of BA-induced injury in the biliary system and small intestine under physiological circumstances. In *Mdr2*-knockout mice and patients lacking the canalicular phosphatidylcholine transporter MDR3 (*Mdr2* in rodents), phospholipid secretion is markedly impaired, leading to an increase in the concentration of monomeric BAs, which causes damage to biliary epithelial cells^[48].

BILE-ACID-INDUCED INJURY IN OTHER CELL TYPES

Biliary cells

In addition to the cytoprotective role of biliary phospholipids against the detergent activity of BAs, other mechanisms prevent cell damage, necrosis and apoptosis in the biliary epithelium even through this is exposed to high concentrations of these compounds. In immortalized mouse cholangiocytes it has been demonstrated that hydrophobic BAs cause apoptosis when they are taken up and accumulate inside the cell. This is, in part, inhibited by the activity of *Mrp3*, an export pump able to reduce intracellular BA concentrations^[50]. However, when the accumulation of hydrophobic BAs occurs in chronic or subchronic cholestasis, this triggers cholangiocyte proliferation^[51]. This stimulation capacity has been observed both in normal cholangiocytes and in cholangiocarcinoma cells^[52,53]. Hydrophobic BA-induced cholangiocellular proliferation occurs by transactivation of EGFR^[37,54], which stimulates a phosphatidylinositol 3-kinase-dependent pathway^[55].

Gastrointestinal cells

In humans, an increased incidence of cancer of the laryngopharyngeal tract, esophagus, stomach, pancreas, small intestine and colon is associated with high levels of hydrophobic BAs^[34]. DCA has been proposed to be a cancer promoter in these organs^[56]. These BAs cause DNA damage in several human colon cancer cell lines^[57,58], probably through an indirect mechanism involving the induction of oxidative stress and the production of ROS. Repeated DNA damage is likely to increase the mutation rate in several genes, including those coding for tumour suppressor factors and oncogenes. Moreover, hydrophobic BAs may behave as selection agents for apoptosis-resistant cells. The subpopulation of cells with a reduced apoptosis capability may be selected to survive and proliferate versus the more fragile normal cells. Indeed, long-term exposure of a human colonic epithelial cell line to sublethal concentrations of DCA has been reported to result in the selection of a population of cells partially resistant to DCA-induced apoptosis^[59].

Kidney and lung cells

Regarding extrahepatic tissues, BA accumulation in the systemic circulation may contribute to endothelial

injury in the kidney and lungs^[60]. Hydrophobic BAs cause oxidative damage to tubular cell membranes by stimulating the generation of ROS from mitochondria, as well as promoting their release from neutrophils and macrophages. Oxidative stress can promote the formation of a variety of vasoactive mediators including endothelin-1, cysteinyl leukotrienes, F₂-isoprostanes and endogenous products of lipid peroxidation. These mediators can affect renal function directly by causing renal vasoconstriction or by decreasing the coefficient of glomerular capillary ultrafiltration, and thus reducing the glomerular filtration rate. Collectively, these factors contribute to the onset of renal failure in patients with biliary obstruction^[61]. Disturbances in the biochemical functions of the kidney glomeruli and tubules have been reported in patients with intrahepatic cholestasis during pregnancy^[62].

BAs may reach the lung accidentally by aspiration of gastrointestinal contents or by direct uptake from blood. Bile aspiration produces a severe chemical pneumonitis in a porcine lung model^[63] and intratracheally injected BAs have been shown to produce severe pulmonary edema in rabbits^[64]. When intratracheal instillation of taurocholic acid (TCA, Figure 1) in rabbits was studied, microscopic evidence of widespread atelectasis, the pooling of eosinophilic substances in the intra-alveolar spaces, and the formation of hyaline membranes were found. The authors speculated that surfactant activity might be inhibited by BAs^[65].

Placental and fetal cells

The accumulation of BAs may also have deleterious effects on the fetal-placental unit. The excretion of BAs produced by the fetus is performed by the placenta and the maternal liver, therefore impairment in biliary excretion may lead to the accumulation of BAs, in particular in the fetal liver-placenta-maternal liver trio^[66]. An interesting issue that has recently been addressed is how this accumulation of BAs can affect fetal and placental tissues. In cholestatic pregnant rats, hydrophobic BA accumulation induces impairment of the placental antioxidant system and oxidative damage. These alterations are accompanied by enhanced activation of the mitochondrial pathway of apoptosis^[67]. Moreover, the accumulation of hydrophobic BAs in the fetal compartment also causes marked oxidative damage and apoptosis in the fetal liver^[68]. This is evidenced by enhanced lipid peroxidation and protein carbonylation, a pro-apoptotic imbalance in the Bax- α /Bcl-2 ratio, caspase-3 activation, and DNA fragmentation. Different sensitivities to BAs have been reported in fetal and maternal hepatocytes in short-term primary culture^[69]. Although the basal production of ROS by fetal hepatocytes has been found to be higher than by maternal hepatocytes, ROS production is higher in maternal hepatocytes in response to exposure to relatively high concentrations of GCDCA^[69].

BA accumulation can cause other complications in extrahepatic fetal tissues. TCA (Figure 1) impairs neonatal rat cardiomyocyte function by altering calcium

dynamics and impairing the function of gap junctions in these cells, resulting in dysrhythmia. This has been proposed as a mechanism for intrauterine fetal death in obstetric cholestasis^[70]. Recently, it has been reported that BAs can induce lung injury in newborn infants because these compounds are detectable in the bronchoalveolar lavage fluid of newborns from mothers with intrahepatic cholestasis during pregnancy affected by respiratory distress syndrome. Elevated serum BA levels in these infants can reach the lung after uptake from the circulation. These findings support the concept of a role of BA in the etiopathogenesis of some types of pneumonia^[71].

BILE-ACID-INDUCED CELL PROTECTION

UDCA: protective effects and mechanisms of action

UDCA (Figure 1) and its taurine-conjugated derivative, tauroursodeoxycholic acid (TUDCA) are hydrophilic BAs that have become highly popular owing to their low toxicity and efficiency in the treatment of several cholestatic liver diseases, such as cholelithiasis, primary biliary cirrhosis, primary sclerosing cholangitis, cystic fibrosis and intrahepatic cholestasis of pregnancy^[2,3]. These BAs may be also useful to protect organs other than the liver, such as the brain^[72] or placenta^[67,73-75].

UDCA is a major primary BA in some species of bears^[76]. In fact, for centuries dried bear bile has been used in traditional Chinese medicine as a remedy for liver disorders^[76]. In humans, UDCA is considered as a minor secondary BA because it is formed by 7 β -epimerization of CDCA in the gut by intestinal bacteria. The abundance of UDCA in the total BA pool is less than 3%^[77]. Despite its clinical efficacy, the precise mechanism by which UDCA improves liver function during cholestasis is still under study. It is considered that the choleric effect of UDCA, together with its ability to cause a marked shift in the composition of the BA pool towards hydrophilicity, accounts for the beneficial properties of UDCA in the treatment of liver disorders^[2,78]. Moreover, it has recently become evident that UDCA and TUDCA are capable of exerting direct protective effects at the cellular and molecular level, including the stabilization of hepatocyte membranes, the enhancement of defences against oxidative stress and the inhibition of apoptosis induced by several agents^[2]. Other proposed mechanisms of action for UDCA, such as the immunomodulation and stimulation of bile secretion by hepatocytes and bile duct epithelial cells, may also contribute to the cytoprotective effects of this hydrophilic BA^[78]. Nevertheless, the predominant mechanism of action of UDCA may vary, depending on the pathophysiology of the underlying liver disease.

Protection against oxidative stress: UDCA has shown to play an important role in the prevention of the oxidative injury induced by several agents, either through a direct antioxidant effect or an increase in antioxidant defences. The enhancement of glutathione levels, which has been demonstrated using isolated rat

hepatocytes^[79] and rats with bile-duct-ligation-induced secondary biliary cirrhosis^[80], can be included among the beneficial effects of UDCA treatment, and may be due to a higher expression of the enzymes involved in glutathione synthesis. In this sense, an enhancement of γ -glutamylcysteine synthetase at the transcriptional level has been found in isolated rat hepatocytes treated with UDCA, which allows these cells to be more resistant to cadmium- or hydrogen-peroxide-induced oxidative injury^[79]. UDCA treatment of chronic bile-duct-ligated rats also leads to an up-regulation of γ -glutamylcysteine synthetase and prevents the marked increase in the production of mitochondrial peroxide and of hydroxynonenal-protein adducts observed during chronic cholestasis^[80]. The activity of methionine S-adenosyltransferase, another enzyme involved in glutathione (GSH) biosynthesis, has also been found to be increased by UDCA in rat livers^[81].

Pre-treatment of hepatocytes with UDCA also increases the amount of thiol-containing proteins such as metallothioneins, which efficiently scavenge hydroxyl radicals (OH \cdot), the most highly reactive ROS^[79]. In human hepatoblastoma HepG2 cells, UDCA has been shown to be able to activate the metallothionein IIA promoter^[82].

During rat development, there is an excessive mitochondrial response to pro-oxidant stimuli, together with less well developed antioxidant protection mechanisms. This may account for the particularly high sensitivity of the foetal liver to lipid peroxidation^[83]. In rats, obstructive cholestasis during pregnancy (OCP) increases the degree of lipid peroxidation and protein carbonylation in placenta^[67] and in foetal liver^[68]. Treatment of rats with UDCA during pregnancy prevents oxidative injury in the placenta^[67] and foetal liver^[68]. The drop in the activities of the enzymes involved in the mechanisms of resistance against oxidative stress, such as catalase, glutathione peroxidase and glutathione-S-transferase^[67], increases GSH content and the GSH/glutathione disulfide ratio. The impairment in liver structure and function in 4-wk-old pups born from rats with OCP is also partially prevented if the mothers are treated with UDCA^[75].

Hydrophobic BAs stimulate Kupffer cells, increasing their capacity to generate ROS, which in turn attack nucleic acids, thiol proteins or membrane lipids, causing lipid peroxidation. UDCA can block hydrophobic-BA-induced cellular phenomena, therefore, it could also antagonise macrophage activation by hydrophobic BAs to blunt their capacity to generate ROS^[15].

UDCA has direct antioxidant properties, which are evident at therapeutically relevant drug concentrations and are especially relevant against Fe³⁺- and OH \cdot -induced oxidative damage^[84]. The OH \cdot -scavenging efficiency of UDCA appears remarkable, considering that its rate constant for reactions with this radical species is about 10-fold higher than that of the typical pharmacological scavenger (mannitol) and of the physiological scavengers glucose and histidine^[84]. Thus, together with the high therapeutic concentrations of UDCA reached in

human bile, the drug could readily act as an effective OH[•] scavenger, especially in the biliary milieu^[84]. It is therefore possible that UDCA could act not only as a site-specific OH[•] scavenger, but also as an antioxidant against iron (IV)-induced oxidative damage.

Inhibition of apoptosis: UDCA and TUDCA have been shown to prevent *in vitro* apoptosis induced by several agents in both hepatic and non-hepatic cells^[27]. In rats with OCP, UDCA also prevents apoptosis in the placenta^[67] and fetal liver^[68]. In patients with primary biliary cirrhosis treated with UDCA, this drug shows a potential effect in reducing nuclear DNA fragmentation in biliary epithelial cells^[85]. It has been suggested that UDCA could function as a therapeutic agent in the treatment of neurodegenerative disorders associated with increased levels of apoptosis^[72]. Indeed, UDCA is neuroprotective in pharmacological and transgenic animal models of Huntington's disease^[86]; it inhibits unconjugated bilirubin-induced apoptosis in both glial and neuronal rat cells in culture^[87], improves graft survival in Parkinsonian rats^[86], and protects against neurological injury after acute ischemic and hemorrhagic stroke^[86]. The ability of UDCA to prevent apoptosis induced by a wide variety of compounds, such as hydrophobic BAs, ethanol, transforming growth factor beta 1 (TGFβ1), Fas ligand, and okadaic acid, suggests a mechanism that is common to each of the different apoptotic pathways^[27]. A possible explanation for this ubiquitous antiapoptotic effect of UDCA seems to involve a blockage of mitochondrial dysfunction, mitochondria having a role as integrators of apoptosis signalling pathways^[24,30]. UDCA is involved in both short- and long-term mechanisms in the prevention of the MPT responsible for hepatocyte cell death^[24,30]. UDCA is able to reduce BA-induced disruption of the mitochondrial membrane potential, ROS production, and Bax protein abundance in mitochondria^[30]. UDCA treatment also prevents the marked decrease in cardiolipin levels, which modulate apoptotic processes by inhibiting the MPT of damaged and primarily apoptotic hepatocytes during biliary cirrhosis induced by chronic cholestasis in rats^[88]. Additionally, UDCA prevents the release of cytochrome c from mitochondria to the cytoplasm after mitochondrial injury and the subsequent cytosolic caspase activation and cleavage of the nuclear enzyme poly(ADP-ribose) polymerase^[31].

Nevertheless, the mechanisms by which UDCA prevents cell death involve molecular targets other than mitochondria, such as the ER. In an ER stress model in the liver-derived cell line Huh7, it has been shown that stress in this organelle activates caspase-12 and triggers apoptosis without the involvement of mitochondria^[89]. TUDCA is able to abolish the typical morphological changes of ER stress preceding apoptosis, including activation of caspase-3 and -7, DNA fragmentation, and cleavage of poly(ADP-ribose) polymerase. TUDCA also blocks one of the calcium-mediated apoptotic pathways by a reduction in calcium efflux and the inhibition of

caspase-12 activation^[89].

Apoptosis can be inhibited, not only by blocking pro-apoptotic pathways, but also by promoting survival signals, including the cAMP, Akt, nuclear factor kappaB (NF-κB), mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3-kinase (PI3K) signalling pathways^[90,91]. The antiapoptotic effect of TUDCA against hydrophobic BA-induced apoptosis in rat hepatocytes is independent of caspase-8 inhibition, but results from the activation of the p38 MAPK, extracellular signal regulated-kinase (ERK) MAPK, and PI3K survival pathways^[91]. UDCA stimulates the activation of the intracellular MAPK pathway through the activation of the epidermal growth factor receptor (EGFR)^[90]. TGFβ1-induced hepatocyte apoptosis is associated with the activation of E2F transcription factors and p53 stabilization through its inhibitor Mdm-2. UDCA interferes in the E2F-1 transcriptional activation of apoptosis, thus modulating p53 stabilization, NF-κB activation, and the expression of Bcl-2 family members^[92], through a mechanism that appears to involve nuclear glucocorticoid and mineralocorticoid receptors^[93].

Stimulation of bile flow and detoxification of cholephilic compounds: The impairment of bile formation in cholestasis results in the accumulation of potentially toxic BAs and other biliary constituents in the liver, which can result in necrosis, apoptosis, fibrosis, and ultimately liver cirrhosis^[94]. UDCA stimulates the biliary excretion of BAs and other cholephilic organic anions, even in situations of impaired bile secretion, such as primary biliary cirrhosis and primary sclerosing cholangitis, two disorders in which the effects of UDCA have mostly been studied^[3]. In patients with these diseases, UDCA treatment decreases the serum levels of bilirubin and the accumulation of hydrophobic BAs^[95], probably due to stimulation of the expression of the export pumps involved in the detoxification process of cholephilic organic compounds by hepatocytes^[96], which are down-regulated in cholestatic liver diseases^[94,96].

Transient latent cholestasis in young rats born from mothers with OCP has been reported^[97]. UDCA treatment of rats with OCP has long-term beneficial effects on their offspring by partially preventing the congenital impairment in hepatobiliary function of the pups that affects their biliary lipid secretion^[75]. UDCA also has a beneficial effect on BA transport mechanisms in placentas from patients with intrahepatic cholestasis of pregnancy^[73] and rats with OCP^[74]. UDCA partially prevents OCP-induced impairment in the placenta-maternal liver tandem excretory pathway, by preserving trophoblast structure and function^[74].

In rodents fed for several weeks with diets supplemented with CA, CDCA and LCA, a down-regulation of the expression of xenobiotic-metabolizing enzymes, such as hepatic glutathione S-transferase in mice^[98] and intestinal UDP-glucuronyltransferase in rats^[99], has been found. The prevention by UDCA of the decrease in the activity of phase-II-metabolizing

enzymes by hydrophobic BAs helps to maintain detoxification processes of cholephilic compounds^[98,99].

The secretory capacity of hepatocytes is mainly determined by the number and activity of carrier proteins in the apical membrane. In mouse liver, UDCA stimulates the gene expression of both the canalicular bile salt export pump (Bsep) and the canalicular multidrug resistance associated protein 2 (Mrp2)^[100]. UDCA also stimulates the alternative basolateral ATP-binding cassette proteins Mrp3^[101] and Mrp4^[102], which represent a compensatory overflow system under cholestatic conditions when the function of canalicular export pumps is impaired. UDCA also stimulates murine renal (Mrp2 and Mrp4) and intestinal (Mrp2 and Mrp3) efflux transport proteins, resulting in an increased overall elimination capacity for potentially toxic biliary compounds^[101,102].

Besides the long-term effects of UDCA on the regulation of gene transcription, post-transcriptional events such as increased targeting of transport proteins to the canalicular membrane *via* stimulation of vesicular exocytosis, can be accounted for by the increased expression of hepatobiliary transporters under treatment with UDCA or its conjugated derivatives^[78]. Calcium^{-[103]}, protein kinase C (PKC)^{-[104]} and MAPK-dependent mechanisms^[105] mediate the enhancement of the secretory capacity of cholestatic hepatocytes by TUDCA. Indeed, TUDCA favors the insertion of rat Mrp2 into apical membranes *via* PKC-dependent mechanisms^[104]. Furthermore, p38 MAPK signalling pathways are involved in the enhanced insertion of both rat Bsep^[105] and human BSEP^[106] into the canalicular membrane of hepatocytes by TUDCA. Since the Golgi apparatus may serve as a BSEP pool and since p38 MAPK regulates BSEP trafficking from the Golgi apparatus to the plasma membrane, the activation of p38 MAPK by TUDCA can recruit Golgi-associated BSEP and insert it into the canalicular membrane^[106].

Besides the up-regulation of synthesis, apical targeting and insertion, direct activation of key canalicular transporters through modification of their phosphorylation status may contribute to the anticholestatic action of UDCA. This has been shown for mouse Bsep, whose transport capacity is increased by TUDCA *via* PKC α -mediated phosphorylation^[107].

Protection of cholangiocytes against toxic effects of hydrophobic BAs:

The enrichment of bile with UDCA contributes to the prevention of toxic effects of more hydrophobic BAs, because this compound renders bile more hydrophilic, modifying the structure and composition of mixed phospholipid-rich micelles^[108]. In addition, UDCA and its conjugates exert a direct effect on membranes by stabilizing their structure. This effect appears to be due to the incorporation of UDCA into the non-polar domain of the lipid bilayer and of its conjugates, into the interface^[109].

An experimental model in which the *in vivo* effects of UDCA on the protection of cholangiocytes have been studied is the *Mdr2*-knockout mouse. These

animals lack the ability to secrete phospholipids into bile and develop a chronic cholangitis resembling human chronic cholestatic liver disease^[110]. The feeding of mice with UDCA or TUDCA decreases the degree of cholangiocellular injury, portal inflammation, and ductular proliferation^[110]. 24-norursodeoxycholic acid seems to be superior to UDCA in the treatment of cholangitis in *Mdr2*-knockout mice^[111]. Similar beneficial effects of UDCA have been found in bile-duct-ligated rats^[112]. Likewise, in patients with primary cholestatic liver diseases under treatment with UDCA, the inflammatory reaction around bile ducts has been reported to be less severe^[113].

In addition to stabilizing cell membranes, other molecular mechanisms responsible for the protective effects of UDCA on cholangiocytes have been described recently. BAs are taken up by cholangiocytes mainly *via* the apical sodium-dependent BA transporter (ASBT). The accumulation of BAs in chronic cholestasis triggers cholangiocyte proliferation and secretion through a PI3K-dependent pathway^[55]. UDCA and TUDCA reduce ASBT expression in cholangiocytes isolated from bile-duct-ligated rats, and they inhibit cellular growth and secretion^[114] through Ca²⁺- and PKC α -dependent mechanisms^[115]. TUDCA also inhibits the growth of the cholangiocarcinoma cell line Mz-ChA-1 through a signal-transduction pathway involving MAPK p42/44 and PKC α , which suggests that this compound may be a candidate for the treatment of cholangiocarcinoma^[116].

Immunomodulation: The immunomodulatory mechanism of action of UDCA accounts for the beneficial properties of treatment with this BA against several autoimmune liver diseases, such as primary biliary cirrhosis and chronic viral hepatitis. Among the *in vitro* experimental evidence of the immunomodulatory activity of UDCA reported is its ability to decrease the secretion of interleukins 2 and 4, TNF- α and interferon- γ from activated T lymphocytes, and immunoglobulin production from B lymphocytes^[117]. The suppression of cytokine and immunoglobulin production and T-cell-mediated cytotoxicity in mice by the treatment not only with UDCA^[118] but also with CDCA^[119] has been observed. In patients with primary biliary cirrhosis, the administration of UDCA down-regulates the expression of abnormal major histocompatibility complex (MHC) class I molecules in periportal hepatocytes, whereas the expression of abnormal MHC class II molecules in bile-duct epithelial cells does not change^[120]. However, UDCA suppresses the interferon- γ -mediated induction of MHC class II gene expression *via* the glucocorticoid-receptor-mediated pathway^[121].

Protective effects of other bile acids

Other natural BA molecules or their derivatives have also aroused pharmacological interest owing to their protective properties. Besides the efficiency of CDCA in cholesterol cholelithiasis^[122] and cerebrotendinous xanthomatosis^[48,123], BA replacement therapy with a

mixture of this BA together with CA is used in the treatment of inborn errors of BA biosynthesis involving the A ring^[48]. This treatment suppresses the synthesis of cytotoxic BA precursors and restores the input of primary BAs into the enterohepatic circulation^[48]. Cholyl-N-methylglycine or cholylsarcosine (Figure 1) is a synthetic BA analog that has been shown to prevent the severe fat malabsorption seen in patients with short-bowel syndrome due to a BA deficiency, which leads to impaired micellar solubilization in the proximal intestine^[124].

CONCLUSION

Rapid advances in the understanding of the cellular and molecular pathophysiology of BAs have led to a better knowledge of hepatocyte injury caused by the retention of hydrophobic BAs in cholestatic diseases. These BAs can damage cell membranes and promote the generation of ROS, and eventually cause necrosis and apoptosis. Hydrophobic BAs can also trigger hepatocyte apoptosis by the activation of death receptors, through the mitochondrial pathways, and by the induction of ER stress. The accumulation of hydrophobic BAs in the systemic circulation may have also deleterious effects on extrahepatic tissues such as kidney, lung, placenta, and foetal cells.

UDCA is a hydrophilic BA useful for the treatment of cholestatic liver diseases by its ability to modulate hydrophobic-BA-induced injury in hepatocytes. Underlying mechanisms of their beneficial effects are only now being clarified, and include protection against cytotoxicity due to more toxic BAs, stimulation of bile secretion, immunomodulation, protection against oxidative stress, and the inhibition of apoptosis. Other natural BAs or their derivatives, such as CA, CDCA and cholylsarcosine, have also aroused pharmacological interest owing to their protective properties in several diseases.

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REVIEW

Body weight, lifestyle, dietary habits and gastroesophageal reflux disease

Davide Festi, Eleonora Scaiola, Fabio Baldi, Amanda Vestito, Francesca Pasqui, Anna Rita Di Biase, Antonio Colecchia

Davide Festi, Eleonora Scaiola, Fabio Baldi, Amanda Vestito, Francesca Pasqui, Anna Rita Di Biase, Antonio Colecchia, Department of Clinical Medicine, University of Bologna, Bologna 40138; Department of Pediatrics, University of Modena, Modena 41100, Italy

Author contributions: Colecchia A, Baldi F, Festi D conceived and designed the manuscript; Vestito A, Pasqui F drafted the manuscript; Scaiola E, Di Biase AR acquired data.

Correspondence to: Davide Festi, MD, Department of Clinical Medicine, S. Orsola-Malpighi Hospital, Via Massarenti 9, Bologna 40138, Italy. davide.festi@unibo.it

Telephone: +39-51-6364123 Fax: +39-51-6364123

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Peer reviewer: Wallace F Berman, MD, Professor, Division of Pediatric GI/Nutrition, Department of Pediatrics, Duke University Medical Center, Duke University School of Medicine, Durham, Box 3009, NC27710, United States

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Abstract

While lifestyle modifications are currently used as first-line treatment for subjects with gastroesophageal reflux disease (GERD), the pathogenetic role of lifestyle factors and consequently, the efficacy of lifestyle measures is controversial. Our aim was to systematically review the pathogenetic link between overweight/obesity, dietary habits, physical activity and GERD, and the beneficial effect of specific recommended changes, by means of the available literature from the 1999 to the present. Obesity, in particular, abdominal obesity, plays a key role in determining GERD symptoms and complications through mechanical and metabolic effects. Controlled weight loss (by diet or surgery) is effective in improving GERD symptoms. No definitive data exist regarding the role of diet and, in particular, of specific foods or drinks, in influencing GERD clinical manifestations. Moderate physical activity seems to be beneficial for GERD, while vigorous activity may be dangerous in predisposed individuals. In conclusion, being obese/overweight and GERD-specific symptoms and endoscopic features are related, and weight loss significantly improves GERD clinical-endoscopic manifestations. The role of dietary behavior, mainly in terms of specific dietary components, remains controversial. Mild routine physical activity in association with diet modifications, i.e. a diet rich in fiber and low in fat, is advisable in preventing reflux symptoms.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined as an abnormal reflux of the gastric contents into the esophagus at least once a week, leading to symptoms, such as heartburn and/or acid regurgitation, and/or esophageal mucosal damage, which may also provoke long-term complications, such as Barrett's esophagus^[1,2]. GERD represents a common disorder, particularly in the Western world (about 10%-20% in Western countries and under 5% in Asia) and its prevalence appears to be increasing^[3,4]. The incidence rate, reported by two longitudinal studies^[5,6], was 4.5 and 5.4/1000 people per year, respectively.

GERD is a multifactorial disease in which anatomical and functional factors both play a pathogenetic role. The main pathogenetic mechanism of GERD is considered to be transient lower esophageal sphincter relaxation (TLESR)^[7] which may account for the majority of reflux episodes, in patients with esophagitis and in those with non-erosive reflux disease (NERD). An increased number of TLESR episodes, combined or not with an impaired LES basal tone or with gastric or esophageal motor dysfunction, may lead to GERD, but the underlying causes of these functional disorders are still partially unknown^[2].

However, the role of genetic factors was suggested by twin studies^[8,9] wherein heritability accounted for 31%-43% of the likelihood of reflux disease, which suggests that genetic and environmental factors both

play an important role. Among the environmental factors, lifestyle factors, in particular being overweight/obese, incorrect dietary habits, the lack of regular physical activity and smoking have frequently been suggested to be possible GERD risk factors. However, the exact pathogenetic role of these factors is still under debate and the beneficial effect of specific recommended changes in lifestyle habits is also controversial^[10].

A comprehensive search of the literature (Medline/PubMed databases 1999-June 2008, using the following keywords singly and in different combinations: GERD, food intake, food questionnaire, energy intake, motor activity, exercise, obesity, abdominal obesity) was carried out and the evidence available was critically reviewed.

RELATIONSHIP BETWEEN OVERWEIGHT/OBESITY AND GERD

The observation of a consensual increase in the frequency of obesity and GERD^[7,11,12] in Western countries has suggested a possible pathogenetic link between these two diseases, and it has generated great interest in elucidating the mechanisms demonstrating this association. However, although the relationship between GERD and obesity has been the subject of several studies, conflicting results have been obtained.

In a meta-analysis of epidemiological studies regarding the association between obesity and GERD-related disorders, it was found^[13] that being overweight (BMI, 25-30 kg/m²) and being obese (BMI, > 30 kg/m²) were associated with GERD symptoms (OR, 1.43; 95% CI, 1.158-1.774 and OR, 1.94; 95% CI, 1.468-2.566, respectively), erosive esophagitis (OR, 1.76; 95% CI, 1.156-2.677 in overweight subjects) and esophageal adenocarcinoma (OR, 1.52; 95% CI, 1.147-2.009 and OR, 2.78; 95% CI, 1.850-4.164, respectively). A cross-sectional study on 206 consecutive patients not on acid-suppressing medications who underwent 24-h pH measurement showed^[3] a significant ($P < 0.005$) association between a BMI > 30, a high waist circumference and acid reflux episodes. An additional meta-analysis of clinical studies on the relationship between obesity and reflux symptoms, esophagitis or GERD-related hospitalization documented a positive association between BMI (OR, 1.57; 95% CI, 1.36-1.80 in overweight and OR, 2.15; 95% CI, 1.89-2.45 in obese subjects) and GERD in studies carried out in the USA, but the results were heterogeneous in those carried out in Europe^[14].

Different explanations have been proposed in order to interpret these geographical differences. In fact, the relationship between BMI and the percentage of body fat differs between ethnic groups^[15].

Since there are probably multiple pathogenetic mechanisms of GERD (TLESR, esophageal and gastric motor function, and gastric secretion), it is possible that not all of them are related to, or influenced by, the presence of obesity^[16]. Furthermore, different definitions of GERD (endoscopic, symptom reports by self-administered or validated questionnaires) have

been utilized in the different studies, as well as different measures of obesity (BMI, central adiposity).

RELATIONSHIP BETWEEN BEING OVERWEIGHT/OBESITY AND GERD: SUGGESTED PATHOGENETIC MECHANISMS

The exact pathophysiological mechanisms that demonstrate the association/relationship between being overweight/obese and GERD have not been fully identified, but some hypotheses have been suggested.

It has long been hypothesized^[17-20] that visceral adiposity, expressed by an increased abdominal waist circumference, could be associated with increased intra-abdominal pressure which would, in turn, promote GERD by increasing intragastric pressure (IGP).

Using high-resolution manometry, it was found^[17] that IGP as well as the gastroesophageal pressure gradient (GEPG), during expiration and inspiration, was significantly higher ($P < 0.0001$) in obese and overweight patients, as compared to those with a normal BMI. This showed an IGP increase of 0.3 mmHg per unit of increase in BMI and the association was stronger in men than in women. Similar results were obtained in a recent retrospective analysis^[18] in patients with typical GERD symptoms who underwent pH-monitoring and esophageal manometry. By means of multiple regression analysis, an increase of BMI was independently associated with IGP and with an increase of GEPG during inspiration; furthermore, BMI, IGP and GEPG were strong independent predictors of hiatal hernia, and IGP and GEPG were not independently associated with abnormal acid exposure or esophagitis.

A further pathogenetic mechanism has been suggested by Pandolfino *et al*^[17] who observed that obesity was associated with a separation between LES and the extrinsic crural diaphragm, a disruption which could predispose obese subjects to hiatal hernia. It is known that hiatal hernia is commonly associated with symptomatic GERD, and patients with abnormal esophageal acid exposure have a significantly higher prevalence of hiatal hernia^[21,22]. Furthermore, in patients with hiatal hernia, esophagitis or abdominal low distal esophageal pH are more common than in those without hiatal hernia^[23]. In a retrospective case-control study on 1389 patients, obesity was found to represent an independent risk factor for hiatal hernia^[24]. Therefore, it is possible that obesity, through alteration of normal IGP and separation between LES and the extrinsic crural diaphragm, predisposes to hiatal hernia and consequently, to GERD.

Another plausible mechanism for the association between obesity and GERD is represented by slower esophageal acid clearance, as shown by Quiroga *et al*^[25] in a case-control study using esophageal manometry in normal weight and obese patients with GERD and in healthy subjects. All subjects with GERD showed altered

Table 1 Association between being overweight/obese and GERD-related symptoms

Author	Year	Country	Study design	Population size	Obesity index	Method of data collection	Association
Nilsson <i>et al</i> ^[42]	2003	Norway	Population-based	65 363	BMI	Questionnaire	Yes
Nandurkar <i>et al</i> ^[41]	2004	USA	Population-based	211	BMI	Validated questionnaire	Yes
Hampel <i>et al</i> ^[13]	2005	USA	Meta-analysis		BMI	Validated symptoms score	Yes
Corley <i>et al</i> ^[14]	2006	USA	Meta-analysis		BMI	Questionnaire	Yes
Jacobson <i>et al</i> ^[38]	2006	USA	Cross-sectional, women	10 545	BMI	Questionnaire	Yes
Nocon <i>et al</i> ^[39]	2006	Germany	Population-based	7 124	BMI	Interview	Yes
Corley <i>et al</i> ^[37]	2007	USA	Cross-sectional	80 110	BMI and abdominal diameter	Questionnaire	Yes
Nocon <i>et al</i> ^[40]	2007	Germany	Population-based	6 215	BMI	Validated questionnaire	Yes
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish twin registry	27 717	BMI	Questionnaire, telephone interview	Yes
Andersen <i>et al</i> ^[44]	1991	Denmark	Population-based	1 321	BMI	Validated questionnaire	No
Lagergren <i>et al</i> ^[46]	2000	Sweden	Cross-sectional	820	BMI	Interview	No
Talley <i>et al</i> ^[45]	2004	Australia	Cross-sectional	777	BMI	Questionnaire	No
Zagari <i>et al</i> ^[47]	2008	Italy	Population-based	10 330	BMI	Questionnaire	No

esophageal motility and obese patients also had impaired esophageal acid clearance.

In obese patients, other esophageal motor abnormalities, such as hypotensive LES pressure, nutcracker esophagus and non-specific motility disorders^[26-29] have been observed.

However, the most important reflux mechanism in obese subjects seems to be TLESR^[7]. The main stimulus for generating TLESR episodes is gastric distension, which leads to intense stimulation of both stretch and tension mechanoreceptors in the proximal stomach^[30,31]. In fact, in a recent study^[32], three groups of subjects without GERD (28 obese, 28 overweight and 28 normal) underwent BMI measurements, upper endoscopy, manometry and pH recordings for both the fasting and the postprandial period and were given a symptom questionnaire. During the 2-h postprandial period, both overweight and obese individuals showed a significantly ($P < 0.001$) higher rate of TLESR episodes, and a higher proportion of TLESR episodes accompanied by acid reflux and total acid exposure than normal weight subjects. A direct correlation between increasing BMI, an increased number of TLESR episodes, and an increased number of TLESR episodes associated with acid reflux was identified. Therefore, it seems that obese subjects have a higher postprandial IGP, which provokes more postprandial TLESR episodes.

Another mechanism by which obesity can cause GERD is related to the visceral component of abdominal obesity. In fact, visceral fat is metabolically active^[33] and it has been associated with low serum levels of protective cytokines, such as adiponectin, and high levels of inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6. An increase in these inflammatory cytokines in patients with erosive esophagitis and Barrett's esophagus has also been observed^[34].

In conclusion, although conflicting and non-definitive results exist, it is likely that GERD and obesity are in some way linked; in particular, abdominal obesity seems to play a key role in determining GERD symptoms and complications through mechanical and metabolic effects. Consistent with this evidence, it is

possible to hypothesize that GERD may be a curable condition through the control of body weight and, in particular, by reducing abdominal obesity.

OBESITY AND GERD-RELATED SYMPTOMS

Different studies have analyzed the possible relationship between obesity and GERD by evaluating the clinical manifestation of the disease since reflux symptoms (mainly, heartburn and acid regurgitation) represent the main target for diagnostic evaluation and treatment in GERD patients. However, the factors responsible for the generation of symptoms, in normal or overweight subjects, have not been clearly identified.

Sensitization of esophageal chemoreceptors, either directly by intermittent exposure to refluxed acid or indirectly through esophagitis-associated inflammatory mediators, is thought to be one of the most important mechanisms responsible for symptom generation in GERD^[35,36].

Several authors have evaluated the relationship between obesity and the symptoms of GERD, both to confirm the association between obesity and GERD and to find potential risk factors for symptom generation (Table 1). In all the studies available, only typical GERD symptoms (i.e. heartburn and regurgitation) have been taken into account, with the use of interviews or structured questionnaires.

In a large cross-sectional study^[37] on abdominal obesity, GERD symptoms and ethnicity performed on 80 110 members of a health organization, it was found that increased abdominal diameter, adjusted for BMI, was an independent risk factor for reflux symptoms (OR, 1.85; 95% CI, 1.55-2.21) in the white population but not among blacks and Asians, and this aspect was not influenced by gender.

A recent large cohort study in 10 545 women reported a significant ($P < 0.001$) dose-dependent relationship between increasing BMI and frequent reflux symptoms; this relationship was present even in the normal range of BMI^[38].

Table 2 Association between overweight/obesity and reflux esophagitis

Author	Year	Country	Study design	Population size	Obesity index	Method of data collection	Association
Ruhl <i>et al</i> ^[49]	1999	USA	Cohort	12349	BMI	Upper endoscopy	Yes
Hampel <i>et al</i> ^[13]	2005	USA	Meta-analysis		BMI	Upper endoscopy	Yes
Nocon <i>et al</i> ^[40]	2007	Germany	Population-based	6215	BMI	Upper endoscopy	Yes
Kim <i>et al</i> ^[48]	2007	South Korea	Population-based	27319	BMI	Upper endoscopy	Yes
Kang <i>et al</i> ^[51]	2007	South Korea	Population-based	2457	BMI, waist circumference	Upper endoscopy	Yes
Lee <i>et al</i> ^[50]	2008	South Korea	Population-based	3363	BMI, waist-to-hip ratio	Upper endoscopy	Yes
Chung <i>et al</i> ^[52]	2008	South Korea	Cross-sectional	7078	BMI, waist circumference, visceral adipose tissue	Upper endoscopy	Yes
Furukawa <i>et al</i> ^[58]	1999	Japan	Cross-sectional	6010	BMI	Upper endoscopy	No
Baldi <i>et al</i> ^[59]	2008	Italy	Cohort	1542	BMI	Upper endoscopy	No
Zagari <i>et al</i> ^[47]	2008	Italy	Population-based	1033	BMI	Upper endoscopy	No

Two large population studies^[39,40] and a case-control study^[41] have found a positive association between BMI and reflux symptoms. The same results were previously obtained in a large population-based study carried out in Norway^[42]; the authors found a stronger association among women, especially premenopausally, and that the use of hormone therapy strengthened the association, which suggested that estrogens may play an important role in the etiology of reflux disease. Another recent study^[43] performed in Sweden on a large number of twins, confirmed that BMI seems to be a risk factor for GERD symptoms in monozygotic twins.

However, the association between obesity and GERD symptoms was not confirmed in subjects with benign esophageal disease^[44]. Furthermore, a cohort study from New Zealand found no association between BMI and reflux symptoms^[45] but, as its authors noted, the cohort consisted of young adults, and GERD symptoms do not usually appear until middle age. The same results were produced by a nationwide case-control study in Sweden^[46] and by a recent population-based epidemiological study in Italy^[47].

No data are available yet about the possible correlation between obesity and atypical GERD symptoms.

In conclusion, although further studies are probably needed, most of the available evidence suggests a relationship between obesity, mainly abdominal obesity, and GERD-related typical symptoms.

OBESITY AND REFLUX ESOPHAGITIS

Endoscopic investigation, alone or combined with information regarding specific symptoms obtained by means of structured questionnaires, has been carried out in order to evaluate the possible correlation between GERD-related disorders, such as esophagitis, and obesity (Table 2).

The above mentioned meta-analysis by Hampel *et al*^[13] has documented the association between a BMI ≥ 25 kg/m² and erosive esophagitis. This association was confirmed by Nocon *et al*^[40] using a symptomatic questionnaire and upper endoscopy in 6215 patients and by Kim *et al*^[48] who observed, in 27319 subjects, an association between an increasing BMI and abnormal endoscopic findings, such as erosive gastritis, gastric

ulcer, duodenal ulcer (OR, 1.31; 95% CI, 1.22-1.40; $P < 0.0001$ for overweight subjects; OR, 1.40; 95% CI, 1.14-1.72; $P < 0.001$ for obese patients) and reflux esophagitis (OR, 1.61; 95% CI, 1.42-1.83; $P < 0.001$ for overweight subjects, OR, 2.23; 95% CI, 1.59-3.11; $P < 0.001$ for obese patients). In a long-term follow-up study (median 18.5 years) on 12349 subjects^[49], increased hospitalization rates for esophagitis and hiatal hernia were documented in patients with a BMI > 25 kg/m². Other population-based studies^[50,51] have also confirmed the correlation between obesity, mainly abdominal obesity, and erosive esophagitis.

Furthermore, an association between the metabolic syndrome and reflux esophagitis has recently been documented^[52] in a cross-sectional case-control study on 7078 subjects. In particular, it was observed that, among the single components of the metabolic syndrome, abdominal obesity (in particular visceral fat) and elevated serum triglycerides represented independent risk factors for reflux esophagitis (OR, 1.60; 95% CI, 1.03-2.48). Furthermore, it has been demonstrated that only visceral adipose tissue, evaluated by cross-sectional computed tomography, was an independent risk factor for reflux esophagitis. This tissue, in fact, is recognized to be metabolically active and it has been associated with elevated serum levels of pro-inflammatory adipokines (IL-6, TNF- α and adiponectin), compounds which may play a role in GERD development^[53,54]. It seems that visceral adipose tissue plays a key role in increasing lipolysis and free fatty acid, leading to insulin resistance, which is considered the major pathophysiological factor for the development of the metabolic syndrome^[55-57]. It has been suggested that these substances might alter the LES pressure or affect the esophageal clearance of refluxate^[52].

However, the association between BMI and esophagitis has not been confirmed in a recent Japanese prospective study^[58], as well as in an epidemiological survey^[47] and a multicenter Italian observational study^[59]. The different percentages of body fat in different ethnic groups may represent a possible explanation for these different results^[15].

In conclusion, as occurs for specific GERD symptoms, most of the evidence available also suggests a positive correlation for the association between being

overweight/obese and GERD-related morphological lesions.

WEIGHT CHANGES AND GERD-RELATED SYMPTOMS

The observed relationship between weight gain and an increase in GERD-related symptoms^[60,61], as well as that between weight reduction and a decrease in GERD-related symptoms^[62-64], represents additional evidence of the close relationship between obesity and GERD. However, results from studies of weight loss for the control of GERD-related symptoms in obese subjects are not conclusive^[65].

In an uncontrolled study on selected patients with a BMI > 25 kg/m², Fraser-Moodie *et al*^[64] found that weight loss, induced by general dietary advice, had a beneficial effect on GERD symptoms, as evaluated by a structured questionnaire. Different results were obtained^[66,67] in obese patients with reflux disease who were on a very-low-calorie diet, in which no reduction in reflux symptoms or changes in reflux episodes measured by 24 h pH-monitoring were documented. However, these studies have presented important limitations since they included patients with hiatal hernia, which represents an irreversible condition that contributes to the occurrence of GERD despite the weight loss, and they were carried out on very small numbers of patients, 20 and 15, respectively.

The effect of weight loss induced by endoscopic or surgical procedures has been evaluated. A significant reduction in esophageal acid exposure was documented during weight loss induced by an intragastric balloon^[62,63] in a randomized, double-blind, sham-controlled study. An improvement in GERD symptoms was also found after weight loss induced by bariatric surgery^[68]. In particular, the Roux-en-Y gastric bypass seems to represent the surgical procedure that is most effective in improving GERD symptoms in obese subjects^[68-72]. Roux-en-Y gastric bypass may be successful at reducing GERD symptoms by diverting bile away from the esophagus^[68,73], eliminating acid production in the gastric pouch^[73], and reducing the volume of acid refluxate^[70]. This hypothesis is supported by the observation of rapid symptom improvement after the surgical procedure; long-term symptom improvement is likely to be the result of weight loss^[68].

Studies evaluating other types of bariatric surgery, such as laparoscopic gastric banding or vertical banded gastroplasty, have produced conflicting results^[26,74-78]. In particular, laparoscopic adjustable gastric banding has no, or minimal, effect on GERD-related symptoms but, over time, symptoms increase toward baseline or beyond. This effect is likely caused by slippage of the band distally, which results in more stomach above the band. Therefore, the impact of laparoscopic adjustable gastric banding on GERD is independent of weight loss; instead, it is the direct anatomical alteration of the band that impacts on GERD symptoms^[65].

However, these studies are still limited by small sample sizes, lack of randomization, failure to use control groups, use of retrospective data and inconsistent timing of postoperative re-evaluation. Furthermore, it has yet to be clarified whether the surgical technique itself, and/or weight loss, represents the mechanism of action for symptom improvement.

In obese subjects undergoing weight loss, the effect of changes in meal composition on reflux symptoms has also been investigated^[79,80], although in small studies. In a study on eight obese volunteers, a lower carbohydrate diet reduced reflux symptoms and reflux episodes, which was evaluated by means of 24-h pH monitoring^[79]. A higher frequency of reflux symptoms with a high-fat diet as compared to a low-fat diet has also been demonstrated^[80], using four different calorie/fat composition diets, in 15 patients with GERD. Furthermore, esophageal acid exposure was higher with the high-calorie diet as compared to the low-calorie diet.

Together, these data indicate an effective role for weight loss in improving GERD symptoms as well as the effect of differing meal compositions on reflux disease.

DIET AND GERD

It is a common belief that some foods may induce or worsen GERD symptoms; in fact, in daily clinical practice, this belief leads to advising patients to avoid the suspect foods^[81]. Furthermore, since GERD symptoms are most commonly reported postprandially, the role of diet components in inducing symptoms has been suggested. However, different and conflicting results exist in the literature for identifying the most "refluxogenic" foods (Table 3).

Old experimental and clinical studies have shown a decrease in LES pressure and an increase in esophageal acid exposure in response to the ingestion of food rich in fats, chocolate and carminatives^[82-85]. Nebel *et al*^[86] have demonstrated that fried foods, spicy foods and alcohol are the most common precipitating factors of heartburn; however, this study had no control group and did not quantify the intake of dietary items.

In order to elucidate the association of different nutrients with the risk for GERD, El-Serag *et al*^[87] carried out a cross-sectional study on 915 employees, using a dietary questionnaire to estimate the average food consumption over the previous year, and a GERD questionnaire together with an upper endoscopy for assessing GERD severity. A positive association between high fat intake, and GERD symptoms and erosive esophagitis was observed, while a high-fiber diet seemed to reduce reflux symptoms. However, the effects of fat on GERD symptoms and erosive esophagitis were dependent on BMI since this is statistically significant only in overweight individuals. Furthermore, a higher daily intake of fats and proteins was observed in those participants with erosive esophagitis.

More recently, Shapiro *et al*^[88] observed in 58 subjects with typical heartburn symptoms that increased consumption of cholesterol, saturated fatty acids and an

Table 3 Dietary intake and GERD-related clinical manifestations

Author	Year	Country	Study design	Population size	Method of data collection	Effects
Fatty foods, chocolate, carminatives						
Nebel <i>et al</i> ^[84]	1972	USA	Case series	10	Infused open-tipped system	Lowered LESP
Becker <i>et al</i> ^[82]	1989	USA	Case-control	20	24-h pH-metry	Increased esophageal acid exposure
Nebel <i>et al</i> ^[86]	1976	USA	Population-based	1004	Symptom questionnaire	Worsened reflux symptoms
Iwakiri <i>et al</i> ^[89]	1996	Japan	Population-based	20	pH-monitoring	
Holloway <i>et al</i> ^[90]	1997	Australia	Case-control	23	Esophageal manometry and pH-monitoring	
Meyer <i>et al</i> ^[91]	2001	USA	Population-based	11	Esophageal perfusion with HCl and duodenal perfusion with fat and saline, symptoms interview and scales	
El-Serag <i>et al</i> ^[87]	2005	USA	Cross-sectional	371	Dietary and symptom questionnaire and upper endoscopy	
Shapiro <i>et al</i> ^[88]	2007	USA	Case series	50	GERD symptoms checklist, upper endoscopy, 24-h pH-metry, dietary intake records	
Ruhl ^[49]	1999	USA	Epidemiological	12349	Upper endoscopy, symptoms interview, total dietary servings of high fat foods	No association with GERD symptoms,
Phel ^[92]	1999	Germany	Case series	12	Esophageal manometry, pH-metry	reflux episodes
Colombo ^[93]	2002	Italy	Case series	13	pH-metry in three solid/liquid meals	or lower esophageal sphincter pressure
Nandurkar ^[41]	2004	USA	Population-based, nested case-control	211	GERD, energy expenditure, dietary intake questionnaires	
Zheng ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	
High caloric load						
Colombo ^[93]	2002	Italy	Case series	13	pH-metry in three solid/liquid meals	
Fox ^[80]	2007	UK	Case series	15	pH-monitoring in four dietary conditions	
Fruits, vegetable, high fiber intake						
El-Serag <i>et al</i> ^[87]	2005	USA	Cross-sectional	371	Dietary and symptom questionnaire and upper endoscopy	Improved symptoms
Zheng <i>et al</i> ^[43]	2007	Sweden	Monozygotic co-twin study based on Swedish Twin Registry	27717	Questionnaire, telephone interview	No association
Alcohol						
O'Leary <i>et al</i> ^[97]	2003	Ireland	Randomized, double-blind, placebo-controlled trial	56	Symptom questionnaires and drug-diet-alcohol records	Worsened reflux symptoms
Rosaida <i>et al</i> ^[98]	2004	Malaysia	Cross-sectional	1000	Upper endoscopy and symptom and risk factor questionnaire	
Wang <i>et al</i> ^[96]	2004	China	Epidemiological	2789	Symptom and risk factor questionnaire	
Mohammed <i>et al</i> ^[95]	2005	UK	Population-based	1533	Symptom and lifestyle questionnaire	
Talley <i>et al</i> ^[102]	1994	USA	Cross-sectional	1644	Symptom and lifestyle questionnaire	No association
Stanghellini <i>et al</i> ^[101]	1999	Multinational	Cross-sectional	5581	Symptom and lifestyle questionnaire	
Nilsson <i>et al</i> ^[99]	2004	Norway	Case-control	> 40000	Symptom and lifestyle questionnaire	
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	
Shapiro <i>et al</i> ^[88]	2007	USA	Case series	50	GERD symptoms checklist, upper endoscopy, 24-h pH-metry, dietary intake records	Reduced perception of acid reflux
Coffee						
Price <i>et al</i> ^[103]	1978	USA	Population-based	66	Intraesophageal infusion of coffee, orange juice, spicy tomato drink and HCl	Worsened reflux symptoms
Stanghellini <i>et al</i> ^[101]	1999	Multinational	Cross-sectional	5581	Symptom and lifestyle questionnaire	No association
Boekema <i>et al</i> ^[104]	1999	Netherlands	Randomized controlled crossover study	15	24-h pH-monitoring and coffee and water drinking	
Wang <i>et al</i> ^[96]	2004	China	Epidemiological	2789	Symptoms and risk factor questionnaire	
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	Protective factor

higher percentage of calories from fats was significantly associated with an increased likelihood of having reflux events. The role of fats in symptom generation has been confirmed by some^[89-91], but not by others^[41,49,92,93]. Ruhl *et al*^[49] failed to document an association between dietary fat intake and erosive esophagitis and reflux symptoms,

although higher reflux disease hospitalization rates were associated with an increased BMI. These results are consistent with those produced by Nandurkar *et al*^[41], who analyzed potential risk factors for reflux among 211 community subjects and concluded that only BMI, and not diet, may influence symptomatic GERD.

Also Pehl *et al*^[92] did not find differences in reflux parameters comparing a high-fat meal with a low-fat meal; similar results were obtained by Colombo *et al*^[93], although they found that a caloric load increased esophageal acid exposure.

Furthermore, none of the dietary items evaluated (i.e. vegetables, fruits, fish, meat, rice, milk, grilled and fried food), including alcohol, was associated with the risk of GERD symptoms in the above-mentioned monozygotic co-twin study based on the Swedish Twin Registry^[43], which investigated lifestyle factors that potentially cause GERD.

Considering the recommended advice of controlling alcohol drinking and reducing, or avoiding, coffee to prevent GERD symptoms^[81,94], several studies investigated the role of alcohol and coffee in GERD. While some authors have suggested that alcohol is an independent risk factor for GERD-related symptoms^[95-98], others have not found such a relationship^[99-102]. In their study about the lifestyle habits and GERD symptoms of the participants in two consecutive public health surveys in Norway (> 40000 people), Nilsson *et al*^[99] did not find that alcohol, coffee or tea were risk factors for reflux-related symptoms. Shapiro *et al*^[88] not only confirmed these results, but also documented that alcohol was associated with a reduced perception of intra-esophageal acid reflux events.

Despite the observation that the intraesophageal infusion of coffee in patients with acid sensitivity may induce heartburn^[103], two large epidemiological studies have found no association between coffee drinking and GERD^[96,101]. Boekema *et al*^[104] have found that coffee does not alter postprandial acid reflux time or the number of acid reflux episodes, and others^[41] have noted that coffee consumption is lower in subjects with reflux symptoms. However, the latter result might reflect avoidance of coffee by those who suffer from reflux because the beverage aggravates symptoms.

Furthermore, in their recent study of twins, Zheng *et al*^[43] have found that coffee intake might be a protective factor for GERD symptoms in men, but not in women. The authors have suggested that the differences observed might be caused by sex differences regarding caffeine metabolism. In fact, it has been demonstrated that the conversion of caffeine to paraxanthine, which accounts for 84% of the primary degradation of caffeine in humans^[105], is markedly inhibited by exogenous estrogen in women taking oral contraceptives^[106] or in postmenopausal women on hormone replacement therapy^[107]. Given the conflicting data reported, the relationship between coffee and GERD remains unclear; as a consequence, there is insufficient evidence to support the routine recommendation of avoiding such beverages for patients with GERD.

In conclusion, no definitive data exist regarding the role of diet and, in particular, of specific foods or drinks, in GERD clinical manifestations. Despite the insufficient evidence to support an association between dietary behavior and GERD, some dietary interventions continue to be recommended as first-line therapy^[81]. Larger prospective controlled trials are required to

conclusively recommend dietary modifications in the treatment of GERD.

SMOKING AND GERD

Population-based and epidemiological studies have suggested that tobacco smoking may represent a risk factor for GERD^[9,39,108-113]. Studies using specific questionnaires have reported that smoking is significantly associated with GERD-related symptoms (OR, 1.35; 95% CI, 1.01-1.82)^[114-116]. Furthermore, a recent monozygotic co-twin study^[43] has provided compelling evidence that tobacco smoking increases the risk for the occurrence of frequent GERD symptoms, and a case-control study^[99] on 3153 patients with severe GERD-related symptoms has shown that the duration of smoking was associated with increasing reflux symptoms (OR, 1.7; 95% CI, 1.5-1.9 in subjects who had smoked for > 20 years). Different mechanisms have been suggested to justify the association between smoking and GERD. Cigarette smoking can reduce the LES pressure^[117-119] and decrease salivary bicarbonate secretion, thus reducing the physiological neutralizing effect of saliva on intraesophageal acid and prolonging acid clearance^[120,121]. Furthermore, abrupt increases in intra-abdominal pressure, as occur during coughing or deep inspiration, have been associated with reflux symptoms in smokers^[122]. However, studies that have examined acid perfusion using the Bernstein test or esophageal pH^[123-125] have reported that smokers compared to non-smokers do not show an increased esophageal acid exposure time, despite having more "reflux episodes"^[126]. Furthermore, two old case-control studies evaluating, on very small samples, the effect of smoke cessation on GERD outcomes^[126,127] were unable to document an improvement in GERD symptoms after the cessation of tobacco use.

PHYSICAL ACTIVITY AND GERD

Since previous investigations have demonstrated that strenuous exercise may induce GERD^[128-130] and that GERD symptoms are common among athletes^[131], it has been suggested that physical activity represents another risk factor for GERD. However, available evidence indicates that a positive association between exercise and GERD is present in vigorous, but not in moderate, exercise^[41,132,133]. In fact, Clark *et al*^[134] have reported that running, cycling and weight lifting increase GERD in asymptomatic volunteers. Furthermore, these authors have found that specific types of exercise are more likely to induce reflux symptoms, with running and resistance exercises being more refluxogenic than cycling.

Similar results have been obtained by Peters^[135], in addition, this author has found increased reflux using high-carbohydrate sport drinks with respect to water, which demonstrates a possible role of sport drinks in facilitating reflux symptoms. Therefore, it seems that a hierarchy of exercises in inducing reflux symptoms exists^[134]. However, there is no general agreement with respect to the mechanism by which vigorous exercise

Table 4 Physical activity and GERD-related clinical manifestations

Author	Year	Country	Study design	Population size	Method of data collection	Association/Effect
Vigorous/agonistic exercise						
Clark <i>et al</i> ^[134]	1989	USA	Crossover	12 asymptomatic volunteers	pH-monitoring, 1 h exercise period (bicycling, running and weight routine)	Yes
Schoeman <i>et al</i> ^[132]	1995	Australia	Randomized controlled crossover	10 healthy subjects	Perfused sleeve sensor for 24 h during moderate physical activity, rest and sleep, standardized meals, and standardized exercise	Yes
Peters <i>et al</i> ^[135]	2000	Netherlands	Randomized controlled crossover	7 males triathletes	pH-monitoring, 50 min of running, cycling and supplementation of conventional sport drinks and tap water	Yes
Collings <i>et al</i> ^[128]	2003	USA	Case series	30 athletes	pH-monitoring, evaluation of clinical symptoms during standardized exercise	Yes
Pandolfino <i>et al</i> ^[129]	2004	USA	Case-control	20	pH-monitoring, 60 min of exercise (running and resistance exercise), upper endoscopy and manometry	Yes
Nandurkar <i>et al</i> ^[41]	2004	USA	Population-based, nested case-control	211	GERD, energy expenditure, dietary intake questionnaires	Yes
Ravi <i>et al</i> ^[130]	2005	Ireland	Randomized controlled crossover	135	Esophageal manometry and pH-monitoring before, during and immediately after moderate exercise	Yes
Physical activity at work/postprandial exercise						
Zheng <i>et al</i> ^[43]	2007	Sweden	Swedish Twin Registry	27717	Questionnaire, telephone interview	Yes
Emerenziani <i>et al</i> ^[140]	2005	Belgium-Germany	Clinical trial	37 GERD pz	pH-impedance-monitoring, upper endoscopy, scintigraphic gastric emptying	Yes

induces reflux. Exercise may alter esophageal motility and worsen symptoms of the upper gastrointestinal tract^[136]. Clark *et al*^[134] have speculated that “body agitation” may be important in inducing reflux. Soffer *et al*^[137] have focused on a decreased duration, amplitude and frequency of esophageal contractions with increasing exercise intensity. This suggestion has not been confirmed by Choi *et al*^[138] who reported increased frequency, but not duration or amplitude, of peristaltic contractions. Furthermore, Pandolfino *et al*^[129] have suggested that the anatomical compromise of the esophagogastric junction, as a consequence of frequent abdominal straining associated with strenuous exercise, may predispose to exercise-induced reflux. Other studies have suggested that GERD may be increased in athletes because of a decreased gastrointestinal blood flow, alterations of hormone secretion, changes in the motor function of the esophagus and the ventricle, and constrained body position during exercise^[139].

All these studies suggest that a specific physical activity, i.e. an agonistic activity, plays a possible pathogenetic role in inducing GERD symptoms. However, these results should not be extended to normal physical activity, which has been demonstrated to have a protective effect against GERD^[99] (Table 4). In particular, in a large population-based study^[99], a protective effect of physical activity was observed, documenting a correlation between the number of exercise sessions lasting at least 30 min and a decreased risk of GERD symptoms (OR, 0.5; 95% CI, 0.4-0.7). Therefore, a mechanism of an exercise-strengthened antireflux barrier, possibly constituted by striated muscle was suggested. The same results were produced by Nocon *et al*^[39], who have also found that subjects with typical GERD symptoms are physically less active than those without symptoms.

Furthermore, in their monozygotic co-twin study, Zheng *et al*^[43] have provided evidence that physical activity at work increases the risk of GERD symptoms, whereas physical activity at leisure time decreases this risk. The authors have suggested that physical activity at work might be linked with postprandial exercise, which has been found to be a risk factor for the development of GERD symptoms^[140]. Indeed, physical exercise at leisure time is predominantly performed at times without a feeling of stomach fullness and is, therefore, most unlikely to be a reflux-provoking postprandial exercise^[140].

In conclusion, the relationship between exercise and GERD is also controversial. It may be a consequence of differences in the populations studied (age, race), evaluation of exercise (short-term, long-term), assessment of physical activity (different questionnaires) and diagnosis of the disease (symptom scale or pH-metry). However, mild routine physical activity in association with diet modifications, i.e. a diet rich in fiber and poor in fat, seems to be advisable to prevent reflux symptoms.

CONCLUSION

There is sufficient evidence to support the relationship between being obese/overweight and GERD, expressed as specific symptoms and endoscopic features. Furthermore, available evidence suggests that controlled weight loss (by diet or surgery) is able to induce a significant improvement in GERD symptoms and/or in GERD clinical-endoscopic manifestations. Definitive data still do not exist regarding the association between dietary behavior, mainly in terms of specific dietary components and GERD manifestations. Moderate

physical activity seems beneficial, while vigorous activity may be dangerous in predisposed individuals. However, owing to the evidence that incorrect dietary habits and the absence of regular physical activity represent important risk factors for the development of the so-called “non-communicable diseases”^[141], lifestyle changes are recommended in patients with or at high risk for GERD. According to the recent proposal by a panel of international experts of a new algorithm for GERD management^[142], life-style factors (i.e. meal size and timing, not lying down after a meal or lying down where the head is in a non-elevated position, not smoking, not consuming alcohol, not eating heavily spiced or fatty food and having a physically active life) are important instruments for the overall management of GERD. Additional clinical studies are required.

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REVIEW

Reactive oxygen species: A double-edged sword in oncogenesis

Jin-Shui Pan, Mei-Zhu Hong, Jian-Lin Ren

Jin-Shui Pan, Jian-Lin Ren, Division of Gastroenterology, Zhongshan Hospital Xiamen University, Xiamen 361004, Fujian Province, China; Gastroenterology Institute of Xiamen University, Xiamen 361004, Fujian Province, China; Gastroenterology Center of Xiamen, Xiamen 361004, Fujian Province, China

Mei-Zhu Hong, Division of Infectious Diseases, the 174th Hospital of PLA, Xiamen 361003, Fujian Province, China

Author contributions: Pan JS and Hong MZ contributed equally to this work; Pan JS and Hong MZ generated the idea, wrote the paper; Ren JL proofread the paper.

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Correspondence to: Jian-Lin Ren, Professor, Division of Gastroenterology, Zhongshan Hospital Xiamen University, Xiamen 361004, Fujian Province, China. jianlinr@msn.com

Telephone: +86-592-2993170 Fax: +86-592-2993170

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Abstract

Reactive oxygen species (ROS) are molecules or ions formed by the incomplete one-electron reduction of oxygen. Of interest, it seems that ROS manifest dual roles, cancer promoting or cancer suppressing, in tumorigenesis. ROS participate simultaneously in two signaling pathways that have inverse functions in tumorigenesis, Ras-Raf-MEK1/2-ERK1/2 signaling and the p38 mitogen-activated protein kinases (MAPK) pathway. It is well known that Ras-Raf-MEK1/2-ERK1/2 signaling is related to oncogenesis, while the p38 MAPK pathway contributes to cancer suppression, which involves oncogene-induced senescence, inflammation-induced cellular senescence, replicative senescence, contact inhibition and DNA-damage responses. Thus, ROS may not be an absolute carcinogenic factor or cancer suppressor. The purpose of the present review is to discuss the dual roles of ROS in the pathogenesis of cancer, and the signaling pathway mediating their role in tumorigenesis.

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Key words: p38 mitogen-activated protein kinases; Reactive oxygen species; Signal transduction; Tumorigenesis

Peer reviewer: Hartmut Jaeschke, Professor, Liver Research Institute, University of Arizona, College of Medicine, 1501 N Campbell Ave, Room 6309, Tucson, Arizona 85724, United States

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INTRODUCTION

Reactive oxygen species (ROS) are molecules or ions formed by the incomplete one-electron reduction of oxygen. These reactive oxygen intermediates include singlet oxygen, superoxides, peroxides, hydroxyl radical, and hypochlorous acid. They contribute to the microbicidal activity of phagocytes, regulation of signal transduction and gene expression, and induce oxidative damage to nucleic acids, proteins, and lipids. Peroxidation by ROS alters the amounts of unsaturated fatty acids and proteins in the cell membrane and thus affects membrane fluidity. In addition, with aging, humans tend to show an increased affectability of lipid peroxides caused by ROS^[1]. Recent research has indicated that ROS also play a critical role in the energy dysfunction of mitochondria caused by ethanol-induced gastric mucosa injury^[2]. In addition, oxidative damage caused by ROS and other free radicals is involved in a number of pathological conditions including cancer. Data presented herein is consistent with this opinion. Yagoda *et al*^[3] found erastin interacted with voltage-dependent anion channel proteins to induce mitochondrial dysfunction, release of oxidative species and, ultimately, non-apoptotic, oxidative cell death. This process has a degree of selectivity for cells with activated Ras-Raf-MEK signaling. ROS production also involves the induction of autophagy, which contributes to caspase-independent macrophage cell death^[4]. ROS, produced in the redox cycle, contribute to p53 mutations, which are dominated by G-to-T transversions. These mutations are suppressed by ROS attenuators^[5,6]. The mutations of p53, a well characterized tumor suppressor, are believed to relate to carcinogenesis. Since oxidative stress comprehensively

damages cells and tissues, it is reasonable that factors which induce ROS would contribute to the occurrence and development of tumors, while antioxidant agents that scavenge ROS may inhibit this process. To the best of our knowledge, the former inference is consistent with previous reports^[7,8], however, there is little supportive evidence for the latter^[9,10]. Vitamins C and E, two reducing agents and antioxidants, show no additional benefit in the chemoprevention of gastric cancer^[11,12]. Inadequate dose, heterogeneous research, poor compliance and multiple effects of antioxidants may lead to this paradox. Is there anything more to be elucidated on this subject?

REGULATION OF ROS PRODUCTION BY RAS

Oxidative stress and Ras activation lead to the production of ROS^[13]. Introduction of ROS by Ras may occur at the transcription level. GATA-6 is a component of the specific protein-DNA complexes at the nicotinamide adenine dinucleotide phosphate oxidase (Nox) 1 promoter, and is able to trans-activate the Nox1 promoter. GATA-6 is phosphorylated at serine residues by MEK-activated extracellular signal-regulated kinase (ERK), which enhances GATA-6 DNA binding. The site-directed mutation of the consensus ERK phosphorylation site (PYS(120)P to PYA(120)P) of GATA-6 abolishes its trans-activation activity, suppressing the growth of CaCo-2 cells. By MEK-ERK-dependent phosphorylation of GATA-6, oncogenic Ras signaling enhances the transcription of Nox1^[14]. A regulatory subunit, Rac, of the NADPH oxidase complex also involves the regulation of ROS^[15]. Other factors that regulate the production of ROS will not be discussed here.

ROS INVOLVE TUMORIGENESIS THAT RELATES TO THE RAS-RAF-MEK-ERK PATHWAY

Growth factors, cellular stress, and γ radiation stimulate oncogenic Ras-Raf-MEK signaling, which plays a crucial role in tumorigenesis. As an important mediator of physiological and pathological signal-transduction pathways, ROS is also involved in Ras-Raf-MEK signaling (Figure 1). The functions of ROS in tumorigenesis relating to this pathway include the following. (1) In cells with activated Ras-Raf-MEK signaling, released ROS cause non-apoptotic, oxidative cell death, as previously mentioned^[3], and the Ras-ERK pathway is critical in mediating protection against apoptotic cell death induced by increased oxidative stress^[16]. (2) The activity of the ROS-generating enzyme Nox1 is required for vascular endothelial growth factor (VEGF), a potent stimulator of tumor angiogenesis. However, if extracellular signal-regulated kinase (ERK)-dependent phosphorylation of the transcription factor

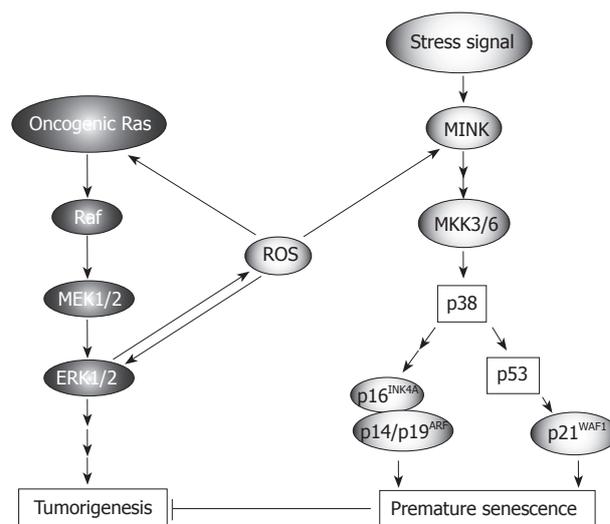


Figure 1 Roles of ROS in the Ras-Raf-MEK-ERK signaling and p38 pathway (Modified from Ref. 31, with permission).

Sp1 and Sp1 binding to a VEGF promoter is inhibited, this activity does not occur. Nox1 mediates oncogenic Ras-induced upregulation of VEGF and angiogenesis by activating Sp1 through Ras-ERK-dependent phosphorylation of Sp1^[8]. (3) Ras (p19) interaction with p73 β , a structural and functional homolog of p53, amplifies p73 β -induced apoptotic signaling responses including Bax mitochondrial translocation, cytochrome c release, increased production of ROS and loss of mitochondrial transmembrane potential. After taxol treatment, endogenous expression of Ras and p73 β significantly increase, and taxol-enhanced endogenous p73 β transcriptional activities are further amplified by p19, which markedly increases cellular apoptosis in the p53-null SAOS2 cancer cell line^[17]. (4) In human U937 monocytes, hydrogen peroxide (H₂O₂) evokes Ca²⁺ influx through TRPM2 to activate Ca²⁺-dependent tyrosine kinase Pyk2 and amplify ERK signaling *via* Ras GTPase. TRPM2 Ca²⁺ influx controls the ROS-induced signaling cascade responsible for chemokine production, which aggravates inflammation^[18].

In contrast, the released ROS have complicated effects on Ras-Raf-MEK signaling, which may occur on several levels (Figure 1). ROS directly enhance the activation of Ras^[19], and augment ERK1/2^[20]. Melatonin, a natural antioxidant, inhibits the activation of Ras in H4IIE hepatoma cells^[21]. Generation of ROS is required for Ras transformation phenotypes including anchorage-independent growth, morphological transformation, and tumorigenesis^[22]. In diabetes-related angiogenesis of the retina, activation of H-Ras and its downstream signaling pathway may be under the control of superoxide, and H-Ras activation in diabetes can be prevented by inhibiting superoxide accumulation^[23]. H₂O₂ activates H-Ras and its downstream signaling pathway, including Raf-1 and phosphorylation of p38 MAP kinase. Inhibition of superoxide significantly attenuates glucose-induced activation of H-Ras, Raf-1 and p38 MAP kinase^[23]. PI3K is a mediator in the

E-Ras-PI3K-Akt signaling pathway, which leads to tumor-like properties in embryonic stem cells. AKR1C2 and AKR1C3 mediated prostaglandin D(2) metabolism augments the PI3K/Akt proliferative signaling pathway in human prostate cancer cells^[24]. Activated Ras is usually associated with cancer, but it also produces paradoxical premature senescence in primary cells by inducing ROS followed by the accumulation of tumor suppressors p53 and p16^{INK4A}^[25].

ROS INVOLVE TUMOR SUPPRESSION VIA THE P38 PATHWAY

Oncogenic Ras sequentially activates MEK, p38 and two p38 downstream kinases, MAPK-activated protein kinase2 (MK2) and p38 regulated/activated protein kinase (PRAK), which in turn suppress Ras-induced cell proliferation by blocking activation of Jun N-terminal kinase (JNK). Increased intracellular levels of ROS, induced by the Ras-Raf-MEK-ERK signaling cascade, may mediate the activation of the p38 pathway and act as an intermediate signal between the MEK-ERK and MKK3/6-p38 pathways (Figure 1). On the one hand, the activation of p38 mitogen-activated protein kinase (MAPK) is a prerequisite for ROS-mediated functions such as apoptotic cell death in cancer cells^[26], and adrenal steroidogenesis^[27]. On the other hand, inhibiting or scavenging ROS may attenuate the activation of p38-dependent pathways^[28,29]. Since Ras induces the production of ROS and the latter activates p38, a conclusion can be derived theoretically that the inhibition of Ras may weaken the tension of p38. This inference is supported by research which involved H4IIE hepatoma cells^[21]. However, in some cases, it is not certain that increased intracellular ROS should enhance the activation of p38^[30]. The p38 MAPK pathway negatively regulates cell proliferation and tumorigenesis. The involvement of the p38 pathway in the regulation of cellular processes that directly contribute to tumor suppression includes oncogene-induced senescence (OIS), replicative senescence, contact inhibition and DNA-damage responses, which have been discussed in detail^[31]. Recently, we found that p38 also plays an important role in inflammation-induced cellular senescence^[32], which is believed to be a process related to tumor suppression. Several reports have shown that ROS mediate OIS *via* p38-dependent pathways^[33-35]. The accumulation of intracellular ROS induced by oncogenic Ras is ERK-dependent during the activation of p38 and the induction of senescence. After sensing the oxidative stress induced by activated Ras, p38 directs cells to undergo apoptosis^[36]. Human cancer cell lines with high ROS levels display enhanced tumorigenicity and impaired p38 α activation by ROS. p38 α has also been reported to antagonize oncogenic transformation induced by activated N-Ras in murine fibroblasts^[37] and by activated K-Ras in colon cancer cell lines^[38]. Activated components of the p38 pathway phosphorylate multiple residues on p53, including Ser33 and Ser46 (by p38),

Ser37 (by PRAK), and possibly others, leading to increased transcriptional activity of p53 and induction of a transcriptional target of p53 and p21^{WAF1}^[31]. Through an unknown mechanism, activated p38 also induces the expression of p16^{INK4A} and p14/p19^{ARF}, which, together with the p53-p21^{WAF1} cascade, cause premature senescence that serves as a tumor-suppressing defense mechanism both in cell culture and *in vivo*^[31]. Ras also involves senescence, in which Seladin-1 acts as a key mediator of oxidative stress^[39]. Seladin-1 has previously been implicated in Alzheimer's disease and cholesterol metabolism. Following oncogenic and oxidative stress, Seladin-1 binds to the p53 amino terminus and displaces E3 ubiquitin ligase Mdm2 from p53, thus resulting in p53 accumulation. Ablation of Seladin-1 causes the bypass of Ras-induced senescence in rodent and human fibroblasts, and allows Ras to transform these cells. Wild-type Seladin-1, but not mutants that disrupt its association with either p53 or Mdm2, suppresses the transformed phenotype. The same mutants are also inactive in directing the p53-dependent oxidative stress response^[39]. p38 related replicative senescence, contact inhibition and DNA-damage responses will not be discussed here, refer to Ref. 31.

ROS INVOLVE APOPTOSIS THAT RELATES TO THE P38 PATHWAY

Numerous researchers have shown that ROS relate to apoptosis that is processed through the mitochondrial pathway, which depends on the activation of p38 (Figure 2)^[40-44]. Apoptosis signal-regulating kinase 1 (ASK1) is an evolutionarily conserved mitogen-activated protein 3-kinase that activates both JNK and p38 MAPK, which may also be triggered by ROS^[45-49]. However, activation of MAPKs (JNK, p38, ERK) is differentially regulated by cleavage size (40 kDa and 36 kDa) of mammalian sterile 20-like kinase 1, which is controlled by caspase-7 and -3^[50]. ASK1-induced and ROS-dependent activation of MAPKs is crucial for apoptosis^[43,51], and for TLR4-mediated mammalian innate immunity^[52]. In the case of oxidative stress, a positive feedback may form in the ASK1-p38-TNF- α pathway, which enhances ROS-mediated apoptosis (Figure 2). Ask1 activates both JNK and p38 MAPK, then the activated p38 translocates into the nucleus and stimulates the expression of MK2. After moving out of the nucleus, MK2 increases TNF- α production. On the other hand, enhanced TNF- α and ROS activate ASK1 activity^[46], which leads to the activation of JNK. JNK abrogates Bcl-2, which is believed to be a protector away from mitochondria-related apoptosis, although Bcl-2 may manifest opposing phenotypes in text of interacting with other proteins^[53]. In addition, this positive feedback is required for ROS-mediated apoptosis (Figure 2). Functional analyses have revealed that the initial ROS-independent activations of JNK, Bax, and caspase-3 are not sufficient for cell death, and thus, should be re-activated by ROS in order to kill the cells^[54]. ROS do

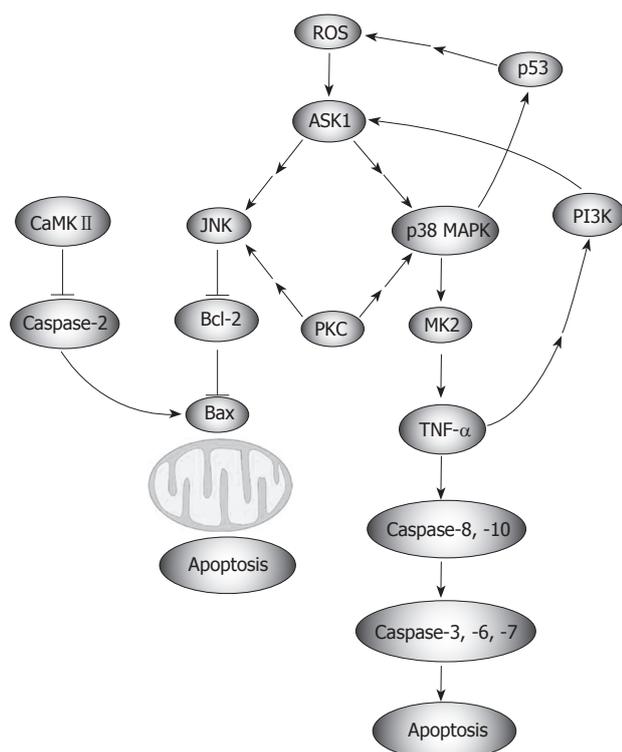


Figure 2 ROS-mediated apoptosis.

not simply mediate the lethal action of γ radiation, but actually amplify it by forming a feedback loop between a downstream effector (caspase) and the upstream initiation signals leading to the activation of JNK. This role of ROS appears to allow Bcl-2 to block the signaling events, which are initially induced upstream^[54]. p38 α MAPK contributes to the further activation of p53, which also leads to a positive feedback loop, p38 α MAPK/p53. The p53/ROS/p38 α MAPK cascade is essential for cisplatin-induced cell death in HCT116 cells, and the subsequent p38 α /p53 positive feedback loop strongly enhances the initial p53 activation^[55]. Of interest, p38 may stimulate indirectly the production of ROS *via* p53. Glioma pathogenesis-related protein 1 (GLIPR1), a novel p53 target gene, is down-regulated by methylation in prostate cancer and has p53-dependent and -independent pro-apoptotic activities in tumor cells. Overexpression of GLIPR1 in cancer cells leads to suppression of colony growth and induction of apoptosis. Mechanistic analysis indicates that GLIPR1 up-regulation increases the production of ROS, leading to apoptosis through activation of the JNK signaling cascade^[56]. However, in p38-related apoptosis that is independent of ROS generation, JNK seems to execute a reverse function. Inhibition of JNK by SP600125 significantly enhanced apoptosis^[57].

Via regulation of MAPKs, the protein kinase C (PKC) δ -mediated pathway also involves ROS related apoptosis. As a tentative stimulator of p38, JNK1/2 and MEK/ERK signaling^[58], PKC- δ regulates cell apoptosis and survival in diverse cellular systems. Knock down of PKC- δ suppresses p38 MAPK phosphorylation. *Via* p38 MAPK, activated PKC- δ

regulates the phosphorylation of heat shock protein (HSP) 27. Attenuated phosphorylation of HSP27 correlates with tumor progression in patients with hepatic cell cancer^[59]. PKC- δ translocates to different subcellular sites in response to apoptotic stimuli. The localization of PKC- δ differentially affects the activation of downstream signaling pathways. PKC- δ -cytosol increases the phosphorylation of p38, whereas PKC- δ -nucleus increases c-JNK phosphorylation. Moreover, p38 phosphorylation plays a role in the apoptotic effect of PKC- δ -cyto, whereas c-JNK activation mediates the apoptotic effect of PKC- δ -Nuc^[60]. Recent evidence has shown that calcium/calmodulin (Ca^{2+} /CaM)-dependent protein kinase II (CaMK II) activity is also enhanced by pro-oxidant conditions. CaMK II is activated by angiotensin II-induced oxidation, leading to apoptosis in cardiomyocytes both *in vitro* and *in vivo* (Figure 2)^[61].

Besides apoptosis, ROS also relate to proliferation. In mice lacking Nrf2 transcription factor, oxidative stress-mediated activation of p38, Akt kinase and downstream targets is impaired, resulting in enhanced death and delayed proliferation of hepatocytes^[62]. p38 MAPK, p53, and p21 also act as molecular mediators on the way from increased ROS levels to the observed growth arrest^[63].

CONCLUSION

Besides their well-known roles, recent studies have demonstrated additional functions of ROS in tumorigenesis. However, the evidence comes from studies performed in cell culture, in addition to data from human tumors. In addition, these cell lines are generally kept in room air, whereas hyperoxic oxygen levels may favor enhanced ROS formation, which is well known. The relevance of ROS in all these events *in vivo*, especially in humans, is not clear. ROS seem to have dual roles in tumorigenesis, cancer promoting and cancer suppressing. ROS participate in both Ras-Raf-MEK1/2-ERK1/2 signaling and the p38 MAPK pathway. However, these two pathways may have inverse functions in tumorigenesis. The former is related to cancer promotion, whereas the latter is associated with a variety of cellular responses such as OIS, replicative senescence, contact inhibition and DNA-damage responses. Thus, regarding ROS as an absolute "carcinogenic factor" or "cancer suppressor" seems to be inappropriate. It seems that more extensive investigations are needed to determine the integrity of ROS in human cancer development. Two aspects of research remain to be carried out in the future. Firstly, we should determine whether ROS directly mediate the Ras-Raf-MEK1/2-ERK1/2 and p38 MAPK signaling pathways, or whether other mediators are needed. Secondly, the definite shunting mechanism, which controls the steering from triggering Ras-Raf-MEK1/2-ERK1/2 signaling to triggering p38 MAPK signaling and *vice versa*, should be determined. The relationship between ROS and p38-pathway-mediated OIS is of particular interest because several reports indicate that part of the OIS pathway is intact at least in certain cancer cells, and that senescence

responses improve the outcome of chemotherapy. Drugs which artificially trigger senescence in tumor cells will thus improve cancer treatment^[31]. Studies on the shunting mechanism would facilitate research on the roles of ROS in tumorigenesis, and could shed light on drug discovery.

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Laser capture microdissection and genetic analysis of carbon-labeled Kupffer cells

Stephan Gehring, Edmond Sabo, Maryann E San Martin, Elizabeth M Dickson, Chao-Wen Cheng, Stephen H Gregory

Stephan Gehring, Maryann E San Martin, Elizabeth M Dickson, Chao-Wen Cheng, Stephen H Gregory, Department of Medicine, Rhode Island Hospital and The Warren Alpert Medical School of Brown University, Providence, RI 02903, United States

Edmond Sabo, Department of Pathology, Rhode Island Hospital and The Warren Alpert Medical School of Brown University, Providence, RI 02903, United States

Stephan Gehring, Zentrum für Kinder- und Jugendmedizin der Universitätsmedizin der Johannes Gutenberg-Universität, 55101 Mainz, Germany

Author contributions: Gehring S, San Martin ME developed the LCM isolation method of Kupffer cells; Gehring S, San Martin ME, Dickson EM, Cheng CW performed the experiments; Sabo E analyzed the microarray data; Gregory SH designed and supervised the study; Gehring S and Gregory SH wrote the manuscript.

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Correspondence to: Dr. Stephen H Gregory, Department of Medicine, Rhode Island Hospital and The Warren Alpert Medical School of Brown University, 432 Pierre M. Galletti Building, 55 Claverick Street, Providence, RI 02903, United States. sgregory@lifespan.org

Telephone: +1-401-4447369 Fax: +1-401-4447524

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Abstract

AIM: To develop a method of labeling and microdissecting mouse Kupffer cells within an extraordinarily short period of time using laser capture microdissection (LCM).

METHODS: Tissues are complex structures comprised of a heterogeneous population of interconnected cells. LCM offers a method of isolating a single cell type from specific regions of a tissue section. LCM is an essential approach used in conjunction with molecular analysis to study the functional interaction of cells in their native tissue environment. The process of labeling and acquiring cells by LCM prior to mRNA isolation can be elaborate, thereby subjecting the RNA to considerable degradation. Kupffer cell labeling is achieved by

injecting India ink intravenously, thus circumventing the need for *in vitro* staining. The significance of this novel approach was validated using a cholestatic liver injury model.

RESULTS: mRNA extracted from the microdissected cell population displayed marked increases in colony-stimulating factor-1 receptor and Kupffer cell receptor message expression, which demonstrated Kupffer cell enrichment. Gene expression by Kupffer cells derived from bile-duct-ligated, *versus* sham-operated, mice was compared. Microarray analysis revealed a significant (2.5-fold, q value < 10) change in 493 genes. Based on this fold-change and a standardized PubMed search, 10 genes were identified that were relevant to the ability of Kupffer cells to suppress liver injury.

CONCLUSION: The methodology outlined herein provides an approach to isolating high quality RNA from Kupffer cells, without altering the tissue integrity.

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Key words: Kupffer cells; India ink; Laser capture microdissection; Bile duct ligation; DNA microarray

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INTRODUCTION

Kupffer cells, resident tissue macrophages that line the liver sinusoids, play a key role in modulating inflammation in a number of experimental models

of liver injury^[1]. Since Kupffer cells represent only a small portion of the entire liver cell population, greatly outnumbered by the parenchymal cells, Kupffer cell isolation faces major technical obstacles. Initial Kupffer cell preparations were heavily contaminated with other cell types or, if sufficiently purified, were only a small fraction of the total cell number and therefore not truly representative. Preparation improved drastically with the introduction of techniques that involved perfusion of the liver with collagenase and pronase^[2].

After enzymatic digestion of the liver, the non-parenchymal liver cells are purified by density gradient centrifugation or centrifugal elutriation^[3]. Neither of these methods is able to separate Kupffer cells from other non-parenchymal cell types since cell size and density exhibit considerable overlap^[4]. Furthermore, Kupffer cell purification can be achieved by adherence to plastic^[5], or positive selection using specific antibodies and magnetic beads^[6].

Laser capture microdissection (LCM) circumvents many of the limitations inherent in conventional isolation methods. LCM was created at the National Institutes of Health, Bethesda, MD, USA and further developed by Arcturus Engineering, Inc., Mountain View, CA, USA^[7]. The principals of LCM entail overlaying the tissue section with a transparent ethylene vinylacetate thermoplastic film. At the point of interest, the film is melted onto the tissue surface with a laser integrated with the microscope optics and then removed, capturing sample areas as small as 3-5 μm from intact tissue sections^[8].

For the analysis of gene expression, an important challenge for LCM remains the specific and rapid labeling of the target cell population, thus minimizing the degradation of RNA. In this regard, classic immunohistochemical staining methods for visualizing Kupffer cells are not generally applicable. In the present study, a well-established and efficient method using India ink to label Kupffer cells *in vivo* was described^[9]. Kupffer cells are mainly located in the periportal areas, where they have ready access to pathogens and particulate antigens entering the liver with portal-venous blood^[10,11]. In contrast to liver sinusoidal endothelial cells (LSECs), which mainly ingest soluble materials *via* pinocytosis, Kupffer cells take up particulate material *via* phagocytosis^[12]. It is relevant to note, therefore, that while colloidal gold ≤ 100 nm diameter particle size is internalized almost exclusively by LSECs, colloidal carbon is taken up primarily by Kupffer cells. This apparent contradiction is explained by the fact that blood platelets bind carbon and the platelet-carbon complexes are subsequently phagocytosed by Kupffer cells^[9]. Importantly, the phagocytic capacity of Kupffer cells is not altered by the ingestion of these complexes^[13].

The ability to isolate carbon-labeled Kupffer cells from intact tissue sections using LCM offers an attractive approach to studying gene expression under diverse conditions. The aim of this study was to validate this approach and to apply it to an animal model of cholestatic liver injury.

MATERIALS AND METHODS

Animals

Wild-type female, C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and used at 8-12 wk of age. The animals were treated in accordance with NIH publications entitled "Principles for Use of Animals" and "Guide for the Care and Use of Laboratory Animals." The mice were housed in well-ventilated rooms maintained at 22°C and an alternating 12-h light and dark cycle; food and water were provided *ad libitum*.

Common bile duct ligation

Ligation of the common bile duct was performed as previously described^[6]. The abdomen of mice under deep anesthesia was disinfected with 70% ethanol. A midline upper abdominal incision was made and the abdominal wall was retracted. The common bile duct was identified, isolated and double-ligated with #4-0 braided silk sutures and divided in between. The fascia and skin of the midline abdominal incision were closed with #6-0 braided silk sutures. Control mice underwent sham operations in which the common bile duct was exposed, but not ligated.

Kupffer cell staining

Frozen, 6- μm thick liver sections were cut with a cryostat (Leica, Wetzlar, Germany). Slides were warmed to room temperature for 30 min, fixed by immersion in ice cold acetone (4°C) for 5 min, and air dried for 30 min. After rinsing the slides three times in PBS to remove the tissue freezing matrix, non-specific binding was blocked with 5% normal rabbit serum and 1% BSA for 60 min. This step was followed by 15 min of avidin blocking and 15 min of biotin blocking (Vector Laboratories, Burlingame, CA, USA). After each step, the slides were rinsed in PBS. The slides were stained first with a 1/10 dilution of rat IgG_{2a} anti-mouse F4/80 (a pan-specific macrophage marker; eBioscience, San Diego, CA, USA) monoclonal antibody for 60 min. Subsequently, the slides were washed three times for 10 min with PBS and then incubated for 45 min on a shaker with a 1/50 dilution of biotinylated goat anti-rat polyclonal antibody (Vector Laboratories). After rinsing the slides, pre-diluted streptavidin-Cy3 (Invitrogen, Carlsbad, CA, USA) was applied to the tissue sections and incubated for 30 min; the slides were then dried and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). All the steps were performed at room temperature.

Processing of the liver tissue and LCM

Mice were inoculated *iv* *via* the tail vein with 200 μL India ink diluted 1:100 in saline 1 d prior to surgery. Immediately following euthanasia, the livers were perfused *in situ* with 20 mL Hank's buffered salt solution to eliminate blood cells, and dissected. Tissue wedges were flash frozen in Tissue-Tek[®] Optimum Cutting Temperature[™] (OCT) compound and stored at -80°C.

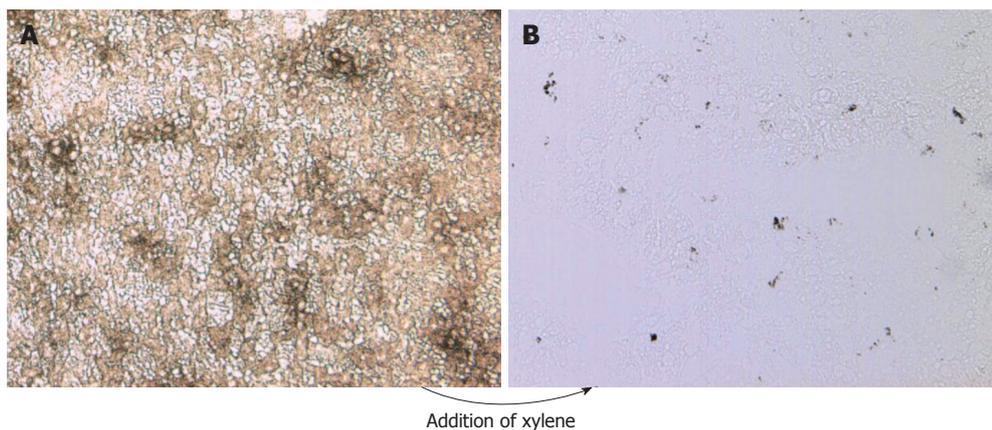


Figure 1 India-ink-positive cells revealed in tissue section overlaid with xylene. A non-stained liver section derived from a mouse inoculated iv with India ink was not mounted (A), or was overlaid with xylene (B).

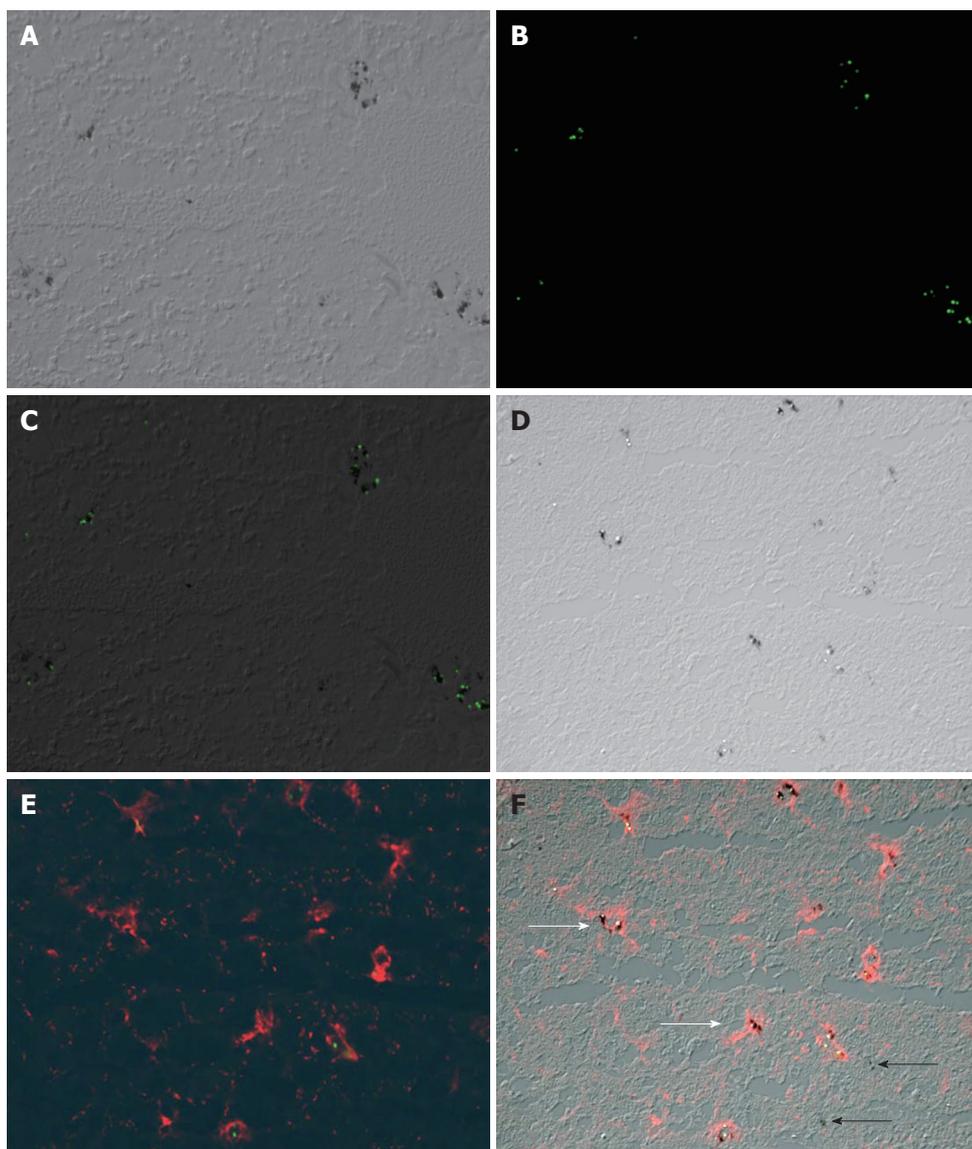


Figure 2 Carbon particles co-localize with ingested fluorescent latex beads and macrophages stained immunohistochemically. A-C: Kupffer cells lining the liver sinusoids were identified by the presence of carbon particles in mice inoculated iv with India ink. Fluorescent latex beads mixed with India ink co-localized with the carbon particles. D-F: A carbon-particle-containing liver section was stained sequentially with streptavidin-conjugated anti-pan macrophage marker F4/80 and biotin-Cy3, and visualized by fluorescence microscopy. An accumulation of carbon particles stained intensely with F4/80 (white arrows), whereas single carbon spots (black arrows) were not stained with F4/80.

Cryostat sections (6 μ m) were prepared under RNase-free conditions at -20°C , fixed immediately in acetone for 2 min at 4°C , dehydrated by sequential immersion for 30 s in 75%, 95% and 100% ethanol, immersed for 2 min in xylene, and then air dried. For each cutting session, new blades, single use staining jars, and fresh (RNase free) solutions were used.

LCM was performed using an AutoPix Automated

Laser Capture Microdissection System equipped with an infrared diode laser according to the protocols and methods provided by the manufacturer (Arcturus Engineering, Santa Clara, CA, USA). Air-dried slides were placed under the microscope of the LCM system and one drop of xylene was applied to visualize the carbon-containing Kupffer cells (Figure 1). A static image was taken and, after the xylene evaporated,

the liver sections were overlaid with thermoplastic membranes mounted on transparent, CapSure Macro LCM Caps (Arcturus). The carbon-labeled (Kupffer) cells were captured by laser activation (12–15 μm laser spot size, 60–80 mW power, 3.0 ms duration) and focal melting of the membrane. At least 300 cells were captured on each cap, and three different caps (equivalent to approximately 1000 cells) were prepared for each sample.

RNA extraction and purification

Cells captured on thermoplastic membranes were immersed in Lysis Buffer (RLT) (Qiagen Inc., Valencia, CA, USA). Subsequently, the RNA was extracted, purified using an RNeasy Micro Kit (Qiagen), and quantified using Lab-on-Chip technology (Agilent Technologies, Palo Alto, CA, USA). Intact 18S and 28S rRNA confirmed the integrity of the RNA extracted. RNA representative of cells constituting the whole liver was extracted from an entire (scraped) tissue section.

RNA amplification and cRNA labeling

Purified total RNA obtained from the microdissected cells was subjected to 1.5 rounds of linear amplification using T7 bacteriophage RNA-polymerase-driven *in vitro* transcription, with materials and protocol provided in the RiboAmpTM HS RNA Amplification Kit (KIT0205; Arcturus). The final amplification and labeling of dsDNA product was performed using the Enzo BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Framingdale, NY, USA).

Microarray hybridization

Microarray analysis was performed using the GeneChip Mouse Genome[®] 430 2.0 array and the recommended instruments (GeneChip Scanner 3000), with reagents and protocols provided by the supplier (Affymetrix, Santa Clara, CA, USA). This GeneChip covers the transcribed mouse genome on a single array with 45 000 probe sets that analyze the expression level of over 39 000 transcripts.

Bioinformatics and data mining

Each group consisted of three samples; each sample was pooled from three sets of captured material derived from individual, sham-operated or bile-duct-ligated mice. The expression signals were normalized using the standardization and normalization of microarray data (SNOMAD) program^[14]. Concordantly absent expression signals were removed from analysis. A *t* test was used to perform paired comparisons of the gene expression levels between sham-operated and bile-duct-ligated animals. A 5% false discovery rate (FDR) correction controlled for multiple comparisons. The *q* value of a test measured the minimum FDR rate incurred when calling that test significant. *q* values were computed from the unadjusted *P* values, using the Q-VALUE program written by Storey and Tibshirani^[15]. Significant, differentially expressed genes were grouped into functional categories using the GenMAPP 2 (<http://www.GenMAPP.org>) and MAPPFinder

programs by integrating the annotations of the Gene Ontology Project (<ftp://ftp.geneontology.org/go/gene-associations>)^[16,17].

Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR)

Purified RNA extracted from microdissected Kupffer cells was treated with DNase (10 U DNase I/ μg total RNA) and reverse transcribed with SensiScript Reverse Transcriptase according to the protocols provided by the supplier (Qiagen). Quantitative gene expression analysis was performed using the MX4000 Multiplex Quantitation QPLR System (Stratagene, La Jolla, CA, USA) and SYBR green technology. QuantiTect SYBR Green PCR Master Mix (12.5 μL ; Qiagen) was mixed in 96-well optical plates with an equal volume of RNase-free water that contained 0.6 $\mu\text{mol/L}$ of the forward and reverse primers, and cDNA corresponding to 70 ng of total RNA input. The plates were heated for 2 min at 50°C, and then 15 min at 95°C to activate the HotStart *Taq* DNA polymerase. Subsequently, 45 cycles consisting of 30 s at 95°C followed by 1 min at 55°C and 30 s at 72°C were run. To verify the generation of single PCR products, melting curves were constructed by heating the samples to 95°C with a ramp time of 20 min at the end of the run. 18S rRNA was used as the housekeeping standard. The threshold cycle (i.e. the number of PCR cycles required in order for the fluorescent signal to reach a fixed intensity) was determined and the number of RNA copies was calculated from standard curves. The mean mRNA copies/ 10^5 18S rRNA copies \pm SD for samples derived from three mice treated comparably was reported. The PCR primers listed in Table 1 were designed using Primer3 software (Whitehead Institute, Cambridge, MA, USA) and purchased from Qiagen.

Statistical analysis

The results were analyzed using the SigmaStat statistics program (Jandel Scientific, San Rafael, CA, USA). Individual means were compared using a non-paired Student's *t* test or a Mann-Whitney rank sum test. Data derived from three or more groups were compared by one-way analysis of variance (ANOVA) followed by a Tukey test, to identify the groups that differed significantly ($P < 0.05$).

RESULTS

Carbon particles co-localize with fluorescent latex beads and anti-F4/80 staining

LCM and analysis of the genes expressed by a specific cell type in a heterogeneous population (i.e. Kupffer cells among other hepatic cells in the study reported here) depends upon the rapid identification of those cells. The intravenous inoculation of India ink and subsequent ingestion of carbon particles by Kupffer cells provides a well-documented means of rapidly labeling Kupffer cells *in vivo* for laser capture. At 18 h or more post-inoculation of India ink, the livers were perfused *in situ* with a balanced salt solution, dissected, and frozen at

Table 1 PCR primers

Gene	GenBank accession size	Orientation	Primer sequence	Amplicon size
KCR	D88577	Forward	AAATGACCTCAGCTCCCAGA	103
		Reverse	TTCACCAGCCCTTTCCATAC	
CSF-1 receptor (CD115)	X06368	Forward	TGGCCTTCCTTGCTTCTAAA	114
		Reverse	ATGTCCCTAGCCAGTCCAA	
HMGCS1	NM_145942	Forward	GCGGCTAGAAGTTGGAACAG	196
		Reverse	AGCATATCGTCCATCCCAAG	
Lipocalin 2	NM_008491	Forward	CCAGTTCGCCATGGTATTTT	169
		Reverse	GGTGGGGACAGAGAAGATGA	
Hck	BC010478	Forward	GCCTCAAAAACAGAGCCAAG	150
		Reverse	GTACAGTGGACCACAATGG	
18S rRNA	XR_000144	Forward	AATGGTGCTACCGGTCATTCC	192
		Reverse	ACCTCTCTACCCGCTCTC	

-80°C, and 6- μ m liver sections were prepared from representative tissue wedges. The carbon particles were visualized microscopically by overlaying the sections with xylene (Figure 1). To ensure the particles were phagocytosed by Kupffer cells, mice were injected simultaneously with India ink and fluorescent latex beads (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), which are often used to track and label Kupffer cells *in vivo*. Using conventional light and UV light to visualize fluorescent emissions, two images were made with a confocal microscope, and both images were merged using Adobe Photoshop. As shown in Figure 2A-C, the carbon particles and fluorescent latex beads co-localized. The liver sections were also stained with a streptavidin-conjugated antibody specific for F4/80, a surface marker expressed by macrophages including Kupffer cells, and biotinylated Cy3 was used to visualize antibody binding. Confocal images of the carbon particles and bound antibody were merged and found to co-localize as well (Figure 2D-F). Notably, areas with an accumulation of carbon particles stained intensely with F4/80 (white arrows), whereas single carbon spots (black arrows) were not stained with F4/80. This observation was considered when selecting areas for LCM.

LCM of carbon-positive areas enriches for Kupffer cell receptor (KCR) and colony-stimulating factor-1 receptor (CSF-1R) mRNA

Labeling Kupffer cells with India ink *in vivo* prior to liver dissection negates *in vitro* staining prior to LCM. This drastically reduced the time between tissue sectioning and actually capturing the cells (approximately 15 min elapsed time) (Figure 3). To ascertain that material was captured, tissue sections and caps were examined by conventional microscopy. A liver section with carbon particles overlaid with xylene is shown in Figure 4A; the red-crossed-through circles show the location of carbon-positive areas on the static image obtained with the LCM system. After microdissection, the same section displayed gaps where the captured areas were removed (Figure 4B). Captured material on caps before RNA extraction with lysis buffer is shown in Figure 4C. Overlaying the cap with water revealed carbon particles associated with this material (Figure 4D).

The RNA was extracted from the captured material

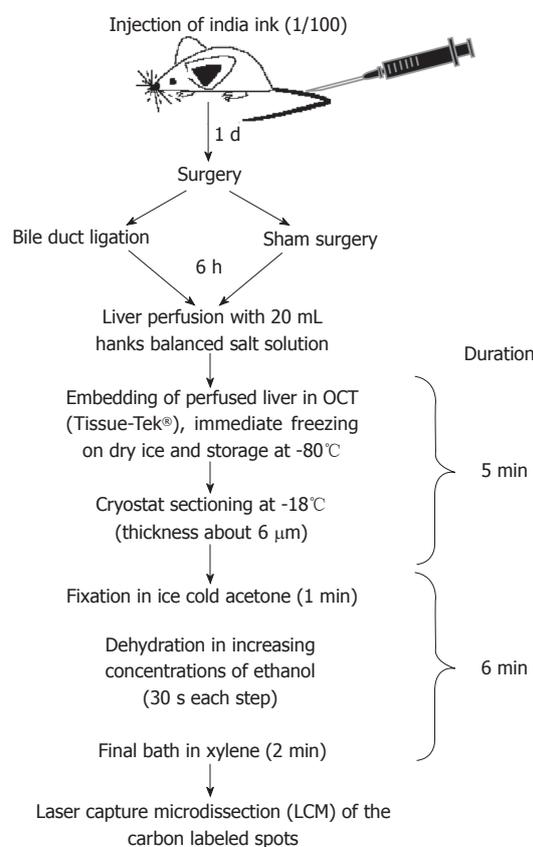


Figure 3 Experimental timeline. Schematic outline of the experimental approach used to isolate Kupffer cells labeled *in vivo* with carbon. Notably, the elapsed time between liver dissection, sectioning and LCM of carbon-labeled Kupffer cells was only about 11 min.

on caps, and the quality and quantity were assessed using a bioanalyzer and Lab-on-Chip technology (Agilent Technologies). Degradation appeared only slight relative to the RNA extracted from an entire tissue section scraped from a slide (Figure 5). Indeed, the quality of RNA obtained by LCM proved to be high and the quantity sufficient for subsequent analyses by real-time RT-PCR and microarray analysis. The amount of RNA isolated ranged from 100 to 1000 pg per 1000 Kupffer cells captured.

The carbon-labeled material obtained by LCM was significantly enriched in KCRs and CSF-1R RNA transcripts relative to the transcripts determined in the

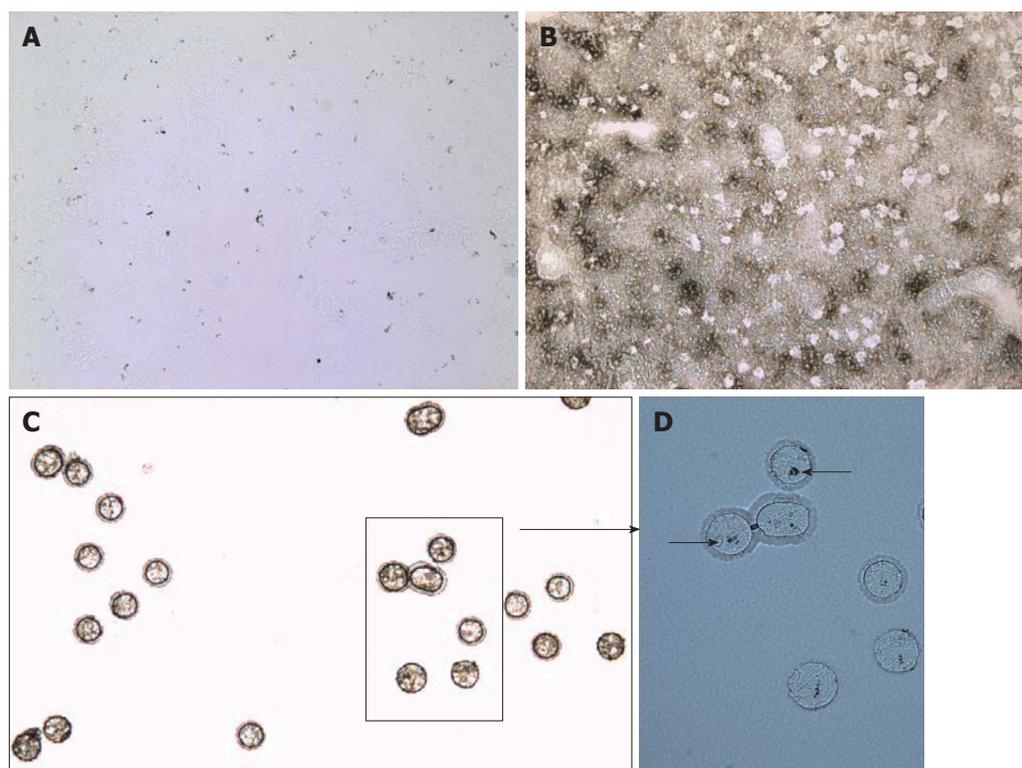


Figure 4 Images documenting LCM of carbon-labeled Kupffer cells. A: Static image of carbon-labeled liver section overlaid with xylene (10 × magnification); B: Same tissue section after the evaporation of xylene and capture of carbon-labeled cells (10 ×); C: Captured cells visualized on the CapSure LCM cap (20 ×); D: Same cap mounted with water and a coverslip showing carbon within microdissected cells (arrows, 40 ×).

tissue scrape (Figure 6). This further substantiates the association of carbon with and preferential dissection of Kupffer cells by LCM in accordance with our previous findings^[6].

Comparison of microarray data generated from Kupffer cells dissected from sham-operated and from bile-duct-ligated mice

Kupffer cells play a vital role in suppressing tissue damage that occurs in a mouse model of cholestatic liver injury. To identify the genes involved, groups of mice inoculated *iv* with India ink 18 h previously underwent bile-duct ligation or sham operation. The livers were dissected at 6 h post-surgery and sectioned; the carbon-labeled cells were obtained by LCM; the RNA was extracted, purified and amplified; and DNA microarray analysis was performed using the GeneChip Mouse Genome[®] 430 2.0 array. A total of 493 genes were found to be more than 2.5-fold up- or down-regulated (q value < 10) when the microarray data from bile-duct-ligated and sham-operated animals were compared. Interestingly, most of the genes that exhibited significant change were involved in cell growth and maintenance (Table 2: Gene ontology). Sixteen of the genes identified were highly up-regulated or down-regulated (> 5-fold change with a q value < 0.5), and the focus of further analyses. Our primary interest in evaluating these genes was to ascertain their role in maintaining the integrity of the liver during periods of cholestasis. Therefore, the 16 highly up- or down-regulated genes were subjected to a standardized PubMed search (<http://www.ncbi.nlm.nih.gov/sites/entrez>) with terms related to our model (Table 3). Abstracts dating back 15 years were reviewed and the publications relevant to our model were selected

Table 2 Significantly up- or down-regulated genes ontology

	Number	Percentage (%)
Cytoplasm	105	16.7
Cell growth and/or maintenance	87	13.8
Integral to membrane	85	13.5
Nucleus	57	9.0
Nucleoside and nucleic acid metabolism	54	8.6
Protein metabolism	54	8.6
Biosynthesis	34	5.4
Purine nucleotide binding	33	5.2
DNA binding	30	4.8
Signal transduction	26	4.1
Response to external stimulus	24	3.8
Transferase activity, transferring phosphorus containing groups	24	3.8
Lipid metabolism	23	3.7
Plasma membrane	19	3.0
Catabolism	18	2.9
Immune response	18	2.9
Phosphorus metabolism	18	2.9
Peptidase activity	16	2.5

(articles of interest). Based upon this search algorithm, the number of genes of interest was further reduced to 10 (Table 4). Three of these genes, *i.e.* 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HM GCS1), lipocalin 2, and hemopoietic cell kinase (Hck), were of particular interest in light of the role of Kupffer cells in abrogating cholestatic liver injury.

DISCUSSION

Non-specific phagocytosis of particles taken up in the liver is mediated primarily by Kupffer cells. Kupffer cells constitute 20%-30% of the non-parenchymal cells of

Table 3 Standardized Pubmed search with terms relevant to Kupffer cells abrogating liver injury during cholestasis: Each gene up- or down-regulated > 5-fold

Gene	Fold change	Search terms									Articles of interest
		Gene	Kupffer cells	Cholestasis	Apoptosis	Cell death	Survival	Inflammation	Liver	Liver repair	
Acetyl-coenzyme A synthetase 2	9.61	179	-	-	-	1	2	-	19	-	2
Hydroxysteroid (17-β) dehydrogenase 2	8.26	668	-	-	4	2	10	3	70	-	1
RIKEN cDNA 1110025G12	7.61	-	-	-	-	-	-	-	-	-	-
HM GCS1	5.81	181	-	-	6	6	2	-	67	-	3
RBP 4	5.76	1044	2	2	10	10	25	42	195	2	3
RIKEN cDNA 1200011D03 gene	5.75	-	-	-	-	-	-	-	-	-	-
Inter-alpha trypsin inhibitor, heavy chain 2	5.46	113	2	-	1	-	3	19	63	-	5
Lipocalin 2	5.35	375	-	1	24	25	15	48	17	1	15
Hck	-5.67	4	-	-	-	-	-	-	-	-	3
Hypothetical protein 5930431H10	-6.15	-	-	-	-	-	-	-	-	-	-
Leucine-rich repeat-containing 5	-6.26	22	-	-	1	1	-	-	1	-	2
SAM domain and HD domain, 1 (SAMHD1)	-6.81	5	-	-	-	-	-	-	-	-	-
Myristoylated alanine rich protein kinase C substrate	-7.14	458	-	-	5	7	10	5	11	-	3
Heterogeneous nuclear ribonucleoprotein A/B (SCAN-KRAB-) zinc finger gene 1	-7.94	50	-	-	2	2	2	-	5	-	3
Inactive X specific transcripts	-9.05	6	-	-	1	-	1	-	-	-	1
	-12.21	3	-	-	-	-	-	-	-	-	-

Table 4 Overview of function, source and involvement of the 10 genes identified by Pubmed-search: Degree of up- or down-regulation

Gene	Fold change	Biological function	Primary cell source	Involvement	Reference
Acetyl-Coenzyme A synthetase 2	9.61	Mitochondrial matrix enzyme involved in CoA ligation	Mitochondrial matrix	Activation during fasting	[50,51]
HM GCS1	5.81	Cholesterol biosynthesis		Inhibition induces apoptosis, up-regulation involved in liver regeneration	[25,26]
RBP4	5.76	An α2-globulin that transports vitamin A from the liver	Parenchymal liver cells, little in Kupffer cells	Known marker for hepatocellular necrosis	[22,23]
Inter-alpha trypsin inhibitor, heavy chain 2	5.46	Plasma protease inhibitor	Kupffer cells	Endothelial growth factor	[27-29]
Lipocalin 2	5.35	Iron-siderophore-binding protein	Macrophages, neutrophils	Suppression of inflammation, tissue involution, apoptosis, differentiation of myeloid cells	[31-40]
Hck	-5.67	Protein-tyrosine kinase	Granulocytes, monocytes	Mitogenesis, differentiation, survival, migration	[41-44]
Leucine-rich repeat-containing 5	-6.26	Interaction with cellular G-proteins		Enzyme inhibition, cell adhesion, cellular trafficking, proliferation and activation of lymphocytes and monocytes	[45,52]
Myristoylated alanine rich protein kinase C substrate	-7.14	Substrate of protein kinase C		Intracellular signaling, brain development, cellular migration and adhesion, phagocyte activation, phagocytosis	[46,47,53-55]
Heterogeneous nuclear ribonucleoprotein A/B (SCAN-KRAB-) zinc finger gene 1	-7.94	Chromatin-associated RNA-binding protein	Ubiquitous	RNA handling, proliferation arrest	[56,57]
	-9.05	Protein interaction domain	Ubiquitous	Cell survival, differentiation	[48,49]

the liver, where they reside within the sinusoidal vascular space, predominantly in the periportal area. Here, they

are perfectly situated to clear endotoxins from the blood, and to phagocytose microorganisms and debris. For

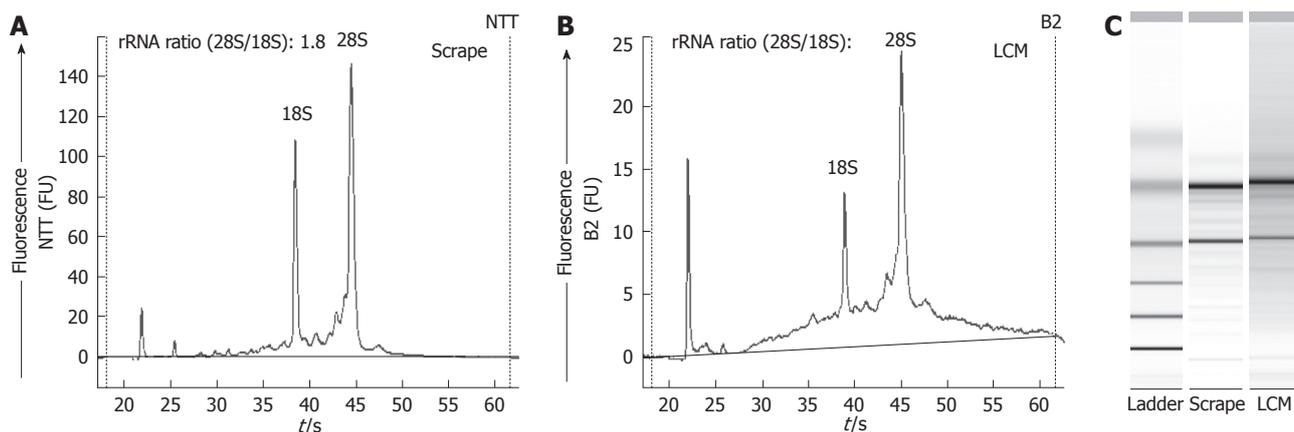


Figure 5 High quality RNA is extracted from material obtained by LCM. RNA size and quality were analyzed with the Agilent 2100 Bioanalyzer. A, B: Profiles of total RNA extracted from scrapes of the entire liver section (A) and from laser-captured cells (B); C: Electrophoresis gel of the same RNA.

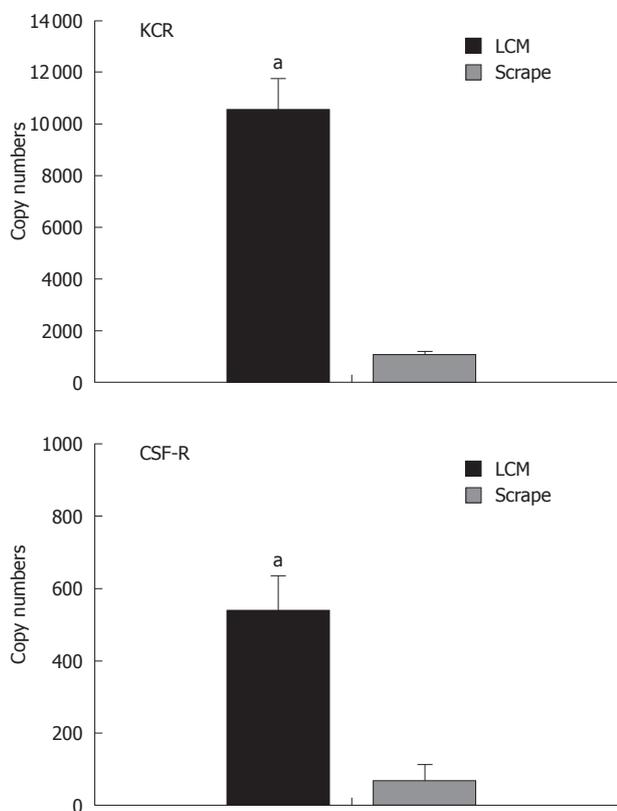


Figure 6 Kupffer-cell-specific mRNA transcripts are enriched in the laser-captured material. Carbon-labelled cells or total liver section scrapes were obtained from three mice; Kupffer cell receptor and CSF-1 receptor mRNA transcripts were quantified by real-time RT-PCR. ^aSignificantly more than extracted from total tissue scrapes ($P < 0.005$; non-paired Student *t* test).

example, latex particles inoculated *iv* distinguish a latex-labeled Kupffer cell population that does not change over a 3-mo period, substantiating the relatively long life span of these resident tissue macrophages^[18]. While endothelial cells lining the blood vessels internalize soluble materials *via* pinocytosis, Kupffer cells generally ingest particulate matter, e.g. colloidal carbon, *via* phagocytosis. The restricted ability to internalize carbon particles was used herein to label and subsequently dissect Kupffer cells by LCM. The specificity of

this approach is demonstrated by co-localization of the particles with fluorescent latex beads inoculated simultaneously *iv*, and with anti-F4/80-stained hepatic macrophages (Figure 2). The specificity is further documented by the elevated levels of macrophage-associated RNA transcripts (i.e. CSF-1R and KCR messages) extracted from the carbon-labeled material obtained by LCM, relative to those levels extracted from whole liver sections.

Unlike the majority of cells that constitute the mononuclear phagocyte system, Kupffer cells synthesize interleukin (IL)-10 (an anti-inflammatory cytokine) when exposed to lipopolysaccharide (LPS), rather than proinflammatory cytokines such as IL-12 and IL-18^[19]. This finding suggests that Kupffer cells, which are continuously exposed to LPS derived from the intestines *via* the hepatic portal vein, generally impose an immunosuppressive effect on the hepatic environment. In this regard, it is relevant to note that the ingestion of endotoxin and debris occurs coincidentally with the ability of Kupffer cells to induce tolerance^[20]. Moreover, in addition to tolerance, our studies involving a mouse model of cholestasis indicate that Kupffer cells play a general role in suppressing inflammation and liver injury^[6].

Differentiating Kupffer cells from non-resident macrophages, which infiltrate the liver during periods of chronic inflammation, and which may or may not display similar effector functions, represents a major challenge in characterizing the activity of Kupffer cells. The relatively short time period between bile-duct ligation and liver dissection enabled the specific identification and isolation of carbon-labeled Kupffer cells in the study reported here. Whether this approach permits the differentiation of Kupffer cells from inflammatory macrophages recruited to cholestatic livers 2-3 d after biliary obstruction is a matter of ongoing investigations in our laboratory.

LCM allows the isolation of tissue sections as small as 8-10 μm ^[8] and avoids procedures such as collagenase digestion that, conceivably, can alter the gene expression profile of Kupffer cells. Since no staining is necessary,

sections derived from the livers of sham-operated and bile-duct-ligated mice could be processed and subsequently dissected within an extremely short period of time. Moreover, while the effects of histological staining on the integrity of RNA are well documented^[21], the quality of RNA obtained using our approach proved to be high and the quantity sufficient enough for subsequent analyses (Figure 5).

Microarray analysis comparing Kupffer cells derived from bile-duct-ligated versus sham-operated mice revealed a total of 493 genes that were differentially expressed (2.5-fold, q value < 10). In an effort to restrict further analysis to genes specifically relevant to Kupffer cells and their response to cholestasis, those genes that were up- or down-regulated more than five-fold were subjected to a standardized PubMed search (Table 3), and 10 genes of interest were selected (Table 4). Notably, the expression of four of these genes [retinol binding protein 4 (RBP4), inter-alpha trypsin inhibitor heavy chain 2, lipocalin 2, and Hck] was previously described in Kupffer cells or cells of the mononuclear phagocyte lineage. Of these, only RBP4 appears more common in hepatocytes than in Kupffer cells^[22]. RBP4 is often up-regulated in response to hepatocellular necrosis^[23] and thus, its expression in Kupffer cells may derive readily from the ingestion of apoptotic hepatocytes^[24]. Two of the other up-regulated genes are involved in tissue regeneration: HMGCS1 is critical for hepatocyte regeneration after partial hepatectomy^[25,26], and inter-alpha trypsin inhibitor, a plasma protease inhibitor, promotes endothelial cell growth^[27-29].

In addition to promoting tissue regeneration and cell growth, Kupffer cells may induce apoptosis by infiltrating inflammatory cells (e.g. neutrophils) and thus, suppress their contribution to cholestatic liver injury^[30]. In this regard, lipocalin 2, the product of one of the up-regulated genes expressed by Kupffer cells present in cholestatic livers induces apoptosis by leukocytes, but not other cell types^[31-34]. Moreover, lipocalin has been implicated in myeloid cell differentiation and innate host defenses to bacterial infections^[35-40].

In contrast to the genes discussed immediately above, five genes expressed by Kupffer cells were significantly down-regulated in response to cholestasis. Hck, a member of the highly conserved sarcoma family of protein-tyrosine kinases that is preferentially expressed by cells of the myeloid lineage, influences cell migration, adhesion, differentiation and survival^[41-44]. Secondly, leucine-rich repeat containing protein 5 promotes the proliferation and/or activation of lymphocytes and monocytes^[45]. Thirdly, myristoylated alanine-rich C kinase substrate (a substrate of protein kinase C contained in large amounts in macrophages) has been implicated in phagocyte activation, endocytosis, exocytosis, phagocytosis, and mobility^[46,47]. Finally, the SCAN domain is a highly conserved motif found near the N terminus of C(2)H(2) zinc finger transcription factors. Some family members play a role in the transcriptional regulation of genes involved in cell differentiation and survival^[48,49].

In summary, LCM offers an effective approach to analyzing gene expression by Kupffer cells without altering liver integrity. Pre-labeling the cells with carbon *in vivo* renders further staining procedures unnecessary and allows the isolation of sufficient amounts of intact RNA. Thus, the contributions of Kupffer cells to various models of liver injury can be delineated. In this regard, using DNA microarray analysis, we identified 16 genes expressed by Kupffer cells that were highly up- or down-regulated following bile-duct ligation in a mouse model of cholestatic liver injury. A standard PubMed search conducted using terms relevant to the model determined 10 of these genes to be of specific interest and germane to the role of Kupffer cells in suppressing tissue damage. These genes are the subject of ongoing investigation in our laboratory.

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COMMENTS

Background

Kupffer cells, resident tissue macrophages that line the liver sinusoids, play a key role in modulating intrahepatic inflammation. Since Kupffer cells represent only a small portion of the entire liver cell population, greatly outnumbered by the parenchymal cells, Kupffer cell isolation faces major technical obstacles. Laser capture microdissection (LCM) offers a method of isolating a single cell type from specific regions of a tissue section. LCM is an essential approach used in conjunction with molecular analysis to study the functional interaction of cells in their native tissue environment.

Research frontiers

LCM circumvents many of the limitations inherent in conventional isolation methods. LCM was created at the National Institutes of Health (Bethesda, MD, USA) and further developed by Arcturus Engineering (Mountain View, CA USA). The principles of LCM entail overlaying the tissue section with a transparent ethylene vinylacetate thermoplastic film. At the point of interest, the film is melted onto the tissue surface with a laser integrated with the microscope optics and then removed, capturing sample areas as small as 3-5 μm from intact tissue sections.

Innovations and breakthroughs

Described herein is a method of labeling and microdissecting mouse Kupffer cells within an extraordinarily short period of time. Kupffer cell labeling is achieved by injecting India ink intravenously, thus circumventing the need for *in vitro* staining. This method provides an approach to isolating high quality RNA from Kupffer cells without altering the tissue integrity.

Applications

The ability to isolate carbon-labeled Kupffer cells from intact tissue sections using LCM offers an attractive approach to studying gene expression under diverse conditions. Thus, the specific contributions of Kupffer cells to various models of liver injury can be delineated.

Terminology

LCM permits under direct microscopic visualization rapid isolation of selected cell populations from a tissue section. A transparent thermoplastic film is applied to the surface of the tissue section, and a laser pulse then specifically activates the film above the cells of interest. The film is melted onto the tissue surface and then removed, capturing the pre-selected cells. Kupffer cells are resident tissue macrophages that line the liver sinusoids. Here, they are perfectly situated to clear endotoxins from the blood, and to phagocytose microorganisms and debris. Kupffer cells play a key role in modulating inflammation in the liver.

Peer review

In the current study, a new method of isolating Kupffer cells is presented based upon labeling the cells with India ink inoculated iv and microdissection of the carbon-labeled cells by laser capture. The study is well-controlled with regard to the specificity of staining of Kupffer cells with India ink. The method was further validated in a model of cholestasis.

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Potential therapeutic significance of increased expression of aryl hydrocarbon receptor in human gastric cancer

Tie-Li Peng, Jie Chen, Wei Mao, Xin Liu, Yu Tao, Lian-Zhou Chen, Min-Hu Chen

Tie-Li Peng, Jie Chen, Wei Mao, Xin Liu, Min-Hu Chen, Department of Gastroenterology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Yu Tao, Department of Pathology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Lian-Zhou Chen, Digestive system tumor tissue bank, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Author contributions: Peng TL and Chen J contributed equally to this work; Peng TL and Chen J performed the research and wrote the paper; Mao W organized the figures and patients' data; Liu X, Tao Y and Chen LZ performed immunohistochemical staining assays; Chen MH designed the research and supervised the writing and organization process.

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Correspondence to: Min-Hu Chen, MD, PhD, Department of Gastroenterology, The First Affiliated Hospital of Sun Yat-Sen University, 58 Zhongshan II Road, Guangzhou 510080, Guangdong Province, China. chenminhu@vip.163.com

Telephone: +86-20-87332916 Fax: +86-20-87332916

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RESULTS: AhR expression was significantly increased in GC tissues and GC cell lines. IHC results indicated that the levels of AhR expression gradually increased, with the lowest levels in CSG, followed by CAG, IM, AH and GC. AhR expression and nuclear translocation were significantly higher in GC than in precancerous tissues. TCDD inhibited proliferation of AGS cells *via* induction of growth arrest at the G1-S phase.

CONCLUSION: AhR plays an important role in gastric carcinogenesis. AhR may be a potential therapeutic target for GC treatment.

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Key words: Apoptosis; Aryl hydrocarbon receptor; Cell cycle; Cell proliferation; Gastric cancer

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Abstract

AIM: To determine the functional significance of aryl hydrocarbon receptor (AhR) in gastric carcinogenesis, and to explore the possible role of AhR in gastric cancer (GC) treatment.

METHODS: RT-PCR, real-time PCR, and Western blotting were performed to detect AhR expression in 39 GC tissues and five GC cell lines. AhR protein was detected by immunohistochemistry (IHC) in 190 samples: 30 chronic superficial gastritis (CSG), 30 chronic atrophic gastritis (CAG), 30 intestinal metaplasia (IM), 30 atypical hyperplasia (AH), and 70 GC. The AhR agonist tetrachlorodibenzo-para-dioxin (TCDD) was used to treat AGS cells. MTT assay and flow cytometric analysis were performed to measure the viability, cell cycle and apoptosis of AGS cells.

INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy and the second most frequent cause of cancer-related death in the world. It is often diagnosed at advanced stages when treatment options are limited, leading to a poor prognosis^[1]. The development of human GC is a multi-step process where normal mucosa progresses to chronic gastritis, precancerous lesions (including gastric atrophy, intestinal metaplasia, dysplasia), and invasive cancer^[2,3]. The carcinogenesis of GC involves numerous genetic and epigenetic alterations, as well as many environmental risk factors^[4]. Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated hydrocarbons (HAHs) are well-known carcinogens that play important roles in GC development^[5,6]. The toxic effects of PAHs and HAHs are mediated by a conserved signaling

pathway that binds and activates the aryl hydrocarbon receptor (AhR)^[7].

AhR is a ligand-activated transcription factor of the basic helix-loop-helix/Per-Arnt-Sim family. PAHs and HAHs are exogenous AhR ligands, among which 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) is the most potent^[8]. The ligand-AhR complex is translocated to the nucleus and heterodimerizes with the AhR nuclear translocator. The complex binds to the cognate enhancer sequence and subsequently activates downstream gene expression. AhR regulates genes that code for xenobiotic metabolizing enzymes, such as cytochrome P450 1A1 (CYP1A1), cytochrome P450 1B1 (CYP1B1), and growth-regulatory proteins^[9]. Inappropriately modified expression, and/or abnormally sustained expression of critical genes by the xenobiotic-activated AhR leads to various toxicities which were observed in exposed organisms: teratogenicity, immunotoxicity, tumor promotion, as well as various metabolic dysfunctions^[10,11].

Many studies in recent years have demonstrated a close relationship between AhR and mammary gland tumorigenesis^[12,13]. AhR gene polymorphisms have been linked to an increased risk of lung and breast cancers^[14,15]. Increased expression of AhR has been reported in lung, breast, and pancreatic cancers in humans^[9,12,16]. Studies also suggest that constitutively active AhR may promote hepatocarcinogenesis in mice^[17]. On the other hand, more and more studies have indicated that AhR-mediated responses are anti-proliferative in some cell types and that AhR might function as a potential target for cancer treatment^[16,18]. However, the role of AhR in gastric tumorigenesis is still unclear. Andersson *et al*^[19-21] reported that constitutively active AhR could induce stomach tumors and mediate down-regulation of osteopontin gene expression in a mouse model. In our previous study, we found increased expression of AhR in two human GC cell lines (RF1 and RF48) using microarray analysis^[22]. A recent study suggested that concurrent expression of AhR and CYP1A1 is correlated with GC development^[23].

The aim of our current study was to further determine the functional significance of AhR in gastric carcinogenesis, and to explore the potential role of AhR as a therapeutic target for GC treatment.

MATERIALS AND METHODS

Tissue specimens

Tissues of chronic superficial gastritis (CSG), chronic atrophic gastritis (CAG), intestinal metaplasia (IM) and atypical hyperplasia (AH) were obtained from 120 patients undergoing upper gastrointestinal endoscopy. Tissues of gastric tumors and their corresponding adjacent non-tumor tissues were collected from 70 GC patients who underwent GC surgery. Written, informed consent was obtained from all patients before sample collection. None of the GC patients had received preoperative chemotherapy or radiotherapy. Tissue samples were fixed in 10% neutralized formalin and embedded in paraffin for histological processing or

Table 1 Clinical and histological characteristics of the study population

Histology type	Patient number	Gender		Age (yr) mean ± SD
		Male	Female	
CSG	30	20	10	50.47 ± 11.63
CAG	30	12	18	53.27 ± 16.36
IM	30	16	14	52.38 ± 10.26
AH	30	15	15	55.67 ± 16.88
GC	70	37	33	56.59 ± 13.24
i-GC	32	17	15	57.27 ± 14.56
d-GC	38	20	18	55.91 ± 11.62

CSG: Chronic superficial gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; AH: Atypical hyperplasia; GC: Gastric cancer; i-GC: Intestinal-type gastric cancer; d-GC: Diffused-type gastric cancer.

snap-frozen in liquid nitrogen and stored at -80°C for RT-PCR and Western blot analysis. All tissue specimens were histologically verified by a pathologist. Chronic gastritis specimens were classified according to the updated Sydney System^[24]. GCs were classified according to the WHO classification^[25] and Lauren's classification^[26]. The clinical and histological characteristics of the study population are shown in Table 1. The study was approved by the Ethics Committee of the university hospital.

GC cell lines

Five GC cell lines- MKN28, MKN45, AGS, NCI N-87 (N87), and KATO III-were obtained from the Riken Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (ATCC, Rockville, MD, USA). All five cell lines were maintained in RPMI-1640 medium (Hyclone, USA) supplemented with 2 mmol/L glutamine, 100 mL/L fetal bovine serum (Hyclone, USA), 1×10^5 U/L of penicillin, and 0.1 g/L of gentamycin. The cellular environment was maintained at 50 mL/L CO₂ and 37°C. Cells were harvested from the exponential growth phase and total RNA and protein were prepared as described below.

RNA isolation, RT-PCR and real-time PCR

Gastric tissue specimens and cell pellets were homogenized with an ultrasound homogenizer. Total RNA in cells and tissues was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was synthesized with 1 µg total RNA using reverse transcriptase, ReverTra Ace™ (Toyobo Co., Osaka, Japan) under the following conditions: 30°C for 10 min, 42°C for 20 min, 99°C for 5 min, and 4°C for 5 min. PCR of cDNA was carried out in a reaction mixture (30 µL) containing 2 µL of template cDNA, 2.5 mmol/L MgCl₂, 200 µmol/L dNTPs, 0.3 µmol/L primer 1 and 2, and 1 U of Taq DNA polymerase (New England Biolabs, China). Amplification was performed using the following conditions: 94°C for 5 min, followed by 25-32 cycles (denaturation for 45 s at 94°C, annealing for 30 s, and extension for 30 s at 72°C), and then 72°C for 7 min. Details of primers, annealing temperature, amplification cycles, and

Table 2 Primer sequences and PCR amplification conditions

Gene	Primers (5'→3')	Annealing temperature (°C)	Cycles	Product size (bp)
AhR	S: ACTCCACTTCAGCC-ACCATC A: ATGGGACTCGGCAC-AATAAA	55	25	204
CYP1A1	S: CCATGTCGGCCAC-GGAGTT A: ACAGTGCCAGGTG-CGGGT	59	32	174
β-actin	S: CTCGCTGTCCAC-CTTCCA A: GCTGTACCTTCA-CCGTTC	52	30	256

S: Sense primer; A: Antisense primer.

PCR product size for each gene are listed in Table 2. The PCR products were electrophoresed on 15 g/L agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator. The positive rate of mRNA expression was calculated. mRNA expression levels of AhR were further detected by quantitative real-time PCR with beta-actin as the internal reference, using the Stratagene MX3000P system (Stratagene, USA). cDNA was mixed with SYBR Green QPCR master mix (Stratagene) and primers. The thermal cycling comprised of an initial step at 95°C for 10 min, then 40 intermediate cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s), and one final cycle (95°C for 1 min, 55°C for 30 s, and 95°C for 30 s). Real-time PCR was performed using AhR primers (5'-TACCCTGGACTTGCCTCTGC-3' and 5'-TGAAGCCAGTCAGCACCTC-3'), and beta-actin primers (5'-TCATGAAGTGTGACGTGGACATC-3' and 5'-CAGGAGGAGCAATGATCTTGATCT-3'). Relative quantitation was calculated using the comparative threshold cycle (C_T) method. C_T indicates the fractional cycle number at which the amount of amplified target genes reaches a fixed threshold within the linear phase of gene amplification, and is inversely related to the abundance of mRNA transcripts in the initial sample. Mean C_T of duplicate measurements was used to calculate ΔC_T as the difference in C_T for target and internal reference (β -actin) genes. ΔC_T for each sample was compared to the corresponding ΔC_T of the experiment control and expressed as $\Delta\Delta C_T$. Relative quantitation was expressed as fold changes of the gene of interest compared to the experimental control according to the formula $2^{-\Delta\Delta C_T}$: fold change = $2^{-\Delta\Delta C_T}$

Western blot analysis

Gastric tissue specimens and cell pellets were homogenized in a lysis buffer containing 20 mmol/L HEPES, 1 mmol/L EGTA, 50 mmol/L β -glycerophosphate, 2 mmol/L sodium orthovanadate, 100 mL/L glycerol, 10 mL/L Triton X-100, 1 mmol/L DTT, and 1 \times Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The lysate was centrifuged at 13000 g and 4°C for 10 min.

The supernatant was the total cell lysate. Protein concentration was measured using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Thirty micrograms of protein was loaded per lane, separated by 100 g/L SDS-PAGE, and transferred onto equilibrated polyvinylidene difluoride membrane by electroblotting. Membranes were blocked with TBS-T buffer containing 50 g/L non-fat dry milk. AhR, CYP1A1, and beta-actin were detected for 2 h using antibodies against AhR (SC-5579, Santa Cruz Biotechnology, USA, working dilution 1:150), CYP 1A1 (AB1258, Chemicon International, USA, working dilution 1:500), and beta-actin (4970, Cell Signaling Technology, USA, working dilution 1:1000). After secondary antibody incubation (working dilution 1:2000), enhanced chemiluminescence (Pierce Biotechnology, Inc., USA) was determined by exposure to x-ray film. Band intensities in Western blotting were quantified using Quantity One imaging analysis software. Band intensities of AhR and CYP 1A1 were normalized with corresponding band intensities of beta-actin. Data was reported as mean \pm SD.

Immunohistochemistry

Paraffin sections (4 μ m thickness) were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was performed by heating the sections for 10 min at 100°C in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 30 mL/L H_2O_2 for 15 min and non-specific staining was reduced using a blocking serum for 10 min. The sections were then incubated with rabbit anti-human AhR antibodies (SC-5579, Santa Cruz Biotechnology, working dilution 1:100) overnight at 4°C. The next day, a two-step detection method (EnVision™ Detection Kit, Gene Tech Company Limited, China) was used according to the manufacturer's instructions. Briefly, after incubation with primary antibodies the tissues were incubated with the ChemMate™ EnVision™/HRP for 30 min at room temperature. The reaction was visualized using the ChemMate™ DAB plus Chromogen. Hematoxylin was used as a counterstain. Negative controls were carried out using a similar process, however, the first antibodies were omitted.

A scoring system with two categories was used to evaluate the immunohistochemical results^[27]. Category A documented the number of immunoreactive cells: 0 (< 5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (> 75%). Category B documented the intensity of the immunostaining: 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). A final score was calculated by adding the individual scores for each category. The staining results were measured semi-quantitatively based on the final combined score: 0 (score less than 2), 1+ (score range from 2 to 3), 2+ (score range from 4 to 5), and 3+ (score range from 6 to 7). Immunostaining was assessed by an experienced histopathologist who was blinded to the clinical data of the patients.

Treatment of cells

TCDD and resveratrol were purchased from Sigma

Chemical Company (Bellefonte, PA, USA). Cells were plated on 60 mm diameter plates (for RNA preparation) and 100 mm diameter plates (for cytosolic preparation) at 80%-90% confluence in RPMI-1640. After incubating for 24 h, one group of cells was treated with TCDD at different concentrations (0, 0.01, 0.1, 1, 10, 100 nmol/L) for 24 h. A second group was also treated for an additional 24 h with TCDD (1 nmol/L) plus resveratrol (0, 1, 5, 10, 20 μ mol/L). Another group was treated with TCDD (1 nmol/L) for different time intervals (0, 1, 6, 24, 48, 72 h), respectively. All drugs were dissolved in dimethyl sulfoxide (DMSO). Control cells received 1 mL/L DMSO only.

MTT Assay

A total of 1×10^4 trypsin-dispersed cells in 0.1 mL culture medium were seeded into each well of a 96-well plate and cultured for 24 h. Next, the cells were incubated with medium alone or with medium plus TCDD at different concentrations (0, 0.01, 0.1, 1, 10, 100 nmol/L) for another 12, 24 or 48 h. Then, 20 μ L of MTT (5 g/L, Sigma) was added to each well and the incubation was continued for 4 h at 37°C. Finally, the culture medium was removed and 200 μ L of DMSO was added to each well. The absorbance was determined with an ELISA reader at 490 nm. The cell viability percentage was calculated as: Viability percentage (%) = (Absorption value of TCDD treatment group) / (Absorption value of control group) \times 100%

Flow cytometric analysis

For flow cytometric analysis, AGS cells were plated on 60-mm diameter culture plates and treated with TCDD at different concentration (0.01, 0.1, 1, 10, 100 nmol/L) for 48 h. The control contained 1 mL/L DMSO only. Prior to harvesting, the cells were washed twice with 0.01 mol/L PBS, trypsinized, and pelleted. The cells were then fixed with ice-cold 700 mL/L ethanol at 4°C overnight. Finally, the cells were washed twice with PBS and dyed with propidium iodide (PI). The DNA content was analyzed with a flow cytometer (Beckman-Coulter, USA). The cell cycle and apoptosis of AGS cells were analyzed using MULTICYCLE and winMDI2.9 software (Phoenix, AZ, USA). The final data was reported as the mean \pm SD for each of the three independent experiments.

Statistical analysis

All quantitative data were expressed as mean \pm SD and analyzed using Student *t*-tests. Immunohistochemical results were analysed using the Kruskal-Wallis test and the Mann-Whitney test. The differences in positive rates were evaluated by Fisher's exact test. All statistical analyses were carried out using the SPSS statistical software package (version 11.0, SPSS Inc.). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of AhR in gastric cancer and pre-malignant tissues

RT-PCR and Western blotting were performed to

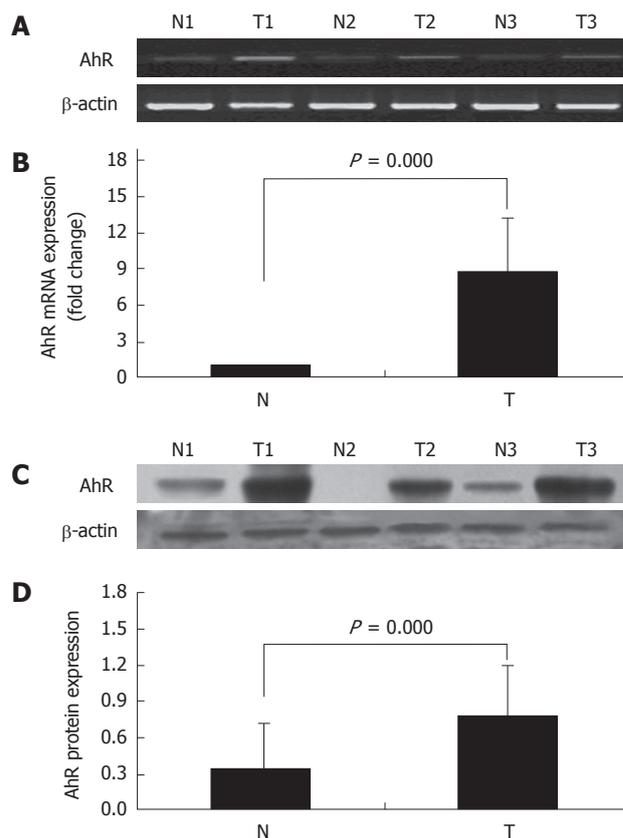


Figure 1 AhR mRNA and protein expression in GC tissues (T) and their corresponding adjacent non-cancerous tissues (N). A: AhR mRNA was detected by RT-PCR; B: AhR mRNA was detected by real-time PCR; C and D: AhR protein expression was detected by Western blotting and band intensities of AhR were normalized with corresponding band intensities of β -actin. A and C represent three cases; B and D summarize the overall mRNA and protein expression levels of AhR in all 39 cases.

analyze AhR mRNA and protein expression in 39 GC tissues and their corresponding adjacent non-cancerous tissues. Five GC cell lines (MKN28, MKN45, AGS, N87, and KATO III) were also analyzed. Compared with non-cancerous tissues both AhR mRNA and protein expression were significantly increased in cancer tissues. The AhR mRNA positive rate was significantly higher in GC tissues compared with their corresponding adjacent non-cancerous tissues (92.31%, 36/39 *vs* 66.67%, 26/39, $\chi^2 = 7.863$; $P = 0.005$). Quantitative real-time PCR and Western blotting results indicated that both AhR mRNA (Figure 1A and B) and protein levels (Figure 1C and D) in cancer tissues were significantly higher than levels in corresponding adjacent non-cancerous tissues ($P < 0.01$). AhR expression was high in MKN28, MKN45, AGS and KATO III cells, but very weak in N87 cells (Figure 2A-D). The five GC cell lines were derived from different sources: MKN45 and AGS were derived from poorly differentiated primary carcinoma of the stomach; MKN28 was derived from a moderately differentiated primary gastric carcinoma; N-87 was derived from a liver metastasis of a well-differentiated carcinoma; KATO-III was derived from metastasis of gastric carcinoma. Tumor stage did not appear to correlate with the level of AhR expression.

Expression of AhR was further detected by

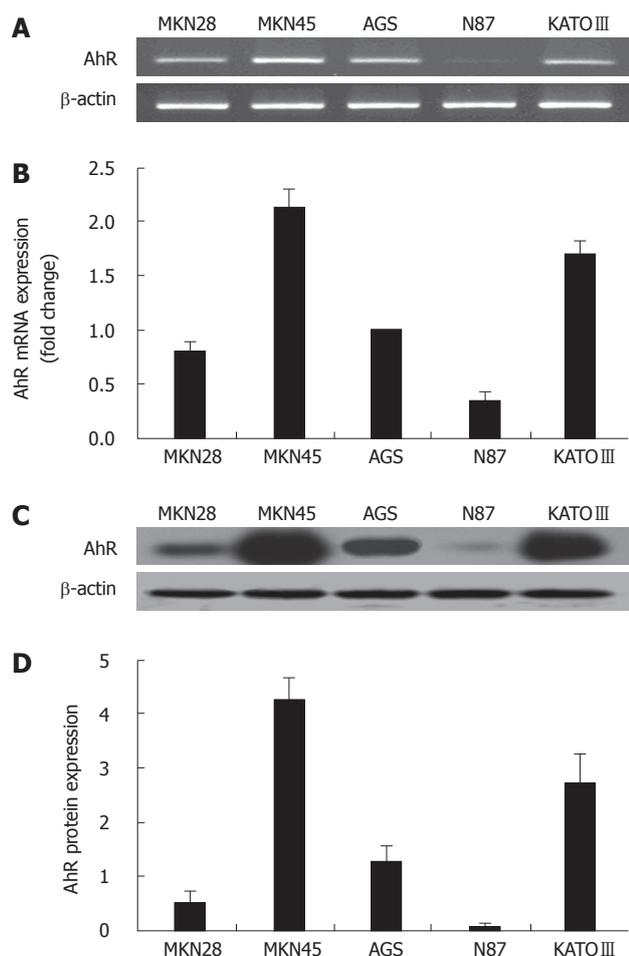


Figure 2 AhR mRNA and protein expression in five GC cell lines. A: AhR mRNA was detected by RT-PCR; B: AhR mRNA was detected by real-time PCR, AhR mRNA of AGS cells was used as the experimental control to calculate the fold changes; C and D: AhR protein expression was detected by Western blotting and band intensities of AhR were normalized with corresponding band intensities of beta-actin.

immunohistochemistry in 190 GC and pre-malignant gastric tissues: 30 CSG, 30 CAG, 30 IM, 30 AH, and 70 GC. Among the 70 GC patients, 38 suffered from Lauren diffuse type and 32 had intestinal type GC^[25]. There were no significant differences in gender or age in the different groups in this study population ($P = 0.095$) (Table 1). Strong nuclear expression and weak cytoplasmic distribution of AhR were observed in epithelial cells of both GC and pre-malignant tissues. Interestingly, AhR expression was also found in some stroma cells of both GC and pre-malignant tissues (Figure 3). The levels of AhR expression gradually increased, with the lowest levels in CSG, followed by CAG, IM, AH and GC (Table 3). Considering the fact that AhR needs to move into the nucleus to trigger expression of its target gene, evaluation of nuclear translocation of AhR may be of more importance than assessing the overall AhR expression in both the nucleus and cytoplasm. Therefore, we further calculated nuclear expression of AhR in GC and pre-malignant tissues. As with overall AhR expression, nuclear translocation of AhR also showed an increasing trend, with the lowest expression in CSG, followed by CAG,

Table 3 Expression of AhR in gastric cancer and pre-malignant tissues

Histology type	Patient number	AhR expression				AhR positive rate (%)
		-	+	++	+++	
CSG	30	16	1	13	0	46.67
CAG	30	8	7	15	0	73.33
IM	30	7	8	15	0	76.67
AH	30	5	5	18	2	83.33
GC	70	2	7	35	26	97.14 ¹
i-GC	32	1	3	16	12	96.88
d-GC	38	1	4	19	14	97.37

¹Compared with CSG, CAG, IM and AH, $P < 0.05$.

Table 4 Nuclear translocation of AhR in gastric cancer and pre-malignant tissues

Histology type	Patient number	Nuclear expression of AhR				AhR nuclear positive rate (%)
		-	+	++	+++	
CSG	30	20	1	9	0	33.33
CAG	30	14	5	11	0	53.33
IM	30	13	6	11	0	56.67
AH	30	5	5	18	2	83.33 ²
GC	70	4	6	34	26	94.29 ¹
i-GC	32	2	3	15	12	93.75
d-GC	38	2	3	19	14	94.74

¹Compared with CSG, CAG, IM and AH, $P < 0.05$. ²Compared with CSG, CAG and IM, $P < 0.05$.

IM, AH and GC (Table 4). Both AhR expression and nuclear translocation were significantly higher in GC than in precancerous tissues. There were no significant differences in AhR expression and nuclear translocation between diffuse type (d-GC) and intestinal type gastric cancers (i-GC) (Tables 3 and 4).

Effects of AhR signal pathway activation in AGS GC cell line

To investigate the potential role of the AhR signal pathway in gastric carcinogenesis, we first treated the GC cell line AGS with a specific AhR agonist, TCDD. CYP1A1, a classic target gene of AhR, was utilized as the indicator of AhR signal pathway activation. Although a baseline level of CYP1A1 expression was observed in AGS cells, RT-PCR and Western blot analysis showed that both CYP1A1 mRNA and protein expression in AGS cells were increased in a dose- and time-dependent manner following TCDD treatment (Figure 4A-D). After TCDD treatment, while CYP1A1 protein expression increased, AhR protein in the total cell lysates gradually decreased (Figure 4C and D). To further confirm the activation of the AhR signal pathway in gastric carcinogenesis, we treated AGS cells with a specific AhR antagonist, resveratrol^[28,29]. Controls included AGS cells treated with DMSO only. Experimental samples included AGS cells treated with resveratrol (10 $\mu\text{mol/L}$) only or TCDD (1 nmol/L) plus different concentrations of resveratrol (0, 1, 5, 10, 20 $\mu\text{mol/L}$), respectively for 24 h (Figure 5). In concordance with previous results, treatment of AGS cells with 1 nmol/L TCDD caused a

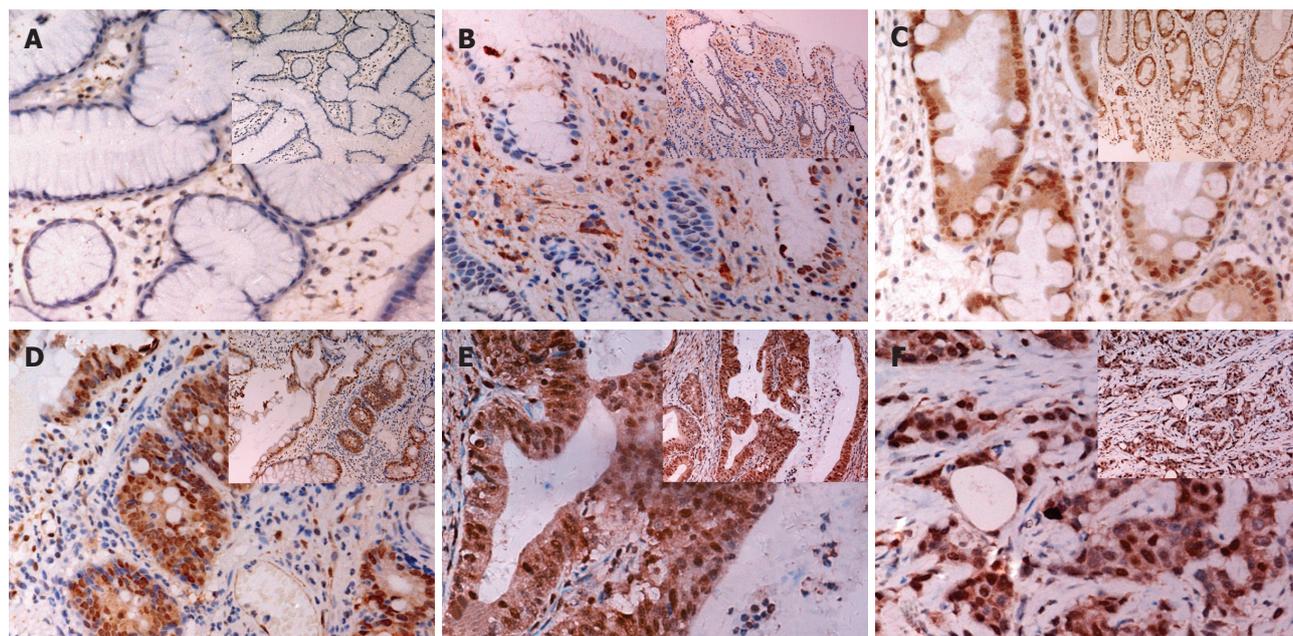


Figure 3 Immunohistochemical staining of AhR in gastric tissues. A: CSG; B: CAG; C: IM; D: AH; E: i-GC; F: d-GC (Original magnification $\times 400$ and $\times 200$). Strong nuclear expression and weak cytoplasmic distribution of AhR were observed in epithelial cells and some stroma cells of both GC and pre-malignant tissues.

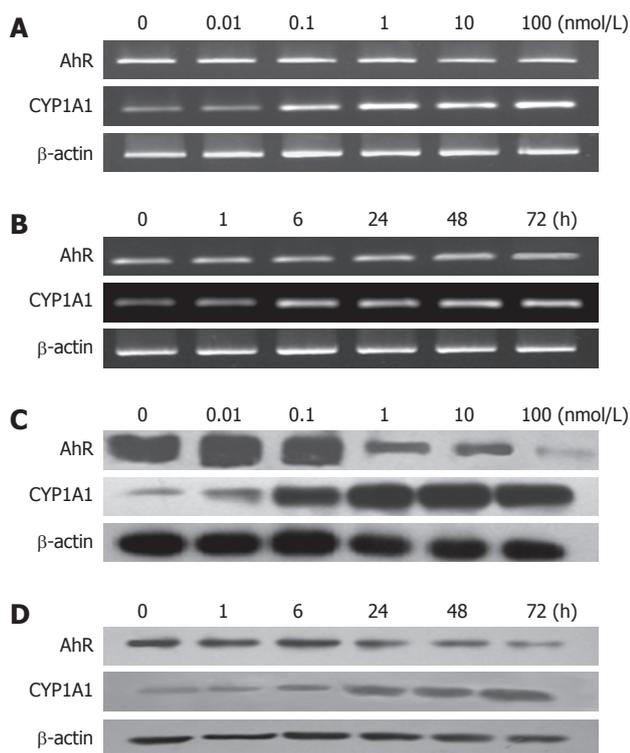


Figure 4 AhR and CYP1A1 expression in AGS cells after TCDD treatment. A and B: RT-PCR; C and D: Western blotting. Treatment of AGS cells with specific AhR agonist TCDD resulted in a dose- (A and C) and time-dependent (B and D) induction of CYP1A1 expression. The results shown are representative of three independent experiments.

remarkable increase in CYP1A1 expression. However, this TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner (Figure 5A and B).

Effects of AhR activation by TCDD on the

proliferation, cell cycle and apoptosis of AGS cells were further analyzed by MTT assay and flow cytometry. MTT assay demonstrated that the viability of AGS cells was significantly decreased in a dose- and time-dependent manner after TCDD treatment (Figure 6). Flow cytometric analysis demonstrated that TCDD caused a dose-dependent alteration in the cell cycle distribution of AGS cells 48 h after treatment. TCDD increased the proportion of cells in the G1 phase and correspondingly decreased the proportion in the S phase of the cell cycle. The proportion of cells in the G2 phase showed no significant change after TCDD treatment (Table 5, Figure 7). However, apoptosis of AGS cells was unable to be detected in this assay (Table 6, Figure 8). Thus, these results suggest that TCDD inhibits proliferation of AGS cells via induction of growth arrest at the G1-S phase.

DISCUSSION

AhR is an evolutionarily conserved ligand-activated transcription factor bound and activated by ubiquitous environmental pollutants. Historically, AhR has been studied for its transcriptional regulation of genes encoding xenobiotic metabolizing enzymes such as cytochrome P450 enzymes, which metabolize many of these chemicals into mutagenic and toxic intermediates. Therefore, it has been suggested that AhR may play a role in oncogenic processes, especially those initiated by environmental carcinogens^[11-13]. Environmental carcinogens such as PAHs and HAHs are well-known exogenous AhR ligands that play important roles in GC^[5,6]. In addition to synthetic and environmental chemicals, numerous naturally occurring dietary and endogenous AhR ligands have also been identified

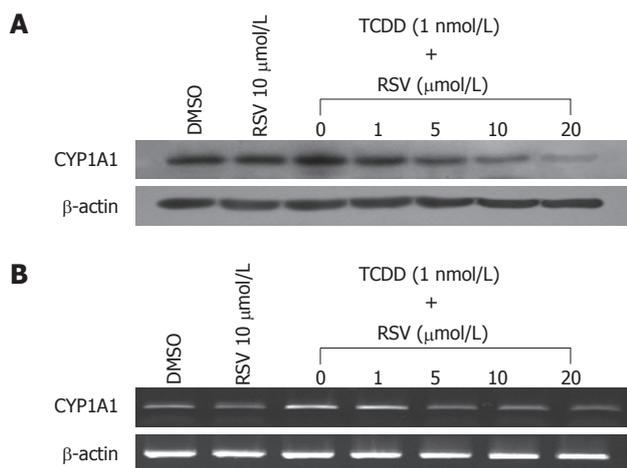


Figure 5 Inhibition of TCDD-induced CYP1A1 mRNA and protein expression by resveratrol. A: CYP1A1 protein was detected by Western blotting; B: CYP1A1 mRNA was detected by RT-PCR. The results shown are representative of three independent experiments. Treatment of AGS cells with 1 nmol/L TCDD caused a remarkable increase in CYP1A1 expression. This TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner.

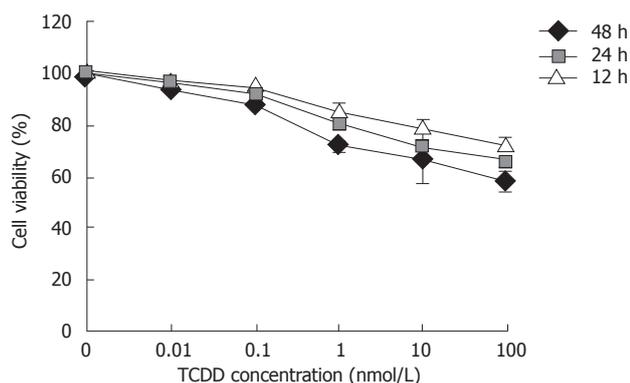


Figure 6 Viability of AGS cells after TCDD treatment was assessed by MTT assay. Viability of AGS cells was significantly decreased in a dose-dependent manner after TCDD treatment.

recently^{8,10}. Since gastric epithelium may be constantly exposed to both exogenous and endogenous AhR ligands, it would be of significance to shed light on the essential role of AhR in gastric tumorigenesis. Andersson *et al*¹⁹⁻²¹ first suggested that constitutively activated AhR could induce stomach tumors in a transgenic mouse model. In our previous study, we found increased expression of AhR in two human GC cell lines (RF1 and RF48) using microarray analysis²². A recent study by Ma *et al*²³ reported that concurrent expression of AhR and CYP1A1 is correlated with GC development. However, the role of AhR in human gastric tumorigenesis is still unclear.

In the current study, we first detected AhR mRNA and protein expression in 39 GC tissues and five GC cell lines using RT-PCR and Western blot analysis. Compared with their corresponding adjacent non-cancerous tissues, both AhR mRNA and protein expression were significantly increased in cancer tissues. Moreover, significantly different AhR levels in GC

Table 5 The effect of TCDD on AGS cell cycle

TCDD concentration (nmol/L)	Percentage of cell cycle (%)		
	G ₀ /G ₁	S	G ₂ /M
Control	54.47 ± 0.45	39.10 ± 1.39	6.43 ± 1.48
0.01	60.47 ± 3.11 ^a	33.20 ± 2.51	6.33 ± 1.12
0.1	66.07 ± 0.80 ^b	28.67 ± 3.08 ^b	5.33 ± 2.34
1	67.53 ± 2.57 ^b	25.73 ± 4.56 ^b	6.73 ± 2.06
10	67.20 ± 4.33 ^b	25.03 ± 5.31 ^b	7.77 ± 1.99
100	68.57 ± 5.57 ^b	25.10 ± 7.41 ^b	6.33 ± 1.96

Values given are the mean ± SD of three independent experiments. ^aP < 0.05, ^bP < 0.01, compared with respective control value.

Table 6 The effect of TCDD on AGS cell apoptosis

TCDD concentration (nmol/L)	Sub-G ₁	P
Control	8.33 ± 1.59	
0.01	9.10 ± 2.46	0.583
0.1	8.20 ± 1.65	0.924
1	7.97 ± 0.31	0.792
10	6.30 ± 1.71	0.161
100	9.57 ± 1.52	0.382

Values of Sub-G₁ given are the mean ± SD of three independent experiments.

cell lines from different derivations suggest that AhR expression may not be correlated with tumor stage. Since the development of human GC is a multi-step process, we further detected the expression and distribution of AhR using immunohistochemistry in a series of GC and pre-malignant gastric tissues. Ma *et al*²³ performed similar examinations in their study. However, their study included only 39 GC tissues, 17 pre-malignant tissues, and six non-cancerous mucosa samples which were detected using immunohistochemistry. In addition, atypical hyperplasia, the most important pre-malignant histology type, was not included in their study. The small sample size in that study may not accurately reflect the real expression pattern of AhR in GC and pre-malignant tissues. In our study, we included a larger sample size and included 30 atypical hyperplasia tissues. Similar to the findings of Ma *et al*²³, our data also demonstrated a close correlation of AhR with tumor formation via enhanced expression levels and frequent nuclear translocation from pre-malignant lesions to GC. Significantly increased nuclear translocation of AhR was found even early in AH (Table 4). There were no significant differences in AhR expression and nuclear translocation between i-GC and d-GC. Our findings suggest that activation of AhR signaling may be an early event in gastric carcinogenesis. Interestingly, besides strong expression of AhR in epithelial cells, AhR expression was also found in some stroma cells of both GC and pre-malignant tissues. Trombino *et al*³⁰ reported similar findings in their study of mammary tumorigenesis. Using a rat model of PAH-induced mammary tumorigenesis, they demonstrated that AhR expression levels were significantly elevated in PAH-induced mammary tumors as well as in stroma elements surrounding these tumors. Since stroma

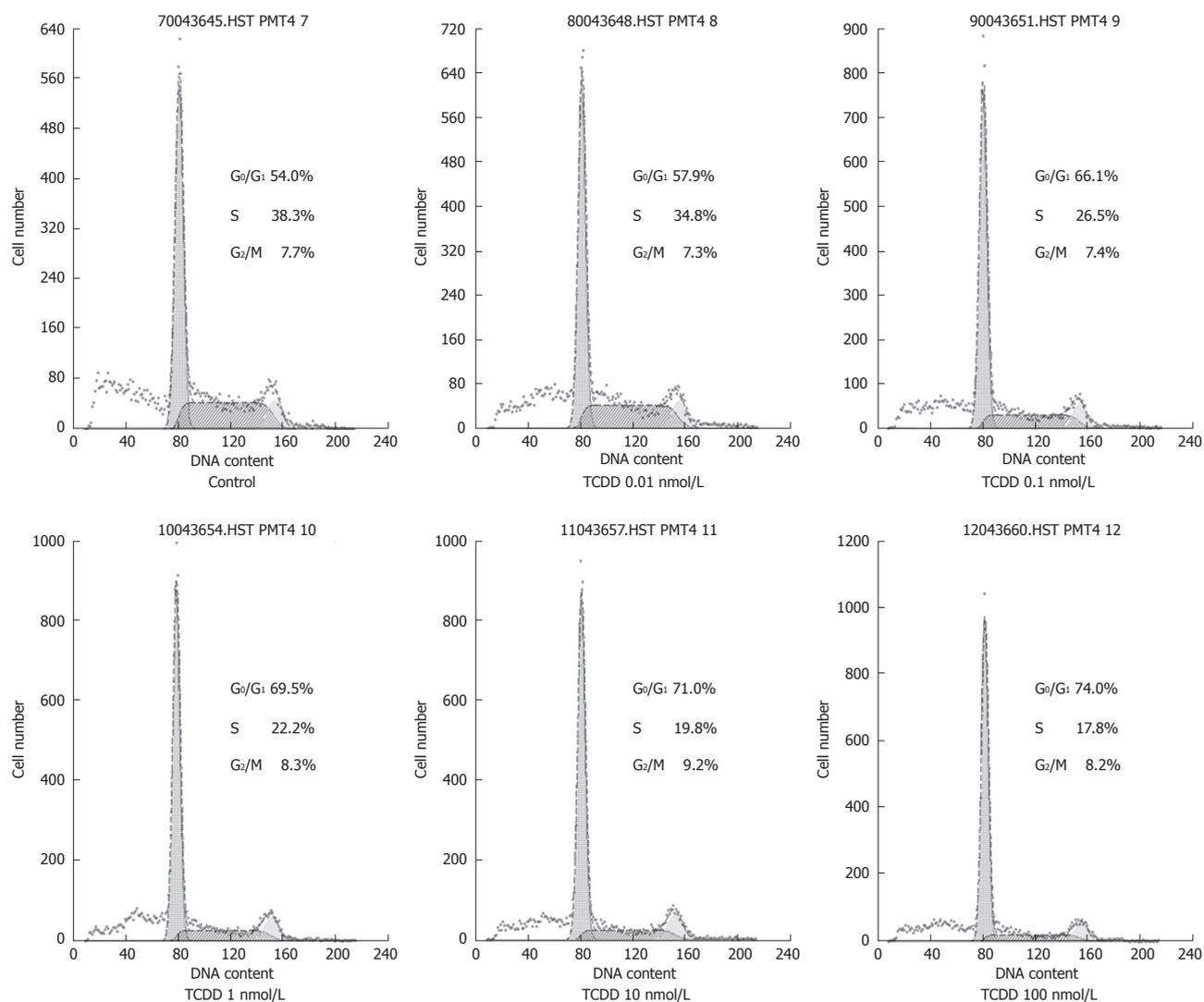


Figure 7 The effect of TCDD on AGS cell cycle distribution. AGS cells were treated with different concentrations of TCDD and subjected to flow cytometric analysis. The percentage of each phase is indicated in each panel. The results shown are representative of three independent experiments.

elements play important roles in maintaining the microenvironment and regulating growth of epithelial cells, expression of AhR in stroma cells may have a bearing on malignant transformation of gastric epithelial cells.

To further investigate the potential role of the AhR signal pathway in gastric carcinogenesis, we treated GC cell line AGS with the most potent AhR agonist, TCDD, and chose CYP1A1, a classic target gene of AhR, as the indicator of AhR signal pathway activation. Although both CYP1A1 and CYP1B1 are classic target genes of AhR, cell-specific expression of these two genes have been reported previously^[30-32]. Over-expression of CYP1A1, but not CYP1B1 in GC has been reported by Ma *et al*^[23] and Zhang *et al*^[33]. Baseline levels of CYP1A1 expression were also observed in AGS cells in the present study. However, expression of CYP1A1 was significantly increased in a dose- and time-dependent manner after TCDD treatment, indicating the activation of AhR. Interestingly, while CYP1A1 protein expression increased, AhR protein in the total cell lysates gradually decreased (Figure 4C and D). Similar phenomena have

been reported by several other groups^[34-36]. Recent studies have demonstrated that the down-regulation of AhR following ligand binding is ubiquitin mediated and occurs *via* the 26S proteasome pathway following nuclear export of AhR. The degradation of AhR is the endpoint and would be one of the key factors controlling gene regulations by the AhR signal pathway^[37]. To confirm the activation of the AhR signal pathway by TCDD, we treated AGS cells with a specific AhR antagonist, resveratrol. Previous studies suggested that resveratrol can regulate the transcription of AhR targeted genes by preventing AhR from binding to the enhancer sequences of the gene promoter^[28,29]. Our results showed that TCDD-induced CYP1A1 expression was partially reversed by resveratrol in a dose-dependent manner. The incomplete reversal of CYP1A1 expression by resveratrol may be due to the fact that AhR is not the only regulator of CYP1A1 transcription^[38,39]. Taken together, these results suggest that the AhR signal pathway could be activated in GC cells and that abnormal activation of the AhR signal pathway may be involved in gastric carcinogenesis.

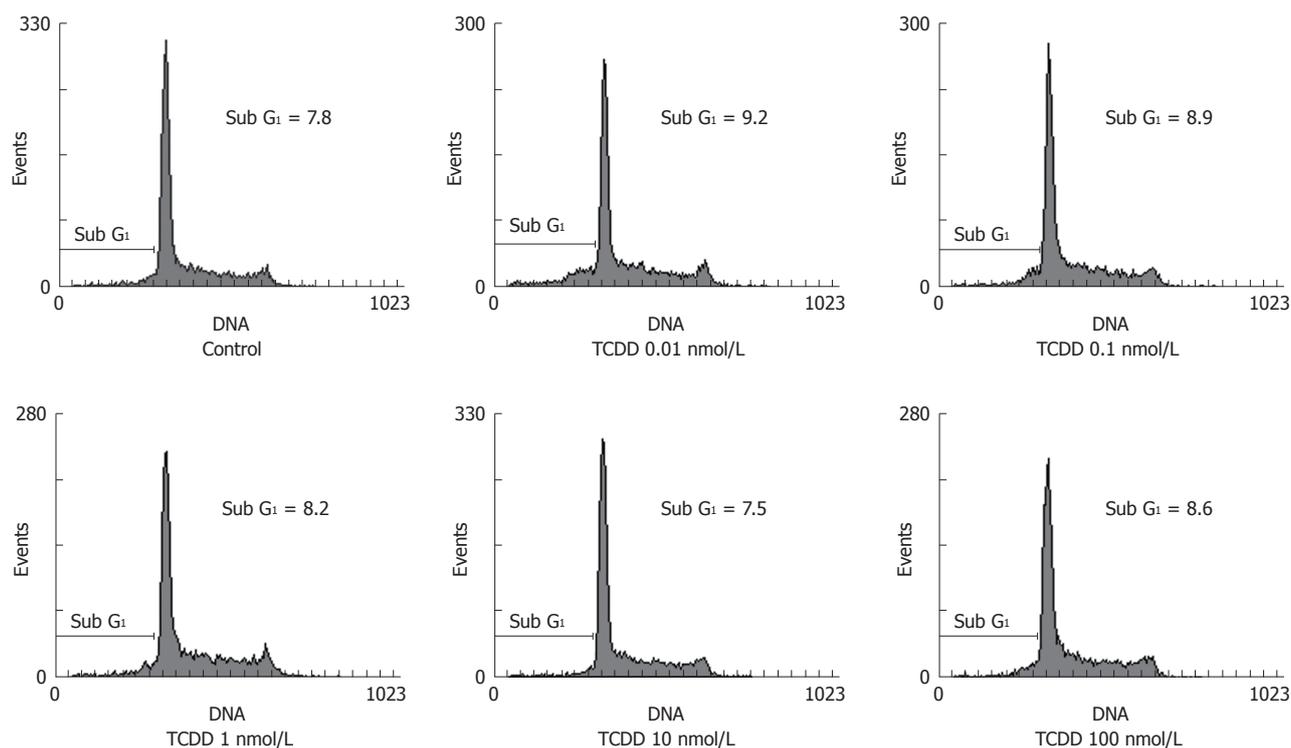


Figure 8 The effect of TCDD on apoptosis of AGS cells. AGS cells were treated with different concentrations of TCDD and subjected to flow cytometric analysis. Cellular apoptosis was evaluated by fragmented DNA (sub-G₁) analysis using winMDI2.9. The results shown are representative of three independent experiments.

Since AhR is significantly up-regulated in GC and may be involved in the early stage of gastric carcinogenesis, regulation of the AhR pathway may have a potential role in the treatment of GC. Interestingly, our MTT assay demonstrated that the viability of AGS cells was significantly decreased in a dose- and time-dependent manner after TCDD treatment. Further flow cytometric analysis indicated that TCDD inhibited growth of AGS cells *via* induction of growth arrest at the G₁-S phase. As far as we know, this is the first report suggesting an inhibitory role of AhR agonists on human GC cell growth. Similar results have been reported in the treatment of pancreatic cancer and mammary tumors by AhR agonists^[16,18,40]. However, previous studies by Andersson *et al.*^[19,20] showed that constitutively active AhR may result in significant proliferation in the parietal/chief cell region of glandular gastric mucosa in transgenic mice. These contradictory outcomes indicate that AhR appears to contribute to processes in both cell cycle arrest as well as cell proliferation. Recent studies on the cellular signal pathway may partly explain this complex phenomenon. As an evolutionarily conserved transcription factor, outside its well-characterized role in the induction of xenobiotic metabolizing enzymes, AhR also functions as a modulator of cellular signaling pathways. By interacting with different signal pathway effectors, AhR activation may result in completely different effects on cell growth^[12,13]. Our present findings on the inhibitory effect of TCDD on GC cell growth suggest that AhR may be a potential therapeutic target for gastric cancer.

ACKNOWLEDGMENTS

This study was accepted and presented orally at the 15th United European Gastroenterology Week (UEGW 2007) in Paris, in October 2007. The authors thank Dr. Robert Wong for his improvements to the English in the manuscript.

COMMENTS

Background

The carcinogenesis of gastric cancer (GC) involves numerous genetic and epigenetic alterations, as well as many environmental risk factors. Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated hydrocarbons (HAHs) are well-known carcinogens that play important roles in GC development. The toxic effects of PAHs and HAHs are mediated by a conserved signaling pathway that binds and activates the aryl hydrocarbon receptor (AhR).

Research frontiers

AhR is a ligand-activated transcription factor and can mediate the carcinogenic and other toxic effects of a variety of environmental pollutants. Many studies in recent years have demonstrated a close relationship between AhR and tumorigenesis. However, the role of AhR in gastric tumorigenesis is still unclear. In this study, the authors demonstrate a close correlation of AhR with GC formation and the potential role of AhR as a therapeutic target for GC treatment.

Innovations and breakthroughs

AhR functions as a modulator of cellular signaling pathways. By interacting with different signal pathway effectors, AhR activation may result in completely different effects on cell growth. This is the first report suggesting an inhibitory role of AhR agonists on human GC cell growth. Furthermore, the present findings suggest that AhR may be a potential therapeutic target for GC.

Applications

By understanding how cell growth of human GC is influenced by AhR activation, this study may represent a future strategy for therapeutic intervention in the treatment of patients with GC.

Terminology

AhR is a ligand-activated transcription factor of the basic helix-loop-helix/Per-

Arnt-Sim family. As an evolutionarily conserved transcription factor, outside its well-characterized role in the induction of xenobiotic metabolizing enzymes, AhR also functions as a modulator of cellular signaling pathways.

Peer review

The authors investigated the functional significance of AhR in gastric carcinogenesis. This paper is interesting and written well.

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ORIGINAL ARTICLES

Effect of Bu-Zhong-Yi-Qi-Tang on deficiency of N-glycan/nitric oxide and islet damage induced by streptozotocin in diabetic rats

Xiao-Qiu Liu, Ling Wu, Xue-Jun Guo

Xiao-Qiu Liu, Ling Wu, Pi-Wei Institute, Guangzhou University of Traditional Chinese Medicine, Guangzhou 510405, Guangdong Province, China

Xue-Jun Guo, Laboratory Animal Research Center, Guangzhou University of Traditional Chinese Medicine, Guangzhou 510405, Guangdong Province, China

Author contributions: Liu XQ took part in the animal experiments, provided the vital reagents and analytical tools, designed the study and wrote the manuscript; Wu L performed the animal experiments; Guo XJ took part in the animal experiments.

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Correspondence to: Xiao-Qiu Liu, Professor, Pi-Wei Institute, Guangzhou University of Traditional Chinese Medicine, No. 12, Jichang Road, Guangzhou 510405, Guangdong Province, China. lxq6298@yahoo.com.cn
Telephone: +86-20-36585077

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Abstract

AIM: To investigate the effect of Bu-Zhong-Yi-Qi-Tang (Decoction for Reinforcing Middle Jiao and Replenishing Qi) on deficiency of N-glycan/nitric oxide (NO) and islet damage induced by injecting two medium doses of streptozotocin (STZ).

METHODS: Diabetes was induced by intraperitoneal injection of STZ at 55 mg/kg on day 1 and day 8. Islet damage was evaluated using a scoring system. Nitrite, nitrate, α -mannosidase and amylase activities were measured by colorimetry. N-glycan patterns of amylase were determined with lectin [ConA, pisum sativum agglutinin (PSA), peanut agglutinin (PNA), and lens culinaris agglutinin (LCA)] affinity precipitation method.

RESULTS: Severe islet necrosis and mild islet atrophy were observed in diabetic rats. The number and size of islets, the activities of α -mannosidase, amylase and nitrite were decreased, while the binding of PNA and LCA to amylase was increased. All of which were improved after treatment with Bu-Zhong-Yi-Qi-Tang. Islet damage was significantly correlated with nitrite, nitrate, α -mannosidase, amylase and the binding of LCA, PNA, and PSA to amylase.

CONCLUSION: STZ-induced islet damage is related to N-glycan deficiency in proteins by blocking α -mannosidase activity and no deficiency, accumulation of unfolded proteins, and endoplasmic reticulum stress and activation of cellular signals, all of which are improved after treatment with Bu-Zhong-Yi-Qi-Tang.

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Key words: N-Glycan; Nitric oxide; Diabetic rats; Islet damage; Alpha mannosidase; Bu-Zhong-Yi-Qi-Tang

Peer reviewer: Atsushi Nakajima, Professor, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

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INTRODUCTION

N-glycan plays an important role in the quality control (QC) of glycoprotein folding both in endoplasmic reticulum (ER) lumen and in ER-associated degradation (ERAD) of proteins by cytosolic proteasomes^[1]. Alpha-mannosidase, a key enzyme converting precursor high-mannose-type N-glycans to matured complex-type structures, contributes to the establishment of an equitable glycoprotein quality control standard, by which the efficiency of Asn-linked glycoprotein conformational maturation results in dislocation of misfolded glycoproteins into the cytosol, where proteins are degraded in proteasomes and maintain the homeostasis of ER^[2].

The unfolded protein response (UPR) is a conserved cellular response designed to alleviate damage and promote survival of cells experiencing stress by either shutting down translation to reduce the protein flow into ER, or by up-regulating molecules that protect cells or clear misfolded proteins from the ER, thus alleviating ER stress. Nitric oxide (NO) is a known activator of the

UPR^[3-5], and NO-induced UPR activation may function to assist in the recovery of β -cells from NO-mediated damage, which is associated with enhanced expression of heat shock proteins and chaperones that assist in protein folding.

Islet β cells have a well-developed ER and secrete a large amount of insulin and glycoproteins. Islet β cells may be at risk of developing ER stress and increase misfolded glycoproteins. Inability to clear misfolded proteins causes early accumulation of unfolded proteins, and ER stress and activation of cellular signals lead to cell death, thus resulting in loss of β cell secretion potential and diabetes.

Xiong *et al.*^[6] showed that the pathogenesis of diabetes is closely correlated to Pixu (insufficiency of the spleen), a major pathogenic factor for diabetes syndrome. Pixu is therefore considered in the treatment of diabetes. Diabetes mellitus is caused by Pixu, when diabetes progresses, deficiency of Yin and dryness-heat exist simultaneously, and when diabetes prolongs, deficiency of Yin becomes the key pathogenesis, finally resulting in impaired Yin and Yang^[7].

The levels of nitrite and nitrate are significantly decreased in stimulated crude whole saliva of diabetic patients with Pixu^[8]. Bu-Zhong-Yi-Qi-Tang, a traditional Chinese medicine, tonifies qi, strengthens the stomach and spleen, and increases sunken Yang qi. It is prescribed mainly for Pixu by improving the deficiency of spleen and stomach qi. It was reported that the lower levels of nitrite, nitrate, and peroxynitrite in gastric mucosa of rats with Pixu can return to normal after treatment with Bu-Zhong-Yi-Qi-Tang^[9].

This study was to examine the effect of Bu-Zhong-Yi-Qi-Tang on deficiency of N-glycan/NO and islets damage in rats with diabetes mellitus induced by streptozotocin (STZ).

MATERIALS AND METHODS

Ethics

This study was approved by Guangdong Science and Technology Committee and Guangdong Management Committee for Medical Experimental Animals.

General procedure

Sprague-Dawley rats were obtained from the Laboratory Animal Research Center of Guangzhou University of Traditional Chinese Medicine. The animals were housed in a plastic cage in an air-conditioned room at $20 \pm 2^\circ\text{C}$ with a humidity of $58\% \pm 5\%$ in a 12-h light and dark cycle, with free access to standard rat food and tap water. The rats were divided into normal control, and diabetic model groups after 1 wk.

Diabetic model

A diabetic model of rats was established by intraperitoneal injection of STZ at 55 mg/kg on day 1 and day 8. The rats were bred for 4 wk. Blood was sampled from the tail vein and blood glucose was determined with the one touch ultra blood glucose monitoring system (LifeScan,

Inc. Milpitas, CA, USA). Animals with their non-fasting blood glucose less than 16.65 mmol/L were excluded from the study. The rats were divided into diabetics groups and Bu-Zhong-Yi-Qi-Tang treatment group.

Preparation and administration of Bu-Zhong-Yi-Qi-Tang

Bu-Zhong-Yi-Qi-Tang was made of *Radix Astragali*, *Codonopsis pilosula*, *Glycyrrhiza uralensis* f. *isob*, *Rhizoma Atractylodis Macrocephalae*, *Radix Angelicae Sinensis*, *Rhizoma Cimicifugae Foetidae*, *Radix Bupleuri Chinensis*, and *Pericarpium Citri Reticulatae*, which were put into a 20-fold volume of distilled water, decocted from 80°C to 100°C , filtered and concentrated at 40°C - 80°C , and stored in a refrigerator until use. Bu-Zhong-Yi-Qi-Tang was administered by gavage, 8 g/kg per day for 4 wk.

Samples

At the end of treatment, the animals were killed by exsanguination from the carotid artery. Blood samples were taken immediately and centrifuged at 3000 r/min for 5 min. Serum was separated for the measurement of nitrite, nitrate, α -mannosidase and amylase activities, and determination of the N-glycan patterns of amylase. The pancreas was removed from each rat and dissected longitudinally. One half was dried and homogenized. Supernatant was removed for the measurement of nitrite, nitrate, α -mannosidase and amylase activities, and determination of the N-glycan patterns of amylase. The other half was fixed in Bouin's solution for 24 h. The pancreatic tissue, embedded in paraffin wax, was cut into 3-4- μm -thick sections which were stained with hematoxylin and eosin (HE) for histopathological examination.

Histopathological examination of pancreatic islet damage

Bouin's-solution-fixed pancreatic tissue was cut into eight sections (approximately 1 mm \times 1 mm \times 1 mm). Four sections were randomly selected, stained with HE, and observed under a microscope with a digital camera (C170 D425, Olympus Imaging Corp). Digital micrographs were analyzed for estimating the number, size, necrosis and atrophy of islets and insulinitis by two observers unaware of the status and/or treatment modalities.

The number of islets was calculated as previously described^[10] with some minor modifications and graded as 0, no visible islets; 1, < 5 islets; 2, 6-10 islets; 3, 11-20 islets; 4, > 20 islets.

The size of islets was measured as previously described^[11] with some minor modifications and graded as 1, 1-40 cells per section; 2, 41-100 cells per section; 3, 101-200 cells per section; 4, 201-400 cells per section; 5, > 400 cells per section.

Insulinitis was evaluated by examining at least 10 islets from each treatment group and graded as 0, no mononuclear cell infiltration; 1, very mild mononuclear cell infiltration in and around the islets; 2, mild cell infiltration in and around the islets; 3, moderate cell infiltration in and around the islets; 4, marked cell infiltration observed as the structure of islets was

destroyed. Pancreas was graded for insulinitis according to the most severely involved islets in each pancreas.

Necrosis of islets was graded as 0, no islet necrosis; 1, very mild islet necrosis; 2, mild islet necrosis; 3, moderate islet necrosis; 4, marked islets necrosis.

Atrophy of islets was graded as 0, no islet atrophy; 1, very mild islet atrophy; 2, mild islet atrophy; 3, moderate islet atrophy; 4, marked islet atrophy.

Determination of nitrite and nitrate in serum and pancreas tissue

Pancreatic tissue was homogenized, supernatant and serum were initially deproteinized with Somogyi reagent^[12] (using 2.0 mL 55 mmol/L NaOH followed by 2.0 mL 75 mmol/L ZnSO₄). After centrifugation, aliquots of supernatant were mixed with an equal volume of Griess reagent^[13]. The absorbance was measured at 546 nm. Nitrite level was measured using 120 μmol/L sodium nitrite solution diluted to 10 μmol/L intermediate dissolution. Results were expressed as μmol/L per gram wet tissue, and serum nitrite was expressed as μmol/L.

Pancreatic tissue was homogenized, supernatant and serum were initially deproteinized with Somogyi reagent^[12] (using 2.0 mL 55 mmol/L NaOH followed by 2.0 mL 75 mmol/L ZnSO₄). After centrifugation, excess nitrite was eliminated with 10 mg/mL ammonium sulfamate and acidified with 1 mol/L hydrochloric acid which prevented interference with hydroxide or carbonate. Aliquots of supernatant nitrate were determined by dual-wavelength ultraviolet spectrophotometry^[14,15]. Nitrate absorbance was calculated by subtracting the absorbance at 275 nm for background correction from the absorbance at 220 nm. Nitrate level was measured using 120 μmol/L standard sodium nitrate solution diluted to 10 μmol/L intermediate dissolution. Results were expressed as μmol/L per gram wet tissue, and serum nitrate was expressed as μmol/L.

Determination of α-mannosidase activity in serum and pancreas tissue

The animals were killed by exsanguination from the carotid artery. Blood samples were taken immediately. The serum was stored at -18°C. Pancreas was removed, fragmented and stored at -20°C until required. Extracts from pancreatic tissue were prepared by homogenization in 0.05 mol/L sodium phosphate (pH 6.5) containing 5 mmol/L MgCl₂ and 0.1% Triton X-100 (a DY89-1 tissue homogenizer) at 4°C. Tissue homogenate was centrifuged at 16000 r/min for 30 min. Clear supernatant and serum were used to determine the enzyme activity. The level of α-mannosidase was measured by spectrophotometry as previously described^[16] with some minor modifications. Fifty microliters of supernatant (or serum) was added to 50 μL 4 mmol/L p-nitrophenyl-α-D-mannopyranoside substrate (Fluka, Bucks, Switzerland) in 0.1 mol/L sodium acetate-acetic acid buffer (pH 5.8). Reaction mixtures were incubated at 37°C for 60 min. The reaction was terminated by adding

3 mL glycine-sodium hydroxide (0.1 mol/L, pH 10.14). The amount of liberated p-nitrophenol was determined by spectrophotometry at 405 nm. One international unit of enzyme was defined as the amount required for catalyzing the release of 1 μmol p-nitrophenol from p-nitrophenyl-α-D-mannopyranoside substrate per hour at 37°C under the conditions described above. Tissue enzyme activities were expressed as U/g wet tissue, and serum enzyme activities were expressed as U/mL serum.

Determination of amylase activity and binding of lectin to amylase in serum and pancreas tissue

Extracts from pancreatic tissue were prepared by homogenization in 9 g/L NaCl (a DY89-1 tissue homogenizer) at 4°C. Tissue homogenate was centrifuged at 16000 r/min for 30 min. Clear supernatant and serum were used to determine the amylase activity and binding of lectin to amylase. Amylase N-glycan patterns were analyzed with lectin [ConA, pisum sativum agglutinin (PSA), peanut agglutinin (PNA), lens culinaris agglutinin (LCA)] affinity precipitation method. The precipitation procedure was performed as previously described^[17,18]. The clear supernatant and serum were mixed with 400 μL of an aqueous solution of ConA, PSA, PNA, LCA (5 g/L in distilled water). The mixture was incubated for 30 min at 37°C and centrifuged at 2000 r/min for 15 min. Without disturbing the precipitate, we removed the supernatant and measured its amylase activity. Binding of lectin to amylase was calculated by subtracting the corrected value from the total amylase activity. ConA, PSA, PNA, LCA were purchased from Sigma (St. Louis, MO, USA).

Statistical analysis

All data were presented as mean ± SD. Statistical significance was calculated using unpaired Student's *t* test. The correlation coefficient between islet histomorphometry and nitrite, nitrate, binding of lectin to amylase, activity of α-mannosidase and amylase was calculated. *P* < 0.05 was considered statistically significant. All analyses were performed using Excel 2003.

RESULTS

Body weight and blood glucose level

The body weight of normal rats increased over the 12 wk experimental period. However, diabetic rats lost 22.1% of their body weight within 12 wk, the non-fasting blood glucose level increased from 16.62 mmol/L to 31.63 mmol/L 4 wk after injection of STZ, and remained significantly elevated for 8 wk thereafter.

Histopathological changes in pancreatic islets

Relatively well documented pancreatic islets and tightly arranged islet cells were observed in the normal control group. Severe islet necrosis and mild islet atrophy were detected in the diabetic group, and improved after treatment with Bu-Zhong-Yi-Qi-Tang compared to the diabetic group (Figure 1). Histomorphometrical analysis

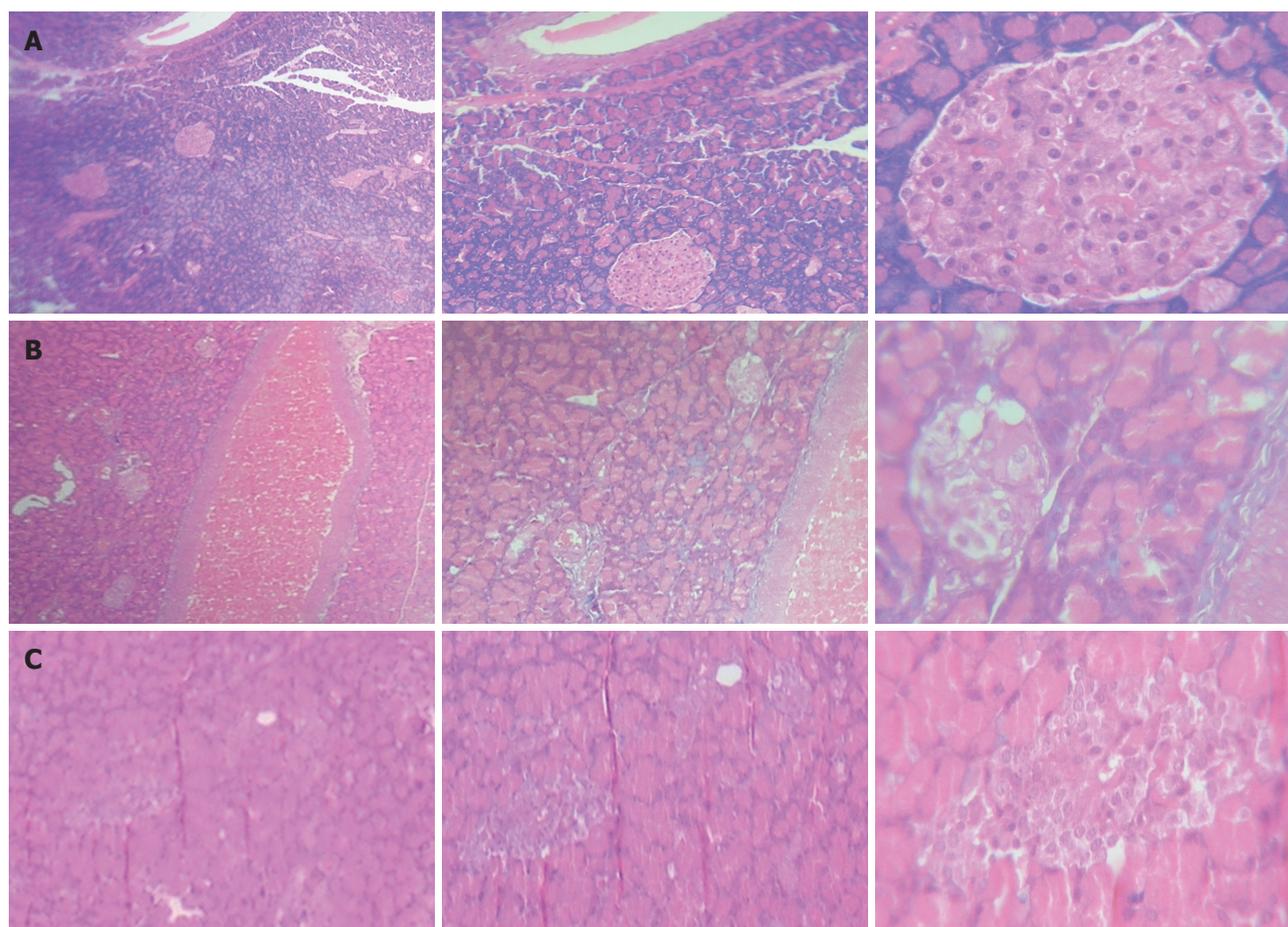


Figure 1 Histological profiles for pancreatic islets in normal rats (A), diabetic rats (B), Bu-Zhong-Yi-Qi-Tang-treated rats (C). Relatively well documented pancreatic islets and tightly arranged islet cells were observed in normal rats. Severe necrosis and mild atrophy of islets were found in diabetic rats, which were improved after treatment with Bu-Zhong-Yi-Qi-Tang. Stained sections showing islet morphology in sections stained with HE. The left region: Low-power magnification view; Middle region: Middle-power magnification view; Right region: High-power magnification view.

Table 1 Changes in histomorphometry of pancreatic islets of diabetic rats after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	Islet number islets/pancrease section	Islet size cells/islet section	Necrosis	Atrophy	Cell infiltration
Normal rats	3.50 \pm 0.84	3.83 \pm 0.45	0	0.17 \pm 0.41	0
Diabetic rats	1.08 \pm 0.29 ^b	1.00 \pm 0.0 ^b	3.25 \pm 0.45 ^b	2.08 \pm 0.29 ^a	0
Bu-Zhong-Yi-Qi-Tang-treated rats	1.17 \pm 0.41 ^b	2.5 \pm 0.55 ^{b,d}	0.33 \pm 0.52 ^d	1.17 \pm 0.41 ^{a,d}	0

^a $P < 0.05$, ^b $P < 0.01$ vs normal control rats; ^d $P < 0.01$ vs diabetic rats.

Table 2 Levels of nitrite and nitrate in serum and pancreas tissue of diabetic rats after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	Nitrite $\mu\text{mol/L}$ serum	Nitrate $\mu\text{mol/L}$ serum	Nitrite $\mu\text{mol/L}$ per gram wet tissue	Nitrate $\mu\text{mol/L}$ per gram wet tissue
Normal rats	21.33 \pm 0.25	98.78 \pm 49.20	146.70 \pm 24.77	13.29 \pm 10.73
Diabetic rats	14.00 \pm 0.19 ^b	85.76 \pm 30.08	95.60 \pm 28.89 ^b	11.59 \pm 9.54
Bu-Zhong-Yi-Qi-Tang-treated rats	25.98 \pm 0.74 ^{b,d}	114.78 \pm 30.09 ^d	132.82 \pm 54.03 ^d	21.30 \pm 18.73

^a $P < 0.05$, ^b $P < 0.01$ vs normal rats; ^d $P < 0.01$ vs diabetic rats.

showed that the number and size of islets were significantly decreased, and the necrosis and atrophy of islets were significantly exacerbated in the diabetic group compared to the normal control group. However, the size of islets was significantly increased, and the necrosis and atrophy of islets were significantly improved in the Bu-Zhong-Yi-Qi-Tang treatment group compared to the diabetic group (Table 1).

Levels of nitrite and nitrate in serum and pancreas tissue

The levels of nitrite and nitrate in serum and pancreas tissue of the diabetic group were significantly lower than those in the normal control group, and significantly increased in the Bu-Zhong-Yi-Qi-Tang treatment group compared to the diabetic group (Table 2).

Table 3 Activity of α -mannosidase and amylase in serum and pancreas tissue of diabetic group after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	Serum enzyme activities (U/mL serum)		Tissue enzyme activities (U/g wet tissue)	
	α -mannosidase	Amylase	α -mannosidase	Amylase
Normal rats	3.60 \pm 2.43	28.71 \pm 11.07	81.78 \pm 30.34	19.53 \pm 3.84
Diabetic rats	3.17 \pm 7.07	17.35 \pm 13.76 ^a	20.26 \pm 20.42 ^b	13.15 \pm 0.98 ^a
Bu-Zhong-Yi-Qi-Tang-treated rats	1.53 \pm 1.72	25.14 \pm 8.76 ^c	44.79 \pm 26.93 ^{b,d}	17.94 \pm 2.20 ^d

^a $P < 0.05$, ^b $P < 0.01$ vs normal rats; ^c $P < 0.05$, ^d $P < 0.01$ vs diabetic rats.

Table 4 Binding of lectin to amylase in serum of diabetic group after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	ConA-binding% (O-binding%)	LCA-binding% (O-binding%)	PNA-binding% (O-binding%)	PSA-binding% (O-binding%)
Normal rats	22.18 \pm 42.27 (71.43)	2.61 \pm 6.78 (85.71)	3.89 \pm 9.92 (85.71)	6.52 \pm 14.22 (78.57)
Diabetic rats	31.18 \pm 43.91 (58.33)	27.84 \pm 44.15 ^a (58.33)	32.86 \pm 41.01 ^a (50.0)	25.0 \pm 45.23 (75.0)
Bu-Zhong-Yi-Qi-Tang-treated rats	1.20 \pm 3.62 ^{b,c} (87.50)	16.41 \pm 34.06 (75.0)	1.78 \pm 5.0 ^c (87.50)	9.09 \pm 16.44 (62.50)

^a $P < 0.05$ vs normal rats; ^c $P < 0.05$ vs diabetic rats.

Table 5 Binding of of lectin to amylase in pancreas tissue of diabetic group after treatment with Bu-Zhong-Yi-Qi-Tang (mean \pm SD)

Treatment	ConA-binding% (O-binding%)	LCA-binding% (O-binding%)	PSA-binding% (O-binding%)
Normal rats	23.53 \pm 27.83 (40.0)	7.06 \pm 15.78 (80.0)	20.0 \pm 44.72 (80.0)
Diabetic rats	20.0 \pm 35.46 (62.5)	47.30 \pm 40.53 ^a (12.5)	14.87 \pm 27.68 (75.0)
Bu-Zhong-Yi-Qi-Tang-treated rats	16.82 \pm 31.18 (50.0)	35.91 \pm 37.08 ^a (30.0)	22.0 \pm 41.46 (50.0)

^a $P < 0.05$ vs normal rats.

Activity of α -mannosidase and amylase in serum and pancreas tissue

The activity of α -mannosidase and amylase in pancreas tissue in the diabetic group was significantly lower than that in the normal control group. However, the serum α -mannosidase activity was not significantly different between the two groups. The activity of α -mannosidase and amylase in pancreas tissue was significantly increased in the Bu-Zhong-Yi-Qi-Tang treatment group compared to the diabetic group (Table 3).

Binding of lectin to amylase in serum and pancreas tissue

Binding of LCA, and PNA to serum amylase and binding of LCA to pancreas amylase in the diabetic group were significantly higher than those in the normal control group, while the binding of ConA and PSA to amylase was not significantly different between the two groups. After treatment with Bu-Zhong-Yi-Qi-Tang, the binding of ConA, PNA and LCA to amylase was significantly lower than that in the diabetic group (Tables 4 and 5).

Correlation between the number, size, necrosis and atrophy of islets and the activity of α -mannosidase, amylase, nitrite, nitrate, and binding of lectin to amylase

The number, size, necrosis and atrophy of islets were significantly correlated with the activity of nitrite, nitrate, α -mannosidase, amylase and binding of LCA, PNA, and PSA to amylase (Table 6).

DISCUSSION

In the present study, severe necrosis and mild atrophy of islets were observed in diabetic rats. The number and size of islets. The activity of α -mannosidase and amylase were decreased, and the binding of PNA and LCA to amylase were increased. All of which were improved after treatment with Bu-Zhong-Yi-Qi-Tang.

Islet damage was found to be related to the levels of nitrite, nitrate, α -mannosidase and amylase, and the binding of LCA, PNA and PSA to amylase.

Trimannosyl oligosaccharide is a unique moiety recognized by concanavalin A lectin (ConA) for high affinity and extended site binding. ConA primarily binds to the outer trimannosyl region of high mannose and bisected hybrid-type glycopeptides rather than to the central trimannosyl region of complex glycopeptides^[19]. Fucosyl residues in the outer chain moieties have a shielding effect on the neighboring α -mannosyl residues and can eliminate this type of interaction^[20]. PNA is a lectin with a high binding affinity for galactose-galactosamine disaccharide^[21]. NeuAc caps the galactose-terminated chains^[22]. Like ConA, LCA is able to bind (or not bind) to the same glycopeptides. LCA additionally requires the fucosyl- N-glycan-asparaginyl core for high-affinity binding. The presence of a core fucose residue greatly enhances recognition of N-linked sugar chains by LCA. Exposure of terminal N-acetylglucosamine (GlcNAc) residues to glycopeptides can enhance the binding of glycopeptide to LCA^[23]. Like ConA, PSA is able to bind (or not bind) to the same glycopeptides. PSA

Table 6 Correlation between the number, size, necrosis and atrophy of islets and levels of α -mannosidase, amylase, nitrite and nitrate and binding of lectin to amylase

Biochemistry index	n	Islet number	Islet size	Necrosis	Atrophy
Serum nitrite	50	0.404 ^b	0.736 ^b	-0.319 ^b	-0.866 ^b
Serum nitrate	50	0.181	0.194	-0.126	-0.245 ^a
Pancreas tissue nitrite	50	0.30 ^a	0.456 ^b	-0.222	-0.480 ^b
Pancreas tissue nitrate	50	0.101	0.157	-0.041	-0.317 ^b
LCA-binding of amylase in serum	61	-0.254 ^a	-0.290 ^a	0.077	0.228 ^a
ConA-binding of amylase in serum	61	-0.048	-0.10	-0.011	-0.016
PNA-binding of amylase in serum	61	-0.114	-0.261 ^a	0.283 ^a	0.041
PSA-binding of amylase in serum	61	-0.143	-0.26 ^a	0.165	0.153
LCA-binding of amylase in pancreas tissue	43	-0.442 ^b	-0.253 ^a	0.174	0.183
ConA-binding of amylase in pancreas tissue	43	-0.117	-0.095	-0.192	0.232
PSA-binding of amylase in pancreas tissue	43	-0.095	-0.037	-0.288 ^a	0.106
The activity of α -mannosidase in serum	61	0.067	0.050	0.026	-0.108
The activity of α -mannosidase in pancreas tissue	43	0.638 ^b	0.747 ^b	-0.508 ^b	-0.714 ^b
The activity of amylase in serum	61	0.158	0.365 ^b	-0.215 ^a	-0.310 ^b
The activity of amylase in pancreas tissue	43	0.428 ^b	0.639 ^b	-0.314 ^a	-0.631 ^b

^a $P < 0.05$, ^b $P < 0.01$, test of significance of correlation coefficient.

additionally requires the fucosyl-N-glycan-asparaginyl core for high affinity binding. The presence of a core fucose residue greatly enhances recognition of N-linked sugar chains by PSA. Exposure of terminal mannose residues to glycopeptides can enhance the binding of glycopeptide to PSA^[23].

These findings indicate that N-glycan processing is deficient in diabetic rats. Amylase core-fucosylate, high-mannose-type, hybrid-type sugar chains are increased while terminal sialic acid and fucose on the sugar chain are decreased, thus resulting in exposure of terminal galactose and GlcNAc residues to PNA and LCA.

N-glycan plays an important role in the quality control of glycoprotein folding in lumen and ERAD of proteins by cytosolic proteasomes^[1]. Alpha-mannosidase, a key enzyme converting precursor high-mannose-type N-glycan to matured complex-type structure, contributes to the establishment of an equitable glycoprotein quality control standard, by which the efficiency of Asn-linked glycoprotein conformational maturation results in dislocation of misfolded glycoproteins into the cytosol where proteins are degraded in the proteasome and maintain the homeostasis of ER^[2].

Lin *et al*^[24] cultured neurons derived from embryonic chicken brains with tunicamycin (TM), an inhibitor of N-linked glycosylation, and found that the light neurons resembled necrotic cells, but the dense neurons exhibited distinct morphological features of necrosis and apoptosis, indicating that TM has an irreversible toxicity to the neurons and a different mechanism underlying neuron death.

Finnie *et al*^[25] showed that cultured rat hepatocytes exposed to TM have degenerative changes characterized by marked cytoplasmic lipid accumulation and dilatation of cisternae of rough ER or necrosis.

Shi *et al*^[26,27] reported that inhibition of 6A8 alpha-mannosidase causes oncosis-like death of BJAB cells with no apoptotic bodies, annexin-V staining DNA fragmentation assay cannot show any evidence of apoptosis in these cells. However, binding of ConA

to the cells transduced with the antisense 6A8 DNA is increased, but ConA-binding to the cells transduced with the sense 6A8 DNA is decreased.

The UPR is a conserved cellular response designed to alleviate damage and promote survival of cells experiencing stress by either shutting down translation to reduce the protein flow into the ER or by up-regulating molecules that protect cells or clear misfolded proteins from the ER and alleviate stress. NO, a known activator of UPR^[3-5], may function to assist in the recovery of β -cells from NO-mediated damage, which is associated with enhanced expression of heat shock proteins and chaperones that assist in protein folding.

Islet β cells have a well-developed ER, reflecting their role in secreting a large amount of insulin and glycoproteins. Islet β cells may be at risk for ER stress. Inability to clear misfolded proteins causes accumulation of unfolded proteins, ER stress and activation of cellular signals, leading to cell death, loss of β cell secretion potential, and diabetes.

These findings indicate that STZ induces islet damage, which might be related to the N-glycan processing deficiency of proteins. Blocking of α -mannosidase activity and NO deficiency can easily cause accumulation of unfolded proteins, ER stress and activation of cellular signals leading to cell death.

Diabetes (Xiaoke) was firstly recorded in the Yellow Emperor's Canon of Medicine (722-221 B.C.), a classic medical book, in which Xiaozhong (polyorexia) and Xiaodan (diabetes) are used to describe diabetes. It points out that the etiologic factors excess fat and sugar, and imbalance of emotion. The General Treatise on the Etiology and Symptomology (618-907 AD) say that diabetes patients often suffer from carbuncle and deep-root carbuncle. The Medical Secrets of an Official (670-755 AD) describes that urine of patients with diabetes is sweet, indicating that glucose can be found in the urine of diabetes patients early.

It has been shown that the pathogenesis of diabetes is closely related to Pixu^[6], a major pathogenic factor

for diabetes syndromes. Improving Pixu is therefore appropriate in the treatment of diabetes. In this study, the nitrite and nitrate levels were significantly decreased in diabetic rats, which is consistent with the reported findings in patients with Pixu^[8].

Bu-Zhong-Yi-Qi-Tang, a traditional Chinese medicine, tonifies qi, strengthens the stomach and spleen, and increases sunken Yang qi, and is prescribed mainly for the deficiency of spleen and stomach qi in the treatment of Pixu. It was reported that the levels of nitrite, nitrate, and peroxyxynitrite in gastric mucosa are lower in Pixu rats, and return to normal after treatment with Bu-Zhong-Yi-Qi-Tang^[9].

In conclusion, Bu-Zhong-Yi-Qi-Tang can improve these abnormal conditions of diabetic rats by increasing the levels of nitric oxide and mannosidase activation, promoting N-glycosylation of proteins, assisting in the quality control of glycoprotein folding in ER lumen, and preventing accumulation of unfolded proteins, ER stress, activation of cellular signals.

COMMENTS

Background

N-glycans, α -mannosidase and nitric oxide (NO) play an important role in the quality control of glycoprotein folding in endoplasmic reticulum (ER) lumen, promote ER homeostasis and prevent ER-stress-related cell damage. There is evidence that ER stress plays a role in the pathogenesis of diabetes, contributing to loss of pancreatic beta-cells and insulin resistance. However, little attention has been paid to islet damage associated with the deficiency of N-glycan/nitric oxide in diabetes mellitus patients. It has been shown that the pathogenesis of diabetes is closely related to Pixu, a major pathogenic factor for diabetes syndromes. Improving Pixu is therefore appropriate in the treatment of diabetes. Bu-Zhong-Yi-Qi-Tang, a traditional Chinese decoction, is prescribed mainly for Pixu. However, little attention has been paid to its activity in islet damage and related mode of action in patients with diabetes.

Research frontiers

N-glycans, α -mannosidase and NO play an important role in the quality control of glycoprotein folding in ER lumen, promote ER homeostasis and prevent ER-stress-related cell damage. There is evidence that ER stress plays a role in the pathogenesis of diabetes, contributing to loss of pancreatic beta-cells and insulin resistance. The results of this study indicate that streptozotocin induces islet damage, which might be related to the N-glycan processing deficiency of proteins. Blocking of α -mannosidase activity and nitric NO easily causes accumulation of unfolded proteins, ER stress and activation of cellular signals leading to cell death. Bu-Zhong-Yi-Qi-Tang could improve these abnormal conditions of diabetic rats by increasing the levels of NO and mannosidase activity, promoting N-glycosylation of proteins, assisting in quality control of glycoprotein folding in ER lumen, and preventing accumulation of unfolded proteins, ER stress, activation of cellular signals leading to cell death.

Terminology

Bu-Zhong-Yi-Qi-Tang: a traditional Chinese medicine, consisting of *Radix Astragali*, *Codonopsis pilosula*, *Glycyrrhiza uralensis* *isch*, *Rhizoma Atractylodis Macrocephalae*, *Radix Angelicae Sinensis*, *Rhizoma Cimicifugae Foetidae*, *Radix Bupleuri Chinensis*, *Pericarpium Citri Reticulatae*, is prescribed mainly for deficiency of spleen and stomach qi in treatment of Pixu. Streptozotocin (STZ): 2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose, is actively transported into pancreatic β cells via the Glut-2 glucose transporter. Alpha-mannosidase: a key enzyme converting precursor high-mannose-type N-glycans to matured complex-type structure, contributes to the establishment of an equitable glycoprotein quality control standard, by which the efficiency of Asn-linked glycoprotein conformational maturation results in dislocation of misfolded glycoproteins into the cytosol, where proteins are degraded in proteasome and maintain the homeostasis of ER.

Peer review

This is a good descriptive study. Experiments were well designed and the data

contained novelty. The authors investigate the deficiency of N-glycan/nitric oxide in diabetic rats, and evaluated the effects of Bu-Zhong-Yi-Qi-Tang on diabetics, showing that STZ can induce islet damage, which might be related to the N-glycan processing deficiency of proteins. Blocking of α -mannosidase activity and nitric oxide deficiency could easily cause accumulation of unfolded proteins, ER stress and activation of cellular signals leading to cell death. Bu-Zhong-Yi-Qi-Tang was found to be effective against these abnormal conditions.

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Expression and location of α -fetoprotein during rat colon development

Xiao-Yan Liu, Dan Dong, Peng Sun, Jun Du, Luo Gu, Ying-Bin Ge

Xiao-Yan Liu, Dan Dong, Peng Sun, Jun Du, Luo Gu, Ying-Bin Ge, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Author contributions: Liu XY and Dong D performed the animal treatment, RT-PCR, immunofluorescent staining and wrote the paper; Sun P did part of RT-PCR and immunofluorescent staining; Du J conducted Western blot and statistical analysis; Ge YB designed the experiment and wrote the paper; Gu L and Ge YB contributed equally to this work.

Correspondence to: Ying-Bin Ge, MD, PhD, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. ybge@njmu.edu.cn

Telephone: +86-25-86862016 Fax: +86-25-86862016

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demonstrated that AFP is localized in the mesenchyme of rat colon from the embryo to the weaning stage by immunofluorescence and presents 72-kDa isoform in the developing rat colons by Western blotting. The dynamic expression of AFP in the various developmental stages of the colon indicates that AFP might be involved in many aspects of colon development.

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Key words: Alpha-fetoprotein; Development; Mesenchyme; Colon; Rat

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Abstract

AIM: To investigate the expression of α -fetoprotein (AFP), a cancer-associated fetal glycoprotein, and its involvement during rat colon development.

METHODS: Colons from Sprague-Dawley rat fetuses, young and adult (8 wk old) animals were used in this study. Expression levels of AFP in colons of different development stage were detected by reverse-transcriptase PCR (RT-PCR) and Western blotting. To identify the cell location of AFP in the developing rat colons, double-immunofluorescent staining was performed using antibodies to specific cell markers and AFP, respectively.

RESULTS: The highest levels of AFP mRNA were detected in colons of rats at embryonic day 18.5 (e18.5). Compared to e18.5 d, the AFP expression was significantly decreased during rat development [85% for e20.5, $P < 0.05$, 58% for postnatal day 0.5 (P0.5), $P < 0.05$, 37% for P7, $P < 0.05$, 24% for P14, $P < 0.05$, and 11% for P21, $P < 0.05$] and undetected in adult rats. Only the 72-kDa isoform of AFP was detected by Western blotting, the expression pattern was similar to AFP mRNA and conformed to the results of mRNA expression. The AFP positive staining was identical to different distribution patterns in fetuses, young and adult animals and positive staining for both AFP and vimentin was overlapped in mesenchymal cells at each stage tested.

CONCLUSION: This study has for the first time

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INTRODUCTION

The mammalian gut epithelium is a highly organized and dynamic system that requires continuous, controlled proliferation and differentiation throughout life. Identification of the growth factors controlling these processes is crucial since the molecular mechanisms regulating organogenesis are often the same as those necessary for repair following injury. Furthermore, mis-regulations of embryonic signaling pathways are often associated with neoplastic diseases.

We assumed that accurate transcriptional profiles over gut development interval could provide fundamental information about underlying mechanisms, and characterized candidate regulators of cell interactions and mucosal differentiation. This resource also can be applied to address long-standing questions about reactivation of fetal genes in cancer.

A number of transcription factors, growth factors, and their receptors have been found to be expressed in the gastrointestinal epithelium or mesenchyme. However, little is known about their specific functions in gastrointestinal development. For those factors where a mutation has been generated by gene targeting,

gastrointestinal development either proceeds normally^[1-3] or the embryos die too early to allow assessment of the gene functions in gut development^[4,5].

Mammalian α -fetoprotein (AFP) is a single-chain glycoprotein with molecular mass ranging from 66 to 72 kDa and 3%-5% carbohydrate (glycan) content. This protein, which expresses at high levels in the fetal liver and yolk sac, constitutes 0.1% of the total mRNA in the fetal gut^[6]. At birth, AFP mRNA declines precipitously in both liver and gut to levels that are barely detectable^[7]. The gut development during late gestation and early neonatal period is accompanied by changes in the synthesis of AFP^[8], and abundance declines significantly during gut development. In this case, AFP is considered as an important growth factor with a specific function in gastrointestinal development.

The ontogeny of AFP gene expression has been examined in the fetal and adult mouse gastrointestinal tract^[9] to understand the basis of the ontogeny of AFP transcription in the gut and its regulatory elements. However, little is known about the expression pattern of AFP genes or its involvement during rat colon development.

MATERIALS AND METHODS

Specimens

Colons from Sprague-Dawley rat fetuses embryonic day 18.5 (e18.5 and e20.5 gestation), young (0 d and 1, 2 and 3 wk old) and adult (8 wk old) animals were used in this study. Five rats were used at each age stage. The embryonic age was determined according to Kaufman^[10]. Mating was performed by housing a male and a female rat together in the same cage overnight. The presence of a vaginal plug was determined the next morning (0.5 d gestation). Rats were housed in plastic cages in an air-conditioned and light controlled room at $24 \pm 2^\circ\text{C}$ and $60\% \pm 5\%$ humidity. The study protocol was approved by the Nanjing Medical University Animal Care and Use Committee.

Reverse-transcriptase PCR (RT-PCR)

Total RNA was extracted from tissues at each time point with TRIZOL reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis using ethidium bromide staining. For each PCR, 2 μg DNA-free total RNA with oligo (deoxythymidine) primers and reverse transcriptase were used. PCR was performed in 50- μL reactions containing 2.5 ng cDNA, 1 μL each primer pair, and 25 μL Premix Taq (TaKaRa, CA, USA). PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for each pair of primers^[11]. The AFP primer pairs used were: 5'-GCTGAACCCAGAGTACTGCAC-3' (forward), and 5'-GACACGTC GTAGATGAACGTG-3' (reverse). Amplification reactions were carried out for 30 cycles at 94°C for 30 s, 58.4°C for 30 s and at 72°C for 1 min.

The amplified products were 443 bp and analyzed on 1% agarose gels and visualized by ethidium bromide staining. Controls omitting RT cDNA or DNA polymerase showed no reaction bands. The data were normalized by 18S RNA.

Western blot analysis

The tissues were homogenized in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The lysate was then centrifuged at $12000 \times g$ for 25 min at 4°C . The total protein concentration of each sample was analyzed by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). An equal amount of protein samples, 60 μg , from each specimen was boiled in $3 \times$ loading buffer (10 mmol/L Tris-HCl, pH 6.8 including 3% SDS, 5% β -mercaptoethanol, 20% glycerol and 0.6% bromophenol blue) for 3 min and separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After transfer, membranes were blocked with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween 20 (TBS-T) overnight at 4°C . The membranes were then incubated with the primary antibody (sc-8108, an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of AFP, diluted 1:500; Santa Cruz, Biotechnology CA, USA) for 2 h at room temperature. After washing in TBS-T three times, the membranes were incubated with the peroxidase-linked rabbit antigoat IgG conjugates (Santa Cruz Biotechnology) for 1 h at room temperature. At the end, they were washed again in TBS-T, incubated in enhanced chemiluminescence reagents (Pierce) for 2 min, and exposed to X-Omat BT film (Eastman Kodak, Rochester, NY, USA). Signal intensity was quantified using a Bio-Rad image analysis system and the results were normalized to band intensities at e18.5. Loading controls of presumably and constantly expressed proteins such as β -actin were used; however, their variability and increase in development precluded their use^[12]. For negative controls, the primary antibody was omitted.

Double fluorescence immunohistochemistry

Tissues were fixed in 4% paraformaldehyde overnight at 4°C followed by a standard protocol of dehydration and paraffin embedding, and 5- μm sections were cut. The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. The non-specific binding sites were blocked in 1% bovine serum albumin (BSA) for 30 min. For AFP and vimentin double immunofluorescence, the goat anti-AFP primary polyclonal antibody was applied and revealed using fluorescein isothiocyanate (FITC)-labeled rabbit antigoat IgG (1:400, sc-2777; Santa Cruz Biotechnology). Mouse anti-vimentin primary monoclonal antibody (1:1000, CBL202; Chemicon International, Inc. Temecula, CA,

USA) was then applied and revealed by rhodamine-labeled anti-mouse IgG (1:400, AP192C; Chemicon International, Inc.). Sections were placed in gel aqueous mounting medium (G0918; Sigma, St. Louis, MO, USA) with a cover glass and were examined under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Controls were treated by omitting the primary or secondary antibody. No staining was observed under the negative control conditions. Images were taken at a magnification $\times 200$.

Statistical analysis

All experiments were done in triplicate. Analysis of the experimental data was carried out using PDQuest 7.0 software (Bio-Rad Laboratories) and one-way analysis of variance and paired *t* test were used. Data are presented as mean \pm SD. $P < 0.05$ was considered statistically significant.

RESULTS

Temporal expression of AFP in the developing rat colons

We carried out RT-PCR and Western blotting to detect the expression of AFP using samples extracted from the colons of fetal e18.5 and e20.5, postnatal day 0 (P0), P7, P14 and P21 and adult rats. As shown in Figure 1, the highest levels of AFP mRNA were detected in colons of rats at e18.5. The AFP mRNA levels in colons declined steadily during rat development and were undetected in adult rats ($P < 0.05$) (Figure 1). The level of AFP mRNA in e20.5 colon was significantly decreased compared with that in e18.5 colon. AFP mRNA in P0 colon was lower than those in e20.5 colon ($P < 0.05$), and AFP mRNA in P7 colon was also lower than in P0 ($P < 0.05$). There was no difference in the levels of AFP mRNA between P14 and P21. The AFP protein had four isoforms: 72, 60, 48 and 37 kDa. In our study, only the 72-kDa isoform of AFP was detected in rat colon (Figure 2A). From the results of the densitometric quantification (Figure 2B), it was seen that the total AFP was the highest at e18.5, after which expression decreased steadily, being the lowest in the adult colons. This result was similar to those of AFP mRNA expression.

Regional and temporal localization of AFP in the developing rat colons

In e18.5 fetus, the colonic mucosa was lined by a stratified epithelium and AFP positive staining was detected in the epithelium and mesenchymal tissue (Figure 3A). In e20.5 fetus, the epithelium has transformed into a simple columnar one. The AFP positive cells located at the bottom of crypt-like structure and the number of positive cells decreased markedly at this time (Figure 4A). After birth, positive cells were scattered on the epithelium during the first 7 d (Figure 4B). By P14 and P21, when the adult crypt structure replaced the villi, positive cells became largely restricted to the base of the crypts, no positive staining of AFP in the adult colonic epithelium was observed (Figure 3C and D).

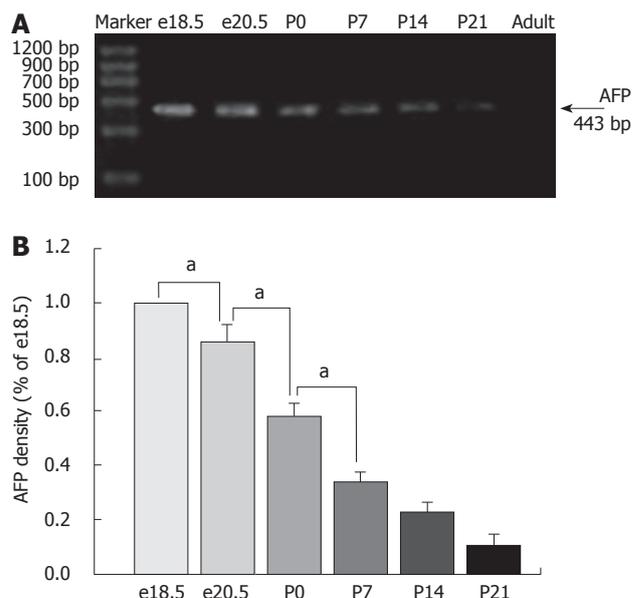


Figure 1 Expression of AFP mRNA using RT-PCR analysis in the developing rat colons as indicated in lanes e18.5, e20.5, P0, P7, P14, P21 and adult rats. The position of molecular weight markers is indicated on the left. A: The highest levels of AFP mRNA were detected in colons of rats at e18.5 and declined steadily during rat development and were undetected in adult rats; B: Results are indicated in percentages above the e18.5 value and are representative of three independent experiments. ^a $P < 0.05$ vs e18.5, e20.5 and P0, respectively.

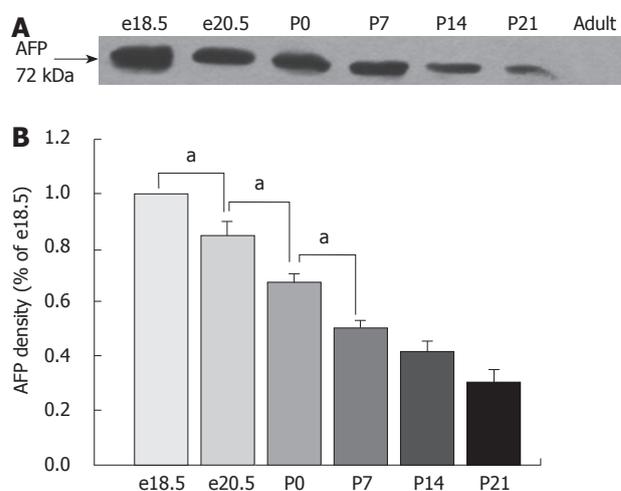


Figure 2 Expression of AFP protein using Western blot analysis in the developing rat colons as indicated in lanes e18.5, e20.5, P0, P7, P14, P21 and adult rats. A: Western blot analysis using AFP (C-19), an affinity purified goat polyclonal antibody against a peptide mapping at the C-terminus of AFP, revealed a 72-kDa isoform. The highest levels of AFP protein were detected in colons of rats at e18.5 and declined steadily during rat development and were undetected in adult rats. B: Results are indicated in percentage above the e18.5 value and are representative of three independent experiments. ^a $P < 0.05$ vs e18.5, e20.5 and P0, respectively.

Cells localization of AFP in the developing rat colons

The pattern of AFP-expressing cells and mesenchymal cells were very similar, which suggested a relationship between them. To identify the cells, an antibody against vimentin, which has been used as a marker for the mesenchymal cells, was used. Double-immunofluorescent staining for the vimentin and AFP showed a complete

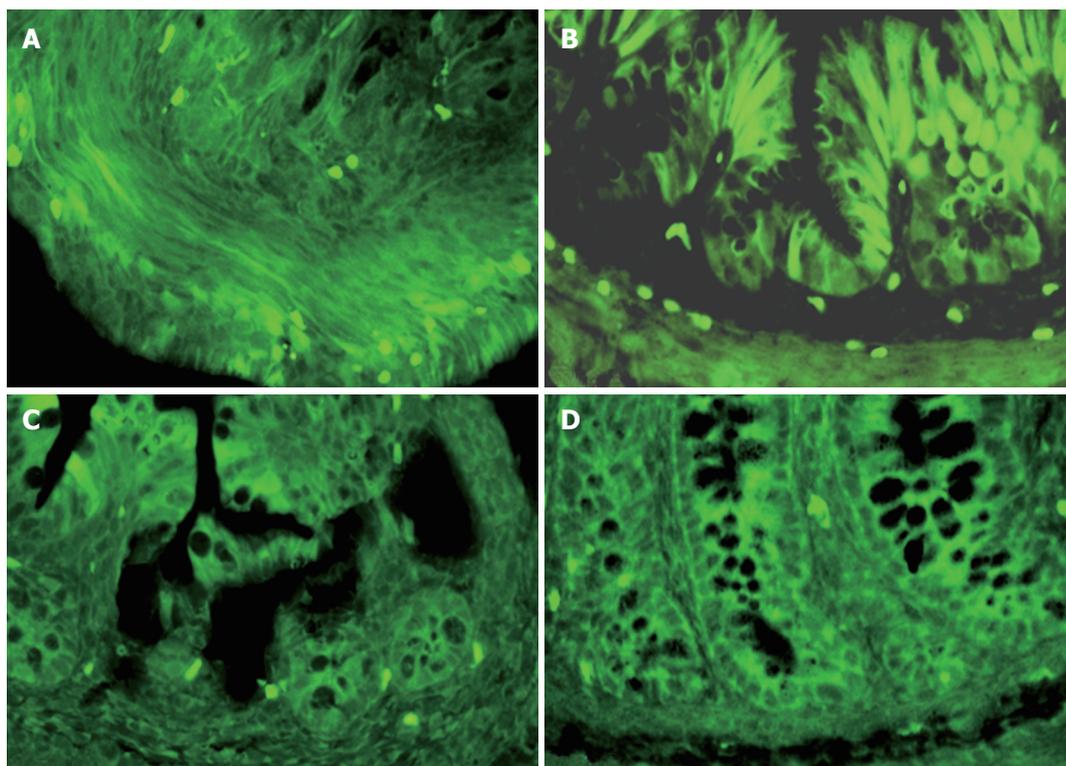


Figure 3 Immunofluorescence localization of AFP in the developing rat colons. A: In e18.5, AFP positive staining can be detected in the epithelium and mesenchymal tissues; B: At P0, positive cells were located at the base of the crypts and scattered on the epithelium; C, D: Only a few positive cells restricted to the base of the crypts between 14 and 21 d, and no positive cells can be detected in adult rat colons. ($\times 200$).

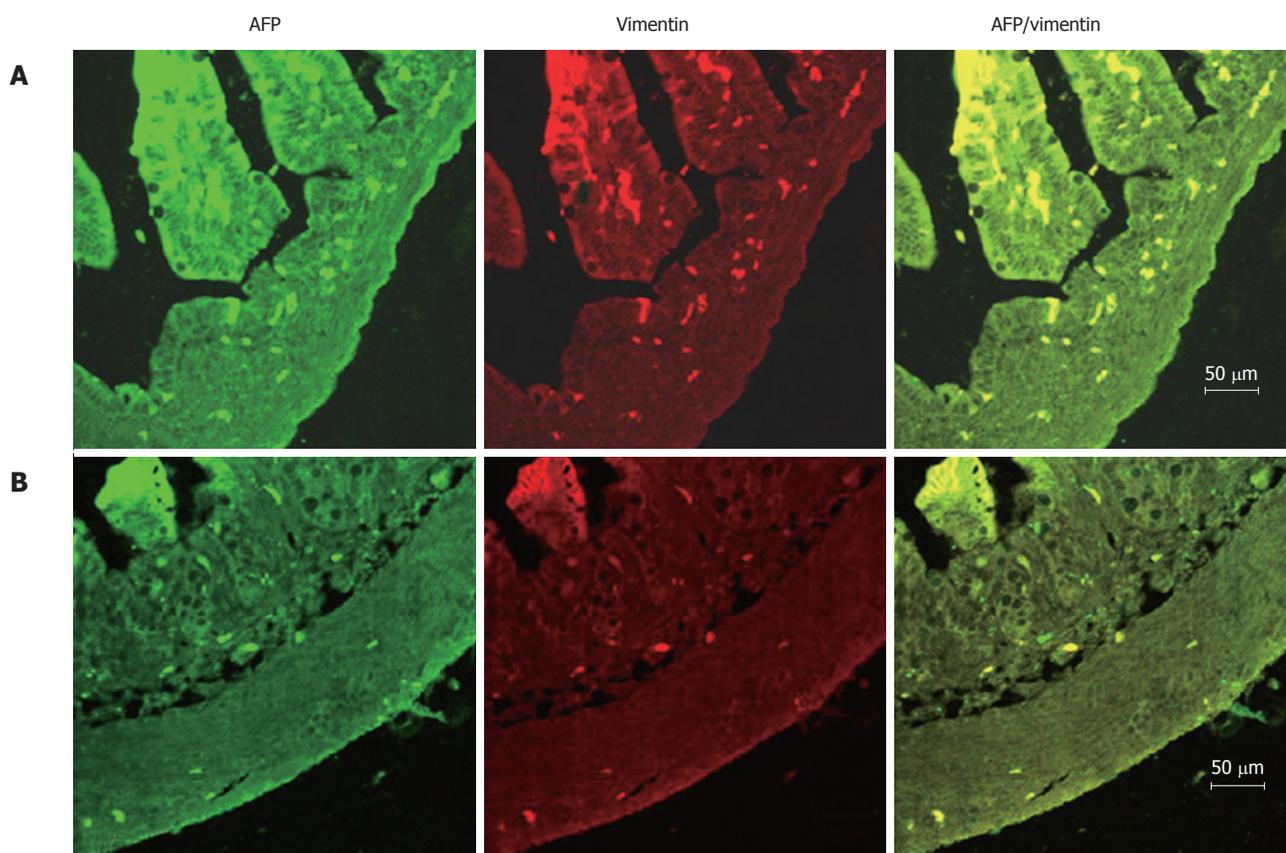


Figure 4 Immunofluorescence localization of AFP and vimentin in the developing rat colons. Labeling by the AFP antibody was detected with an FITC (green)-labeled secondary antibody. Labeling of vimentin was detected with a rhodamine- (red)-labeled secondary antibody on the same section. The overlap of AFP (green) and vimentin (red) labeling appeared orange in color. Double-labeling revealed complete localization of AFP and vimentin in the same colon cells at both e20.5 and P7. A: e20.5; B: P7.

overlap between the AFP positive cells and the antibody staining for vimentin at each stage tested (Figure 4A and B).

DISCUSSION

AFP is known to be associated with the successful completion of term pregnancies in mammals and even minute amounts of AFP may still be necessary during human pregnancy^[13]. The capability of both up and down modulation of growth and differentiation as a dose-dependent function of AFP has been demonstrated in a multitude of cell types including placental, ovarian, uterine, lymphoid, epidermal, endothelial, testicular, breast, and liver^[14-18]. The rat colon undergoes rapid growth and differentiation during the last few days of gestation and the first 2 wk after birth^[19-22]. This maturation process is accompanied by changes in the composition of AFP, which indicates that AFP might be involved in many aspects of colon development. Liu *et al*^[23] studied the changes of the protein levels of AFP in rat pancreas during development also by Western blot analysis and immunohistochemistry. Their results have demonstrated that the expression of AFP protein in the rat pancreas was increased in e18.5 rats and down-regulated after birth. They found a the similar possibility to ours, that the pancreatic cells, which went through dramatic growth, differentiation and proliferation, result from the dynamic expression of AFP in the various developmental stages^[23].

The genetic variants of rat AFP mRNA consist of sizes ranging from 2.2 to 1.35 kb, representing translated proteins ranging from 72 kDa down to 37 kDa, respectively^[24]. The smaller AFP isoforms are found to be truncated from the amino-terminal end. Molecular variants of AFP expressed in liver have long been reported in biomedical studies. All isoforms of the 72-kDa, 60-kDa and 48-kDa AFP protein may be involved in different aspects of liver cell behavior^[25,26]. Using a polyclonal antibody against a peptide mapping at the C terminus of AFP^[27-29], we found that only the 72-kDa isoform in the developing rat colons was detected by Western blotting. These results indicate that the 72-kDa isoform of AFP, with the highest expression in embryonic and regenerated liver, may also play an important role in colon cell proliferation and organ maturation.

Tyner *et al*^[9] have reported that AFP is expressed in a subset of enteroendocrine cells expressing chromogranin A, which suggested that they could be of enteroendocrine origin. AFP is a soluble glycoprotein that is able to bind many ligands including fatty acids, metals, steroids (estrogens), thyroxin, and tryptophan^[6,30]. The identity of the cells that express AFP in the colon cells was tested by double antibody staining with antibodies to chromogranin A (an enteroendocrine cell marker), cytokeratin (a epithelial cell marker), proliferating cell nuclear antigen (a proliferative cell marker) and vimentin (a mesenchymal cell marker and AFP) at each stage in rat development, respectively (data

not shown). Only an overlap of positive staining of AFP and vimentin was found in the same cell, indicating that AFP is indeed expressed and produced in mesenchymal cells. Liu *et al*^[23] reported that, in rat developing pancreas, AFP was also co-expressed with the vimentin, which was similar to our results. These results demonstrated that mesenchymal cell-derived AFP can act as a potent paracrine regulator of colon cell proliferation and organ maturation. The epithelial-mesenchymal interactions play an essential role in the control of gastrointestinal epithelial growth and differentiation not only in fetal stages, but also in adults^[31,32], but the mechanism has not been fully understood. Characterization of AFP expression in mesenchymal cells may help us discern a function in the gastrointestinal tract.

Cancer cells display immature features and dysregulated gene expression, with attenuation of tumor suppressors and aberrant expression of genes that are inactive in normal adult tissues. Since many down-regulated genes during development are re-expressed in tumors, understanding their respective cellular roles can provide information about both development and cancer biology. Despite some recent advances^[33], the extent of embryonic gene expression by tumor cells and the significance of this phenomenon are still unknown. AFP is one such gene and is reactivated in human tumors of the same fetal origin. Further studies are necessary to identify elements in the AFP gene that contribute to its expression. Numerous data support the hypothesis that AFP repression is a part of a global scheme of liver differentiation prematurely activated by glucocorticoids^[34]. The involvement of glucocorticoids in AFP expression in the gut remains an open question. Studies are now in progress in our laboratory using animal and tissue culture models.

In summary, our present study has, for the first time, demonstrated that AFP is localized to the mesenchyme in rat colon from the embryo to the weaning stage (up to 21 d after birth) by immunofluorescence, and presents a 72-kDa isoform in the developing rat colon by Western blotting. The dynamic expression of AFP in the various developmental stages of the colon indicates that AFP might be involved in many aspects of colon development. The exact function of AFP in colon development remains to be determined.

COMMENTS

Background

Mammalian α -fetoprotein (AFP) is a single-chain glycoprotein, developmentally down-regulated and re-expressed in tumors. The authors assumed that its accurate profiles over gut developmental interval could provide fundamental information about underlying mechanisms. This resource also can be applied to address longstanding questions about reactivation of fetal genes in cancer.

Innovations and breakthroughs

This study demonstrated that AFP presented a 72-kDa isoform and localized to the mesenchyme in the developing rat colon. The expression of AFP in the various developmental stages is dynamical. AFP might be involved in many aspects of colon development.

Applications

This animal model is a useful tool for the studies of gastrointestinal mucosal

proliferation and differentiation mechanism *in vivo*. The authors will identify elements in the AFP gene that contribute to its expression.

Terminology

AFP is a single-chain glycoprotein with molecular mass ranging from 66 to 72 kDa and a 3%-5% carbohydrate (glycan) content.

Peer review

The study investigated the expression of AFP and its involvement during rat colon development. It was well designed and conducted adequately.

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Recombinant vascular basement-membrane-derived multifunctional peptide inhibits angiogenesis and growth of hepatocellular carcinoma

You-Hua Wu, Jian-Guo Cao, Hong-Lin Xiang, Hong Xia, Yong Qin, A-Ji Huang, Di Xiao, Fang Xu

You-Hua Wu, Department of Oncology, First Affiliated Hospital of University of South China, Hengyang 421001, Hunan Province, China

Jian-Guo Cao, Hong-Lin Xiang, Di Xiao, Fang Xu, Laboratory of Medicine Engineering, Medical College, Hunan Normal University, Changsha 410006, Hunan Province, China

Hong Xia, Yong Qin, Institute of Cancer Research, University of South China, Hengyang 421001, Hunan Province, China

A-Ji Huang, The Second Xiangya Hospital of Central South University, Changsha 410078, Hunan Province, China

Author contributions: Wu YH, Xia H, Qin Y, Huang AJ, Xiao D, Xu F performed the majority of experiments; Xiang HL provided the vital reagents and analytical tools and was also involved in editing the manuscript; Cao JG designed the study and wrote the manuscript.

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Correspondence to: Jian-Guo Cao, Laboratory of Medicine Engineering, Medical College, Hunan Normal University, Changsha 410006, Hunan Province,

China. caojianguo2005@yahoo.com.cn

Telephone: +86-731-8912434 Fax: +86-731-8912417

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Abstract

AIM: To investigate the anti-angiogenic and anti-tumor activities of recombinant vascular basement membrane-derived multifunctional peptide (rVBMDMP) in hepatocellular carcinoma (HCC).

METHODS: HepG2, Bel-7402, Hep-3B, HUVE-12 and L-02 cell lines were cultured *in vitro* and the inhibitory effect of rVBMDMP on proliferation of cells was detected by MTT assay. The *in vivo* antitumor efficacy of rVBMDMP on HCC was assessed by HepG2 xenografts in nude mice. Distribution of rVBMDMP, mechanism by which the growth of HepG2 xenografts is inhibited, and microvessel area were observed by proliferating cell nuclear antigen (PCNA) and CD31 immunohistochemistry.

RESULTS: MTT assay showed that rVBMDMP markedly inhibited the proliferation of human HCC (HepG2, Bel-7402, Hep-3B) cells and human umbilical

vein endothelial (HUVE-12) cells in a dose-dependent manner, with little effect on the growth of L-02 cells. When the IC₅₀ was 4.68, 7.65, 8.96, 11.65 and 64.82 μmol/L, respectively, the potency of rVBMDMP to HepG2 cells was similar to 5-fluorouracil (5-FU) with an IC₅₀ of 4.59 μmol/L. The selective index of cytotoxicity to HepG2 cells of rVBMDMP was 13.8 (64.82/4.68), which was higher than that of 5-FU [SI was 1.9 (8.94/4.59)]. The VEGF-targeted recombinant humanized monoclonal antibody bevacizumab (100 mg/L) did not affect the proliferation of HepG2, Bel-7402, Hep-3B and L-02 cells, but the growth inhibitory rate of bevacizumab (100 mg/L) to HUVE-12 cells was 87.6% ± 8.2%. Alternis diebus intraperitoneal injection of rVBMDMP suppressed the growth of HepG2 xenografts in a dose-dependent manner. rVBMDMP (1, 3, 10 mg/kg) decreased the tumor weight by 12.6%, 55.9% and 79.7%, respectively, compared with the vehicle control. Immunohistochemical staining of rVBMDMP showed that the positive area rates (2.2% ± 0.73%, 4.5% ± 1.3% and 11.5% ± 3.8%) in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly higher than that (0.13% ± 0.04%) in the control group (*P* < 0.01). The positive area rates (19.0% ± 5.7%, 12.2% ± 3.5% and 5.2% ± 1.6%) of PCNA in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly lower than that (29.5% ± 9.4%) in the control group (*P* < 0.05). rVBMDMP at doses of 1, 3 and 10 mg/kg significantly reduced the tumor microvessel area levels (0.26% ± 0.07%, 0.12% ± 0.03% and 0.05% ± 0.01% vs 0.45% ± 0.15%) in HepG2 xenografts (*P* < 0.01), as assessed by CD31 staining.

CONCLUSION: rVBMDMP has effective and unique anti-tumor properties, and is a promising candidate for the development of anti-tumor drugs.

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Key words: Hepatocellular carcinoma; Recombinant vascular basement membrane-derived multifunctional peptide; Proliferating cell nuclear antigen; CD31; Therapeutic action

Peer reviewer: Francesco Feo, Professor, Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, Via P, Manzella 4, 07100 Sassari, Italy

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INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common cancer in the world, is responsible for over 600 000 deaths annually^[1]. The majority of patients with HCC die within 1 year after the diagnosis. Unfortunately, HCC is often diagnosed at its late stage when potentially curative therapies are least effective. For such patients, medical treatment modalities, including chemotherapy, chemoembolization, ablation, and proton beam therapy, remain disappointing. Most patients show recurrent HCC that rapidly progresses to its advanced stage with vascular invasion and multiple intrahepatic metastases and their 5-year survival rate is only 7%^[2]. Patients with surgically resectable localized HCC have a better prognosis, but their 5-year survival rate is only 15%-39%^[3], showing that new therapies for this aggressive disease are urgently needed.

Angiogenesis plays a critical role in the development of HCC. Antiangiogenesis therapy, which inhibits blood vessel formation, may be a promising treatment modality for HCC, because HCC depends on a rich blood supply^[4].

Tumstatin, a 28-kDa (244 amino acids) peptide fragment derived from the NC1 domain of $\alpha 3$ chain of type IV collagen, is an endogenous angiogenesis inhibitor, and has two binding sites for $\alpha v\beta 3$ integrin. One is in the N-terminal region of the molecule consisting of amino acids 74-98, which is associated with the anti-angiogenic property. The other is in the C-terminal region consisting of amino acids 185-203, which is associated with the antitumor activity^[5-7]. The peptide fragment of tumstatin consisting of amino acids 74-98 binds to both endothelial and melanoma cells, but only inhibits the proliferation of endothelial cells. However, the anti-tumor activity of amino acids 185-203 is not realized until this peptide region is exposed by truncation, a requirement not essential for the anti-angiogenic activity of amino acids 74-98^[5].

By targeting proliferating tumor cells and endothelial cells in a previous study^[8], we have constructed a fusion gene of the human IgG3 upper hinge region with two tumstatin-derived specific sequences, which exhibit anti-proliferation and anti-angiogenic activities. The human IgG3 upper hinge region is composed of 11 amino acids, and has a good flexibility, thus not affecting the spatial conformations of the connected peptides. The fusion sequence is named vascular basement membrane-derived multifunctional peptide (VBMDMP)^[9]. Recombinant

VBMDMP (rVBMDMP) can significantly inhibit tumor growth and metastasis in a mouse lung carcinoma model^[10]. Moreover, rVBMDMP selectively inhibits the proliferation of endothelial and human colon cancer cells, as well as induces apoptosis of endothelial cells *in vitro* and suppresses the growth of human colon cancer xenografts in Balb/c-nude mice^[11]. However, whether rVBMDMP inhibits tumor growth and angiogenesis of human HCC xenografts in a nude mouse model is unknown.

In the present study, we showed that rVBMDMP selectively inhibited the proliferation of HCC cells, using *in vitro* models of tumor growth, and also potently inhibited tumor neoangiogenesis of HepG2 xenografts in a nude mouse model, suggesting that rVBMDMP can be used as a potential agent in the treatment of human HCC.

MATERIALS AND METHODS

Cell culture and reagents

HepG2, Bel-7402, Hep-3B, HUVE-12 and L-02 cell lines, were purchased from the China Center for Type Culture Collection (CCTCC), were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin (Life Technologies) in an incubator containing 50 mL/L CO₂ at 37°C. rVBMDMP was over-expressed in *Escherichia coli* with pGEX-4T-1-VBMDMP and purified as previously described^[10] with a purity of over 95%. Synthetic peptide CNYYSNSYSFWLASLNPER (amino acid 185-203 of tumstatin, T4 peptide) and its rabbit polyclonal antibody were provided by Xi'an Huacheng Biotechnology Co., Ltd (China). Bevacizumab was purchased from Roche (Avastin®, Basel, Switzerland). Mouse monoclonal antibodies against proliferating cell nuclear antigen (PCNA) and CD31, as well as peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

MTT assay

Cells were seeded in a 96-well plate at a density of 1000 cells/well as described previously^[12]. Different concentrations of drugs were added to each well and cultured for 48 h, followed by incubation with 0.5 g/L MTT for 4 h. The supernatant was removed after centrifugation. Finally, 100 μ L DMSO was added and the absorbance at 570 nm wavelength (A_{570}) was measured with an enzyme-labeling instrument (ELX-800 type). Relative cell proliferation inhibition rate (IR) = (1-average A_{570} of the experimental group/average A_{570} of the control group) \times 100%. The IR was analyzed using the Calcsyn program to determine the IC₅₀.

Tumor xenograft experiments

Balb/c-nude female mice (Vital River Laboratory Animal Technology Co., Ltd), used in *in vivo* study, were housed in a sterile room at Institute of Cancer Research, University of South China, with free access to food and water.

Tumors were generated by harvesting HepG2 cells

from mid-log phase cultures using 0.25% trypsin (Life Technologies). Cells were resuspended in PBS to a final cell count of 2.5×10^7 /mL. A cell suspension (0.2 mL) was subcutaneously injected into the back of each mouse. The mice received a total of 10 injections of 1, 3, and 10 mg/kg body weight rVBMDMP (i.p) every other day when their average tumor volume reached 200 mm³.

Tumor dimensions and body weight were recorded every 5 d from the beginning of treatment. Tumor length and width were measured using a Vernier caliper, and tumor volume was calculated as described previously^[13]. Upon termination of treatment, the mice were weighed and sacrificed, and their tumors were excised. The mean tumor weight per group was calculated. The ratio of the mean of the treated tumor weight to the mean of vehicle control tumor weight $\times 100$ was subtracted from 100% to give the tumor growth inhibition rate for each group.

Immunohistochemical staining and quantification

Immunohistochemical staining of paraffin tumor tissue sections was done with rabbit polyclonal anti-T4 peptide antibody (Xi'an Huacheng Biotechnology) at a dilution of 1:50 using the DAB system from DAKO (Carpinteria, CA, USA) according to the manufacturer's instructions.

The tumor tissue sections were viewed at $\times 100$ magnification and images were captured with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA), and analyzed under four fields, excluding peripheral connective tissue and necrotic regions. The total tissue area in each section was 2.576 mm². Areas of rVBMDMP, PCNA or CD31-positive objects were quantified using ImagePro Plus version 3.0 (Media Cybernetics, Silver Spring, MD, USA). Percentage of microvessel area (MVA) in each field was calculated as (area of CD31-positive objects/measured tissue area) $\times 100$. Percentage of rVBMDMP or PCNA-positive staining in each field was calculated as (area of rVBMDMP or PCNA-positive objects/measured tissue area) $\times 100$. Mean values of MVA- or VBMDMP or PCNA-positive area in each group were calculated from six tumor tissue samples.

Statistical analysis

Experimental data in each group were expressed as mean \pm SD. Analysis of variance was performed with SPSS software for Windows 15.0 using one way ANOVA and pairwise comparison with Student's *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of rVBMDMP on proliferation of HCC, endothelial cells (ECs) and L-02 cell lines

MTT assay showed that rVBMDMP markedly inhibited the proliferation of human HCC (HepG2, Bel-7402, Hep-3B) cells and human umbilical vein endothelial (HUVE-12) cells in a dose-dependent manner, with

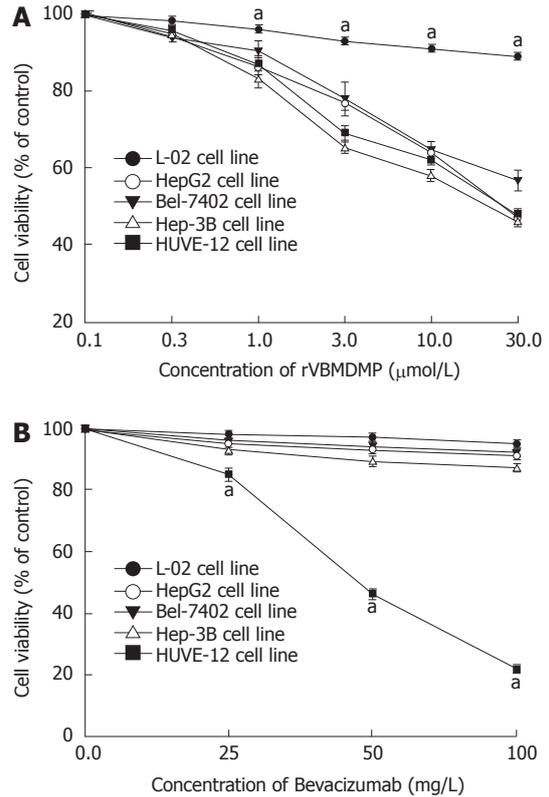


Figure 1 Proliferation of HCC (A) and ECs (B) selectively inhibited by rVBMDMP (mean \pm SD, *n* = 9). ^a*P* < 0.05 vs other cells.

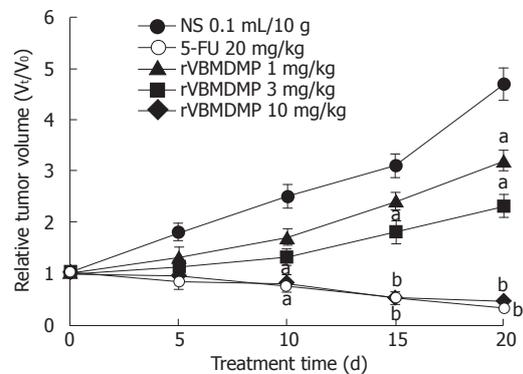


Figure 2 Robust efficacy of rVBMDMP against HepG2 xenografts in nude mice (mean \pm SD, *n* = 6). ^a*P* < 0.05, ^b*P* < 0.01 vs NS.

little effect on the growth of L-02 cells (Figure 1A). When the IC₅₀ was 4.68, 7.65, 8.96, 11.65 and 64.82 μmol/L, respectively, the potency of rVBMDMP to HepG2 cells was similar to that of 5-FU with an IC₅₀ of 4.59 μmol/L. The selective index of cytotoxicity to HepG2 cells of rVBMDMP was 13.8 (64.82/4.68), which was higher than that of 5-FU with a SI of 1.9 (8.94/4.59). Bevacizumab (100 mg/L) did not affect the proliferation of HepG2, Bel-7402, Hep-3B and L-02 cells, but its growth inhibitory rate for HUVE-12 cells was 87.6% \pm 8.2% (Figure 1B).

In vivo efficacy of rVBMDMP against HepG2 xenografts

rVBMDMP inhibited the growth of implanted HepG2 tumor xenografts in nude mice in a dose-dependent

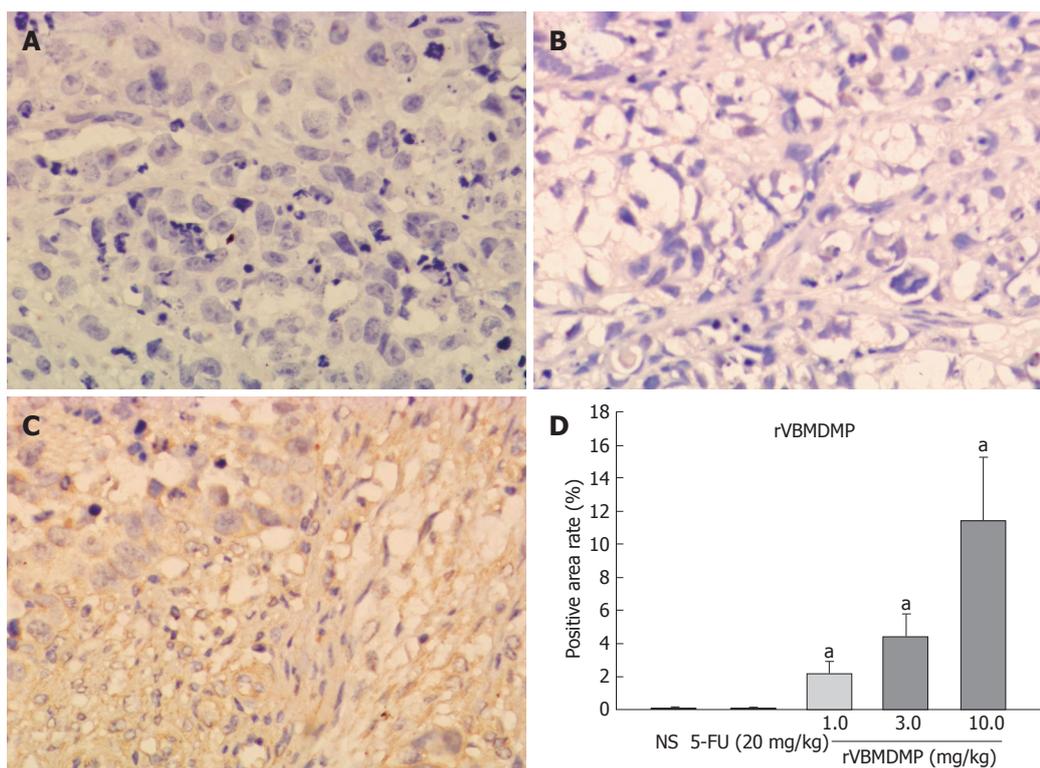


Figure 3 Accumulation of rVBMDMP in HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of rVBMDMP-positive areas in HepG2 xenografts. The data are expressed as mean \pm SD ($n = 6$). ^a $P < 0.05$ vs NS.

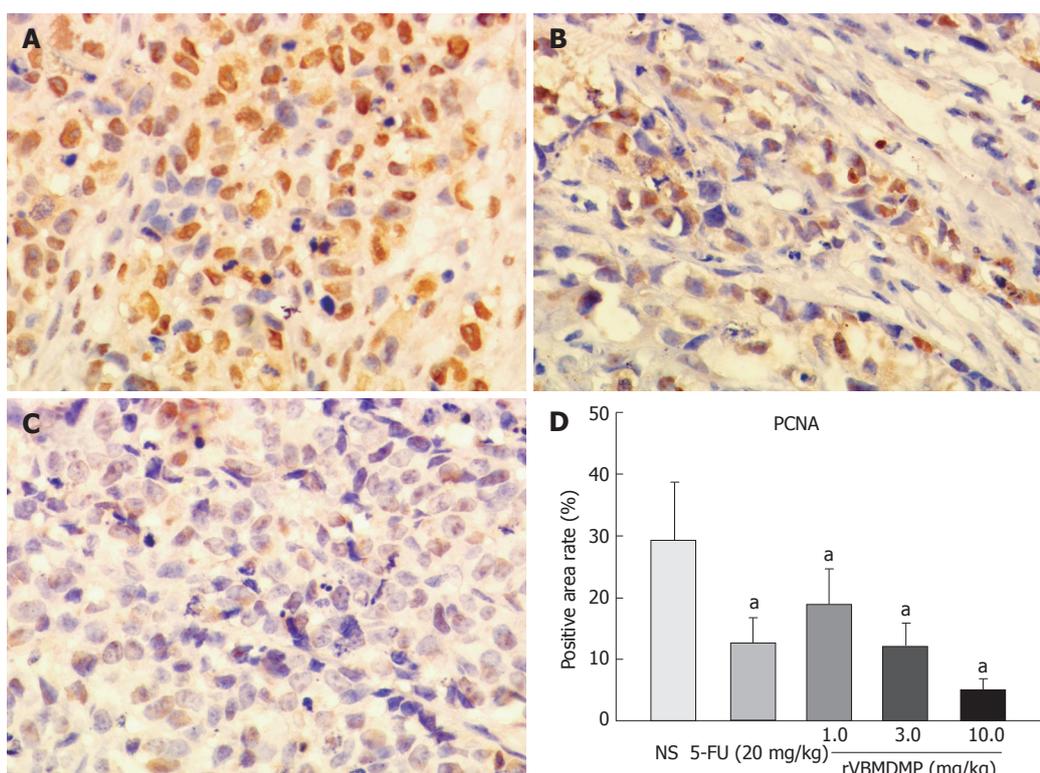


Figure 4 Expression of PCNA in HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of PCNA-positive areas in HepG2 tumors. The data are expressed as mean \pm SD ($n = 6$). ^a $P < 0.05$ vs NS.

manner (Figure 2). Different doses of rVBMDMP (1, 3, 10 mg/kg) decreased the tumor weight by 12.6%, 55.9% and 79.7%, respectively, compared with the vehicle control.

Distribution of rVBMDMP in HepG2 xenografts

Immunohistochemical staining of rVBMDMP showed that the positive area rates ($2.2\% \pm 0.73\%$, $4.5\% \pm$

1.3% and $11.5\% \pm 3.8\%$) were significantly higher in rVBMDMP treated group (1, 3, 10 mg/kg) than that ($0.13\% \pm 0.04\%$) in the control group ($P < 0.01$, Figure 3), indicating that rVBMDMP can accumulate in HepG2 xenografts nude mice.

PCNA expression in HepG2 xenografts

After intraperitoneal injection of rVBMDMP every other

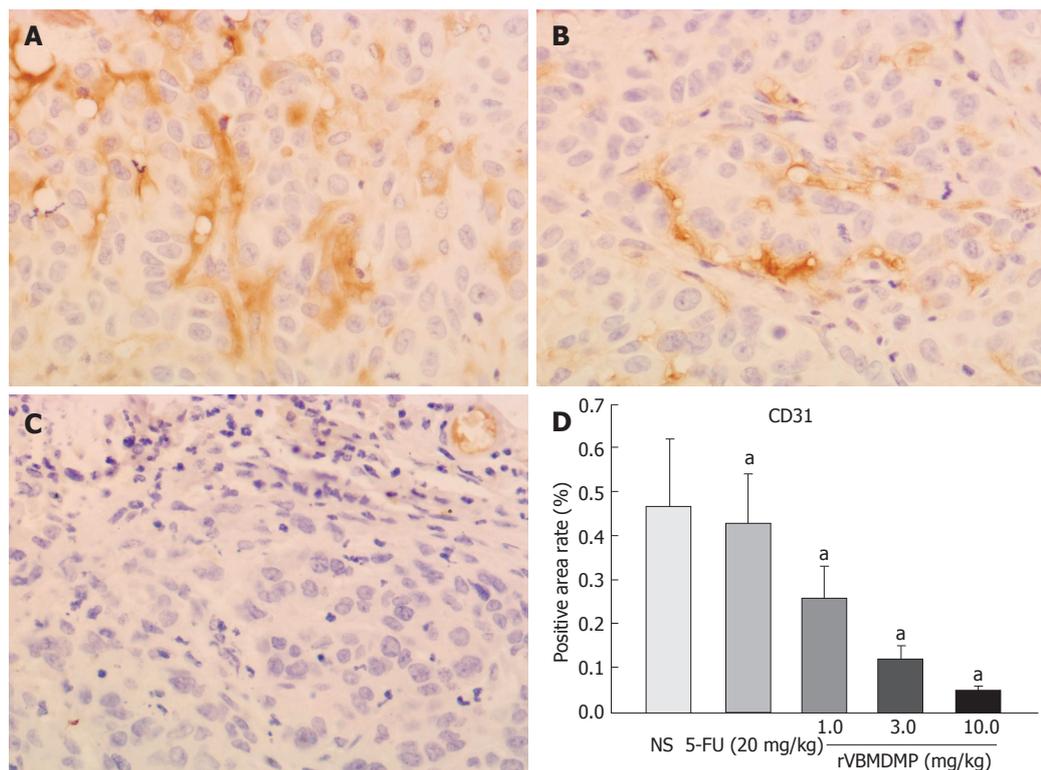


Figure 5 Microvessel density of HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of microvessel density (CD31 staining) positive areas in HepG2 xenografts in nude mice. The data are expressed as mean \pm SD ($n = 6$). ^a $P < 0.05$ vs NS.

day, the positive area rates ($19.0\% \pm 5.7\%$, $12.2\% \pm 3.5\%$ and $5.2\% \pm 1.6\%$) of PCNA in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly lower than that in the control group ($29.5\% \pm 9.4\%$) ($P < 0.05$, Figure 4), suggesting that rVBMDMP inhibits the proliferation of tumor cells in HepG2 xenografts in nude mice.

Effect of rVBMDMP on angiogenesis of HepG2 xenografts

The tumor MVA rates ($0.26\% \pm 0.07\%$, $0.12\% \pm 0.03\%$ and $0.05\% \pm 0.01\%$) were significantly lower in the HepG2 xenografts of the rVBMDMP-treated group assessed by CD31 staining (1, 3 and 10 mg/kg) than that ($0.45\% \pm 0.15\%$) in the control group ($P < 0.01$, Figure 5), demonstrating that rVBMDMP inhibits angiogenesis of HepG2 xenografts in nude mice.

DISCUSSION

It was recently reported that angiogenesis inhibitors may not work well in monotherapy^[14,15]. In contrast, studies conducted in preclinical tumor models showed that angiogenesis inhibitors in combination with cytotoxic chemotherapeutic agents or radiation therapy produce additive or synergistic anti-tumor activities^[12,16,17]. The positive effects of combined chemotherapy with angiogenesis inhibitors have been reported^[18-22], suggesting that the combination therapy of a cytotoxic agent and an angiogenesis inhibitor may be a fruitful topic in future clinical research^[23,24].

In this report, rVBMDMP inhibited the proliferation of human HCC cells selectively *in vitro*. Our previously research also showed that rVBMDMP could inhibit the proliferation of colon cancer cells, but have no effect on the proliferation of normal cells^[11], suggesting that

rVBMDMP can maintain the selective anti-tumor activity of tumstatin amino acids 185-203 fragment, which is consistent with the previously reported findings^[23]. The specific inhibitory effect of rVBMDMP on the proliferation of tumor cells strongly suggests that rVBMDMP functions *via* a tumor-specific cell surface protein or its receptor.

Tumor neoangiogenesis has recently been recognized as an important factor in defining subsets of cancer patients with a poor outcome^[25-27]. A number of angiogenesis inhibitors, discovered in recent years, can inhibit tumor growth by targeting proliferating and migrating ECs. Targeting ECs supports growth of tumor rather than tumor cells directly, which is particularly promising because these ECs are genetically stable and do not develop drug resistance. In this study, rVBMDMP suppressed reduplication in human endothelial HUVE-12 cells, like bevacizumab. By immunostaining of CD31 in tumor tissues, we found that rVBMDMP significantly decreased the microvessel density of human HCC xenografts in a mouse model. It was reported that rVBMDMP can significantly inhibit the proliferation of endothelial cells, blood vessel formation, and tumor growth in *in vitro* and *in vivo* models of angiogenesis, as well as induce EC-specific apoptosis^[11]. These anti-angiogenic properties of rVBMDMP, coupled with its anti-tumor activities, strongly indicate that rVBMDMP acts as a novel inhibitor of angiogenesis and tumor growth.

Since the proliferation velocity of ECs is higher in tumor tissue than in normal tissue, angiogenesis inhibitors may be accumulated in tumor^[28]. Our results show that rVBMDMP was significantly accumulated in human HCC xenografts in a mouse model, indicating

that rVBMDMP is selectively distributed in tumor tissue.

Maeshima *et al.*^[5] demonstrated that tumstatin amino acids 185-203 fragment does not show anti-tumor activity until the peptide region is exposed to truncation, which is not required for the anti-angiogenic activity of tumstatin amino acids 74-98 fragment. A shorter fragment comprising seven N-terminal residues 185-191 (CNYYSNS) shares the same inhibitory profile. The three-dimensional structures of CNYYSNS and tumstatin amino acids 185-203 fragment show a β -turn at the YSNS (188-191) sequence level, which is crucial for its biological activity^[29]. In our study, analysis of the structures of rVBMDMP using the AntheProt software indicated that both ends of the IgG3 upper hinge region sequence were a rarefaction structure, suggesting that rVBMDMP acts as a potent and specific agent against tumor progression^[11].

It has been shown that $\alpha_v\beta_3$ integrin is a putative receptor of tumstatin^[30,31]. Tumstatin fails to suppress neovascularization of Matrigel plugs in β integrin-deficient mice, and tumors in β integrin-deficient mice grow much faster than tumors in wild-type mice^[30,31], strongly suggesting that tumstatin acts via $\alpha_v\beta_3$ integrin as a negative regulator of angiogenesis. We speculate that the anti-tumor activity of rVBMDMP might also be mediated by $\alpha_v\beta_3$ integrin^[32].

In conclusion, rVBMDMP is a novel inhibitor of angiogenesis and tumor growth. Targeting both endothelial and tumor cells can enhance the efficacy of anti-tumor therapy. The mechanism of its action requires further investigation.

COMMENTS

Background

Tumstatin, a 28-kDa (244 amino acids) peptide fragment, derived from the NC1 domain of α_3 chain of type IV collagen, is an endogenous angiogenesis inhibitor. Tumstatin has two binding sites for $\alpha_v\beta_3$ integrin. One is in the N-terminal region of the molecule consisting of amino acids 74-98, which is associated with the anti-angiogenic property. The other is in the C-terminal region consisting of amino acids 185-203, which is associated with the antitumor activity. However, the anti-tumor activity of amino acids 185-203 is not realized until this peptide region is exposed to truncation, a requirement not essential for the anti-angiogenic activity of amino acids 74-98.

Research frontiers

Angiogenesis plays a critical role in the development of hepatocellular carcinoma (HCC). Antiangiogenesis therapy, which inhibits blood vessel formation, may be promising treatment modality for HCC, because HCC depends on a rich blood supply. The strategy of targeting both proliferating tumor and endothelial cells can improve the effectiveness of therapy for HCC.

Innovations and breakthroughs

It was recently reported that rVBMDMP significantly inhibits tumor growth and metastasis in a mouse lung carcinoma model and selectively inhibits the proliferation of endothelial and human colon cancer cells. In this study, recombinant vascular basement membrane-derived multifunctional peptide (rVBMDMP) selectively inhibited the proliferation of HCC cells in *in vitro* and *in vivo* models of tumor growth. Furthermore, our *in vivo* studies suggested that rVBMDMP was significantly accumulated in human HCC xenografts and potently inhibited tumor neoangiogenesis in HepG2 xenografts in nude mice.

Applications

rVBMDMP is a novel inhibitor of angiogenesis and tumor growth. Targeting both endothelial and tumor cells can enhance the efficacy of anti-tumor therapy and can be used as a treatment modality for HCC.

Terminology

VBMDMP is a fusion gene in the human IgG3 upper hinge region with two tumstatin-derived specific sequences (amino acids 74-98 and amino acids 185-203), which exhibits anti-proliferation and anti-angiogenic activities. Recombinant VBMDMP (rVBMDMP) is produced as a recombinant molecule in *E. coli*.

Peer review

The authors demonstrated that rVBMDMP selectively inhibited the proliferation of HCC cells, and was significantly accumulated in human HCC xenografts, and potently inhibited tumor neoangiogenesis in HepG2 xenografts in a nude mouse model by examining the effects of rVBMDMP on tumor growth and angiogenesis of HCC *in vitro* and *in vivo*. The results are interesting and may represent the strategy of targeting both proliferating tumor and endothelial cells, and provide a new treatment modality for HCC.

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Exogenous phosphatidylethanolamine induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway

Yu Yao, Chen Huang, Zong-Fang Li, Ai-Ying Wang, Li-Ying Liu, Xiao-Ge Zhao, Yu Luo, Lei Ni, Wang-Gang Zhang, Tu-Sheng Song

Yu Yao, Department of Oncology, First Affiliated Hospital of Medical College of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Yu Yao, Chen Huang, Ai-Ying Wang, Li-Ying Liu, Xiao-Ge Zhao, Yu Luo, Lei Ni, Tu-Sheng Song, Department of Genetics and Molecular Biology, Medical School, Xi'an Jiaotong University/Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Xi'an 710061, Shaanxi Province, China

Zong-Fang Li, Wang-Gang Zhang, Second Affiliated Hospital of Xi'an Jiaotong University/Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Xi'an 710004, Shaanxi Province, China

Author contributions: Yao Y and Huang C performed the majority of experiments; Yao Y, Huang C and Song TS designed the research; Li ZF, Zhang WG and Liu LY provided the vital reagents and analytical tools and were involved in editing the manuscript; Wang AY, Zhao XG, Luo Y, Ni L analyzed the data.

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Correspondence to: Chen Huang, Department of Genetics and Molecular Biology, Medical School, Xi'an Jiaotong University/Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Xi'an 710061, Shaanxi Province, China. hchen@mail.xjtu.edu.cn

Telephone: +86-29-82657723 Fax: +86-29-82655077

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Abstract

AIM: To investigate the signaling pathways implicated in phosphatidylethanolamine (PE)-induced apoptosis of human hepatoma HepG2 cells.

METHODS: Inhibitory effects of PE on human hepatoma HepG2 cells were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle, apoptosis and mitochondrial transmembrane potential ($\Delta\Psi_m$) were analyzed by flow cytometry. Immunocytochemical assay and Western blotting were used to examine Bcl-2, Bax and caspase-3 protein levels in HepG2 cells treated with PE.

RESULTS: PE inhibited the growth of HepG2 cells in a dose- and time- dependent manner. It did not

affect the cell cycle, but induced apoptosis. PE significantly decreased $\Delta\Psi_m$ at 0.25, 0.5 and 1 mmol/L, respectively, suggesting that PE induces cell apoptosis by decreasing the mitochondrial transmembrane potential. The Bcl-2 expression level induced by different concentrations of PE was lower than that in control groups. However, the Bax expression level induced by PE was higher than that in the control group. Meanwhile, PE increased the caspase-3 expression in a dose- and time-dependent manner.

CONCLUSION: Exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

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Key words: Apoptosis; Bcl-2; Bax; Caspase-3; Phosphatidylethanolamine; Human hepatoma HepG2 cell

Peer reviewer: Fanyin Meng, MD, PhD, Assistant Professor, Department of Internal Medicine, Ohio State University, Room 514A, Medical Research Facility, 420 West 12th Avenue, Columbus, Ohio 43210, United States

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INTRODUCTION

Phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM), are the dominant lipid constituents of the membranes of animal cells, in which they are distributed in an asymmetrical fashion and act as the matrix for the support and organization of different membrane proteins^[1]. In addition to this structural role, many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli^[2-4].

Some studies have provided evidence that membrane

phospholipid asymmetry is disturbed in one of the early stages of apoptosis^[5-7]. Specifically, a translocation of PS and PE from the internal to external surface of the plasma membrane appears to be a fundamental mechanism through which apoptotic cells are recognized and eliminated by phagocytic macrophages^[8-10]. PS and PE externalization could arise either through inactivation of aminophospholipid translocase (APTL), whose normal function maintains the asymmetric distribution of PS and PE in cells^[11,12] or by accelerating reversal of movement of the phospholipid. Inhibition of APTL could induce apoptosis of central nervous system (CNS)-derived HN2-5 and HOG cells, activating caspase-3, indicating that abnormal distribution of cell membrane phospholipid can induce apoptosis^[13]. PS externalization is a characteristic feature of the apoptotic cells. It has been shown that the externalized PS serves as a marker for detecting the apoptotic cells, and PE exposed to the cell surface forms lipid rafts with PS during apoptosis^[14].

It was recently reported that overexpression of Raf kinase inhibitor protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, can inhibit the Raf-ERK1/2 pathway^[15]. However, the effects of PE on cell proliferation or apoptosis remain unclear. Our results in this study suggest that exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

MATERIALS AND METHODS

Materials and agents

RPMI-1640 medium and fetal calf serum were purchased from GIBCO (Canada). PE, MTT and propidium iodide (PI) were purchased from Sigma. Annexin V-FITC apoptosis detection kit was from BD Biosciences (USA). Monoclonal antibodies were obtained from Cell Signaling Technology (USA).

Cell culture

Human hepatoma cell line HepG2 was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SMMC7721, HEK293 and HeLa cells were provided by Molecular Biology Center of the First Affiliated Hospital, Xi'an Jiaotong University. HepG2 cells (5.0×10^4 cells/mL) were cultured in RPMI-1640 supplemented with 100 mL/L fetal bovine serum, containing 2.0 mmol/L glutamine and 20 µg penicillin-streptomycin/mL in 50 mL/L CO₂ at 37°C, and allowed to adhere for 24 h. The experiments were divided into four groups: control group, 0.25 mmol/L PE, 0.5 mmol/L PE, and 1 mmol/L PE treatment groups.

MTT assay for cell viability

HepG2 cells (2×10^4 cells/well) were seeded onto 96-well plates and incubated with test substances for an indicated time at 37°C in an atmosphere containing 50 mL/L CO₂. Then, 20 µL MTT solution (5 g/L) was added into each well and incubated for another 4 h. Supernatants

were removed and formazan crystals were dissolved in 200 µL dimethylsulfoxide. Finally, optical density was determined at 490 nm by POLARstar + OPTIMA (BMG Labtechnologies, Germany).

Cell cycle analysis by flow cytometry

DNA content per duplicate was analyzed by flow cytometry (BD Biosciences). Adherent cells were harvested by brief trypsinization, and washed with PBS, fixed in 700 mL/L ethanol, stained with 20 µg/mL PI containing 20 µg/mL RNase (DNase free) for 30 min, and analyzed by flow cytometry. The number of cells at the G₀/G₁, S, and G₂/M phases was calculated.

Detection of HepG2 cell apoptosis by annexin-V/PI staining

HepG2 cells were treated with PE at 37°C in an atmosphere containing 50 mL/L CO₂ for 48 h, then harvested and washed twice with PBS. The cells were labeled by incubation with 5 µL FITC-annexin V and 10 µL PI at 250 µg/mL for 10 min in the dark at room temperature. The cells were washed with PBS again and examined by flow cytometry. Apoptosis was routinely quantified by counting the number of cells stained with FITC-labeled annexin V.

ΔΨ_m examination

HepG2 cells (1×10^6 /mL) were washed twice with PBS, incubated with rhodamine 123 (10 µg/mL) at 37°C for 30 min, then washed with PBS and analyzed by flow cytometry.

Immunocytochemical assay

HepG2 cells were grown on glass culture slides coated with poly-lysine in a 24-well plate and treated with PE. Slides with cells were fixed in 40 mg/L paraformaldehyde for 20 min, and then incubated with monoclonal antibody (anti-Bcl-2, anti-Bax, anti-caspase-3), which was labeled with FITC-conjugated goat anti-rabbit IgG. Positive-staining-area percentages were calculated under SP2 confocal microscope (Leica, Germany). Additionally, fluorescence intensity in 20 positive cells was evaluated.

Western blotting

HepG2 cells were plated at 5×10^4 cells/tissue culture dish in six-well plates with RPMI-1640. After exposure to the inhibitor at different concentrations, the cells were washed with PBS and subsequently lysed in 200 µL of a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1 mg/L sodium azide, 10 mL/L NP-40, 1 mmol/L phenylmethylsulfonyl fluoride. Insoluble material was removed by microcentrifugation at 13000 r/min for 15 min at 4°C. Cell lysates (80 µg of protein/lane) were subjected to electrophoresis on 100 mg/L SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA, USA).

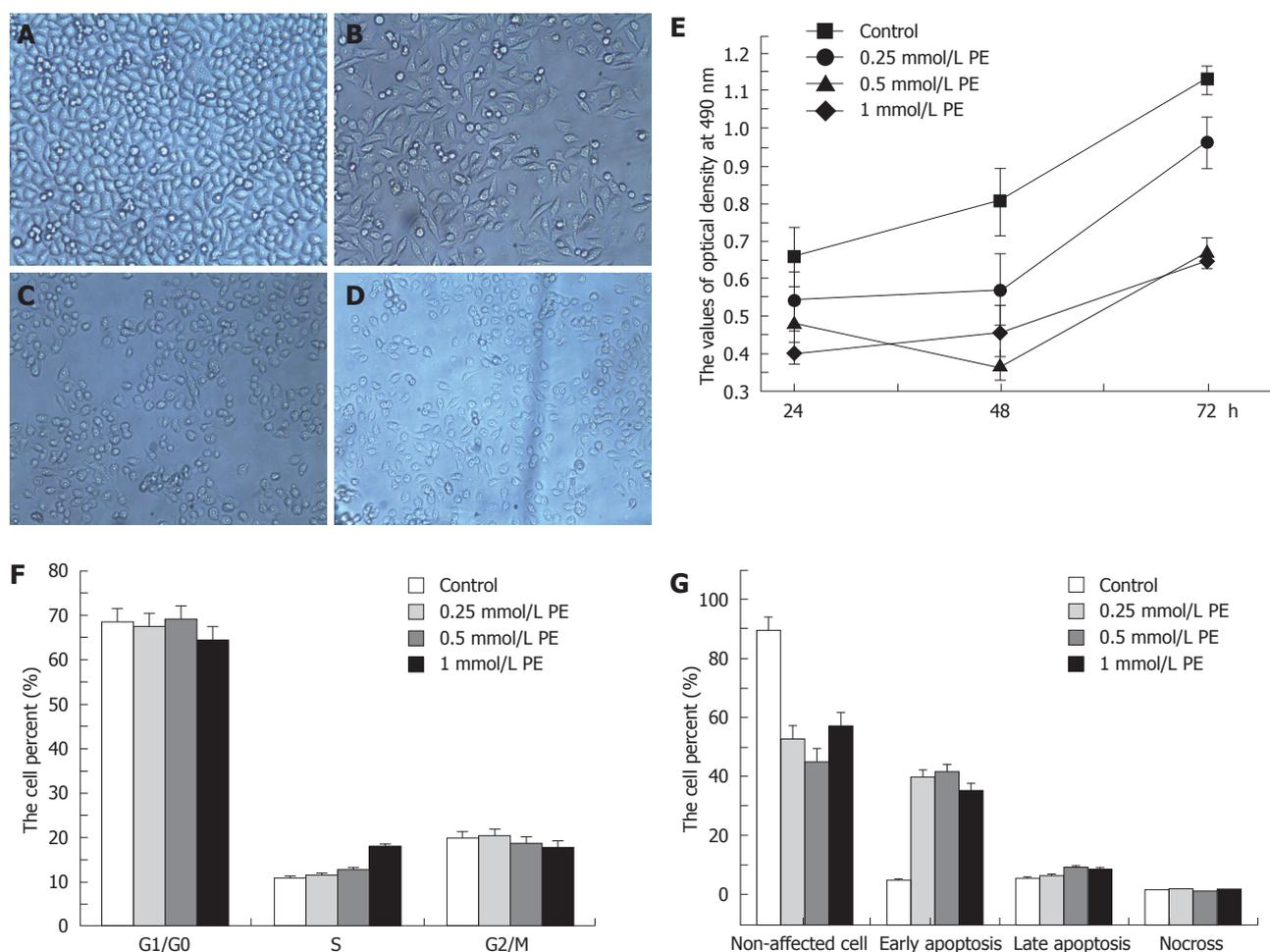


Figure 1 Effect of exogenous PE on the growth and apoptosis of human hepatoma HepG2 cells. A: Control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: 48 h after treatment, inhibition of cell growth shown by MTT assay; F: Cell cycle in human hepatoma HepG2 cells shown by PI staining 24 h after PE treatment, with data showing the cell percentages at G1/G0, S and G2 phases; G: Apoptosis of human hepatoma HepG2 cells shown by annexin-V/PI staining 24 h after PE treatment, with data showing the percentages of non-affected, early and late apoptotic cells and necrosis. The results are given as mean \pm SD from three experiments.

After blocked with Tris-buffered saline containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5 mL/L Tween 20, 10 mg/L bovine serum albumin, the membrane was incubated with different monoclonal antibodies (R&D Systems) and anti-glyceraldehyde-3-phosphate dehydrogenase, respectively. For chemiluminescence detection, WB membranes were incubated in the dark with ECL (Amersham) which is a luminol-based enhanced chemiluminescence substrate for horseradish peroxidase. The luminescent signal was recorded and quantified with the Syngene G box (Syngene, UK) which consists of a high-performance CCD videocamera with focus stabilized optics, in a mini darkroom enclosure. The instrument is linked to a computer that controls the instrument and handles the data. The luminescent signal is detected by the CCD camera and transmitted to the controller unit and the data are sent to the computer for analysis and documentation.

Statistical analysis

All data were expressed as mean \pm SD and analyzed by

SPSS 11.0 software. Analysis of data was performed using *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

PE inhibited growth of HepG2 cells

The growth of SMMC7721, HEK293, HeLa and HepG2 cells was detected by MTT assay at different time points after treatment with PE at different concentrations. The results showed that PE inhibited the growth of these cells as well as normal cells (HEK293), suggesting that the inhibition is non-specific (Table 1). HepG2 cells were chosen in our study.

Polygon or fusiform HepG2 cells were observed in the control group with intact and distinct peripheria (Figure 1A), but round HepG2 cells were found in the PE treatment groups with ambiguous peripheria (Figure 1C and D). PE inhibited the growth of HepG2 cells in a dose-and-time dependent manner (Figure 1E). PE did not affect the cell cycle (Figure 1F), but induced apoptosis (Figure 1G).

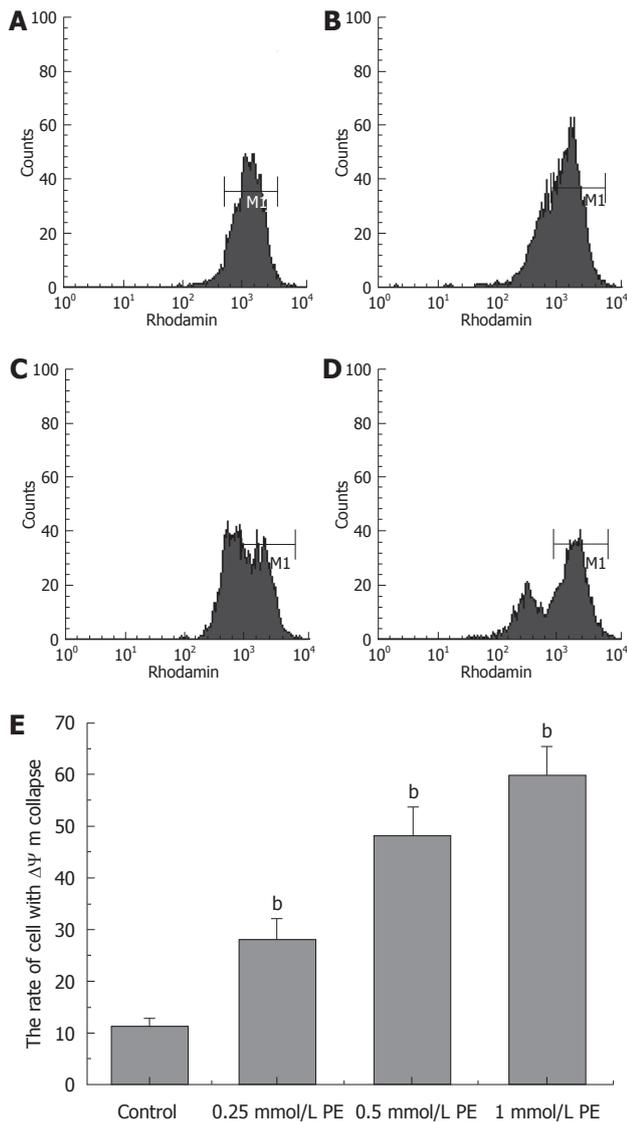


Figure 2 Effects of exogenous PE on the $\Delta\Psi_m$ of human hepatoma HepG2 cells. A: Control group; B, C, D: Groups treated with 0.25, 0.5 and 1 mmol/L PE at 24 h; E: Flow cytometry analysis of cells with $\Delta\Psi_m$ collapse in human hepatoma HepG2 cells shown by rhodamine staining at 24 h. The results are given as mean \pm SD from three repeat experiments. ^b $P < 0.01$ vs control group.

Effect of PE on $\Delta\Psi_m$ of HepG2 cells

The uptake of rhodamine123, a lipophilic fluorescent dye absorbed by mitochondria, is positively correlated with $\Delta\Psi_m$. The descent of rhodamine can reflect the collapse of $\Delta\Psi_m$ ^[16]. In order to determine the effect of PE on $\Delta\Psi_m$, the uptake of rhodamine was detected by flow cytometry. PE significantly decreased the $\Delta\Psi_m$ at 0.5 mmol/L and 1 mmol/L (Figure 2C-E), suggesting that PE induces cell apoptosis by decreasing the $\Delta\Psi_m$.

PE induced apoptosis of Bcl-2/Bax in HepG2 cells

Bcl-2 and Bax are involved in the maintenance of mitochondria membrane stability^[17,18]. Immunocytochemical assay showed that Bcl-2 expression in HepG2 cells was significantly suppressed 24 h after treatment with

PE, showing a negative correlation with the PE dosage (Figure 3A-E), while Bax expression was significantly increased 24 h after treatment with PE, showing a positive correlation with the PE dosage (Figure 4A-E). The results of Western blotting and immunocytochemical assay were similar. The Bcl-2 expression level was lower in different PE treatment groups than in the control group at different time points (Figure 3F). However, the Bax expression level was higher in different PE treatment groups than in the control group (Figure 4F).

Involvement of caspase-3 in HepG2 cell apoptosis induced by PE

Caspase-3, a key regulatory protease from which many signaling pathways merge for the execution of apoptosis, participates in apoptosis induced by bcl-2/bax, p38 and JAK-STAT^[19,20]. We detected the caspase-3 expression in HepG2 cells after treatment with PE. Immunocytochemical assay showed that caspase-3 expression was significantly increased, showing a positive correlation with the PE dosage 24 h after treatment (Figure 5A-E). The caspase-3 expression level was higher in PE treatment groups than in the control group (Figure 5F). However, PE increased the caspase-3 expression in a dose- and time-dependent manner (Figure 5F).

DISCUSSION

PE is an important phospholipid component, which is involved in the formation of membrane asymmetry. PE locates at the intracellular leaflet of normal cell membranes, and is exposed to the cell surface during apoptosis. It has been shown that externalization of PE is a signal of early apoptosis^[21]. However, the effect of PE on cell apoptosis remains unclear.

In this study, PE inhibited the growth of HepG2 cells (Figure 1A-E) in a dose-dependent manner. Because the cell cycle and apoptosis are involved in the regulation of cell growth, they were detected with a flow cytometer 24 h after treatment with PE in our study. PE did not significantly affect the cell cycle (Figure 1F), but induced apoptosis of HepG2 cells (Figure 1G), suggesting that PE induces apoptosis by inhibiting the growth of HepG2 cells.

At the early stage of cell apoptosis, $\Delta\Psi_m$ was decreased before chromatin condensation and DNA fragmentation. It was recently reported that mitochondrial dysfunction is essential to the apoptotic pathway, and loss of $\Delta\Psi_m$ may be an early event in the apoptotic process^[22]. Reduced $\Delta\Psi_m$ induces cytochrome C release from the mitochondria, and causes apoptosis^[23-25]. In this study, 0.5 and 1 mmol/L of PE significantly decreased the $\Delta\Psi_m$ (Figure 2C-E), suggesting that PE induces apoptosis of HepG2 cells *via* the mitochondrial pathway. Loss of $\Delta\Psi_m$ was found to be closely associated with the expression of Bcl-2 and Bax. It is generally thought that the expression of Bax increases following death stimulation, and then translocates at the mitochondria to induce cytochrome

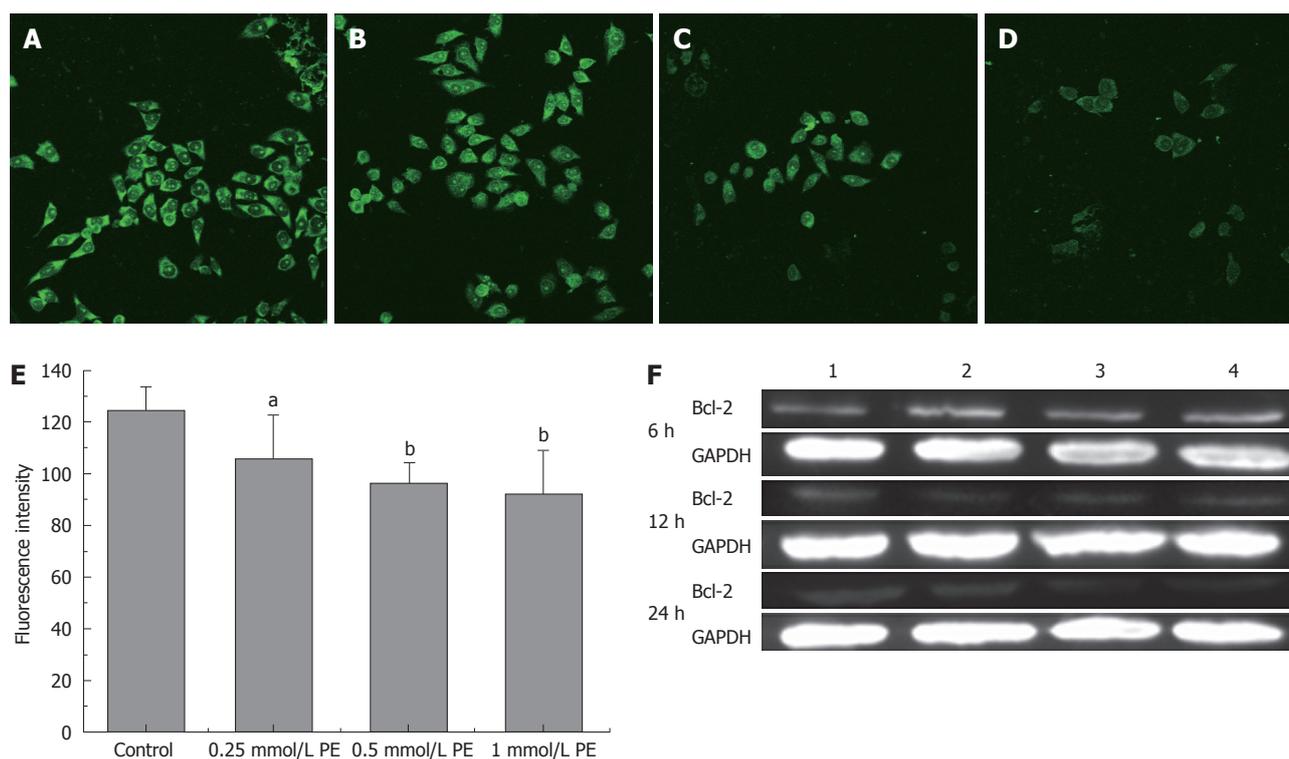


Figure 3 Inhibitory effect of exogenous PE on bcl-2 expression in human hepatoma HepG2. A: Cells in control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean \pm SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Table 1 Inhibitory effect of PE on the growth of different types of cells (mean \pm SD)

Time of treatment	24 h				48 h			
	0	0.25	0.5	1	0	0.25	0.5	1
SMMC7721	0.75 \pm 0.06	0.59 \pm 0.03 ^b	0.42 \pm 0.02 ^b	0.30 \pm 0.02 ^b	0.96 \pm 0.03	0.42 \pm 0.02 ^b	0.36 \pm 0.02 ^b	0.30 \pm 0.02 ^b
HepG2	0.66 \pm 0.08	0.54 \pm 0.08 ^b	0.48 \pm 0.07 ^b	0.40 \pm 0.03 ^b	0.81 \pm 0.09	0.57 \pm 0.10 ^b	0.36 \pm 0.03 ^b	0.45 \pm 0.08 ^b
HEK293	0.63 \pm 0.02	0.39 \pm 0.10 ^b	0.40 \pm 0.01 ^b	0.35 \pm 0.02 ^b	0.80 \pm 0.03	0.40 \pm 0.02 ^b	0.31 \pm 0.02 ^b	0.24 \pm 0.03 ^b
HeLa	1.28 \pm 0.09	1.16 \pm 0.06 ^a	1.08 \pm 0.04 ^a	0.95 \pm 0.09 ^b	1.66 \pm 0.11	1.29 \pm 0.01 ^b	1.15 \pm 0.05 ^b	1.01 \pm 0.06 ^b

^a $P < 0.05$ and ^b $P < 0.01$ vs control group.

C release^[26,27]. It is also known that Bax undergoes post-translational modification during apoptosis of HepG2 cells in response to various stimuli with interferon alpha and chemotherapeutic drugs, and the cleaved form of Bax is a potent inducer of apoptosis^[28-30]. Anti-apoptotic Bcl-2 inhibits the pro-apoptotic function of Bax. In the present study, PE up-regulated the expression of Bax and down-regulated the expression of Bcl-2 in HepG2 cells in a dose-and time-dependent manner. Bax expression was observed 12 and 24 h after treatment with 0.5 and 1 mmol/L PE (Figure 4E). The expression of Bax was negatively correlated with decreased $\Delta\Psi_m$, suggesting that increased Bax expression may be involved in the decreased $\Delta\Psi_m$. Increased Bax/Bcl-2 proportion will lead to release of cytochrome C from the mitochondria, thus inducing cell apoptosis^[31]. Experiments *in vitro* have proved that PE plays a key role in cytochrome C transmembrane transport in liposomes composed of acid-phospholipid and neutro-phospholipid^[32]. When the amount of the PE in PA/PE/PC system is increased, cytochrome C transmembrane

transport increases in liposome^[32].

Caspases are cystein proteases that play a key role in cascade activation during apoptosis induced by many stimuli^[33-36]. Activation of initiator of caspases (procaspases 8-10) leads to proteolytic activation of downstream effector caspases (caspase-3, -6, -7). The activation of caspase-3 is a common event in two major pathways, death receptor and mitochondrial pathways^[37-40]. In the present study, we proved that PE up-regulated caspase-3 expression in a dose-dependent manner, suggesting that exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

COMMENTS

Background

Phosphatidylethanolamine (PE) is one of the dominant lipid constituents in the membranes of animal cells in which it is distributed in an asymmetrical fashion. Many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli, but the specific signaling function of PE is largely unknown.

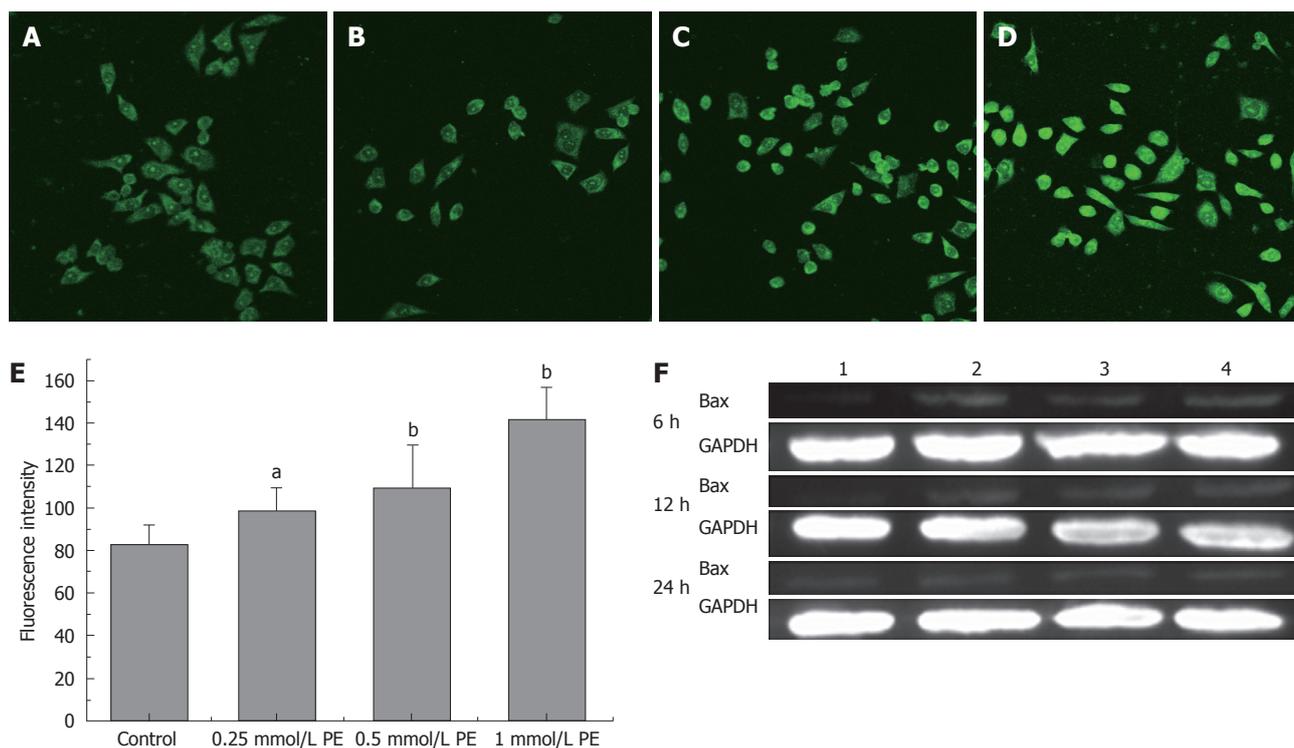


Figure 4 Inhibitory effects of exogenous PE on bax expression in human hepatoma HepG2 cells. A: Control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean ± SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. ^a*P* < 0.05, ^b*P* < 0.01 vs control group.

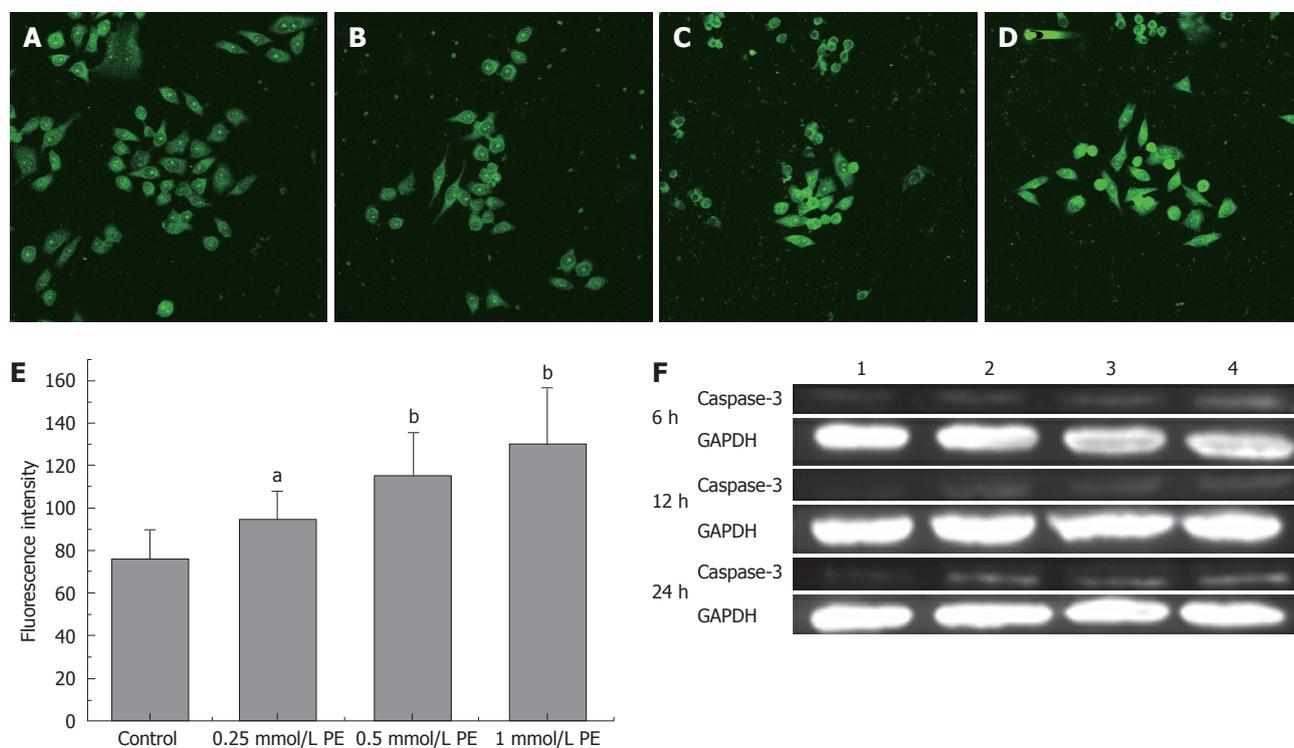


Figure 5 Inhibitory effect of exogenous PE on caspase-3 expression in human hepatoma HepG2 cells. A: control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean ± SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. ^a*P* < 0.05, ^b*P* < 0.01 vs control group.

Research frontiers

Translocation of phosphatidylserine (PS) and PE from the internal to external surface of plasma membrane appears to be a fundamental mechanism underlying apoptotic cells. Aminophospholipid translocase involving the

asymmetric distribution of PE in cells was inhibited to induce apoptosis in our study, implying that PE externalization leads to apoptosis. The authors of this paper demonstrated that exogenous PE could induce apoptosis of human hepatoma HepG2 cells.

Innovations and breakthroughs

PS externalization is a typical feature of apoptotic cells. It has been shown that externalized PS serves as a marker for detecting apoptotic cells. PE exposed to the cell surface forms lipid rafts with PS during apoptosis. This is the first study to report that the bcl-2/bax pathway is involved in PE-induced apoptosis of HepG2 cells.

Applications

PE is a dominant component of liposome. This study revealed the mechanism of liposome cytotoxicity to cells.

Peer review

In the present work, the authors showed that PE, one of the important phospholipid components in cell membrane, inhibited the growth of HepG2 cells by inducing apoptosis, but did not change the cell cycle. Furthermore, PE down-regulated Bcl-2 expression, and up-regulated Bax expression in HepG2 cells, induced $\Delta\Psi_m$ collapse, and increased the caspase-3 expression level. Therefore, exogenous PE could induce apoptosis of human hepatoma HepG2 cells via the bcl-2/bax pathway. So it is an interesting work.

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Addition of senna improves quality of colonoscopy preparation with magnesium citrate

Stergios Vradelis, Evangelos Kalaitzakis, Yalda Sharifi, Otto Buchel, Satish Keshav, Roger W Chapman, Barbara Braden

Stergios Vradelis, Evangelos Kalaitzakis, Yalda Sharifi, Otto Buchel, Satish Keshav, Roger W Chapman, Barbara Braden, Department of Gastroenterology, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Evangelos Kalaitzakis, Section of Gastroenterology and Hepatology, Department of Internal Medicine, Sahlgrenska University Hospital, 41345 Gothenburg, Sweden

Author contributions: Vradelis S and Kalaitzakis E contributed equally as first authors in data analysis and editing the manuscript; Braden B designed the study, performed the data collection and wrote the manuscript; Sharifi Y, Buchel O, Keshav S and Chapman RW were involved in editing the manuscript; Kalaitzakis E also provided financial support for publishing this work.

Correspondence to: Dr. Barbara Braden, Professor, Consultant Gastroenterologist, Gastroenterology Department, John Radcliffe Hospital, Headley Way, Oxford OX3 9DU, United Kingdom. braden@em.uni-frankfurt.de

Telephone: +44-1865-227860 Fax: +44-1865-2274

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However, abdominal cramps were observed more often under the senna protocol (29.2%) compared to the magnesium citrate only protocol (9.9%, $P < 0.0003$).

CONCLUSION: The addition of senna to the bowel preparation protocol with magnesium citrate significantly improves the cleansing outcome.

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Key words: Colonoscopy; Bowel preparation; Senna; Magnesium citrate; Polyp

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Abstract

AIM: To prospectively investigate the effectiveness and patient's tolerance of two low-cost bowel cleansing preparation protocols based on magnesium citrate only or the combination of magnesium citrate and senna.

METHODS: A total of 342 patients who were referred for colonoscopy underwent a colon cleansing protocol with magnesium citrate alone ($n = 160$) or magnesium citrate and senna granules ($n = 182$). The colonoscopist rated the overall efficacy of colon cleansing using an established score on a 4-point scale. Patients were questioned before undergoing colonoscopy for side effects and symptoms during bowel preparation.

RESULTS: The percentage of procedures rescheduled because of insufficient colon cleansing was 7% in the magnesium citrate group and 4% in the magnesium citrate/senna group ($P = 0.44$). Adequate visualization of the colonic mucosa was rated superior under the citramag/senna regimen ($P = 0.004$). Both regimens were well tolerated, and did not significantly differ in the occurrence of nausea, bloating or headache.

INTRODUCTION

Good bowel preparation is mandatory for optimal intraluminal visualization during colonoscopy. Inadequate bowel cleansing has a negative impact on completion rate^[1] and polyp detection rate^[1,2]. It also increases procedure time^[1,3] and difficulty^[1], and it may affect the procedure safety profile^[4]. All these factors negatively affect therapeutic efficiency and diagnostic accuracy and increase colonoscopy costs^[5].

Magnesium citrate is an osmotic saline agent that increases intraluminal volume resulting in a secondary increase of intestinal motility^[6]. According to a prospective study of colonoscopy practice in 68 hospitals in the UK, magnesium salts are used as bowel cleansing agents in 36.8% of colonoscopies^[7]. Magnesium citrate must be used cautiously in patients with impaired renal function.

Senna, an anthraquinone derivative, is a stimulant laxative stimulating intestinal motility^[8]. Senna has been reported to be a useful adjunct to polyethylene glycol

(PEG) colonoscopy preparation regimens^[9,10]. However, the role of senna as a potential adjunct of magnesium citrate for bowel preparation is yet to be explored.

In two prospective clinical audits, we compared the colon cleansing efficacy and patient tolerability of two colon preparation regimens for adults undergoing colonoscopy: (a) magnesium citrate; and (b) magnesium citrate combined with senna granules.

MATERIALS AND METHODS

Patients

A total of 345 consecutive adult out patients referred to the endoscopy unit of the John Radcliffe hospital for colonoscopy on routine clinical indications were prospectively invited to participate. Exclusion criteria were ileus or suspected bowel obstruction, significant gastroparesis or gastric outlet obstruction, toxic colitis or megacolon, pregnancy, or lactation. Patients included were referred mainly due to alarm symptoms such as altered bowel habit in patients > 45 years of age, blood per rectum, iron-deficiency anaemia, unintentional weight loss, and/or other symptoms suggestive of malignant disease. No patient refused to participate. Patients who could not be sedated for colonoscopy were excluded from further evaluation. All patients gave informed consent before participation.

From July to November 2006, we prospectively audited the performance of the large bowel cleansing with magnesium citrate (citramag-only group). The colon preparation protocol with magnesium citrate and senna (citramag/senna group) was audited from August 2007 to January 2008.

Bowel preparation regimens

All patients were asked to refrain from taking iron tablets 7 d prior to colonoscopy and any medications reducing gastrointestinal motility, e.g. loperamide, 4 d before colonoscopy, but continued all other medications. Two days before colonoscopy, all patients were instructed to eat a low-fiber diet such as white fish, chicken, white bread, eggs, cheese, or potato without skins. High-fiber kinds of food such as red meat, fruit, or vegetables, were to be avoided and patients were advised to consume plenty of fluids. On the day before colonoscopy patients, were instructed to have a low-fiber breakfast. Following that, patients were instructed not to eat any solid food until after colonoscopy and to consume plenty of clear fluids. On the day before colonoscopy at 5:00 pm, patients were instructed to dissolve one sachet of citramag in 200 mL of hot water, which was to be consumed half an hour later when cool. One sachet contains 11.6 g magnesium carbonate and 17.8 g anhydrous citric acid. At 7:00 pm, the patients were instructed to dissolve half of the second sachet of citramag in 100 mL of water and consume it once cooled. Patients were instructed to drink clear fluids (at least a cupful every 30 min) throughout the day and evening before colonoscopy. On the day of colonoscopy, at 6:00-7:00 am in the case of a morning appointment

or at 9:00-10:00 am in the case of an afternoon appointment, patients were instructed to consume the other half of a sachet of citramag as described above.

Patients in the citramag-only group underwent colonoscopy after bowel cleansing as described above (two sachets of citramag). Patients in the citramag/senna group were instructed to follow the above instructions but they were also asked to consume one sachet of senna in a cup of warm water and consume it at 2:00 pm on the day before colonoscopy (two sachets of citramag and one sachet of senna).

Colonoscopy

In order to eliminate interobserver variability, all colonoscopies were performed by the same experienced endoscopist who was, however, not blinded as to the cleansing regimen used. The colonoscopist rated the overall cleansing of the bowel on a 4-point Likert scale as in previous studies^[11-13]: 1 = "unacceptable" (large amounts of solid and semisolid fecal residue requiring additional cleansing resulting in rebooking); 2 = "poor" (enough feces or fluid to prevent a completely reliable examination); 3 = "satisfactory" (small amounts of feces or fluid not interfering with the exam); 4 = "good" (no more than small bits of adherent feces/fluid). For the primary efficacy variable, scores of 3 and 4 were considered "adequate" and scores of 1 or 2 were considered "inadequate".

Symptom score

Prior to colonoscopy, patients were asked to describe their tolerance to the cleansing protocol. Symptoms of nausea, vomiting, bloating, abdominal pain/discomfort, and headache were documented. Patients were asked to rate their symptom severity on a 5-point Likert scale as in previous bowel cleansing studies^[12-14]: 1 = "none"; 2 = "mild"; 3 = "moderate"; 4 = "severe"; and 5 = "extreme". Any adverse event reported during bowel cleansing and/or during the procedure and/or during stay in the recovery room after the procedure was also recorded.

Statistics analysis

Data are shown as mean and standard deviation or as median and ranges, as appropriate. The χ^2 test was used for comparisons between categorical variables and the Student's *t* test was used for comparisons between quantitative variables. All tests were two-tailed and conducted at a 5% significance level.

RESULTS

Overall, 345 patients underwent colonoscopy. Three patients in the citramag-only group could not be sedated for colonoscopy and were excluded from further evaluation. Data analysis was based on 160 patients who underwent colonoscopy after using the citramag bowel cleansing regimen and on 182 patients who performed bowel preparation for colonoscopy according to the combined citramag/senna regimen. Demographic and

Table 1 Demographic and clinical data

	Citramag-only (<i>n</i> = 160)	Citramag/senna (<i>n</i> = 182)
Male/female	76/84	97/85
Age	60 ± 13	58 ± 14
History of bowel resection	6	9
Sedation		
Fentanyl iv (µg)	75 (0-200)	75 (0-200)
Midazolam iv (mg)	4 (0-10)	4 (0-10)
Polyp detection rate	37 (23.1%)	63 (34.6%) ¹
Colorectal cancer	17	16
Completion rate	86% (adjusted 92%)	92% (adjusted 97%) ²

Data are presented as mean and standard deviation or *n* and percentage as appropriate. ¹*P* < 0.03, ²*P* = 0.07.

Table 2 Quality of colon cleansing as evaluated by the endoscopist *n* (%)

	Citramag-only (<i>n</i> = 160)	Citramag/senna (<i>n</i> = 182)
Good	20 (12.5)	41 (22.5)
Satisfactory	88 (55.0)	107 (58.8)
Poor	41 (25.6)	26 (14.3)
Unacceptable	11 (6.9)	8 (4.4)

clinical data of the patients are given in Table 1. The two groups did not differ regarding age, gender, previous history of colorectal surgery, caecal intubation rate, and sedation required (Table 1).

Patients following the citramag-only bowel cleansing regimen

In six of 160 patients (3.8%) using the citramag only cleansing protocol, bowel preparation was so poor with solid fecal residues that the procedure was abandoned in the rectosigmoid, and we did not attempt further insertion. In five other patients the cecum was reached but the view was unacceptable due to fecal residues, especially on the right side. Therefore, a repeat colonoscopy was indicated in these patients as well. Totally, 11/160 (6.9%) of the patients had to be rebooked for colonoscopy or virtual colonoscopy because of insufficient bowel cleansing after the citramag regimen.

Complete colonoscopy with visualization of the cecum was reached in 137/160 colonoscopies (86%). Adjustment for tumor strictures (4), cancellation of the procedure due to poor bowel preparation (6) or severity of colitis (1) raised the completion rate to 92% (137/149).

Patients following the citramag and senna bowel cleansing regimen

Five out of 182 patients (2.7%) were not examined further than the rectosigmoid because of insufficient bowel cleansing after taking the senna protocol, and three had to be rebooked because of insufficient views despite intubation of the cecum. Thus, eight of 182 patients (4.4%) had unacceptable bowel cleansing under the senna/citramag regimen.

The unadjusted completion rate for cecal intubation

was 92%. Adjustment for tumor strictures^[4] and exclusion of procedures abandoned in the rectosigmoid due to poor bowel preparation^[5] resulted in a completion rate of 97%.

Table 2 shows the quality of colon cleansing results for the two regimens used in the current study as assessed by the endoscopist. The combined citramag/senna regimen proved superior in bowel cleansing as it achieved “adequate” colon visualization (quality of colon cleansing rated as “good” or “satisfactory” in 148/182 (81.3%) compared to 108/160 (67.5%) colonoscopies using the citramag protocol (*P* = 0.004; Table 3). The colonic polyp detection rate was higher in the citramag/senna group compared to the citramag-only group (*P* < 0.03; Table 1).

Side effects and tolerability

Both protocols were well tolerated. None of the side effects observed were categorized as extreme, and five patients reported severe side effects during bowel preparation. The two bowel cleansing regimens did not significantly differ in the occurrence and intensity of nausea, vomiting, bloating or headache (Table 3). Abdominal cramps occurred more often in the citramag/senna group (*P* < 0.003; Table 3). Two patients reported “severe” abdominal pain/cramps both of whom had a stricturing tumor in the rectosigmoid.

DISCUSSION

According to our findings, the overall cleansing results were superior using the combination of senna compared to the citramag-only regimen. Although we did not perform a segmental evaluation of colon cleansing, the general impression was that particularly the right colon was better visualized in the citramag/senna group, while in the citramag-only group the cecum and ascending colon were often still covered in sticky solid fecal layers. Having reviewed the relevant bibliography and to the best of our knowledge, the current study is the first to evaluate the efficiency of senna as an adjunct to magnesium citrate for large bowel preparation prior to colonoscopy.

The rationale for using an osmotic agent such as magnesium citrate, together with a stimulant laxative such as senna for colonoscopy preparation, is that increased fluid bowel content produced by the osmotic agent may be more readily evacuated upon bowel stimulation by the stimulant agent^[15]. Previous studies have shown that the combination of PEG with stimulant bisacodyl allows for less volume of PEG to be used for colonic cleansing^[14,16]. Furthermore, the adjunctive use of senna with PEG has been shown to improve the quality of bowel preparation^[9] and to reduce the amount of PEG required for colonic cleansing^[10]. Similarly, we could demonstrate that the combination of senna with magnesium citrate was associated with improved quality in bowel preparation as assessed by a single experienced endoscopist. Although no cost-effectiveness analysis was undertaken, considering the relatively low price of senna^[13] and that bowel preparation has been reported

Table 3 Proportions of patients with side effects reported under bowel cleansing using citramag only or the combination of senna and citramag

Side effect	Treatment group	None (%)	Mild (%)	Moderate (%)	Severe (%)	Extreme (%)	P-value ¹
Nausea	Citramag	83.5	7.9	7.9	0.7	0	NS
	Citramag/senna	76.5	10.3	12.6	0.6	0	
Vomiting	Citramag	94.8	2.6	2.6	0	0	NS
	Citramag/senna	92.6	3.4	4	0	0	
Bloating	Citramag	88.1	5.3	6.6	0	0	NS
	Citramag/senna	81.7	7.4	10.3	0.6	0	
Abdominal pain	Citramag	90.1	6	3.9	0	0	P < 0.0003
	Citramag/senna	70.8	16	12	1.2	0	
Headache	Citramag	95.4	3.9	0.7	0	0	NS
	Citramag/senna	93.7	3.4	2.9	0	0	

¹P-value (significant at the 0.05 level) was obtained using a χ^2 -test to compare the frequency of side effects in treatment groups.

to have an impact on colonoscopy costs^[5], our results suggest that it may be a useful adjunct to magnesium citrate for outpatient colonoscopy preparation.

Bowel preparation was well tolerated in both the magnesium citrate-only and the magnesium citrate/senna group with no self-reported “extreme” symptoms during preparation in any one group. The prevalence of gastrointestinal symptoms did not differ between the two groups with the exception of an about three times increased frequency of abdominal pain noted in the magnesium citrate/senna group which, however, did not seem to affect compliance. No serious adverse events were recorded in either preparation group. Nevertheless it must be noted that no monitoring of laboratory values and electrolytes was performed, which is a limitation of the current study.

Although the majority of patients enrolled had alarm symptoms suggestive of colonic neoplasia (which may explain the relatively high polyp detection rate in both patient groups), our aim was not specifically to explore the potential role of the addition of senna to magnesium citrate in polyp detection. However, more colonic polyps were found in the group receiving bowel preparation with the addition of senna to magnesium citrate, which is in accordance with previous studies showing that the detection of polyps is dependent on bowel cleansing quality^[1,2].

There are certain limitations to the evaluation of the present audits. Although it was conducted in a prospective manner, no placebo was used and the endoscopist was not blinded to the patient group that was colonoscoped. Second, the scales used for the assessment of bowel cleansing and patient symptom severity were not previously validated. Third, no segmental assessment of bowel cleansing quality was performed, and finally, no monitoring of electrolyte levels was performed. As all consecutive patients underwent the same bowel preparation protocol during the audit periods, we can consider the allocation of the bowel cleansing regimen a block randomization.

In conclusion, the addition of senna to bowel preparation protocol with magnesium citrate significantly improves the cleansing outcome. It produces no major side effects but abdominal pains occur more often.

Senna might be a useful adjunct to magnesium citrate for colonoscopy preparation.

COMMENTS

Background

Adequate colon preparation is essential for the quality of colonoscopy. The completion and polyp detection rate as well as the complication risk and the duration of the colonoscopy are strongly affected by poor bowel cleansing.

Innovations and breakthroughs

As far as we know, this is the first study to evaluate the efficacy of senna as an adjunct to magnesium citrate for large bowel preparation prior to colonoscopy. Our findings demonstrate that the addition of senna to the bowel cleansing protocol with magnesium citrate significantly improves the cleansing outcome, but also increases the occurrence of side effects such as (mild) abdominal pains and discomfort.

Applications

Our study results suggest that senna can be used as a helpful adjunct to a cleansing protocol based on magnesium citrate and will increase the efficacy of the bowel preparation before colonoscopy.

Terminology

Magnesium citrate acts as an osmotic laxative which is often used for bowel preparation; senna is a stimulative agent increasing peristaltic movement.

Peer review

This interesting study evaluates how the addition of senna alters the cleansing outcome and the occurrence of side effects under a bowel preparation protocol based on magnesium citrate. The results suggest that senna might be a helpful adjunct to standard bowel preparation protocols.

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Prevalence of linked angina and gastroesophageal reflux disease in general practice

Hirohito Kato, Takamasa Ishii, Tatsuo Akimoto, Yoshihisa Urita, Motonobu Sugimoto

Hirohito Kato, Takamasa Ishii, Tatsuo Akimoto, Yoshihisa Urita, Motonobu Sugimoto, Department of General Medicine and Emergency Care, Toho University School of Medicine, Omori Hospital, 6-11-1, Omori-Nishi, Ota-Ku, Tokyo 143-8541, Japan

Author contributions: Kato H and Ishii T collected all the human material and performed the majority of data collection; Akimoto T and Sugimoto M provided analytical tools and were also involved in editing the manuscript; Kato H and Urita Y designed the study and wrote the manuscript.

Correspondence to: Dr. Yoshihisa Urita, Department of General Medicine and Emergency Care, Toho University School of Medicine, Omori Hospital, 6-11-1, Omori-Nishi, Ota-Ku, Tokyo 143-8541, Japan. foo@eb.mbn.or.jp

Telephone: +81-3-37624151 Fax: +81-3-37656518

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CONCLUSION: The present study suggested that ischemic heart disease might be found although a patient was referred to the hospital with a complaint of GERD symptoms. Physicians have to be concerned about missing clinically important coronary artery disease while evaluating patients for GERD symptoms.

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Key words: Linked angina; Epidemiology; General practice; Electrocardiography; Gastroesophageal reflux disease

Peer reviewer: Tomohiko Shimatani, Assistant Professor, Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Kato H, Ishii T, Akimoto T, Urita Y, Sugimoto M. Prevalence of linked angina and gastroesophageal reflux disease in general practice. *World J Gastroenterol* 2009; 15(14): 1764-1768 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1764.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1764>

Abstract

AIM: To evaluate the association between gastroesophageal reflux diseases (GERD) and coronary heart diseases.

METHODS: One thousand nine hundred and seventy consecutive patients who attended our hospital were enrolled. All of the patients who first attend our hospital were asked to respond to the F-scale questionnaire regardless of their chief complaints. All patients had a careful history taken, and resting echocardiography (ECG) was performed by physicians if the diagnostic necessity arose. Patients with ECG signs of coronary artery ischemia were defined as ST-segment depression based on the Minnesota code.

RESULTS: Among 712 patients (36%) with GERD, ECG was performed in 171 (24%), and ischemic changes were detected in eight (5%). Four (50%) of these patients with abnormal findings upon ECG had no chest symptoms such as chest pain, chest oppression, or palpitations. These patients (0.6%; 4/712) were thought to have non-GERD heartburn, which may be related to ischemic heart disease. Of 281 patients who underwent ECG and did not have GERD symptoms, 20 (7%) had abnormal findings upon ECG. In patients with GERD symptoms and ECG signs of coronary artery ischemia, the prevalence of linked angina was considered to be 0.4% (8/1970 patients).

INTRODUCTION

Chest pain often precedes echocardiographic coronary angiography, electrocardiography (ECG), and scintigraphy for the diagnosis of angina. Many patients presenting to a hospital emergency room for chest pain turn out not to have coronary artery disease (CAD)^[1]. According to data from a large university, 81%-86% of patients evaluated in an emergency room for acute chest pain did not have coronary ischemia^[2,3]. It is well known that non-cardiac chest pain is closely related to gastroesophageal reflux diseases (GERD)^[4,5]. Similarly, erosive esophagitis is not found in some patients with persistent GERD symptoms. Although GERD symptoms affect 10%-30% of the population in Western countries^[6], endoscopic esophagitis is less prevalent, and is reported to occur in up to 2% of individuals^[7-8]. Only one-third of GERD patients have endoscopic positive findings, while others have no obvious mucosal breaks, even though GERD symptoms are present^[9]. Chest pain of esophageal origin can be difficult to distinguish from that caused

by cardiac ischemia because the distal esophagus and the heart share a common afferent vagal supply, and GERD can cause episodes of non-cardiac chest pain that resemble ischemic cardiac pain^[10,11]. It is possible that GERD may be misclassified as angina pectoris and vice versa in clinical practice. The aim of this study was to evaluate the association between GERD and coronary heart disease and to clarify the presence of non-GERD heartburn.

MATERIALS AND METHODS

Ethics

The study was carried out in accordance with the Declaration of Helsinki, and approved by the ethical committee at Toho University.

Patients

Between October 2005 and May 2006, 1970 consecutive patients (934 men and 1036 women with a mean age of 43 years) who first attended the Outpatient Department of General Medicine and Emergency Care of Toho University Omori Hospital were enrolled. Informed consent was obtained from all the patients. None of the patients had a history of use of proton pump inhibitors, H₂-receptor antagonists, antibiotics, steroids, or nonsteroidal anti-inflammatory drugs for a period of at least 2 mo before the investigation. Patients who had a previous history of partial gastrectomy were also excluded from the study.

Questionnaire

All of the patients who attended our hospital were asked to respond to the F-scale questionnaire regardless of their chief complaints. The questionnaire is a self-report instrument, written in a simple and easy-to-understand language, which contained 12 questions. As reported previously by Kusano *et al*^[12], the following definitions were used to identify symptoms in the F-scale: (1) "Do you get heartburn?"; (2) "Is your stomach bloated?"; (3) "Does your stomach ever feel heavy after meals?"; (4) "Do you sometimes subconsciously rub your chest with your hand?"; (5) "Do you ever feel sick after meals?"; (6) "Do you get heartburn after meals?"; (7) "Do you have an unusual sensation in your throat?"; (8) "Do you feel full while eating meals?"; (9) "Do some things get stuck when you swallow?"; (10) "Do you get bitter liquid coming up into your throat?"; (11) "Do you belch a lot?"; and (12) "Do you get heartburn if you bend over?". Symptoms frequency was measured on the following scale: 0, never; 1, occasionally; 2, sometimes; 3, often; and 4, always. A score of more than 7 points, was considered positive for GERD.

Electrocardiogram

All patients had a careful history taken, and resting ECG was performed by physicians if diagnostic necessity arose. Patients with ECG signs of coronary artery ischemia were defined as having ST-segment depression based on the Minnesota code^[13].

Table 1 Characteristics of study participants

Cardiogram	Abnormal	Normal	P
No. of patients	456	1514	
Age (yr)	48.2 ± 17.6 (16-91)	40.7 ± 15.7 (15-93)	< 0.001
Male/female	231/225	703/811	< 0.05
Hypertension	49/407	84/1430	< 0.001
Diabetes	36/420	45/1469	< 0.001
Hyperlipidemia	79/377	146/1473	< 0.001
Current smoker	156/300	488/1026	NS

Table 2 Relationship between GERD symptoms and ECG abnormalities

Cardiogram	Abnormal	Normal
GERD (+)	8	166
GERD (-)	20	261

Statistical analysis

All values are expressed as means ± SD. Comparisons of groups were made using Student's *t* test or chi-square tests as appropriate. *P* < 0.05 was considered statistically significant.

RESULTS

Overall, ECG was performed in 456 patients (23%, ECG group). The remaining 1514 patients were defined as controls. Patients in the ECG group were significantly older and the male to female ratio was significantly higher than in controls (Table 1). The prevalence of hypertension, diabetes mellitus, and hyperlipidemia was also significantly higher in the ECG group. There was no difference in the proportion of current smokers between the two groups.

Among 712 patients (36%) with GERD, ECG was performed in 171 patients (24%) and ischemic changes were detected in eight patients (5%). Four (50%) of these patients with abnormal findings upon ECG had no chest symptoms such as chest pain, chest oppression, or palpitations. These patients (0.6%; 4/712) were thought to have non-GERD heartburn, which may be related to ischemic heart disease. Of 281 patients who underwent ECG and did not have GERD symptoms, 20 (7%) had abnormal findings upon ECG (Table 2). No significant differences in the prevalence of ischemic changes were found between patients who underwent ECG and those who did not. As shown in Table 3, an exercise thallium test was performed in 12 GERD patients, of whom, one had ischemic coronary artery disease that was proven angiographically. In patients with GERD symptoms and ECG signs of coronary artery ischemia, the prevalence of linked angina, was considered to be 0.4% (8/1970 patients) (Figure 1). In contrast, patient with angina not related to GERD was found in 20 (1.0%).

DISCUSSION

Screening asymptomatic patients is an area of

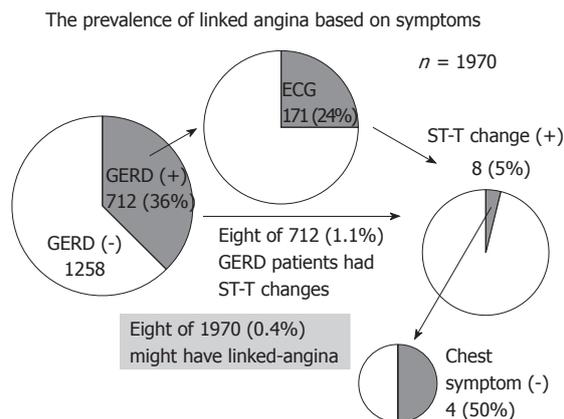
Table 3 Number of patients undergoing additional cardiological tests

	GERD (+) <i>n</i> = 171 Abnormal findings		GERD (-) <i>n</i> = 285 Abnormal findings	
	(+)	(-)	(+)	(-)
ECG	8	163	20	265
Exercised thallium test	2	10	5	13
Cardioangiography	1	0	4	3

considerable interest because silent CAD is an important cause of premature death; many of these patients have three-vessel CAD or left main coronary artery stenosis^[14]. Little is known, however, about the effects of intervention on asymptomatic disease, and screening strategies for asymptomatic CAD have been difficult to justify. Of patients with a low pre-test probability of CAD, only about 20% with a positive exercise test will have angiographically verified CAD^[15]. Little evidence exists currently to support doing resting ECGs^[16] or exercise tests^[17] in patients without clinical evidence of CAD or cardiovascular risk factors.

When coronary arteries appear normal upon angiography, particularly when there is no other cardiac disorder or objective evidence of ischemia, a diagnosis of non-cardiac chest pain is made. In that particular study^[18], cardiac mortality was 0.09% and coronary event rate was 0.65% per year, but 81% of patients had chest pain after 9 years follow-up. The clinician often is faced with the problem of a patient with comparatively mild coronary disease but persistent severe symptoms despite anti-anginal drugs. This subset of patients presents a difficult clinical problem because both the patient and physician usually perceive the pain to be of cardiac origin. It is probable that many of these patients have coexisting coronary disease and a symptomatic esophageal disorder. Symptoms of chest pain are a major source of concern for patients and physicians alike because they can sometimes be the harbinger of acute life-threatening events. However, many patients who describe chest pain that sounds identical to the type associated with significant cardiac disease can actually be free of such disease. After appropriate evaluations, about 40% of patients admitted for potential acute coronary syndromes turn out not to have CAD^[19,20]. A study at the Emergency Room Assessment of Sestamibi for Evaluating Chest Pain Trial (ERASE trial), and a second study at a large hospital center in Philadelphia, showed that 81%-86% of all patients presenting to the emergency department with chest pain did not have a final diagnosis of cardiac ischemia^[3,21]. Thus, differentiation between cardiac- and esophageal-origin chest pain is especially difficult since both organs have overlapping sensory pathways: Th1-Th4 for the heart and C8-TH10 for the esophagus. Moreover, they have autonomic nerve reflex, the so-called vagal visceral reflex function between the heart and organs of the gastrointestinal tract^[22].

Direct mechanical effects between the esophagus

**Figure 1** Prevalence of linked angina based on symptoms.

and the heart are thought to be compression of the left atrium by a huge hiatus hernia or extrinsic esophageal compression by cardiac enlargement. Other than these direct effects, a neural reflex, mediated by the vagus nerve, exists that allows changes in esophageal function to affect cardiac physiology^[22]. Atrial tachycardia can be triggered by swallowing and belching, although the precise neural mechanism remains unclear^[23]. Furthermore, bradycardia occurs in most people during balloon inflation within the esophagus and this may be blocked by atropine^[24]. It has been suggested that esophageal dysfunction can itself trigger myocardial-ischemia-linked angina^[25]. Smith *et al*^[25] have coined the term “linked angina”, which implies that gastrointestinal factors bring on attacks of genuine angina in patients with established CAD. They have explained this on the basis of cardiovascular changes, although gastroesophageal acid reflux may equally explain the phenomenon. Instillation of acid into the esophagus has been shown to significantly reduce the exertional angina threshold at which angina occurs or can provoke angina with ischemic ECG changes^[26]. A previous animal study has demonstrated the reduced coronary flow caused by distension of the stomach that does not occur after vagal section or after the administration of atropine. This suggests that reflex coronary vasoconstriction is initiated by vagal irritation in the gastrointestinal tract^[27]. Mellow *et al*^[28] have demonstrated that acid perfusion of the esophagus results in reduced coronary blood flow in patients with proven CAD. It should be emphasized that 67% of patients felt chest pain and two-thirds of them developed ischemic ST-segment changes upon ECG. Dobrzycki *et al*^[29] have also reported that GERD patients have a larger total ischemic burden and higher incidence of ST depression. Thus, spontaneous GER may have a role to play in the causation of or provocation of cardiac chest pain. Linked angina can be defined as that induced by GER. However, most patients with asymptomatic angina or symptoms, that do not reach the threshold required to define disease, do not refer to a hospital. Therefore, the present study was designed in the Department of General Medicine to evaluate the association between GERD and coronary heart disease in the general population because general

practice registers offer the best means of sampling the general population^[30]. We hypothesized that the prevalence of CAD in GERD patients who were referred for the first time to the gastroenterology outpatient clinic because of heartburn may be substantial.

The present study revealed a small number of patients with abnormal ECG findings but without chest symptoms such as chest pain, chest oppression, or palpitations. This suggests that the extra-esophageal condition causes GERD symptoms and that angina may be misclassified as GERD. Such patients are often treated with proton pump inhibitors and chest pain disappears, which suggests that CAD may be overlooked. The results may therefore have clinical relevance, as it has been reported in a previous population-based, nested case-control study that patients with GERD have an increased risk of angina pectoris in the year after GERD diagnosis^[31]. Actually, the incidence of reflux esophagitis is increasing as the population grows older, which makes it likely that such disease may be present in patients with CAD^[32]. Physicians have to be concerned about missing clinically important CAD while evaluating patients for GERD symptoms.

COMMENTS

Background

Screening asymptomatic patients is an area of considerable interest because silent coronary artery disease (CAD) is an important cause of premature death, although many patients presenting to a hospital emergency room for chest pain turn out not to have CAD. It is well known that non-cardiac chest pain is closely related to gastroesophageal reflux diseases (GERD) and GERD symptoms affect 10%-30% of the population. Little is known, however, about the prevalence of linked angina, which is defined as angina induced by GER.

Research frontiers

In patients with GERD symptoms and echocardiography signs of coronary artery ischemia, prevalence of linked angina, was considered to be 0.4% (8/1970 patients). The prevalence may be increased when more sensitive tests such as angiography are used for diagnosis of angina.

Innovations and breakthroughs

Recent reports have highlighted the association between non-cardiac chest pain and GERD because both organs have overlapping sensory pathways. However, there have been few reports that GERD symptoms may be caused by ischemic heart disease. The present study is believed to be the first to evaluate the presence of non-GERD heartburn. GERD symptoms induced by coronary artery ischemia, so-called linked angina, certainly exist.

Applications

The study results suggest that an extra-esophageal condition causes GERD symptoms and that angina may be misclassified as GERD. Since patients with GERD have an increased risk of angina pectoris in the year after GERD diagnosis, physicians have to be concerned about missing clinically important CAD while evaluating patients for GERD symptoms.

Terminology

Linked angina is defined as angina induced by GER. GERD symptoms typically include heartburn and regurgitation. GERD is diagnosed according to symptoms regardless of the presence of endoscopically proven esophagitis.

Peer review

The authors examined the prevalence of linked angina. The results suggest the possibility that GERD patients also have coronary heart disease.

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Lower gastrointestinal bleeding secondary to a rectal leiomyoma

Giovanni D De Palma, Maria Rega, Stefania Masone, Saverio Siciliano, Marcello Persico, Francesca Salvatori, Francesco Maione, Dario Esposito, Antonio Bellino, Giovanni Persico

Giovanni D De Palma, Maria Rega, Stefania Masone, Saverio Siciliano, Marcello Persico, Francesca Salvatori, Francesco Maione, Dario Esposito, Antonio Bellino, Giovanni Persico, Department of Surgery and Advanced Technologies, Center for Technical Innovation in Surgery, University of Naples Federico II, School of Medicine, Via Pansini 5, Naples 80131, Italy

Author contributions: All the authors contributed to ideation, drafting, and revision of text and composition of figures and research of references.

Correspondence to: Giovanni D De Palma, Professor, Department of Surgery and Advanced Technologies, Center for Technical Innovation in Surgery, University of Naples Federico II, School of Medicine, Via Pansini 5, Naples 80131, Italy. giovanni.depalma@unina.it

Telephone: +39-81-7462773 Fax: +39-81-7462752

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Abstract

The occurrence of leiomyoma of the rectum is uncommon. Most of these lesions are clinically silent and are found incidentally during laparotomy or endoscopic procedures for unrelated conditions. Symptomatic leiomyomas of the rectum are encountered less frequently, with only sporadic reports in the literature. We describe a case of a leiomyoma of the rectum presenting as recurrent lower gastrointestinal hemorrhage and secondary anemia.

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Key words: Rectal leiomyoma; Gastrointestinal bleeding; Endoscopy; Endoscopic ultrasonography; Immunohistochemistry

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De Palma GD, Rega M, Masone S, Siciliano S, Persico M, Salvatori F, Maione F, Esposito D, Bellino A, Persico G. Lower gastrointestinal bleeding secondary to a rectal leiomyoma. *World J Gastroenterol* 2009; 15(14): 1769-1770 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1769.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1769>

INTRODUCTION

Smooth muscle tumors may occur throughout the entire gastrointestinal tract, but are rarely seen in the colon and rectum. Most of these lesions are clinically silent and are found incidentally during laparotomy or endoscopic procedures for unrelated conditions. Symptomatic leiomyomas (LMs) of the rectum are encountered less frequently, with only sporadic reports in the literature. We describe a case of LM of the rectum, presenting as recurrent lower gastrointestinal hemorrhage and secondary anemia.

CASE REPORT

A 55-year-old woman presented to our unit complaining of recurrent rectal bleeding and secondary sideropenic anemia. Colonoscopy revealed the presence of a polypoid, submucous, ulcerated lesion in its vertex (2 cm from the anal margin) (Figure 1).

An endoanal ultrasound scan showed a mass located in the anterior wall of the rectum, approximately 7 cm in size, with no infiltration of perirectal fat (Figure 2). A biopsy was made and the pathological study showed a proliferation of fusiform, elongated spindle cells arranged in fascicles. The nuclei were elongated and cigar-shaped, and there was minimal nuclear pleomorphism. No mitotic figures were seen (Figure 3). Immunohistochemistry was positive for smooth muscle actin (SMA) and desmin and negative for CD117.

With a preoperative diagnosis of rectal LM, the mass was removed by local excision with preservation of the rectum. The patient is currently in the 12th mo of follow-up, and has no signs or symptoms of relapse.

DISCUSSION

Primary LMs present most commonly in the female genital tract and as skin lesions. This tumor is seldom encountered in the gastrointestinal tract. The most common localization is the stomach, followed by the small intestine. The colon, rectum and esophagus are less likely sites. LM of the anorectal region represent 3% of all gastrointestinal LM, and less than 0.1% of rectal tumors^[1-6].

Most reported LMs are sessile intraluminal or intramural tumors. They can also present as pedunculated extra luminal mass of the colon^[7]. LM often remain asymptomatic until they have reached a fairly large size. The clinical manifestations of these smooth muscle



Figure 1 Endoscopic view of a polypoid, submucosal, ulcerated lesion in its vertex.

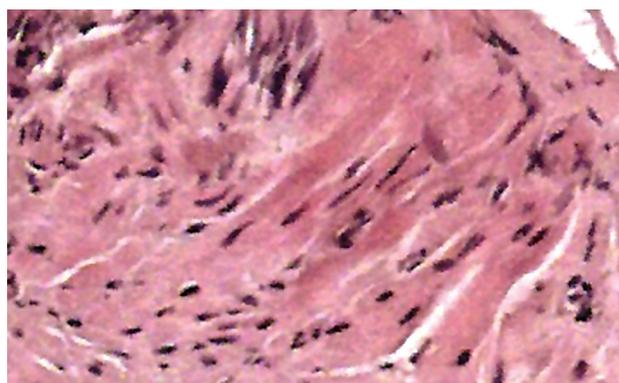


Figure 3 Microscopic findings showing a proliferation of fusiform, elongated spindle cells arranged in fascicles.



Figure 2 Endoanal ultrasound scan showing a mass located in the anterior wall of the rectum.

tumors depend on the location, size and direction of tumor growth. They include intestinal obstruction, hemorrhage, and perforation into the peritoneal cavity.

Intraluminal lesions can be detected earlier because of the earlier presentation of symptoms. Many of these tumors are discovered incidentally on routine endoscopic examination of the large bowel. Endoscopically, these tumors can present as pedunculated intramural or intraluminal polyps, and they may look like the more usual adenomas. Complementary investigation, such as with computed tomography, endoscopic ultrasonography, and magnetic resonance imaging, strongly corroborates the diagnosis. Endorectal ultrasound can help to define the extent of disease and may be a useful adjunct in deciding about the appropriate surgical procedure^[8].

The biological behavior of smooth muscle tumors varies from benign to locally aggressive and highly malignant. The biological behavior may not be reflected by the histology, as even benign-looking smooth muscle tumors may metastasize. Thus, a combination of site, tumor size, histological appearance and mitotic count give the best prediction of behavior^[9].

LM should be separated from gastro-intestinal stromal tumors (GISTs). LMs are positive for actin and desmin and negative for CD34 and CD117 (KIT), and

GISTs have the opposite pattern^[10]. Surgical excision is the treatment of choice for most LMs. Snare polypectomy is an adequate treatment, but large LMs are believed to be best treated by surgical resection, because conventional colonoscopic resection of large and deep-seeded tumors poses a high risk of perforation^[11]. Ensuring the complete removal and follow-up are necessary precautions for tumors with any atypia or mitotic activity.

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Infliximab to treat severe ulcerative colitis

Dídia Bisamra Cury, Marcelo de Souza Cury, Geraldo Vinicius Hemerly Elias, Sender Jankiel Mizsputen

Dídia Bisamra Cury, Marcelo de Souza Cury, Geraldo Vinicius Hemerly Elias, Sender Jankiel Mizsputen, Department of Gastroenterology, Federal University of São Paulo, rua Botucatu, 740 São Paulo, SP 04023-900, Brazil
Author contributions: Cury DB wrote the article; Cury MS performed the second colonoscopy and collated the bibliographic references; Elias GVH performed the first colonoscopy; Mizsputen SJ helped to conduct this case, and reviewed the article.

Correspondence to: Dídia Bismara Cury, Department of Gastroenterology, Federal University of São Paulo, rua Botucatu, 740 São Paulo, SP 04023-900, Brazil. didia_cury@uol.com.br
Telephone: +55-67-33256040 Fax: +55-67-33256040
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Abstract

A 48-year-old female with severe ulcerative colitis refractory to conventional therapy was referred to our facility for management. The patient showed extensive ulcerative colitis since the age of 20 years and had failed therapy with 5-aminosalicylic acid agents and azathioprine. The disease remained active despite treatment with steroids and cyclosporine. The clinical and endoscopic parameters were consistent with severe disease. Infectious precipitants were ruled out. Given the severity of the disease and in order to avoid a colectomy, we started the patient on infliximab therapy. A dramatic clinical and endoscopic response was observed and she remained in remission at the end of a 1-year follow-up period. We discuss findings in the literature regarding the use of infliximab therapy in patients with ulcerative colitis who have failed steroids and cyclosporine.

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Key words: Cyclosporine; Infliximab; Treatment failure; Ulcerative colitis; Inflammatory bowel diseases

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INTRODUCTION

Infliximab is a monoclonal antibody against tumor necrosis factor alpha (TNF- α) used in the treatment of Crohn's disease and ulcerative colitis. It has demonstrated efficacy in patients with moderate to severe ulcerative colitis, as well as in those with severe disease who have failed intravenous (IV) steroids^[1]. According to historical data, patients with severe disease who fail IV steroids have a risk of colectomy of up to 60%. Currently, there are three therapeutic options: cyclosporine, infliximab and colectomy^[2]. Such patients are often treated empirically with cyclosporine or infliximab. If the patient fails to respond to either, colectomy is typically performed. Using infliximab to treat patients who have failed steroids and cyclosporine is controversial, since the risk of opportunistic infection is considered to be high. We report a case in which infliximab was safely introduced and successfully used in a patient with severe ulcerative colitis who would otherwise have undergone colectomy.

CASE REPORT

A 48-year-old female with ulcerative colitis presented to our facility for the management of refractory disease. She had extensive ulcerative colitis since the age of 20 years and had a number of flares requiring IV and oral (PO) steroids, having failed 5-aminosalicylic acid agents. In the prior 6 mo, the patient had been treated with azathioprine at 2 mg/kg per day and both IV and subsequent PO cyclosporine (8 mg/kg per day). She was referred for further management as she was passing in excess of six stools per day, as well as presenting bloody diarrhea and abdominal pain. She had severe anemia, with repeated need for blood transfusions. Sigmoidoscopy confirmed active ulcerative colitis (Figures 1 and 2). This clinical spectrum was despite current therapy with cyclosporine and azathioprine.

At the time of presentation, her blood pressure was 160/100 mmHg, with a pulse rate of 150 bpm, and an intense generalized abdominal pain. She had a white blood cell count of 161 000/mm³, hematocrit of 13%, hemoglobin of 5.0 g/dL, erythrocyte sedimentation rate of 130 mm in the first hour, C-reactive protein of > 5 mg/dL, iron of 10 mg/dL, albumin of 2 g/dL, and alpha 1-acid glycoprotein of 230 mg/dL. A colonoscopy revealed severe ulcerative colitis extending from the rectum (Figure 1). Biopsies confirmed chronic active colitis.



Figure 1 Rectal view during treatment with azathioprine.

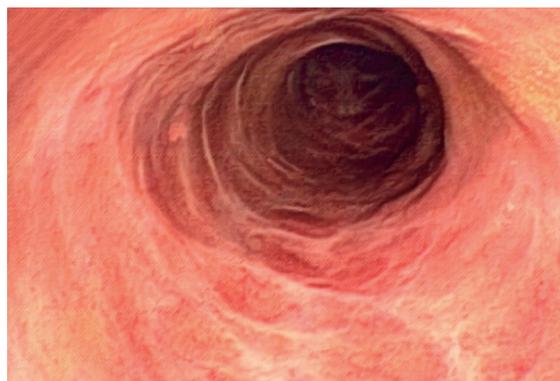


Figure 3 Colonoscopy: Rectal view after 18 mo of treatment with infliximab.



Figure 2 Colonoscopy: Sigmoid view during treatment with azathioprine.



Figure 4 Colonoscopy: Sigmoid view after 18 mo of treatment with infliximab.

	Azathioprine	Cyclosporine	Infliximab (after 18 mo of treatment)
Hemoglobin, g/dL	5.0	8.0	13.0
Hematocrit (%)	13	19	40
Erythrocyte sedimentation rate, mm in the first hour	130	230	7
Albumin, g/dL	2	2.5	3.5
Iron, mg/dL	10	15	55
α -1 glycoprotein, mg/dL	230	245	< 100
C-reactive protein, mg/dL	> 5	> 5	< 5

	Azathioprine	Cyclosporine	Infliximab
Frequency of evacuations	3	3	0
Rectal bleeding	3	3	0
Endoscopic findings	3	3	0
Overall medical evaluation	3	3	0

After 18 mo of infliximab treatment, the patient was in remission, with one stool per day and no abdominal pain. A repeat colonoscopy demonstrated marked endoscopic improvement (Figures 3 and 4). There was a dramatic improvement in the overall nutritional status of the patient and in the serum levels of all parameters, as shown in Tables 1 and 2. She remained in remission on maintenance infliximab and azathioprine at the last assessment.

DISCUSSION

Refractory ulcerative colitis is currently defined as an inadequate response to conventional treatment. In cases of ulcerative colitis, the symptoms used to determine whether an individual is refractory to treatment include

fever, diarrhea three or more times per day, bleeding, and fecal urgency^[3]. Immunomodulators, such as azathioprine, have been used as adjuvant therapy in patients with ulcerative colitis who are classified as non-responders to oral steroids, the recommended initial dose being 2 mg/kg per day, which can be gradually increased if a satisfactory response is not achieved^[4].

Cyclosporine has been used in refractory patients, as well as in patients classified as non-responders, who typically present extensive or severe colitis. For such patients, treatment with cyclosporine appears to control the disease in approximately 90% of cases^[5-8]. In the present case, the patient did not exhibit a sustained response to cyclosporine, and there was recurrence within 1 mo, at which point infliximab was initiated.

Infliximab, a chimeric monoclonal antibody to TNF- α , was originally believed to bind biologically active TNF- α freely present in the lamina propria and expressed on inflammatory cells. However, it soon became clear that this binding phenomenon had to be

followed by complement binding and activation, leading to apoptosis of the activated inflammatory TNF- α -bearing cells, as well as inducing apoptosis of T cells^[9-11].

In two large-scale studies, designated Active Ulcerative Colitis Trial 1 and Active Ulcerative Colitis Trial 2, patients with ulcerative colitis were treated with infliximab, together with corticosteroids or the 6-mercaptopurin/azathioprine combination, and were monitored/evaluated using the Mayo scale^[12,13]. The authors found that the rate of remission was higher in patients treated with infliximab than in those receiving placebo.

Currently, the use of infliximab in patients with ulcerative colitis is recommended for those with corticosteroid dependence, refractory pouchitis and pouchitis in the maintenance phase, although there is still controversy regarding the appropriate duration of treatment^[14,15]. Treating patients with infliximab after failing steroids and cyclosporine is controversial, as one study reported a serious adverse event rate of 15%, due to opportunistic infections^[16-18]. In the case described here, we opted to maintain the infliximab, taking into account the quality of life that could be sustained without surgical intervention. The patient remained under treatment with infliximab every 8 wk for 2 years, at a dose of 5 mg/kg without adverse events and recurrence of the symptoms.

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CASE REPORT

A case of small bowel adenocarcinoma in a patient with Crohn's disease detected by PET/CT and double-balloon enteroscopy

Chise Kodaira, Satoshi Osawa, Chihiro Mochizuki, Yoshihiko Sato, Masafumi Nishino, Takanori Yamada, Yasuhiro Takayanagi, Kosuke Takagaki, Ken Sugimoto, Shigeru Kanaoka, Takahisa Furuta, Mutsuhiro Ikuma

Chise Kodaira, Satoshi Osawa, Masafumi Nishino, Takanori Yamada, Yasuhiro Takayanagi, Kosuke Takagaki, Ken Sugimoto, Mutsuhiro Ikuma, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

Chihiro Mochizuki, Yoshihiko Sato, Department of Gastroenterology, Seirei Hamamatsu General Hospital, 2-12-12 Sumiyoshi, Naka-ku, Hamamatsu 431-3192, Japan

Shigeru Kanaoka, Molecular Diagnosis, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

Takahisa Furuta, Center for Clinical Research, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

Author contributions: Kodaira C and Osawa S wrote the paper; Mochizuki C and Sato Y contributed to the PET/CT and imaging diagnosis; Nishino M, Yamada T, Takayanagi Y, and Takagaki K performed the double-balloon enteroscopy; Sugimoto K, Kanaoka S, Furuta T, and Ikuma M designed the paper.

Correspondence to: Satoshi Osawa, MD, PhD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. sososawa@hama-med.ac.jp

Telephone: +81-53-4352261 Fax: +81-53-4349447

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the small bowel appeared to be associated with the cancer development. Previous reports suggest the risk of SBA in patients with CD is higher than in the overall population. Since early diagnosis is extremely difficult in these cases, novel techniques, such as PET/CT and DBE, may be expected to help in making a preoperative diagnosis of the development of SBA in CD.

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Key words: Crohn's disease; Double-balloon enteroscopy; Positron emission tomography; Small bowel adenocarcinoma; Surveillance

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Kodaira C, Osawa S, Mochizuki C, Sato Y, Nishino M, Yamada T, Takayanagi Y, Takagaki K, Sugimoto K, Kanaoka S, Furuta T, Ikuma M. A case of small bowel adenocarcinoma in a patient with Crohn's disease detected by PET/CT and double-balloon enteroscopy. *World J Gastroenterol* 2009; 15(14): 1774-1778 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1774.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1774>

Abstract

Small bowel adenocarcinoma (SBA) in patients with Crohn's disease (CD) is quite rare, difficult to diagnose without surgery, and has a poor prognosis. Here, we report a 48-year-old man with SBA and a 21-year history of CD who was diagnosed by a combination of positron emission tomography/computed tomography (PET/CT) and double-balloon enteroscopy (DBE). Since the age of 27 years, the patient had been treated for ileal CD and was referred to our hospital with persistent melena. Multiple hepatic tumors were found by CT. PET/CT detected an accumulation spot in the small bowel. DBE revealed an ulcerative tumor in the ileum about 100 cm from the ileocecal valve. An endoscopic forceps biopsy specimen showed poorly differentiated adenocarcinoma. There were some longitudinal ulcer scars near the tumor, and the chronic inflammation in

INTRODUCTION

Previous studies have documented that patients with inflammatory bowel disease (IBD) are at increased risk of developing colorectal and intestinal cancer^[1-6]. However, because of its rarity, there is limited data on the precise cancer risk of small bowel adenocarcinoma (SBA) in patients with Crohn's disease (CD). To date, recommendations for screening and surveillance have been supported by very limited data^[7]. Even in extensive colitis, a recent report found that colonoscopy surveillance may not improve survival^[8], and other tools are needed to detect cancer development in the early stages. Here, we report a patient with a 21-year history of CD who developed a SBA that was detected by 18F-fluorodeoxyglucose (FDG) positron emission

tomography/computed tomography (PET/CT) and double-balloon enteroscopy (DBE) without conventional intestinal examinations.

CASE REPORT

A 48-year-old man who was diagnosed with ileal CD as a 27-year-old was referred to our hospital with persistent melena. His family history was negative for this disease. He had no evidence of gluten intolerance. He had been treated with 5-aminosalicylates (5-ASAs) combined with an elemental-diet (ED) therapy and had several hospitalizations for temporary melena in the past two decades. He had not agreed to have an intestinal examination at regular intervals. In January 2007, he was examined by abdominal CT due to persistent melena for 1 year, and multiple metastatic tumors were discovered in the liver (Figure 1A). Blood tests showed a slight anemia (Hb 11.8 g/dL), but he was negative for inflammatory factors. The serum carcinoembryonic antigen (CEA) was elevated (31.6 ng/mL; normal range, < 2.5 ng/mL), and wall thickening in part of the ileum was detected by CT (Figure 1B). PET/CT showed accumulations in the multiple hepatic tumors and the wall thickening of the ileum (Figure 2). DBE showed an ulcerative tumor in the ileum about 100 cm away from the ileocecal valve (Figure 3A). There were some longitudinal ulcer scars near the tumor (Figure 3B). An endoscopic forceps biopsy specimen showed microscopically poorly-differentiated adenocarcinoma (Figure 4A and B). Immunohistochemistry of the tumor cells was negative for p58 and positive for β -catenin (Figure 4C and D). Accordingly, he was diagnosed with SBA with hepatic metastases. Although he was treated by chemotherapy with S-1 and cisplatin (CDDP), there was no effective response. He died 4 mo after the diagnosis because of liver failure due to progression of the hepatic metastases.

DISCUSSION

Primary SBA is rare and the incidence is reported to be 1 to 5% of all gastrointestinal tract malignancies^[9,10], and the most important known risk factor for this malignant tumor is previous CD^[5,11]. SBA in CD was reported for the first time by Ginzburg in 1956^[12]. The risk for SBA is reportedly higher in patients with CD than in the overall population. Recent meta-analysis revealed the relative risk of SBA in patients with CD as 28.4 (95% CI, 14.5-55.7)^[13] to 33.2 (95% CI, 15.9-60.9)^[14]. In clinical settings, SBA in CD is difficult to diagnose, and most previous cases were diagnosed after surgery without suspicion of malignancy. Therefore, novel tools are needed to detect and survey the development of this cancer. In our case, we were able to diagnose SBA non-surgically by a novel approach using PET/CT and DBE.

Based on previous reports, Dossett *et al*^[3] summarized the 154 cases of SBA in CD reported in Europe and America. SBA in CD occurred more frequently in males than females (M:F ratio of 2.4:1). The age at diagnosis

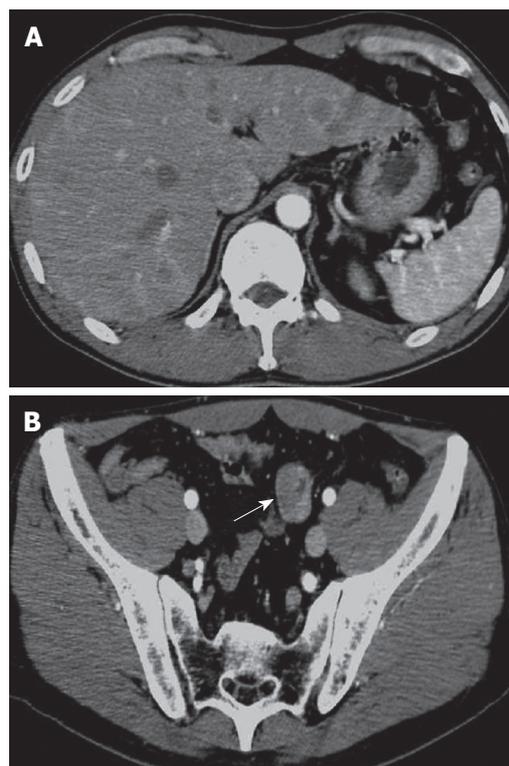


Figure 1 CT findings. A: Abdominal CT showed the multiple hepatic tumors with ring enhancement; B: Wall thickening of a part of the small bowel (arrow).

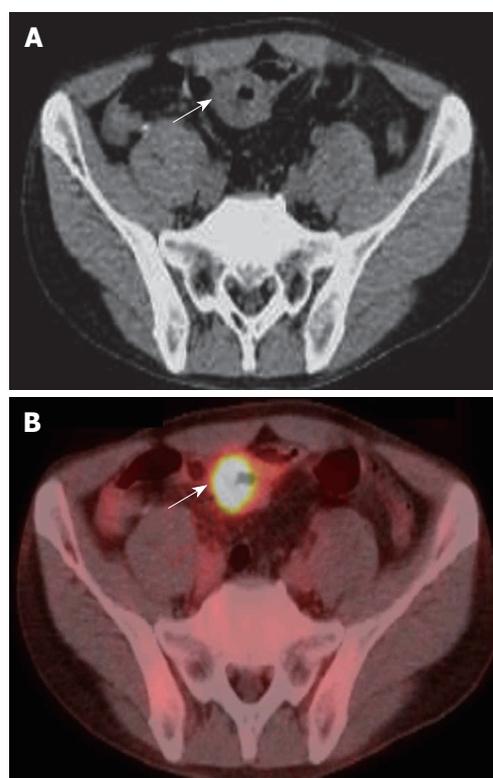


Figure 2 PET/CT findings. A: Wall thickening of a part of ileum (arrow). B: PET/CT showed ¹⁸F-FDG accumulation in the site of wall thickening of ileum (arrow).

ranged from 21 to 86 years (mean age, 51.3), and the average duration of CD was 24.5 years (0-45 years)^[3]. SBA in CD was observed at a younger age^[11] as compared

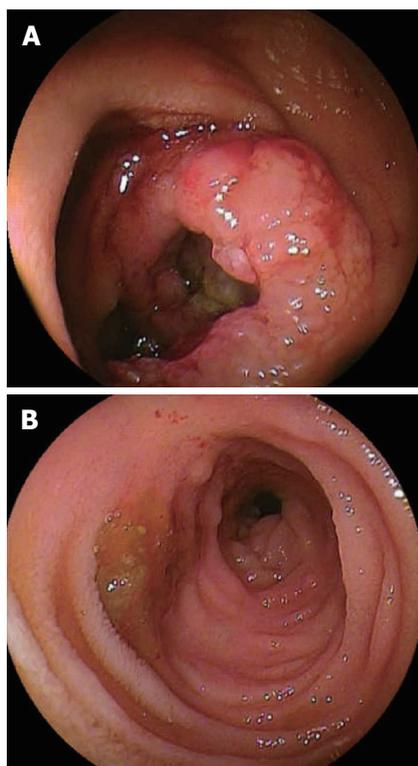


Figure 3 DBE findings. A: DBE showed ulcerative mass located in the ileum at about 100 cm away from ileocecal valve; B: Some longitudinal ulcer scars near the tumor.

with *de novo* cancers that were found in 60-69-year-olds^[15]. Tumors were found in the ileum at a rate of 75%. The presence of previously bypassed segments of the intestine was 20.3%. Obstruction was the most common manifestation (76%), whereas hemorrhage, fistula, and perforation were observed in 3.9%, 3.9%, and 5.4% of cases, respectively. The majority of diagnoses were made at the time of operation (35.4%) or postoperatively (61.5%). Only 3.1% of the cases could be diagnosed preoperatively. Survival rates after 1 and 2 years were 49.6% and 27%, respectively^[3].

In the present case, there were some longitudinal ulcer scars near the tumor, suggesting that chronic inflammation is associated with the development of SBA. Because it is well known that a combination of immunohistochemically strong p53 expression and absent or weak β -catenin expression in ulcerative colitis (UC) patients is evidence for colitis-associated dysplastic lesions^[16], we examined the immunohistochemical characteristics of the tumor^[17]. In this case, there was no detectable p53 expression, but strong β -catenin expression. Chemotherapy and radiation for SBA have produced disappointing results, and only a few studies have evaluated chemotherapy for unresectable SBA in CD^[18]. There have been no controlled studies recommending an effective treatment regimen. Although we treated this patient by systemic chemotherapy with S-1 and CDDP in agreement with the patient, the therapy was not effective and did not seem to prolong the survival time.

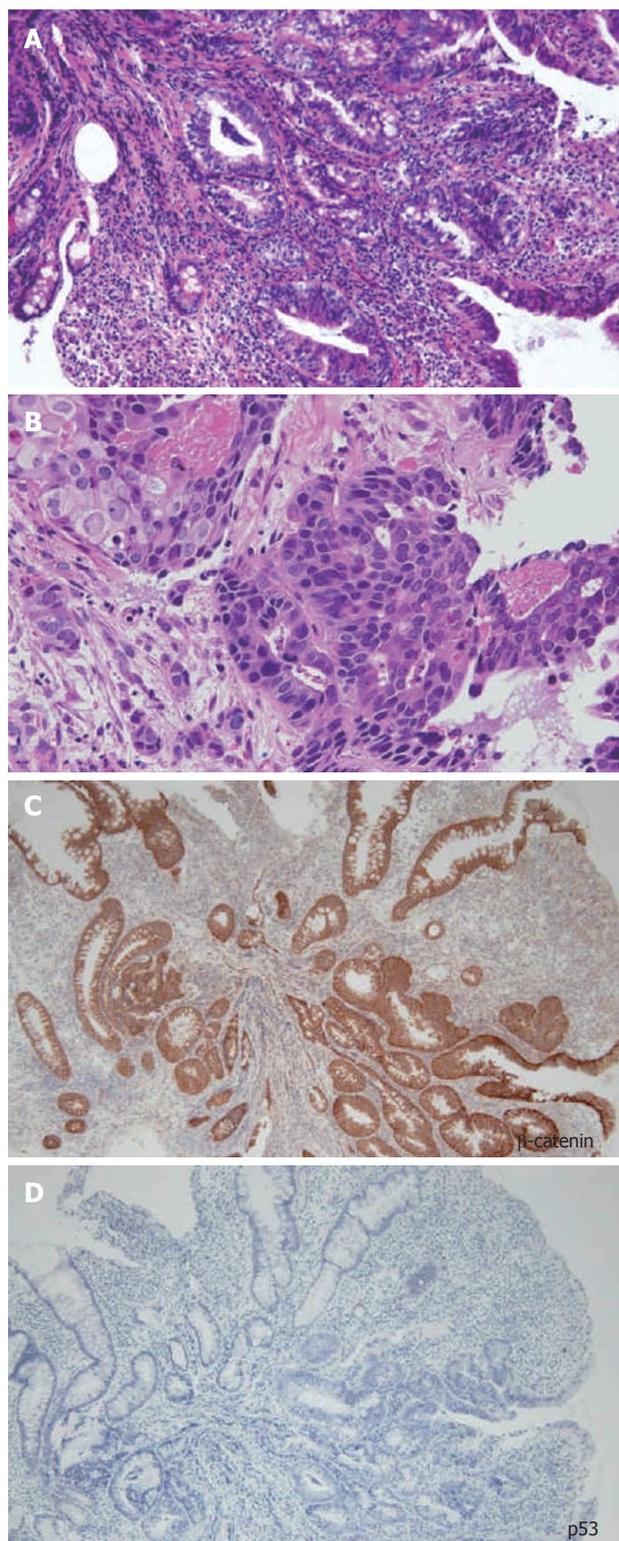


Figure 4 Histopathological findings of the biopsy specimen showed poorly differentiated adenocarcinoma. A: HE ($\times 100$), B: HE ($\times 400$), C: Immunohistochemical staining using β -catenin antibody ($\times 100$), D: Immunohistochemical staining using p53 antibody ($\times 100$).

There are several previous reports of long-standing risk factors for SBA, including onset of the disease before the age of 30 years, presence of a bypassed segment, chronic active course with stricture and fistulas, male gender, and smoking^[1-3,5,11,19-21]. Corticosteroid and

azathioprine therapy to treat CD are also considered potential risk factors. However, 5-ASAs prevent the development of intestinal adenocarcinoma in IBD^[1,22-25]. There is also a theoretical risk for an increased rate of malignancies due to antagonism of TNF- α , but to date, there is no clear proof of such an effect^[26,27].

Recommendations for screening and surveillance of SBA in CD have been supported by only very limited data^[8]. The overall risk of colorectal cancer for UC patients is estimated to be 3.7% (95% CI, 3.2%-4.2%); 2% at 10 years, 8% at 20 years, and 18% at 30 years^[28], whereas reported cancer in CD is 0.63%-3.1%^[29]. Although diagnostic investigations using conventional modalities such as small bowel series, double-contrast enteroclysis, and upper and lower gastrointestinal endoscopies have been performed on patients with a high cancer risk, a recent report found that colonoscopy surveillance may not improve survival even in studying extensive colitis, thus other tools are needed to detect cancer development^[8]. CT and MRI are now considered the common imaging modality of choice for abdominal malignancy. Endoscopy of the small intestine, especially capsule endoscopy and DBE, are promising new diagnostic tools^[30]. Capsule endoscopy is not invasive, but has a risk of retention for patients with CD who have a stenosis in the intestinal tract^[31,32]. DBE is a new endoscopic method that provides complete visualization, the ability to biopsy the small bowel, and provides diagnostic and therapeutic information^[33].

In this case, PET/CT was effective for the discovery of a small bowel tumor at the initial examination. Although the localization of lesions may not be completely accurate, PET/CT provides accurately fused morphological and functional imaging within a single examination. Today, PET imaging has a high sensitivity for detecting colorectal cancer (CRC) and is superior to conventional CT staging when CRC patients are assessed for local and distant metastases^[34,35]. PET/CT also offers a better non-invasive tool for identifying and localizing active intestinal inflammation in patients with CD^[36]. Although PET/CT is not able to replace conventional studies due to its high cost, it may be useful when conventional studies cannot be performed or are not completed in CD patients. In this case, the tumor was detected at an advanced stage with multiple liver metastases. Although it is still uncertain whether combining PET/CT and DBE will enable us to make an early diagnosis for SBA, this case certainly indicates the possibility of combining PET/CT and DBE without conventional modalities, such as a small bowel series, to detect a primary malignant tumor of the small bowel without surgery. One strategy to monitor high-risk patients might be to perform PET/CT at an initial examination, and then conduct a DBE examination for the patient who has abnormal regions suspected as being malignant.

The recent development of innovative imaging techniques involving PET/CT and DBE has opened a new area in the exploration of the small bowel in CD patients. Each of these techniques is characterized by

its own profile of favorable and unfavorable features^[30]. Future clinical studies are expected to demonstrate strategies to monitor SBA in CD patients.

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Successful treatment of multiple lung metastases of hepatocellular carcinoma by combined chemotherapy with docetaxel, cisplatin and tegafur/uracil

Atsunori Tsuchiya, Michitaka Imai, Hiroteru Kamimura, Tadayuki Togashi, Kouji Watanabe, Kei-ichi Seki, Toru Ishikawa, Hironobu Ohta, Toshiaki Yoshida, Tomoteru Kamimura

Atsunori Tsuchiya, Michitaka Imai, Hiroteru Kamimura, Kouji Watanabe, Kei-ichi Seki, Toru Ishikawa, Hironobu Ohta, Toshiaki Yoshida, Tomoteru Kamimura, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Nishi-Ku, Teraji 280-7, Niigata 950-1104, Japan

Atsunori Tsuchiya, Hiroteru Kamimura, Tadayuki Togashi, Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Science, Niigata University, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan

Author contributions: Tsuchiya A, Imai M, and Togashi T performed research; Kamimura H, Watanabe K, Seki K, Ishikawa T, Ohta H, Yoshida T and Kamimura T designed research. Tsuchiya A wrote the paper.

Correspondence to: Atsunori Tsuchiya, Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Science, Niigata University, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510,

Japan. atsunori@med.niigata-u.ac.jp

Telephone: +81-25-2272207 Fax: +81-25-2270776

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Peer reviewers: Satoshi Osawa, MD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, 431-3192, Japan; Hitoshi Yoshiji, MD, PhD, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan; Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

Tsuchiya A, Imai M, Kamimura H, Togashi T, Watanabe K, Seki K, Ishikawa T, Ohta H, Yoshida T, Kamimura T. Successful treatment of multiple lung metastases of hepatocellular carcinoma by combined chemotherapy with docetaxel, cisplatin and tegafur/uracil. *World J Gastroenterol* 2009; 15(14): 1779-1781 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1779.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1779>

Abstract

We report the successful treatment of multiple lung metastases after hepatic resection for hepatocellular carcinoma (HCC) with combined docetaxel, cisplatin (CDDP), and enteric-coated tegafur/uracil (UFT-E). A 68-year-old man was diagnosed with multiple lung metastases of HCC 7 mo after partial hepatectomy for HCC. Oral UFT-E was given daily and docetaxel and CDDP were given intra-arterially (administered just before the bronchial arteries) every 2 wk *via* a subcutaneous injection port. One month after starting chemotherapy, levels of tumor marker, protein induced by vitamin K absence II (PIVKA-II), decreased rapidly, and after a further month, chest X-ray and computed tomography revealed the complete disappearance of multiple liver metastases. Two years after the combined chemotherapy, HCC recurred in the liver and was treated but no pulmonary recurrence occurred. In the absence of a standardized highly effective therapy, this combined chemotherapy with docetaxel, CDDP and UFT-E may be an attractive option for multiple lung metastases of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide^[1]. Prognosis has been improved by careful follow-up of the high-risk patient, progress in diagnostic techniques, and combined modality therapy such as surgery, chemotherapy including transarterial chemoembolization (TACE), percutaneous ethanol injection therapy, and radiofrequency ablation. While recurrence of HCC is generally intrahepatic, long-term therapy increases the risk of extrahepatic metastases, and more than 50% of these occur in the lung^[2,3]. While the most effective therapy for lung metastasis is surgical resection^[4-7], Kawamura *et al*^[5] reported that only 2.6% of patients with lung metastases can undergo this type of surgery. Most patients are not deemed suitable because of multiple lung metastases, uncontrolled intra-hepatic HCCs, and deterioration in liver function. Since the prognosis of extrahepatic tumors is poor, options are limited to palliation for many people. Most people will be forced to depend on the few available chemotherapy protocols suitable for this situation; there is no standardized effective chemotherapy regime. A recent pilot study found that S-1, a novel oral dihydropyridine dehydrogenase

(DPD) inhibitor, and interferon-alpha (IFN- α) were associated with objective response in three of 12 patients with lung metastasis of HCC^[8]. Accumulation of such reports may lead to a standard therapy being confirmed in the future. Ishikawa *et al*^[9] previously reported combined chemotherapy with docetaxel, combined docetaxel, cisplatin (CDDP) and enteric-coated tegafur/uracil (UFT-E) for lung metastases of HCC. This chemotherapy regime was effective for lung metastases of HCC but not for the primary hepatic lesion; however, the reasons for this difference remain unknown. We report a case in which multiple lung metastases of HCC were completely resolved by modified combined chemotherapy with docetaxel, CDDP and UFT-E.

CASE REPORT

A 68-year-old man diagnosed with multiple lung metastases of HCC was admitted to Saiseikai-Niigata Daini Hospital in February 2006. He had undergone right hepatic lobectomy to remove three hepatocellular carcinomas (6 cm, 5 cm and 4 cm in diameter) 7 mo before the admission. Pathological diagnosis was moderately differentiated HCC, and clinical stage at the time of surgery was stage IVa according to the TNM classification. Tests for hepatitis B and C viral markers were all negative, but examination of the resected liver indicated cirrhosis, possibly caused by alcohol. Liver function test disclosed the following values: alanine aminotransferase 137 IU/L, alkaline phosphatase 503 IU/L, serum albumin 4.4 g/dL, and PT-INR 1.19 (Child-Pugh class A). While there was no elevation of tumor marker alpha-fetoprotein (4.3 ng/mL), protein induced by vitamin K absence II (PIVKA-II) was markedly elevated at 6280 mAU/L. Chest X-ray and computed tomography (CT) showed at least 50 metastases spread diffusely throughout the lungs (Figure 1). Since no treatment guidelines were available, we modified the combined chemotherapy with docetaxel, CDDP and UFT-E reported by Ishikawa *et al*^[9] after obtaining informed consent. First, 400 mg/d of oral UFT-E was started. We then placed an intra-arterial catheter that delivered medication into the aorta just before the bronchial arteries, and docetaxel (80 mg/body initially, followed by 40 mg/body) and CDDP (50 mg/body initially, followed by 20 mg/body) were administered every 2 wk *via* a subcutaneous injection port (Figure 2). This was performed on an outpatient basis from the third injection. The serum level of PIVKA-II decreased 1 mo after starting chemotherapy and normalized after 4 mo. Two months after starting chemotherapy, chest X-ray and CT showed complete disappearance of the lung metastases (Figure 1). Adverse events during the therapy were grade 3 leukocytopenia (minimum white blood cell count was 2500/ μ L), grade 2 fatigue and anorexia, and grade 1 alopecia according to Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Therapy was stopped after the fourth administration and only UFT-E was continued, in accordance with the patient's wishes. This was also stopped 5 mo after starting chemotherapy, when chest X-ray and CT remained

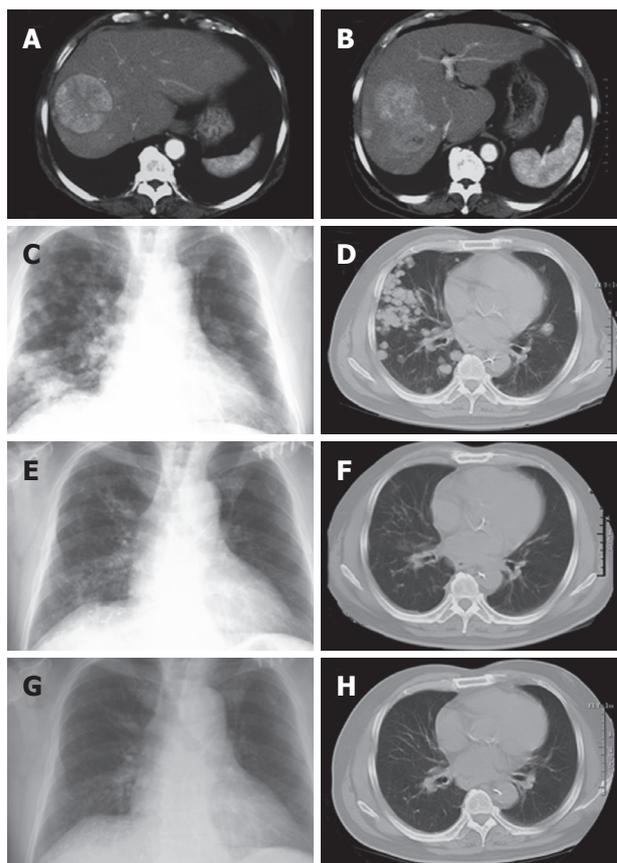


Figure 1 Chest X-ray and CT images before and after chemotherapy (A-H). Seven months before the chemotherapy, huge multiple HCCs were apparent in the right lobe of the liver (A, B). On admission before chemotherapy, multiple lung metastases were seen in the bilateral lung fields (C, D). Two months after starting chemotherapy, the multiple lung metastases had disappeared completely (E, F). Five months after starting chemotherapy, the tumors had not recurred (G, H).

negative. Approximately 2 years after hepatic surgery, HCC reappeared in the liver and was treated with TACE; however, lung metastasis did not recur. The patient remains well and is being followed-up as an outpatient.

DISCUSSION

The most effective therapy for lung metastasis of HCC is thought to be pulmonary resection^[4-7], and this has been reported to prolong life in some cases^[10]. However, the present patient was unsuitable for pulmonary resection because of multiple lung metastases. Reports of chemotherapy for lung metastasis of HCC are rare and most are case reports, indicating the absence of a highly effective standard therapy. Nonetheless, the literature contains reports of successful treatment of lung metastasis of HCC using tegafur/uracil^[11], 5-fluorouracil (5-FU) + IFN^[12], and 5-FU + CDDP + IFN^[13]. Moreover, a pilot study of S-1 and IFN- α in patients with pulmonary metastases of HCC showed that three of 12 patients responded objectively^[8]. There is also a report of the complete disappearance of multiple lung metastases with combined chemotherapy with docetaxel, CDDP and UFT-E^[9]. In the present case, multiple lung metastases also disappeared in response to modified

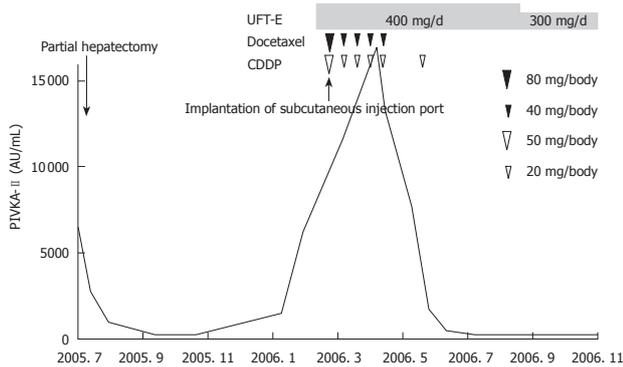


Figure 2 Clinical course of this patient. First, 400 mg/d of oral UFT-E was started. We then placed an intra-arterial catheter that delivered medication into the aorta just before the bronchial arteries, and docetaxel (80 mg/body initially, followed by 40 mg/body) and CDDP (50 mg/body initially, followed by 20 mg/body) were administered every 2 wk via a subcutaneous injection port. Levels of PIVKA-II, a tumor marker, decreased rapidly 1 mo after starting chemotherapy; levels continued to fall to normal and were maintained.

combined chemotherapy with docetaxel, CDDP, and UFT-E, suggesting that the effects of this combined chemotherapy are reproducible. We chose to modify the original intravenous combined chemotherapy including the docetaxel dose (original recommended dose was 60 mg/m² of docetaxel, 80 mg/m² of CDDP on day 1 and 400 mg/m² per day of tegafur/uracil in consideration of adverse effects^[9]. This original dose was determined by modifying the regime for advanced lung cancer^[14]) since two patients who received this could not be treated effectively because of adverse events attributed to docetaxel (personal communication). To reduce the adverse events and increase treatment efficacy, we used an intra-arterial catheter that delivered medication to just before the bronchial arteries. In this way, docetaxel and CDDP could be administered in high concentration to the lung.

The precise mechanisms underlying the effectiveness of this therapy for lung metastasis of HCC are not known. It has been reported previously that the growth inhibition of hepatoma cells induced by docetaxel was mediated through G2/M-phase arrest, caspase activation and DNA fragmentation^[15]. It is uncertain whether a single drug is sufficient, whether combinations of these agents are necessary, or whether docetaxel is the key drug. Furthermore, we do not know whether repeated doses or administration *via* the intra-arterial route improved the outcome for this patient. Further study is needed into the tumor characteristics of lung metastasis and immune responses in patients such as this one. While many issues must be resolved, this report is the second to document the effectiveness of combined chemotherapy with docetaxel, CDDP and UFT-E for lung metastasis of HCCs. This suggests that the present regime could be useful in this situation, as no highly effective standard therapy has been reported.

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CASE REPORT

Primary gastric teratoma on the cardiac orifice in an adult

Liu Liu, Wen Zhuang, Zhong Chen, Yong Zhou, Xiao-Ran Huang

Liu Liu, Wen Zhuang, Yong Zhou, Department of Gastrointestinal Surgery, West China Hospital/West China Medical Center, Sichuan University, Chengdu 610041, Sichuan Province, China

Zhong Chen, Department of Pediatrics, The 2nd West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Xiao-Ran Huang, Department of Pathology, West China Hospital/West China Medical Center, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Liu L and Chen Z collected the clinical data; Liu L and Huang XR collected the pathological data and wrote the paper; Zhou Y and Zhuang W revised the paper.

Correspondence to: Wen Zhuang, Department of Gastrointestinal Surgery, West China Hospital/West China Medical Center, Sichuan University, Guo Xue Xiang NO. 37, Chengdu 610041, Sichuan Province, China. zhuangwen2008@126.com

Telephone: +86-28-85422872 Fax: +86-28-85164035

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Abstract

Gastric teratoma (GT) is a seldom seen congenital abnormality. GT always occurs in children. The greater curvature and posterior wall of the stomach are the most common sites involving GT. We diagnosed a case of GT located on the inferior wall of the cardiac orifice in a 20-year-old man. To the best of our knowledge, this is the first case of GT located on the wall of the cardiac orifice in an adult in the English literature. We report this unusual case as an addition to this rare disease usually found in children. Computed tomography combined with endoscopic ultrasonography can be selected to diagnose GT.

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Key words: Adult; Cardiac orifice; Endoscopic ultrasonography; Stomach; Teratoma

Peer reviewer: Luis Bujanda, MD, PhD, Avda, Sancho El Sabio, 21-3°C, 20010 San Sebastián, Spain

Liu L, Zhuang W, Chen Z, Zhou Y, Huang XR. Primary gastric teratoma on the cardiac orifice in an adult. *World J Gastroenterol* 2009; 15(14): 1782-1785 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1782.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1782>

INTRODUCTION

Gastric teratoma (GT) is a seldom seen congenital abnormality. It has been reported in the world literature that GT always occurs in children^[1,2], and an increasing mass in the epigastric region is the main sign of GT^[1,3,4]. Only six cases of GT in adults were reported in Medline^[5-8] between 1922 and 2007. Yoon *et al*^[9] reported the second case of GT in a child in Korea in 2000. GT is usually located on the posterior wall or greater curvature of the stomach^[3,7]. To the best of our knowledge, this is the first report of GT located on the wall of the cardiac orifice in an adult in the English literature. Calcifications in the cystic-solid mass may be features of GT on computed tomography (CT)^[3,4,10]. Endoscopic ultrasonography (EUS) may help to correctly diagnose GT before surgery.

CASE REPORT

The patient was a 20-year-old man. Due to slight pain and distention of the upper abdomen of 3 mo duration, he was admitted to our hospital. Physical examination was carried out and no mass was found in the epigastrium. The level of serum alpha-fetoprotein (AFP) was normal. CT showed a spherical mass on the inferior wall of the cardiac orifice of the stomach. The mass was about 4.8 cm × 5.2 cm, and there was no clear border between the mass and the wall of the stomach. The density of the mass was uneven. High-density and low-density substances were found in the mass (Figure 1). EUS demonstrated a giant mass on the inferior wall of the cardiac orifice. The mucosa of the mass was normal (Figure 2), and there was a lobulated polyp near the mass. The five-layer structure of the stomach was clearly manifested in the mass on ultrasonography (Figure 2). The mass had formed from the outer layer of the stomach. The mass was completely excised with a partial gastrectomy and the digestive tract was rebuilt. Macroscopically, the mass was about 5.0 cm × 5.3 cm × 2.3 cm, and was derived from the inferior wall of the cardiac orifice (Figure 3A). The mass contained small cystic tissue and a large solid area. Microscopically, the tumor was resected completely in one piece with a tumor cell-free margin. Cartilage, squamous cells and respiratory epithelium were observed in the mass (Figure 3B-F). No immature tissue was observed in the mass. The diagnosis of a primary mature GT derived from the cardiac orifice was established. The syndromes

Table 1 Five reported cases of GT in Medline between 1922 and 2007

Author (yr)	Age (yr)	Signs and symptoms	Location	Type	Size
Eustermann <i>et al</i> ^[6] 1922	31	?	Posterior wall	Mature	7 cm × 6 cm × 5 cm
Fadeeva <i>et al</i> ^[12] 1960	25	?	Anterior wall	Mature	4 cm × 3 cm
Gray <i>et al</i> ^[8] 1964	40	?	Lesser curvature	Mature	7 cm × 6 cm
Matsukuma <i>et al</i> ^[5] 1995	83	Tarry stool and anemia	Lesser curvature	Immature	12 cm × 10 cm × 6 cm
Joo <i>et al</i> ^[7] 1999	27	Fever, pain and vomiting	Greater curvature	Mature	9.5 cm × 7.5 cm × 5 cm

Note: Five patients were all male. Complete resection of the tumor was carried out in all patients and all patients recovered.

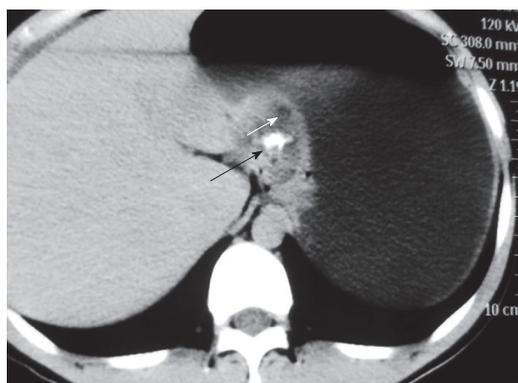


Figure 1 Axial CT shows a large soft tissue mass located on the inferior wall of the cardiac orifice of the stomach. The border of the mass is clear and the mass is from the stomach. The density of the mass is uneven. Low density (white arrow) indicates cystic tissue and the high density (black arrow) indicates calcifications.

in this patient disappeared within 1 wk after surgery. In the 10-mo follow-up period, the patient was well without abdominal discomfort.

DISCUSSION

Teratoma usually occurs in the gonads (mainly between the age of 6 mo and 15 years), the sacrococcygeal region (mainly in infants)^[1,2], and in the retroperitoneum, cranium or mediastinum^[5]. Teratoma derived from the stomach is very rare, and was first reported by Eustermann and Sentry in 1922^[6]. Up to 2002, only 107 cases of GT had been reported in the English literature^[1]. Surprisingly, GT always occurs in male children, especially around the age of 1 year^[1-3,5,11]. GT in an adult is unusual. To the best of our knowledge, there were just six cases of GT in adults reported in MEDLINE between 1922 and 2007, and five of these cases are listed in Table 1^[5-8,12]. GT is commonly located on the greater curvature and the posterior wall of the stomach^[3,7]. In the five cases listed in Table 1, two cases originated from the lesser curvature, and three cases from the posterior wall, anterior wall and the greater curvature, respectively. To the best of our knowledge, we report the seventh case of GT in an adult in the English literature and the first case originating from the cardiac orifice. Partly because of the rarity of GT in adults, the preoperative diagnosis of GT is difficult for surgeons^[7].

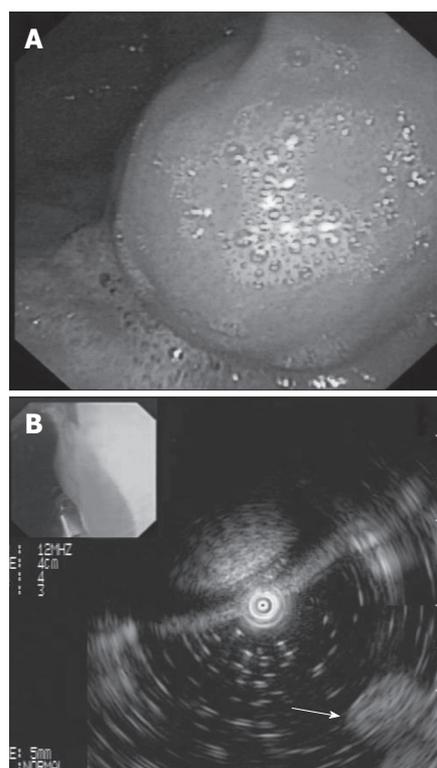


Figure 2 EUS was used to diagnose the gastric teratoma. A: A spherical mass was noted on the inferior wall of the cardiac orifice, and the mass mucosa was normal; B: The heterogeneous mass was formed from the outer layer and the five-layer structure of the stomach in the mass was clearly detected (white arrow).

Typical radiographic findings of GT^[3,4,10], such as calcifications, uneven density of the tumor and an intratumoral solid area with mixed cysts, were predominant in our case (Figure 1). EUS was first used to diagnose GT. A mass from the inferior wall of the cardiac orifice was observed. EUS demonstrated a heterogeneous mass from the outer layer of the stomach, and the five layers of the stomach were clearly detected (Figure 2). The echo pattern in the mass and the mass from the outer layer of the stomach were different from those of other common submucosal tumors, such as lipoma, myogenic tumor and lymphangioma. The use of EUS alone cannot help to diagnose GT qualitatively, but could provide useful information for preoperative diagnosis.

Cartilage, squamous cells and respiratory epithelium were found histologically (Figure 3F). According to pathological criteria, cartilage, squamous cells and respiratory epithelium in the mass are interesting

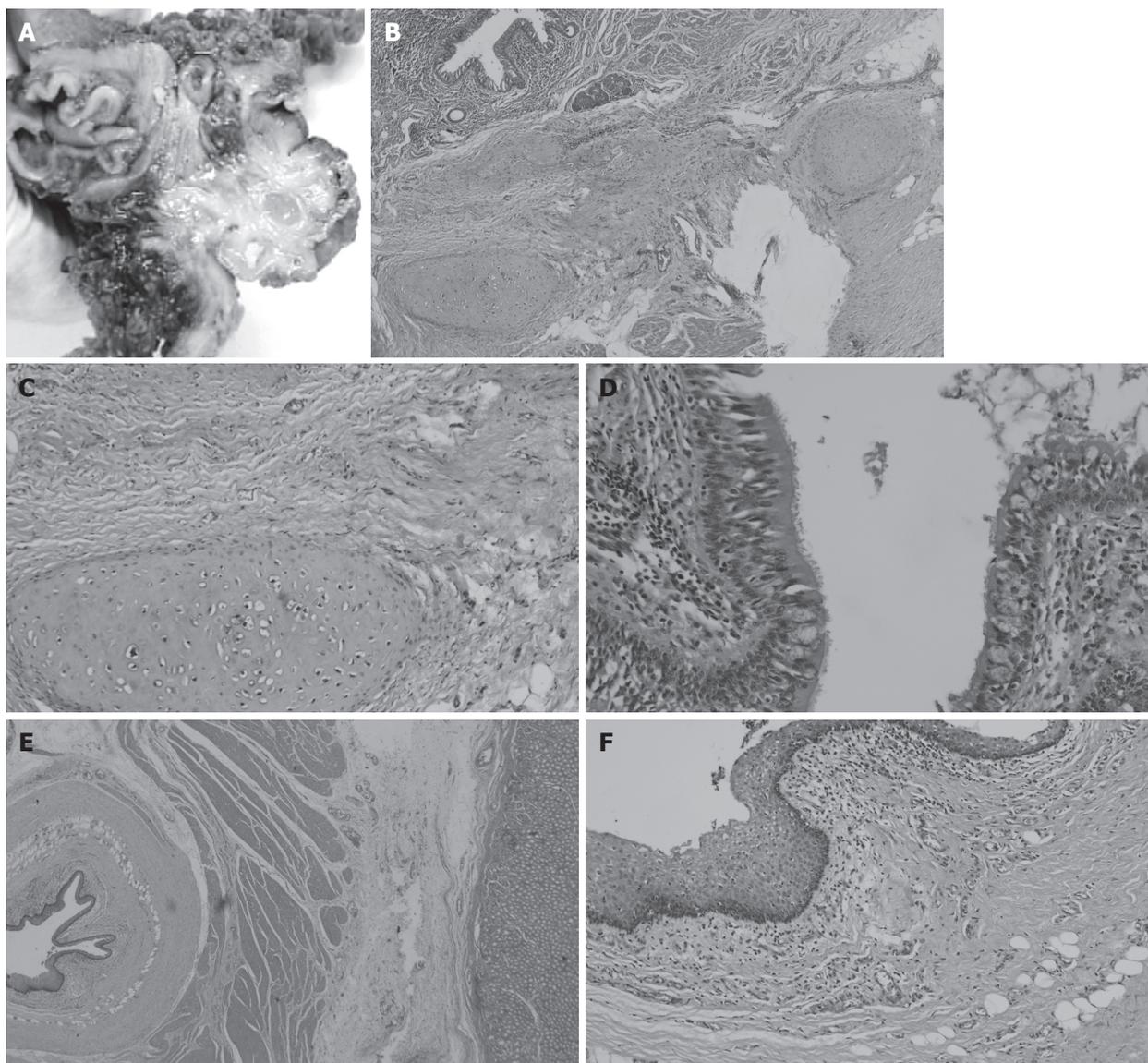


Figure 3 GT was pathologically examined under macroscopy and microscopy. A: The mass was about 5.0 cm × 5.3 cm × 2.3 cm, and it derived from the inferior wall of the cardiac orifice; B: Photomicrograph of an area composed respiratory epithelium and cartilage without immature teratoma components (HE, × 20); C, D: The cartilage (C) and the respiratory epithelium (D) were amplified (HE, × 100); E: The three layers, mucous layer, submucous membrane, and muscular layer, of the wall of the stomach were clear. The tissue of GT was derived from the muscular layer of the stomach (HE, × 20); F: The Squamous cell was amplified (HE, × 100).

histological features of teratoma. The diagnosis of GT from the cardiac orifice was therefore exclusively established. Complete resection is an effective method to treat both benign and malignant GT. Compared to traditional surgery, laparoscopic surgery has many advantages, and has been used to treat teratomas^[13], and will be used to treat GT in the future. AFP is a good indicator of the recurrence of malignant GT^[1,3,11]. Subsequent chemotherapy might be required when the level of AFP is high after surgery.

In conclusion, GT is rare and always occurs in infancy or childhood. The greater curvature and the posterior wall of the stomach are the most common sites involving GT. GT involving the cardiac orifice in an adult is seldom seen. To the best of our knowledge, this is the first report involving GT on the cardiac orifice in the English literature. EUS is helpful in diagnosing GT.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM

Hepatotoxicity associated with weight-loss supplements: A case for better post-marketing surveillance

Ano Lobb

Ano Lobb, Public Health Consultant, 80 Lepage Rd, Barre, VT 05641, United States

Author contributions: Lobb A interpreted and synthesized the data, and wrote this commentary.

Correspondence to: Ano Lobb, Public Health Consultant, 80 Lepage Rd, Barre, VT 05641,

United States. ano.lobb@gmail.com

Telephone: +1-802-2797625 Fax: +1-802-4793280

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Abstract

There is a growing number of case reports of hepatotoxicity from the widely marketed weight-loss supplement Hydroxycut, which contains the botanical ingredient *Garcinia cambogia*. These case reports may substantially undercount the true magnitude of harm. Based on the past experience with harmful dietary supplements, US regulators should assume the more precautionary approach favored by Canada and Europe. Lacking effective adverse event surveillance for supplements, or the requirements to prove safety prior to coming to the market, case reports such as those summarized here assume added importance.

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Key words: Hydroxycut; Dietary supplements; *Garcinia cambogia*; Liver failure; Weight loss; Super citrimax; Hca-sx

Peer reviewer: Einar S Björnsson, Professor, Department of Internal Medicine, Section of Gastroenterology and Hepatology, Sahlgrenska University hospital, Med pol II, SE-413 45 Gothenburg, Sweden

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TO THE EDITOR

Dara *et al*^[1] report on a case series of two patients with hepatotoxicity associated with the weight-loss

supplement Hydroxycut, so named because it contains potentially hepatotoxic hydroxycitric acid derived from the tropical fruit *Garcinia cambogia*^[1]. Two earlier case reports in 2005 were also referenced^[2]. To this count should be added two additional case reports of hepatotoxicity associated with Hydroxycut^[3,4]. An estimated 15% of the US population uses dietary supplements for weight loss^[5], and Hydroxycut is the top selling product in this class and market, with roughly a million units sold per year^[6]. With such wide usage, these six cases may underestimate the true incidence of hepatotoxicity by several degrees of magnitude.

Each case report has similarities both in reported liver screening abnormalities and symptoms reported by patients, all of whom were otherwise healthy and experienced normalized hepatic function once they stopped taking the supplement. Table 1 synthesizes key laboratory findings and reported symptoms.

Poor regulation of dietary supplements in the US has been noted by consumer advocates, researchers and policymakers^[7-10]. US manufacturers of dietary supplements are not required to conduct trials establishing safety or efficacy prior to marketing; only provide a copy of their label for the Food and Drug Administration (FDA) to review^[7,9,11]. Ingredients do not need to be considered “generally regarded as safe” as pharmaceuticals or food additives do, and the FDA must prove that a supplement is harmful before taking regulatory action^[9,11]. This means consumers in effect become unwitting subjects in a large scale post-marketing trial of a product’s safety. Unfortunately, the FDA does a generally poor job of post-marketing monitoring of adverse events from supplements, only receiving reports of an estimated 1% of such events^[11]. A recent search of FDA’s adverse events surveillance database for “Hydroxycut”, “hydroxycitric acid”, “*Garcinia cambogia*”, or “SuperCitrimax” (the proprietary blend of *Garcinia cambogia* used in Hydroxycut) yielded no reports^[12]. Furthermore, the nation’s poison control centers, which receive far more supplement adverse event reports than the FDA, lack the necessary coordination to act in a surveillance role^[11]. Supplement manufacturers may not be forthcoming with information about emerging health risks from their products. The makers of the weight-loss supplement Metabolife 356, for example, withheld over 14000 reports they had received over 5 years documenting serious adverse events associated with

Table 1 Patients, symptoms and laboratory values reported with hydroxycut associated hepatotoxicity

Citation	Dara <i>et al</i> ^[11]	Jones <i>et al</i> ^[4]	Shim <i>et al</i> ^[3]	Stevens <i>et al</i> ^[2]		
Patient age (yr)	40	33	19	28	27	30
Patient gender	F	F	M	M	M	M
Reported symptoms/ duration	Fatigue, nausea, vomiting, cramping, fever, chills, anorexia/3 d	Fatigue, nausea, cramping, abdominal pain/2 wk	Nausea, vomiting, jaundice/6 d	Fatigue, dyspnea on exertion, jaundice/ 3 wk	Fatigue, jaundice/8 d	Fatigue, vomiting, jaundice, fever/10 d
Aspartate aminotransferase (U/L)	1020	934	1981	1049	1808	59
Alanine aminotransferase (U/L)	1150	570	1143	2272	3131	45
Serum alkaline phosphatase (U/L)	299	112	153	153	171	530
Serum bilirubin (mg/L)	6.7	209	117	181	78	78
Serum direct bilirubin (mg/L)	Not reported	142	68	90	Not reported	Not reported
Prothrombin time (s)	Not reported	Not reported	17.1	12.8	16	15

their ephedra-containing product, including myocardial infarction, stroke, seizure and death^[7]. (Though not legally mandated to report serious adverse events until 2007^[9,11], failure to act on such reports suggests an ethical lapse.) To be fair, the FDA has taken action to protect the public health from dangerous supplements, banning ephedra in 2004 due to its cardiac risk^[13]. Formerly a common ingredient in numerous weight-loss supplements, ephedra was banned only after 155 deaths were associated with its use^[14]. In contrast, regulators in Canada, the UK and Europe appear to take a more precautionary approach. The supplement kava-kava was banned in Canada in 2002 after 29 cases of hepatotoxicity were associated with its use^[15], and in the UK and Europe the following year after some 40 more cases were reported^[16]. In the US, the FDA has issued a consumer advisory about possible hepatotoxicity associated with kava-kava use, but has not banned the substance^[17].

Faced with the aforementioned difficulties ensuring the safety of widely used dietary supplements, reliable and transparent case reports such as those cited above assume added importance. They may also underscore the need for US regulators to adopt the more precautionary post-marketing practices of their European and Canadian counterparts. Authorities should also exercise their responsibilities to the public's health by being empowered to practise more stringent and evidenced-based regulation of these products, many of whom could be considered pharmaceuticals due to their clear pharmacological effects and potent risks^[9].

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Rakesh Aggarwal, Additional Professor

Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

Ramsey Chi-man Cheung, MD, Professor

Division of GI & Hepatology, VAPAHCS(154C), 3801 Miranda Ave, Stanford University School of Medicine, Palo Alto, CA 94304, United States

Benedicte Y De Winter, President, MD, PhD, Professor, Associate Professor, Chief

Laboratory of Gastroenterology, University of Antwerp, Campus Drie Eiken, Universiteitsplein 1, 2610 Antwerp, Belgium

Robert JL Fraser, Associate Professor

Investigations and Procedures Unit, Repatriation General Hospital, Daw Park, Australia

Yik-Hong Ho, Professor

Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Liang-Ping Hu, Professor

Consulting Center of Biomedical Statistics, Academy of Military Medical Sciences, Beijing 100850, China

Mercedes Susan Mandell, MD, PhD

Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

Jose JG Marin, Professor

Head of the Departamento Physiology and Pharmacology,

University of Salamanca, CIBERehd, Campus Miguel de Unamuno, ED-S09, Salamanca 37007, Spain

Naofumi Mukaida, MD, PhD, Chairperson and Professor

Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan

Fabio Pace, Professor

Division of Gastroenterology, "L. Sacco" University Hospital, University of Milan, Via G. B. Grassi, 74, Milano 20157, Italy

Raffaele Pezzilli, MD

Department of Internal Medicine and Gastroenterology, Sant'Orsola-Malpighi Hospital, Via Massarenti, 9, Bologna 40138, Italy

Raymund R Razonable, MD

Division of Infectious Diseases, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, United States

Norbert Senninger, Professor

Department of General Surgery, University Clinics, Westphalian-Wilhelm's-University, Waldeyerstrasse 1, D-48149 Muenster, Germany

Mitsuo Shimada, Professor

Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

Tadashi Shimoyama, MD

Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan

Ana Cristina Simões e Silva, MD, PhD, Professor, Faculdade de Medicina UFMG

Departamento de Pediatria, sala 267, Avenida Professor Alfredo Balena, 190, Bairro Santa Efigênia, Belo Horizonte, Minas Gerais 30130-100, Brazil

Frank I Tovey, OBE, ChM, FRCS, Honorary Research Fellow

Department of Surgery, University College London, London, United Kingdom

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.apdwcgress.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.

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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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 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]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

^[3]Passed away on June 14, 2008



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EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No.62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
 Telephone: +86-10-59080039
 Fax: +86-10-85381893
 E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

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Evolution of gastroenterology training

Hanna Telleman, Trevlyn Felicity Burger, Chris Jacob Johan Mulder

Hanna Telleman, Chris Jacob Johan Mulder, Department of Gastroenterology and Hepatology, VU Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands

Trevlyn Felicity Burger, Department of Gastroenterology Witwatersrand University, Private Bag 3, Wits 2050, Johannesburg, South Africa

Author contributions: Telleman H, Burger TF and Mulder CJJ wrote the paper.

Correspondence to: Chris Jacob Johan Mulder, Department of Gastroenterology and Hepatology, VU Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands. cjmulder@vumc.nl

Telephone: +31-20-4440613 Fax: +31-20-4440554

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Abstract

There have been rapid developments in gastroenterology (GE) over the last decade. Up until the late 1980s, GE-training was incorporated in Internal Medicine training. The introduction of endoscopy has necessitated the need for additional training. Around the world different national boards have developed their own curricula which will be discussed in this paper. Emphasis will be placed on the curriculum recently introduced in The Netherlands. The internal medicine component has become a two-year requirement (Common Trunk) and the duration of training in GE has been extended to four years. Because of the growing complexity of GE, there are now four subspecialties: Interventional Endoscopy, Neuromotility, Oncology and Hepatology that trainees can choose from. These subspecialties each have predefined specific requirements. The World Gastroenterology Organization has drawn up a standard curriculum which can be of help to the boards in different countries. The curriculum emphasizes the knowledge and skill components. The curriculum also defines the training recommendations, the requirements of training facilities and competence evaluation of fellows and facilities, while less is said about research, finance and the number of gastroenterologists required. In the coming decades the curriculum will need to be revised continuously. Personalization of the curriculum will be the next challenge for the years to come.

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Key words: Career; Common Trunk; Curriculum;

INTRODUCTION

Since the late 1960s, there have been rapid developments in the field of gastroenterology (GE). Up until the late 1980s, GE was considered a subspecialty of internal medicine, along with other subspecialties such as cardiology, pulmonology and rheumatology. However, since then, GE has become more complex, with advancements in both diagnostic and management procedures, and now incorporates hepatology. In this fast moving field, it is challenging to develop a comprehensive GE training program to enable gastroenterologists to become competent in all aspects of GE by the completion of their training program. In earlier years, one to two years of GE training was incorporated into Internal Medicine. Today, gastroenterologists have less exposure to general medicine, with more emphasis placed on GE. Endoscopy has completely revolutionized GE. In 1961, Basil Hirschowitz published the first description of flexible endoscopy of the stomach and duodenal bulb - a gastroduodenoscopy^[1].

In many hospitals, endoscopy was first introduced by performing gastroduodenoscopies in an all-purpose room. Due to the rapid development in endoscopy, it was necessary to implement training requirements. In the last decade, many national boards of GE developed their own curriculum handbook^[2,3].

Because of the world wide need to standardize procedures, we will attempt to give an overview of the different approaches to training. Our focus has been on the first world. However, developing areas, where access to high-quality training in GE is sometimes problematic, have some guidelines that are worth considering. We will also discuss the curricula of a number of countries, with emphasis on The Netherlands. Recently, the World Gastroenterology Organization (WGO) published a

document about the basic standards of a GE training program, which will be reviewed and discussed^[4].

GENERAL

In Europe, as in many other parts of the world, GE was considered a subspecialty of internal medicine. This was and still is hampering the development of GE in advanced care and skills, especially in rapidly developing fields such as hepatology, interventional endoscopy, motility, GE-oncology and -immunology.

In the 1970s and 1980s, the majority of gastroenterologists were trained as internists, with a fellowship of one to two years in GE. In general, the fellowship focused on 'on-the-job' training in an endoscopy unit, which was necessary due to the extensive workload in the different countries by either colorectal cancer screening programs or the enormous amount of people with chronic hepatitis and other GE infections in developing countries. High-quality training in GE has been a secondary consideration and unfortunately, in most countries, a two-year fellowship in endoscopy is still the recognised standard (Table 1).

Since hepatology has been incorporated into GE, additional training seems mandatory. The duration of specific training in GE in the late 1980s and early 1990s was extended from two to three years. Recently, in some countries, including The Netherlands and the USA, GE training has been increased to four years. Training in internal medicine has now been limited to a Common Trunk. The Common Trunk is a two to three year basic training in internal medicine; similar to cardiology and pulmonology.

United States of America

Four American GE-related societies have joined together to develop the GE curriculum. Together, they have structured the American GE Core Curriculum. Their first document was published in 1996, and thereafter an updated edition has been released every five years^[2]. A detailed description of requirements in seventeen GE fields is given in the latest release, the third edition (2007). GE trainees are required to select a subspecialty^[5,6].

United Kingdom

The British Society of Gastroenterology developed their National Training Program in a similar manner. They emphasized a comprehensive, well structured program that encourages flexibility in content and duration. The core curriculum offers various options of training in specialised fields, such as hepatology, advanced endoscopy and research^[3].

The Joint Advisory Group on GE Endoscopy (JAG) developed their programs in the late 1990s in UK. Interestingly, it defines standards of endoscopy training which are not specific to a medical specialty. JAG developed and organizes endoscopy training. This organization is responsible for the accreditation of endoscopic units and the certification of trainees. They developed the criteria for accreditation of

endoscopies: colonoscopy, endoscopic retrograde cholangiopancreatography (ERCP), data of cumulative life and year time number of procedures, complication thresholds and cecal intubation percentages *etc*^[7].

Global standardization

Unfortunately, there is no global standardization. In contrast to these high-quality training programs, many countries have no detailed training guidelines and only specify the duration of training. The duration of basic training in internal medicine prior to training in GE varies from one to six years^[8]. The duration of training in GE varies from one to four years. It is also variable as to whether there are entrance examinations, or examinations on completion of the curriculum. The European Union, a Free Labour Market, has not standardized their training in GE (Table 1).

The implementation of high-quality training in GE remains problematic. Boards such as the United European Gastroenterology Federation and the WGO should regularly collaborate and present their recommendations at the American, Pacific and World Digestive Disease Weeks. The WGO Training Centers in the developing world should focus on preventing the loss of skills from their countries, therefore lessening the impact of these losses on digestive health care in Asia, Africa and South America^[9].

THE DUTCH MODEL FOR TRAINING IN GE

Common Trunk

The Dutch Gastro and Hepatology Board concluded that high-quality training in GE could not be accomplished within a three year period. A working committee developed a new curriculum^[10]. In 2002, the working committee extended GE training from 3 to 4 years, with two years of Internal Medicine as a Common Trunk. This Common Trunk may be completed at either a university hospital or a general hospital. At least 12 mo should be completed in a ward of internal medicine during this Common Trunk period^[11]. Optional training periods can be completed in intensive care, emergency medicine, oncology or cardiology. The Board of Internal Medicine is responsible for this Common Trunk period. During the four years of training in GE, fellows may train for two years in a general hospital. Requirements include a six months rotation in the GE ward and two and a half years in outpatient care and endoscopy.

Subspecialisation

In the fourth so called final year of training, the fellow can choose from the subspecialties as accredited by the Board. These include interventional endoscopy, neuromotility, oncology or hepatology. All the subspecialties require research, preferably resulting in the publication of at least one article in a peer-reviewed journal and, if possible, a PhD-thesis. International courses and conferences should be attended, such as the American Society of Clinical Oncology, the American Association for the Study of Liver Disease *etc*.

Table 1 Different curricula

	Duration (yr)		Minimal numbers of endoscopy					Exam		Protocols	English protocols
	Internal medicine	GE	Gastro	Colo	Abdominal			Entrance	Final		
					ERCP	Echo	EUS				
Austria	6	2	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt	Nt
Canada	4	2	100	200	200	0	200	No	Yes	Yes	Yes
UK	2.5	2.5	200/yr	100/yr	No	No	No	Yes	No	Yes	Yes
Finland	3	3	Nt	Nt	Nt	Nt	Nt	Yes	No	No	No
Hungary	Nt	2-3	Nt	Nt	Nt	Nt	Nt	Oral	No	No	No
Iran	4	2	> 400	100-150	40-60	No	No	Yes	Yes	No	No
Italy	1	3	300	100	No	300	No	Yes	Yes	Nt	Nt
NL	2	4	300	200	100	No	100	No	No	Yes	No
Romania	2	3	> 150	100	No	No	No	Yes	Yes	No	No
South Africa	4	2	500	75	50	No	No	Yes	No	No	No

No: Not required; Nt: Not known.

Hepatology

Training in hepatology has been well defined. During the 12 mo of subspecialisation, the fellow needs to complete at least four months liver transplant training in a certified transplantation unit. Beside these four months, the fellow must attend at least two out-patient clinics a week for four to six months and consultations in the clinics. Skills like the endoscopic treatment of esophageal varices and paracentesis are components of this subspecialisation. In some university clinics, abdominal ultrasonography will be included.

Motility

Motility, as a subspecialty, incorporates outpatient clinic and laboratory skills in motility and neurogastroenterology, such as 24-h pH measurements. Like the other subspecialties, an article should be written and an abstract presented at an international neurogastroenterology conference.

Oncology

Oncology training should be carried out in a hospital where at least two gastroenterologists in the team are specialised in oncology. Added emphasis is placed on interventions and rare tumors, beside the knowledge of screening and primary prevention. Special interventions like endoscopic ultrasound (EUS) must be done on a regular basis. In addition, specific fellowships in related specialties are a part of the training. The fellow is required to work for at least one month in a multidisciplinary setting. This setting should include oncologists, radiotherapeutics, pathologists and clinical geneticists. All the main surgical procedures, for example sigmoid resection and esophagogastrectomy should be observed. Interventional endoscopy is a primary skill required in the oncology subspecialisation.

Interventional endoscopy

Another subspecialisation is interventional endoscopy. The unit where the fellow works in this sixth year should do at least 250 ERCP and 250 EUS procedures a year, as well as a minimum of 5000 endoscopies a year. During this fellowship, the candidate should perform at least 100

ERCP and 100 EUS procedures of the esophagus, and another 100 EUS procedures of the pancreas/duodenum. Experience can also be gained in endoscopic procedures for Zenker diverticulum, and in double balloon enteroscopy and endomucosal resection of the esophagus.

Trainees

The duration of this new curriculum is thus six years on a full-time basis, with a minimum of 38 h of work and 10 h of teaching a week. Training in GE is an ongoing dynamic process and the program should be individualized if possible for each fellow. Fellows are required to keep a portfolio, so that their progress and accomplishments can be evaluated. This portfolio must include numbers of (interventional) endoscopies done, 360-degree interviews, evaluations of skills, short clinical observations, direct observations during clinical rounds, saved files of oral lectures given during courses, conferences and published articles. Management is a major part of daily practice after becoming a medical doctor (MD). This skill is slowly introduced into the program.

WGO

The WGO Education & Training Committee formulated a document outlining the standards of GE training^[4]. The WGO reviewed the composition of programs in a number of countries. They reviewed both comprehensive curricula in the USA and UK as well as curricula in developing countries, which have a number of shortcomings.

Curriculum

The WGO curriculum includes recommendations on training standards and accreditations of institutions (Table 2). After completion of this curriculum, an MD will be a generalist in GE. Local facilities will dictate what the fellow is exposed to. Fellows may be able to include abdominal ultrasonography and/or EUS in their portfolio. In some countries, ERCP is not included in the curriculum as it is done by radiologists or surgeons. In the former Soviet-Union, endoscopy was considered a specialty independent of GE. These endoscopists are also performing bronchoscopies^[12].

Table 2 Standard curriculum

Knowledge
Anatomy, histology, molecular biology, embryology and development of the GI tract
Physiology and pathophysiology
Pharmacology
Epidemiology
Nutrition, metabolism and endocrinology
Diagnose and evaluate patients
Timely surgery and other therapeutic interventions
Cost-effective management
Prevention
Complications and disinfection of endoscopy procedures
Educator of chronic patients and support family
Bioethics
Conduct, write and publish research
Skills
Professional principles: behavior, commitment highest standard, responsive
Effectively and efficiently
Lead multidisciplinary teams
Maintain skills in general medicine
Appropriate communications
Information-science resources
Interpret laboratory data
Interpret radiographic data
Endoscopic skills

Training

The recommendation of the WGO is to complete three years of internal medicine (Common Trunk), before fellows enter GE. The standard training includes both knowledge and skill components that must be fulfilled (Table 2). The WGO proposes a minimum number of each procedure that must be completed by the fellow. This is in contrast with higher numbers required in the UK, The Netherlands and the USA (Tables 3 and 4). The WGO differentiates between level I and level II endoscopists. These endoscopists have different skills. Level II includes ERCP, stenting of the esophagus and colon, diagnostic laparoscopy and EUS. Techniques that are still being developed for clinical practice like natural orifice transluminal endoscopic surgery (NOTES) have not yet been included. We suggest that Level I endoscopists should also have defined training in hepatology, oncology and motility. Although we do recognise that training in endoscopy level I might be sufficient for some developing environments.

Outpatient clinic, clinic and endoscopy

These three components form the foundation of the training required to become an all-round gastroenterologist. The WGO advises that 150 new patient contacts must be seen a year. They do not however, define how these outpatient contacts should be structured. We suggest that at least one outpatient clinic session is attended by a fellow each week.

Trainees and trainers

WGO suggests that attitudes, experience in patient and non-patient care as well as management skills should be more precisely defined by the different societies. The

Table 3 Endoscopic skills level I

	Numbers			
	WGO	UK	NL	USA
Esophagoduodenoscopies	100	> 200	300	130
Esophagoduodenoscopies therapeutic interventions	-	> 30	25	-
Treatment of non-variceal bleeding	20/10 active	-	-	25
Treatment of variceal bleeding	15/5 active	-	-	20
Esophageal dilation	15	-	-	20
Lifetime serious complications	-	< 10%	-	-
Flexible sigmoidoscopy	25	-	200	-
Colonoscopy	100	> 200	200	140
Lifetime perforations	-	< 0.5%	-	-
Cecal intubations	-	> 90%	> 90%	-
Ileum intubation (when indicated)	-	-	> 50%	-
Polypectomy	20	> 20%	-	30
Percutaneous endoscopic gastrostomy (PEG)	10	-	-	15
Liver biopsy	20	-	-	-
Paracentesis	50	-	-	-
Foreign body removal	-	-	-	-
Videocapsule endoscopy	-	-	-	25

WGO states that at least three years of training in GE is required. During this time, fellows should be present in the GE training unit for at least 33 to 44 h a week. Part-time fellows who work fewer hours are required to amend the time period of their training accordingly. The most efficient and reliable manner in which to hand over patient details to doctors starting the next work shift is not detailed in the guidelines. This is a challenge for both full and part-time fellows. Such a curriculum could not be implemented if the training period for internal medicine remains at four to five years.

Training institutes require a number of components in order to deliver a comprehensive curriculum. The training team should require a ratio of one trainer to one and a half trainees. At least one trainer should be a hepatologist. Training for endoscopic procedures is graded and staged, and involves the progression from observation, to working under supervision and finally performing procedures independently. The trainer should have at least five years experience as a gastroenterologist. He or she should be a role model, participating in all gastroenterology activities including research meetings, scientific societies *etc.*

Competency evaluation

The competence of the fellow is based on the various areas of expertise that are evaluated. A wide variety of evaluation methods are recognised around the world. These include direct observations, patient based exams, portfolios and final assessments. The WGO committee has not initiated guidelines regarding entrance and final exams. In some countries, candidates are accepted into training programs based upon results of an entrance exam. These examinations usually comprise a multiple choice format; but some centers also require clinical assessment examinations.

Table 4 Endoscopic skills level II

	Numbers			
	WGO	UK	NL	USA
ERCP	-	> 200	100	200
Sphincterotomy	-	-	25	-
Stone extraction	-	-	-	-
Stenting	-	-	25	-
Complications	-	< 5%	-	-
Satisfactory completion of intended therapeutic procedure	-	> 80%	-	-
Diagnostic laparoscopy	-	-	-	-
EUS	-	-	100	150

Research

The WGO has not, as yet, incorporated research training, despite the recognition that there has been a significant decline in the number of physicians-scientists in GE^[8,13].

In order to increase the trainees devoted to research, it is advocated to increase medical student research, and improve mentoring and funding for GE-fellows. Unfortunately, there are no available data in this regard. Training in GE should offer research opportunities with an emphasis on intestine, liver or pancreas. Up until now, the limited time in the GE curriculum hampers the participation in research. An alternative is to identify talent and increase interest among undergraduates and develop and integrate a formal undergraduate GE research program in medical school^[14-16]. In The Netherlands, and especially in our department, we encourage students to complete a two year GE research program prior to entering the Common Trunk of internal medicine. Involvement in these programs unquestionably strengthens the physician-scientist pool of the future. Such programs are comparatively inexpensive as the students are a relatively inexpensive investment as they have not yet been formally trained in GE. Clinicians with scientific training will be valuable members of university and general hospitals.

Public health

Public health care is not specifically highlighted in the WGO curriculum. Education and global standardization, is, however, emphasized in the training program. The WGO documents the importance of preventative medicine, for example colorectal carcinoma screening and viral hepatitis prevention; but no protocols are given in this regard. Going forward, further emphasis should be placed on the anagement of these public health issues.

Finance and requirements of gastroenterologists

The burden of disease is markedly different between developing and developed countries. Thus, the numbers of gastroenterologists required and their expertise will vary. This issue has not been addressed. The reasons for this are multifactorial^[17]. By way of illustration, intestinal infectious disease was the fifth leading cause of death

in South Africa, a developing country, in 2006. In The Netherlands, a developed country, diarrhoeal disease does not feature in the 10 most common causes of mortality. Colon and rectal cancer were the seventh leading cause of death in 2006^[18,19]. Recently, Everhart *et al*^[20] showed an increase of 35% in medical care for digestive diseases in the United States between 1998 and 2004.

The WGO committee does not give a recommendation for the numbers of gastroenterologists required in different countries. In South Africa, there is only one gastroenterologist per one million inhabitants; while in the USA there is one gastroenterologist per twenty thousand inhabitants. Similarly, there are no recommendations regarding the number of doctors required to carry out colorectal cancer screening programs. We should mention the shortage of skills in sub-Saharan Africa, for example there are no doctors performing ERCP in Nigeria.

The committee also does not outline the financial implications of new curricula^[21].

Maintenance certification

The WGO curriculum is written to formulate a standard GE training program. Learning is on a continuum following certification, and the quality thereof is of importance. Less is known about maintaining this certification or re-validation thereof. Although many countries like the US have their own continuing medical education program, there is no proper data regarding the number of endoscopies required to maintain a licence. In The Netherlands 200 h training in five years is required and at least 100 colonoscopies, 200 gastroduodenoscopies and 30 ERCPs should be done per year. This continuing certification should be defined and evaluated in the near future.

CONCLUSION

Reviewing all recently published papers on the subject, a new trend in training of GE fellows has been identified. In the last ten years, GE has emerged as a leading branch of internal medicine. The developments seen over this period are expected to continue, ensuring exponential growth in the discipline for years to come. A new curriculum will, therefore, need to be revised on a continuing basis. It is envisaged that GE will become more sub-specialised, although more generalised skills will also be necessary to ensure a complete service delivery in a number of areas, especially in developing countries. In the curricula written so far, little is mentioned about part-time contracts, continuing certification or special public health needs. The daily transition is something which has to be learned. Beside the part-time issues, it may also be possible to develop a flexible training program, where the length of the training is dependent on the skills of the trainee. The personalization of the curriculum for GE is the next major challenge in the curriculum, which will develop the GE in the coming years.

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Use of new once-daily 5-aminosalicylic acid preparations in the treatment of ulcerative colitis: Is there anything new under the sun?

Peter Laszlo Lakatos

Peter Laszlo Lakatos, 1st Department of Medicine, Semmelweis University, Koranyi st. 2/A, H-1083, Budapest, Hungary

Author contributions: Lakatos PL wrote this paper.

Correspondence to: Peter Laszlo Lakatos, MD, PhD, 1st Department of Medicine, Semmelweis University, Koranyi st. 2/A, H-1083, Budapest, Hungary. kislakpet@bell.sote.hu

Telephone: +36-1-2100278 Fax: +36-1-3130250

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Abstract

5-aminosalicylate (5-ASA) agents remain the mainstay treatment in ulcerative colitis (UC). A number of oral 5-ASA agents are commercially available, including azobond pro-drugs, as well as delayed- and controlled-release forms of mesalazine. However, poor adherence due to frequent daily dosing and a large number of tablets has been shown to be an important barrier to successful management of patients with UC. Recently, new, once-daily formulations of mesalazine, including the unique multi-matrix delivery system and mesalazine granules, were proven to be efficacious in inducing and maintaining remission in mild-to-moderate UC, with a good safety profile comparable to that of other oral mesalazine formulations. In addition, they offer the advantage of a low pill burden and might contribute to increased long-term compliance and treatment success in clinical practice. This editorial summarizes the available literature on the short- and medium-term efficacy and safety of the new once-daily mesalazine formulations.

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Key words: Ulcerative colitis; 5-aminosalicylate; Mesalazine; Multi matrix system; Therapy; Once-daily; Compliance

Peer reviewer: NKH de Boer, PhD, MD, Gastroenterology and Hepatology, VU University Medical Center, PO Box 7057, 1007 MB, Amsterdam, The Netherlands

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INTRODUCTION

The pathogenesis of ulcerative colitis (UC) has only been partly elucidated. Inflammatory bowel disease (IBD) is a multifactorial entity with both genetic and environmental factors contributing to disease pathogenesis^[1]. Worldwide, the incidence rates for UC vary from 0.5 to 24.5 per 100 000 person-years^[2]. Recent reports from China and Korea also present an increase in patient numbers^[3]. The classical presentation is that of rectal bleeding and diarrhea, with other symptoms such as urgency, tenesmus, and abdominal cramping also being common. The disease might be limited to the rectum or extend proximally to include the entire colon and is characterized by a remission-relapse course in most patients.

5-aminosalicylic acid (5-ASA) remains the mainstay treatment in mild to moderate UC^[4]. In left-sided and extensive cases, a combination of oral and topical mesalazine appears to be more effective than either alone; however, this is probably not a simple dose-response effect, as higher topical 5-ASA doses do not improve efficacy^[5]. A number of oral 5-ASA agents are commercially available, including azo-bond prodrugs, such as sulfasalazine, olsalazine and balsalazide, and delayed- and controlled-release forms of mesalazine. Overall, the safety profile of oral 5-ASA agents is favorable and similar to that of a placebo in large clinical trials^[6]. In addition, the use of sulfasalazine is mainly limited by its side effects (including nausea, vomiting, abdominal pain, fever, skin rash, agranulocytosis, neutropenia, male infertility, folate deficiency, neuropathy, autoimmune hemolysis, and, rarely, nephrotoxicity, hepatotoxicity or pancreatitis) and the high rate of intolerance (up to 20%). Somewhat in contrast, although mild side effects are more common with sulfasalazine, some of the more severe side effects, for example pancreatitis, are more common with mesalazine (OR: 7.0), with interstitial nephritis being exclusively described for mesalazine^[7]. Conclusions from this study, however, were criticized due to incomplete data collected through spontaneous reporting. Interestingly,

in a recent review by the Cochrane group, mesalazine was not superior compared to sulfasalazine for inducing response or remission (OR = 0.83, 95% CI: 0.60-1.13)^[6], but was better tolerated.

Much emphasis has been placed on the manner in which different delivery systems may influence response to 5-ASAs; however, evidence in clinical practice for variability in efficacy is rather weak. Delivery systems can be divided into azo-compounds, controlled release, pH-dependent (either pH 6 or 7) and composite (pH-dependent combined with controlled release)^[8]. In addition, the effectiveness of oral therapy relies on good compliance, which may be adversely affected by frequent daily dosing and a large number of tablets. Recent studies have shown that poor adherence has been an important barrier to the successful management of patients with UC. Only 40% to 60% of the patients who are newly diagnosed or have longstanding disease are adherent to therapy^[9,10]. Hence, once-daily oral formulations of 5-ASA are likely to be a better therapeutic option in clinical practice, partly due to improved adherence. Furthermore, when assessing remission and response rates, one must be aware that the placebo group rates may vary anywhere from 0 to 40% according to the definition used for response and remission. In a recent review^[11], a significant heterogeneity was reported among studies using different criteria [e.g. UC Disease Activity Index (UC-DAI), Rachmilewitz]. Thus, the direct comparison of studies using different criteria is difficult to interpret.

A new, oral delayed-release formulation of mesalazine utilizing Multi Matrix System (MMX) technology (hereafter referred to as MMX mesalazine) was recently approved in the US for the induction and maintenance of remission in patients with active, mild-to-moderate ulcerative colitis^[12]. It is a high dose (mesalazine 1.2 g/tablet), delayed-release form that permits once-daily administration. The MMX technology involves incorporating mesalazine into a lipophilic matrix, which itself is dispersed within a hydrophilic matrix, to delay and prolong dissolution. A gastro-resistant polymer film prevents initial drug release until exposed to a pH < 7; thus, the film coat normally starts to dissolve only in the terminal ileum. The hydrophilic matrix is then exposed to intestinal fluids and swells, resulting in the formation of a viscous gel mass with a slow and gradual release of mesalazine throughout the length of the colon. This editorial will focus on the efficacy and tolerability of the new, once-daily mesalazine formulations.

EFFICACY AND SAFETY OF THE MMX MESALAZINE IN INDUCTION AND MAINTENANCE OF REMISSION

First, a preliminary randomized, double-blind, double-dummy clinical study compared the efficacy of MMX mesalazine *versus* topical mesalazine in 79 patients with active, left-sided, mild-to-moderate UC^[13]. Comparable

clinical remission rates were achieved; 60% of the patients in the MMX mesalazine group and 50% of the patients in the enema group were in clinical remission at the end of week eight. Endoscopic remission rates were also not significantly different. Overall compliance was 97% for oral administration and 87.5% for the enema. In a subsequent Phase II, randomized, double-blind, dose-ranging study, D'Haens *et al*^[14] evaluated three different doses of MMX mesalazine (1.2, 2.4, and 4.8 g/d) given once daily for the induction of remission in 38 patients with mild-to-moderate UC in an eight week trial. Remission at the end of week eight was defined as a UC-DAI score of 1 or less, a score of 0 for rectal bleeding and stool frequency, and at least a 1-point reduction in sigmoidoscopy scores from baseline. Remission was achieved in 0% (0/12), 30.8% (4/13), and 18% (2/11) of the patients receiving MMX mesalazine 1.2, 2.4, and 4.8 g/d, respectively, with no statistically significant differences ($P = 0.13$). Improvements in physician's global assessment (PGA), stool frequency, and rectal bleeding were similar in all treatment arms.

The FDA's approval of MMX mesalazine (SPD476, MezavantTM, LialdaTM) was based on the two randomized, double-blind, placebo-controlled Phase III trials^[15,16]. The first trial investigated the efficacy of MMX mesalazine 1.2 g twice daily and 4.8 g once-daily compared with the placebo for eight weeks, for the induction of remission in 280 patients with mild-to-moderate UC. The primary endpoint was endoscopic and clinical remission at week eight, defined as a modified UC-DAI ≤ 1 with a subscore of 0 for rectal bleeding and stool frequency, a combined PGA and sigmoidoscopy score of ≤ 1 , a sigmoidoscopy score reduction of ≥ 1 from baseline, and no mucosal friability. Secondary endpoints included clinical improvement (reduction in modified UC-DAI scores from baseline of ≥ 3 points) and clinical remission (scores of 0 for stool frequency and rectal bleeding). At the end of week eight, both MMX mesalazine groups achieved statistically significant clinical and endoscopic remission compared with the placebo (34.1% and 29.2% *vs* 12.9%, 2.4 g/d and 4.8 g/d *vs* placebo, $P < 0.001$ and $P = 0.009$, respectively). A statistically significant proportion of patients receiving either dose of MMX mesalazine achieved clinical improvement and clinical remission (37.5%, 32.6% *vs* 18.8%, $P < 0.05$) compared with the placebo. The median time to initial clinical remission (lasting ≥ 3 consecutive days) was 43 and 44 d for the 2.4 g/d and 4.8 g/d MMX mesalazine groups, respectively; in contrast it was not reached for the placebo.

In the second Phase III double-blind, placebo-controlled, multicenter clinical trial, Kamm *et al*^[16] randomized 343 patients with active, mild-to-moderate UC to receive MMX mesalazine 2.4 g once daily, MMX mesalazine 4.8 g once daily, placebo, or a delayed-release mesalazine (AsacolTM) 800 mg, 3 times daily. The Asacol group served as a reference arm in the study. Due to the study's double-dummy design, all patients received 4 tablets and 2 capsules in the morning, 2 capsules at

lunch time, and 2 capsules in the evening. Significantly more patients achieved clinical and endoscopic remission at week eight in the MMX mesalazine groups compared with the placebo group (40.5% and 41.2% *vs* 22.1% with 2.4 g/d, 4.8 g/d *vs* placebo; $P = 0.01$ and $P = 0.007$). In contrast, the Asacol group demonstrated only a trend for improvement (32.6% *vs* 22.1%, $P = 0.124$). MMX mesalazine was not directly compared with Asacol. Interestingly, endoscopic remission rates (69% for MMX 2.4 g/d, 77.6% for MMX 4.8 g/d, 61.6% for Asacol, and 46.5% for the placebo) exceeded clinical remission rates for both active treatment and placebo groups and were much better than previously reported for 5-ASA.

In a combined analysis^[17] of the two trials, data from 517 patients were analyzed. Eight-week remission rates were 37.2% and 35.1% in the MMX mesalazine 2.4 g/d and 4.8 g/d groups, respectively, *versus* 17.5% in the placebo group ($P < 0.001$, for both). The respective rates for clinical improvement were 58%, 62%, and 33%. The eight-week, complete mucosal healing rates were 32% in both MMX mesalazine groups compared with 16% in the placebo group. In an intent-to-treat analysis, the median time to resolution of symptoms (stool frequency and rectal bleeding) was 25, 26, and 44 d, respectively^[18]. The median time to resolution of rectal bleeding was seven, eight, and 16 d, while the median time to normalization of stool frequency was 19, 20, and 34 d.

In a subsequent analysis^[19], the authors stratified the data according to disease extent, severity, gender and prior 5-ASA use. The percentage of patients in clinical and endoscopic remission was not different according to disease extent and severity and among patients who did not previously receive low-dose 5-ASA. Among patients transferring directly from prior low-dose oral 5-aminosalicylic acid, MMX mesalazine 4.8 g/d was significantly ($P = 0.018$) more effective than the placebo in inducing clinical and endoscopic remission. Efficacy over the placebo did not reach significance in patients transferring directly to MMX mesalazine 2.4 g/d. Interestingly, remission rates were higher in females in both active treatment groups and placebo groups (44.8% for MMX 2.4 g/d, 41.4% for MMX 4.8 g/d, and 20.7% for the placebo) compared to males (29.4%, 28.7%, and 14.3%, $P = 0.008$). Nevertheless, in a logistic regression analysis, the authors excluded the gender effect. It is not clear, however, which other possible confounding variables were included in the analysis.

If patients were not in remission after an eight-week treatment with either MMX mesalazine 2.4 g once daily, MMX mesalazine 4.8 g once daily, placebo, or a delayed-release mesalazine (AsacolTM) 800 mg 3-times-daily, patients were offered an open-label extension treatment with 4.8 g MMX mesalazine for another eight weeks^[20]. Out of the 304 patients who entered the extension study, 59.5% of patients achieved remission at the end of the extension treatment irrespective of prior therapy. Normal mucosal appearance was seen at sigmoidoscopy in 42.4% of the patients at the end of the extension study *vs* 3.3% prior to the extension phase.

Upon completion of the remission induction

trials, eligible patients could enter a Phase III open-label extension study to evaluate the long-term efficacy and safety of MMX mesalazine in the maintenance of remission. Patients who were not in remission at the end of the original induction trial were offered an additional eight-week open label MMX mesalazine 4.8 g/d treatment administered twice daily^[21]. Those who were in remission at either eight or 16 wk were then randomized to MMX mesalazine 2.4 g/d given once or twice daily for 12 mo. Two-hundred-twenty-five and 234 patients were randomized into the two treatment groups. At the end of the 12-mo follow-up, 67.8% and 72.3% of the patients were strictly defined to have clinical and endoscopic remission in the per-protocol population. 88.7% and 92.5% of the patients were not considered to have relapsed based on the physician's clinical assessment and the need for alternative therapy. These data are comparable to other mesalazine agents, with reported remission rates of 60%-70% after 6-12 mo of maintenance therapy^[22].

In a post-hoc analysis^[23], the authors did not find differences in the relapse rate according to the initial treatment; relapse rates at 12 mo were 6.3%, 10.8%, and 5.6% for patients initially treated with MMX mesalazine 2.4 g/d, 4.8 g/d or Asacol 2.4 g/d, respectively. No significant differences were found in remission rates in a similar sub-group analysis in patients with baseline mild or moderate (71% *vs* 64%) and left-sided or extensive (67% *vs* 65%) ulcerative colitis. Similarly, relapse rates were independent of previous relapse history, although there was a trend for increased frequency of relapses in patients with a higher number of prior relapses (< 3 prior relapses: 70.1% *vs* ≥ 3 prior relapses: 59.8%). In contrast, the degree of initial mucosal inflammation (mild: 68.6%, moderate: 68.1%, and severe: 43.3%) and time needed to induce remission (remission at week eight: 75.8% *vs* remission at week 16: 55.9%) were significantly associated with decreased remission rates at 12 mo^[21,24,25].

In a subsequent Italian multicenter study^[26], the authors preliminarily reported on the efficacy of once-daily 2.4 g MMX mesalazine *vs* 2.4 g delayed-release mesalazine (AsacolTM) maintenance therapy taken twice daily in 323 mild-to-moderate patients with left-sided ulcerative colitis in clinical remission without mucosal friability. At 12 mo, 30.8% *vs* 43.2% of the patients relapsed in the two groups in a per-protocol analysis, resulting in an 11.3% difference in long-term remission rates in favor of the once-daily treatment (95% CI: -0.01-22.7).

MMX mesalazine was generally well tolerated in all controlled clinical trials, with most adverse events being of mild or moderate severity. Of the 434 MMX mesalazine recipients evaluated for safety in the four published controlled trials^[13-21], only two patients had serious adverse events that were considered treatment-related; both included pancreatitis caused by hypersensitivity to mesalazine. There was no evidence of a dose-response relationship with MMX mesalazine for any tolerability parameter in either trial.

Table 1 Efficacy of the MMX mesalazine formulations for induction and maintenance of remission in mild-to-moderate UC

Study	Phase	Patient number (n)	Dosing regimen (g/d)	Duration	Remission rates (%)	
					Treatment	Placebo
Induction D'Haens ^[14]	II	38	MMX 1.2	8 wk	0	-
			MMX 2.4		30.8	-
			MMX 4.8		18	-
Lichtenstein ^[15]	III	280	MMX 2.4	8 wk	34.1 ^a	12.9
			MMX 4.8		29.2 ^a	
Kamm ^[16]	III	343	MMX 2.4	8 wk	40.5 ^a	
			MMX 4.8		41.2 ^a	22.1
Sandborn ^[17] , combined ^[25,26]	III		Asacol TM 2.4	8 wk	32.6	
			MMX 2.4		37.2 ^a	17.5
			MMX 4.8		35.1 ^a	
Maintenance					Patients still in remission	
Kamm ^[21]	III	459	MMX 2.4 <i>od</i>	12 mo	67.8	
			MMX 2.4 <i>bid</i>		72.3	
Prantera ^[26]	III	325	MMX 2.4 <i>od</i>	12 mo	69.2 ^b	
			Asacol TM 2.4 <i>bid</i>		56.8	

^a $P < 0.01$ vs placebo; ^b $P < 0.05$, *od* vs *bid* remission rates: endoscopic and clinical remission rates.

Table 2 Efficacy of other, new mesalazine formulations for induction and maintenance of remission in mild-to-moderate UC

Study	Phase	Patient number (n)	Dosing regimen (g/d)	Duration	Remission rates (%)
Induction Kruis ^[27]	III	388	Granules 3 <i>od</i>	8 wk	79.1
			Granules 3 <i>tid</i> Salofalk®		75.7
Maintenance					Patients still in remission
Dignass ^[30]	III	388	Granules 2 <i>od</i>	12 mo	73.8 ^a
			Granules 2 <i>bid</i>		63.6
Kruis ^[29]	III	647	Pentasa®	12 mo	
			Granules 3.0 <i>od</i>		74.7
			Granules 1.5 <i>od</i>		60.8
			Granules 1.5 <i>tid</i>		68.8
			Salofalk®		

^a $P < 0.05$, *od* vs *bid*.

The most common treatment-related adverse events were headache, flatulence, and abdominal pain. Severe events were more common in the placebo recipients (6.1%) than in patients receiving MMX mesalazine 2.4 or 4.8 g/d (1.1% and 2.2%), and mostly consisted of gastrointestinal events related to the underlying disease.

The efficacies of the new MMX mesalazine formulations for the induction of remission in mild-to-moderate UC are summarized in Table 1.

THERAPEUTIC EFFICACY AND SAFETY OF OTHER ONCE-DAILY MESALAZINE FORMULATIONS

Another once-daily preparation (SalofalkR granules) also proved to be efficacious in inducing remission in mild-to-moderate active ulcerative colitis in a double-blind, randomized, Phase III clinical trial, termed SAG-26^[27]. Three-hundred-eighty patients were randomized to receive 3 g/d mesalazine granules either once daily (OD) or three times per day (TID). At week eight, treatment groups achieved comparable clinical (as defined by a CAI ≤ 4 at the final/withdrawal visit, 3 g OD: 79.1% vs TID: 75.7%)

with comparable endoscopic (71% vs 70%) remission and histological remission (35% vs 41%) rates. OD treatment was more effective in patients with proctosigmoiditis (86% vs 73%, $P = 0.02$); but, efficacy was not different according to baseline severity and disease duration.

In a combined analysis of three Phase III clinical trials (SAG2, SAG15, and SAG26)^[28], the efficacy of the 3 g/d mesalazine (Salofalk granules) treatment was not affected by gender, duration since first symptom, disease location or disease duration (new vs established disease). In contrast, significantly lower remission rates were achieved in patients with moderate disease (66% vs 89%, $P = 0.0009$) and in patients relapsing on 5-ASA maintenance therapy (67% vs 82%, $P < 0.0001$).

The once-daily maintenance treatment was also shown not to be inferior for the mesalazine, 3 g Salofalk granules in a double-blind, double-dummy, randomized, controlled, dose-ranging study^[29]. Six-hundred-forty-seven patients who have achieved clinical (CAI ≤ 4) and endoscopic remission (EI ≤ 3) within 12 wk from baseline were randomized to 3 g once daily, 1.5 g once daily, and 1.5 g three-times-daily mesalazine treatment. At 12 mo, 74.7%, 60.8%, and 68.8% of patients were in clinical remission pointing toward a statistically

significant superiority of the once-daily 3 g treatment group. All treatment groups showed excellent safety profiles and there were no indications for increased risk in patients treated once daily or with the higher dose.

The use of once-daily treatment for maintenance of remission is further supported by a recent randomized, multicentre, investigator-blinded study of 362 patients who were randomised to receive mesalazine granules (PentasaR) 2 g once daily or 1 g twice daily. It showed an 11.9% greater remission rate at one year (73.8% *vs* 63.6%, respectively) in the single daily dose group^[30]. The 95% CI values for the treatment difference (1.4%-22.5%, $P = 0.024$) in intent to treat analysis were completely above the non-inferiority limit of -10.0 and did not cross 0. Therefore, once-daily administration of the drug proved to be superior to twice-daily treatment. Normal mucosa was found in 49.3% and 46.2% of the patients with once-daily or twice-daily treatment and there was a trend toward less friability in the once-daily group (9.7% *vs* 15.9%). In addition, subjects undergoing once-daily treatment had a lower likelihood of rectal bleeding (20.4% *vs* 29.3%) and also increased rates of normal stool frequency (81.5% *vs* 61.7%) at 12 mo. Patient questionnaires showed significantly greater self-reported compliance ($P < 0.05$) and acceptability ($P < 0.001$) in the once-daily group. High compliance rates were reported for the once-daily MMX mesalazine^[17]; therefore, the effect is likely to be generic rather than compound-specific. The efficacy of the other new mesalazine formulations for the maintenance therapy in mild-to-moderate UC is summarized in Table 2.

CONCLUSION

MMX mesalazine and the newly developed mesalazine granules were all shown to be efficacious in inducing and maintaining remission in mild-to-moderate UC in large clinical trials. However, existing data are insufficient to make a comparison between new and "conventional" 5-ASA formulations. Short-term evaluation reveals that the new formulations are at least as effective as other oral 5-ASA formulations. Recently, MMX mesalazine has been approved in the US for the induction of remission in adult patients with active, mild-to-moderate ulcerative colitis. In Europe, it is indicated for both induction and maintenance of remission. The safety profile is favorable and comparable to that of other mesalazine formulations. In addition, new mesalazine formulations offer a simplified dose regime, resulting in presumably improved long-term compliance that can be considered an important advantage in the management of UC patients. This is of great importance in everyday clinical practice, because only 40% to 60% of the patients who are newly diagnosed or have longstanding disease are adherent to therapy. While patients at all stages of UC are affected by non-adherence, those in symptomatic remission are particularly at risk of poor adherence, often taking less than 70% of their prescribed medication, with non-adherent patients being more likely to relapse. Therefore, improving adherence to therapy has become

one of the most important goals of patient management. However, once-daily administration was never tested for conventional 5-ASAs. Furthermore, ulcerative colitis patients (except proctitis) are at an increased risk for colorectal cancer^[31,32], and according to a recent meta-analysis^[33], the incidence of colon cancer is approximately 50% lower in aminosalicilate users. Thus, improved compliance might further contribute to decreasing the likelihood of the colorectal cancer burden in UC.

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Emmet B Keeffe, MD, Professor, Series Editor

Treatment of chronic viral hepatitis with nitazoxanide and second generation thiazolides

Emmet B Keeffe, Jean-François Rossignol

Emmet B Keeffe, The Romark Institute for Medical Research, Tampa, Florida and Sausalito, California, United States and the Division of Gastroenterology and Hepatology, Department of Medicine, Stanford University Medical Center, Stanford, California 94304-1509, United States

Jean-François Rossignol, The Romark Institute for Medical Research, Tampa, Florida, United States and the Division of Gastroenterology and Hepatology, Department of Medicine, Stanford University Medical Center, Stanford, California 94304-1509, United States

Author contributions: Keeffe EB and Rossignol JF solely contributed to this article.

Correspondence to: Emmet B Keeffe, MD, Romark Laboratories, L.C., 2320 Marinship Way, Suite 250, Sausalito, California, 94965, United States. emmet.keeffe@romark.com

Telephone: +1-415-3321060 Fax: +1-415-3321067

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Abstract

Nitazoxanide, the first thiazolide, was originally developed for the treatment of *Cryptosporidium parvum*. More recently, antiviral activity of nitazoxanide against hepatitis B virus (HBV) and hepatitis C virus was recognized in *in vitro* systems. These basic studies led to phase II clinical trials that demonstrated the safety and efficacy of nitazoxanide in combination with peginterferon, with or without ribavirin, in the treatment of chronic hepatitis C genotype 4. The sustained virologic response rate was 79% and 80% in two studies, which was higher than the response rate of 50% with the standard of care with peginterferon plus ribavirin. In very preliminary studies of patients with chronic hepatitis B, nitazoxanide suppressed serum HBV DNA and led to loss of hepatitis B e antigen in the majority of patients and hepatitis B surface antigen in approximately a quarter of patients. Randomized controlled studies of naive and nonresponder patients with chronic hepatitis C genotype 1 are underway, new second generation and controlled release thiazolides are being developed, and future studies of patients with chronic hepatitis B are planned.

Key words: Hepatitis C; Hepatitis C virus; Nitazoxanide

Peer reviewer: Dr. Vicente Carreño, Fundacion Estudio Hepatitis Virales, C/ Guzman el Bueno 72, Semisotano, Madrid 28015, Spain

Keeffe EB, Rossignol JF. Treatment of chronic viral hepatitis with nitazoxanide and second generation thiazolides. *World J Gastroenterol* 2009; 15(15): 1805-1808 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1805.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1805>

INTRODUCTION

Nitazoxanide (Alinia[®], Romark Laboratories, L.C., Tampa, FL, USA), the first thiazolide, was licensed in the USA for the treatment of *Cryptosporidium parvum* and *Giardia lamblia* in immunocompetent adults and children in 2002^[1]. A number of emerging basic and clinical studies support an additional role of nitazoxanide in the treatment of chronic hepatitis C virus (HCV) and chronic hepatitis B virus (HBV) infection. This brief review summarizes current data from emerging phase II studies related to this new potential use of nitazoxanide combined with peginterferon and ribavirin for the treatment of chronic hepatitis C and very preliminary experiences with nitazoxanide for the treatment of chronic hepatitis B.

ANTIVIRAL MECHANISM OF ACTION OF NITAZOXANIDE

The antiviral mechanism of action of nitazoxanide is different from the mechanism of action in protozoa and anaerobic bacteria *via* direct inhibition against the pyruvate-ferrodoxin oxidoreductase reaction^[1], and appears in recent studies to involve activation of the protein kinase activated by double-stranded RNA (PKR), an interferon-induced mediator of the cellular antiviral response^[2]. The activation of PKR results in phosphorylation of its substrate, eukaryotic initiation factor 2 alpha (eIF2 α). Nitazoxanide, thus, represents a new class of small molecules that modulate host antiviral pathways. By targeting a host function, the barrier to development of

antiviral resistance is significantly higher than for drugs directly targeting a viral function.

IN VITRO ACTIVITY OF NITAZOXANIDE AGAINST HBV AND HCV

After nitazoxanide was serendipitously suspected as active against viral hepatitis in patients with acquired immune deficiency syndrome (AIDS) treated with nitazoxanide for *Cryptosporidium*, the antiviral activities of nitazoxanide and its metabolite, tizoxanide, were confirmed in standard antiviral assays *in vitro*^[3,4]. Both nitazoxanide and tizoxanide are potent inhibitors of HBV, and in combination with other antiviral agents such as lamivudine or adefovir, show synergistic effects. Nitazoxanide and tizoxanide are also effective against HBV-resistant mutants to lamivudine and adefovir. Additionally, both nitazoxanide and tizoxanide are potent inhibitors of HCV in genotype 1a- and 1b-derived replicon cells and genotype 2a cell culture models, and synergistic effects are observed when tizoxanide is combined with interferon^[3]. Three days of pretreatment of the HCV replicon model with nitazoxanide sensitizes the virus to the effects of subsequent treatment with interferon, providing support to the clinical studies underway using a nitazoxanide lead-in phase prior to combination therapy.

LACK OF DIRECT ANTIVIRAL RESISTANCE TO NITAZOXANIDE

Studies were carried out in HCV replicons exposed to increasing concentrations of nitazoxanide or tizoxanide over 24 wk in an attempt to produce resistance to nitazoxanide and tizoxanide^[5]. This serial passage did not reduce the susceptibility of HCV replicons to interferon, ribavirin, or 2'-C-methyl-cytidine, indicating that nitazoxanide and tizoxanide do not induce resistance to these agents.

TREATMENT OF CHRONIC HEPATITIS C

Completed studies

Romark made the decision to initially focus on the potential treatment of chronic hepatitis C with nitazoxanide. Three phase II studies of nitazoxanide for the treatment of chronic hepatitis C have been completed and communicated in publications or presentations at national and international meetings^[6-8]. The first study was a randomized, double-blind, placebo-controlled study of the treatment of chronic hepatitis C with nitazoxanide 500 mg twice daily in 50 adult patients with chronic hepatitis C infected with genotype 4^[6]. Seven of 23 patients (30%) had a virologic end-of-treatment response (ETR) with undetectable virus, and a sustained virologic response (SVR) with undetectable virus 24 wk after the completion of treatment was observed in 4 of 23 patients (17%). All responders had low serum HCV RNA levels less than 400 000 IU/mL. This study was the first use of nitazoxanide in patients with chronic hepatitis C and for

a longer time period than for its use for cryptosporidiosis and giardiasis, and the drug was well tolerated with the same number of mild gastrointestinal adverse events in the treated and placebo groups.

A second randomized, double-blind, placebo-controlled study (STEALTH C-1) evaluated the effects of nitazoxanide plus peginterferon alfa-2a (dual regimen) or nitazoxanide plus peginterferon alfa-2a and ribavirin (triple regimen) *versus* the standard of care (SOC) with peginterferon alfa-2a and ribavirin in 96 treatment-naive patients with chronic hepatitis C infected with genotype 4^[7]. Nitazoxanide 500 mg twice daily was administered over a 12-wk lead-in followed by an additional 36 wk in combination with peginterferon alfa-2a 180 µg weekly with or without ribavirin 1000-1200 mg daily. The SOC group received the same doses of peginterferon alfa-2a and ribavirin for 48 wk. A rapid virologic response (RVR; undetectable serum HCV RNA after 4 wk of combination therapy) occurred in 38%, 54%, and 64% of patients receiving the SOC, dual regimen and triple regimen, respectively ($P = 0.048$, SOC *vs* triple regimen). A complete early virologic response (cEVR; undetectable serum HCV RNA at week 12 of combination therapy) occurred in 70, 68 and 86% of the SOC, dual regimen and triple regimen, respectively. The SVR rates at 24 wk post-treatment were 50%, 61% and 79%, respectively, demonstrating a 29% difference between the SOC and the triple regimen with nitazoxanide ($P = 0.023$).

A third open label study was carried out in 44 patients; 40 were infected with genotype 4, and 3 were infected with genotype 1, and 1 infected with genotype 2^[8]. This study evaluated a shorter 4-wk lead-in phase with nitazoxanide 500 mg twice daily followed by 36 wk of treatment with a combination of nitazoxanide with peginterferon alfa-2a 180 µg weekly without ribavirin, and the results were compared with the historical results from the STEALTH C-1 study. The RVR, cEVR, and SVR rates were 59%, 82% and 80%, respectively, and the SVR rate of 80% in this study was significantly higher than the historical SVR rate of 50% in the SOC group ($P = 0.006$). The 3 patients infected with genotype 1 and the single patient infected with genotype 2 all had an SVR. The SVR rate of 80% raises the possibility using nitazoxanide in place of ribavirin, which requires further study.

In both this study and the STEALTH C-1 study, the administration of nitazoxanide in combination with peginterferon, with or without ribavirin, was associated with a low relapse rates (3 of 38 in the current study; and 3 of 20 patients receiving dual therapy and 1 of 23 patients receiving triple therapy, compared with 10 of 30 patients in the SOC arm in the STEALTH C-1 study). These low relapse rates, with or without ribavirin, suggest the possibility that nitazoxanide might play a similar role as ribavirin in reducing the relapse rate and be a possible substitute for ribavirin in combination with peginterferon for the treatment of chronic hepatitis C.

The results of these preliminary studies in Egypt in patients predominantly infected with genotype 4 were met with considerable interest in the hepatology community; but, studies conducted in the United States and the

response to nitazoxanide in combination with the SOC in patients with genotype 1 were recognized as the next steps required to confirm these initial interesting findings.

Ongoing studies

A phase II randomized, double-blind, placebo-controlled study with of nitazoxanide or placebo monotherapy (2:1 randomization) over a 4-wk lead-in phase followed by nitazoxanide or placebo in combination with peginterferon alfa-2a plus ribavirin is currently underway in 112 naive patients infected with genotype 1 at 13 USA study sites. The preliminary results of the early virologic responses will be communicated in early 2009.

A second phase II, randomized, double-blind, placebo controlled study of nitazoxanide with peginterferon and ribavirin is being conducted in 10 USA sites in 64 patients who are prior nonresponders to peginterferon and ribavirin. Preliminary results from this study will also be communicated in 2009.

CONTROLLED RELEASE TABLET

Studies have recently been initiated to study the pharmacokinetics, viral kinetics, and tolerability of a controlled release tablet of nitazoxanide in adult healthy volunteers as well as a phase II study in patients with chronic hepatitis C^[9]. The new nitazoxanide controlled release tablet contains 675 mg of the drug, and kinetics has been evaluated using either 675 mg or 1350 mg twice daily for 7 d in a phase I study. The pharmacokinetics profile is substantially improved compared to the standard tablet, with higher blood levels and an increased area under the curve of approximately 70%.

In a subsequent randomized, controlled trial, 40 treatment-naive patients with chronic hepatitis C genotype 4 have been allocated to receive either 675 mg or 1350 mg or placebo twice daily for 4 wk followed by the same regimen plus the addition of peginterferon alfa-2a 180 µg weekly and ribavirin 1000 or 1200 mg daily based on body weight. An early interim analysis has shown that the mean reduction in serum HCV RNA from baseline to week 8 (after 4 wk of combination therapy) were 5.45, 5.25, and 2.75 in the high-dose, low-dose and placebo groups, respectively^[9]. Nitazoxanide was well tolerated without gastrointestinal toxicity.

TREATMENT OF CHRONIC HEPATITIS B

Nitazoxanide alone has shown preliminary evidence of efficacy in the treatment of chronic hepatitis B over a one year course of therapy^[10]. Nitazoxanide 500 mg twice daily resulted in a decrease in serum HBV DNA in all of 4 HBeAg-positive patients, with undetectable HBV DNA in 2 of 4 patients, loss of HBeAg in 3 patients, and loss of HBsAg in one patient. Seven of 8 HBeAg-negative patients treated with nitazoxanide 500 mg twice daily had undetectable HBV DNA and 2 had loss of HBsAg. Additionally, nitazoxanide monotherapy in one case and nitazoxanide plus adefovir in another case resulted in undetectable HBV DNA, loss of HBeAg and loss of

HBsAg^[11]. These preliminary studies showed a higher rate of HBsAg loss than any currently licensed therapy for chronic hepatitis B. The similar mechanism of action of interferon and nitazoxanide suggest that stand-alone nitazoxanide therapy or nitazoxanide in concert with nucleos(t)ide analogs have the potential to increase loss of HBsAg, which is the ultimate end-point of therapy. A formal phase II study is being planned for 2009.

SECOND GENERATION THIAZOLIDES

Newer thiazolide analogs have been identified with higher specific activity against HBV and HCV. The objective of this search for alternative thiazolidines is to identify compounds that do not have antiparasitic or antibacterial activity *via* direct inhibition against the pyruvate-ferredoxin oxidoreductase reaction and increased antiviral activity. There are several lead compounds that have undergone preliminary study in experimental replicon models and have the potential to move into clinical trial.

CONCLUSION

Nitazoxanide shows significant activity against HBV and HCV, both in cell culture models as well as in patients with chronic hepatitis B and C. The HCV antiviral mechanism of action has been elucidated to involve up-regulation of PKR and eIF2 α phosphorylation, which enhances natural cellular antiviral mechanisms without induction of antiviral resistance. Second generation molecules in controlled release formulations are in development. Interim results of the American phase II trial involving treatment of naive and nonresponder patients with chronic hepatitis C infected with genotype 1 will be reported in early 2009. The thiazolidines have a very favorable toxicity profile with a very low incidence of mild gastrointestinal side effects.

There are many future potential uses of nitazoxanide in treatment regimens for chronic hepatitis C. Nitazoxanide might allow reduction in the duration of a standard peginterferon-based regimen, as the results in the phase II studies of patients with genotype 4 were achieved with 36 wk of combination therapy versus the usual 48 wk used in the current SOC. Further studies using even shorter duration of combination therapy, i.e. 24 wk, are warranted. It is also possible that the interferon-like mechanism of action of nitazoxanide will allow use of a reduced dose of peginterferon in combination treatment regimens. The findings of high SVR rates with nitazoxanide in combination with peginterferon without ribavirin require confirmation, but raise the possibility that nitazoxanide might be used in the place of ribavirin and avoid the substantial hematologic and other side effects of this drug. Finally, nitazoxanide might be used with a reduced dose of peginterferon or even without peginterferon in combination with a protease inhibitor and/or polymerase inhibitor as part of an all-oral cocktail for treatment of chronic hepatitis C, avoiding the need for injectable interferon with all of its side effects.

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Olive oil consumption and non-alcoholic fatty liver disease

Nimer Assy, Faris Nassar, Gattas Nasser, Maria Grosovski

Nimer Assy, Liver Unit, Ziv Medical Center, Safed, Israel;
Faculty of Medicine, Technion Institute, Haifa 32000, Israel

Faris Nassar, Gattas Nasser, Department of Medicine,
Western Galilee Hospital, Nahariya 22356, Israel

Maria Grosovski, Department of Biotechnology, Ort-Braude
College, Karmiel 20100, Israel

Author contributions: Assy N wrote the paper; Nasser G and
Nassar F helped in collecting data; Grosovski M analyzed the
data.

Correspondence to: Dr. Nimer Assy, Liver Unit, Ziv Medical
Centre, Safed 13100, Israel. assy.n@ziv.health.gov.il

Telephone: +97-246828441/5 Fax: +97-246828442

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Abstract

The clinical implications of non-alcoholic fatty liver diseases (NAFLD) derive from their potential to progress to fibrosis and cirrhosis. Inappropriate dietary fat intake, excessive intake of soft drinks, insulin resistance and increased oxidative stress results in increased free fatty acid delivery to the liver and increased hepatic triglyceride (TG) accumulation. An olive oil-rich diet decreases accumulation of TGs in the liver, improves postprandial TGs, glucose and glucagon-like peptide-1 responses in insulin-resistant subjects, and upregulates glucose transporter-2 expression in the liver. The principal mechanisms include: decreased nuclear factor-kappaB activation, decreased low-density lipoprotein oxidation, and improved insulin resistance by reduced production of inflammatory cytokines (tumor necrosis factor, interleukin-6) and improvement of jun N-terminal kinase-mediated phosphorylation of insulin receptor substrate-1. The beneficial effect of the Mediterranean diet is derived from monounsaturated fatty acids, mainly from olive oil. In this review, we describe the dietary sources of the monounsaturated fatty acids, the composition of olive oil, dietary fats and their relationship to insulin resistance and postprandial lipid and glucose responses in non-alcoholic steatohepatitis, clinical and experimental studies that assess the relationship between olive oil and NAFLD, and the mechanism by which olive oil ameliorates fatty liver, and we discuss future perspectives.

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Key words: Liver steatosis; Fatty liver; Olive oil; Fatty

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Peer reviewer: Eddie Wisse, Professor, Irisweg 16,
Keerbergen 3140, Belgium

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consumption and non-alcoholic fatty liver disease. *World J
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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) occur in 10%-24% of the general population^[1]. The potential to progress to fibrosis (20%-40%), cirrhosis (30%) and hepatocellular carcinoma^[1-3] makes these conditions clinically important. Obesity, diabetes, hyperlipidemia, and the intake of soft drink beverages are risk factors frequently associated with NAFLD^[4,5].

The pathogenesis of NASH includes insulin resistance, increased inflammation, tumor necrosis factor (TNF)- α , interleukin (IL)-6 and increased oxidative stress^[6]. The etiologic mechanism of NAFLD includes increased influx of free fatty acids (FFAs) to the liver from dietary triglycerides (TGs) and from FFAs that are released from adipocytes during fasting, reduced FFA β -oxidation, reduced hepatic secretion of TG-rich very low density lipoprotein (VLDL), and increased lipid peroxidation^[6]. An impaired postprandial TG response has been recently reported in patients with NASH and may play a pathophysiologic role by favoring TG accumulation in the liver^[7].

Diet and nutrition, in particular the amount and type of fat intake, has been linked to insulin resistance, an increased risk of developing type 2 diabetes and impaired postprandial lipid metabolism^[8,9]. In addition, animal models and human studies suggest that dietary factors can affect fatty infiltration and lipid peroxidation in various types of liver disease including NAFLD^[10,11]. More recently, increased ingestion of soft drinks was found to be linked to NAFLD^[5]. Although few studies of the effects of different diets on NAFLD have been performed in humans, a Mediterranean diet has been proposed for the prevention of metabolic syndrome, hypertension and cardiovascular disease^[12]. The major

part of its beneficial effect is a high supply of energy coming from monounsaturated fatty acids (MUFAs), mainly from olive oil. The principle fatty acid esters present in normal liver are palmitate (16:0) and oleate (18:1 n-9). In patients with alcoholic fatty liver, the proportion of linoleate (C18:2 n-6) and linolenic acid (C18:3 n-3) decreases and the proportion of oleate (C18:1 n-9) increases compared with diabetics with fatty liver and control subjects who underwent liver biopsies^[13].

In this review, we describe dietary habits and their relationship to insulin resistance and postprandial glucose and TG levels in NASH, the mechanism by which olive oil ameliorates fatty liver, experimental and clinical studies of olive oil and NAFLD, and future perspectives.

COMPOSITION OF OLIVE OIL

Each 100 g of olive oil contains the following fatty acids: MUFA 73.7 g (n-9 oleic acid 18:1); saturated fatty acids (SFA) 13.5 g (16:0 palmitic acid); polyunsaturated fatty acids (PUFA) 7.9 g (n-6 linoleic acid 18:2, and n-3 alpha-linolenic acid 18:3)^[14].

MUFAs include palmitic (C16:1), oleic (C18:1), elaidic (C18:1) and ventic acids (C18:1). The most abundant MUFA in the diet is oleic acid (C18:1 n-9)^[15]. In Mediterranean countries, the main source of MUFA is olive oil (74 g/100 g). Other oil sources of MUFAs are canola (59 g/100 g), peanut (46 g/100 g), sunflower (32 g/100 g), corn (29 g/100 g), soybean (24 g/100 g) and safflower oils (14 g/100 g)^[16]. Additionally, new oil variants, rich in oleic acid have been developed including high-oleic acid sunflower oil (84 g/100 g) and high oleic acid safflower oil (74 g/100 g)^[17]. In addition to a high MUFA content, virgin (unrefined) olive oil contains a significant amount of antioxidants and α -tocopherol and phytochemicals. However, when refined or heated, olive oil loses these natural compounds^[18].

Olive oil is graded according to its acidity. Extra virgin olive oil, the first pressed oil, having maximum free acidity, contains an abundance of squalene and phenolic antioxidants including simple phenols (hydroxytyrosol, tyrosol), aldehydic secoiridoids, flavonoids and lignans (acetoxypinoresinol, pinoresinol). Interestingly, it contains significantly higher concentrations of phenolic antioxidants and squalene than refined virgin and seed oils. In addition, seed oils, which contain very low amounts of squalene, have none of the phenolic antioxidants that are present in virgin and refined olive oils^[19]. The exact composition of olive oil depends not only on the growth conditions in the year preceding the harvest, but also on the degree of ripeness of the fruit and the technical processing (cold pressing, refining)^[20].

PATHOPHYSIOLOGY OF NAFLD, DIETARY FAT AND HEPATIC LIPIDS

Fat metabolism in fatty liver

Excessive inappropriate dietary fat intake combined with peripheral insulin resistance, continued TG hydrolysis

via lipoprotein lipase and other genetic alterations in key lipid metabolic pathways results in increased blood FFA concentration^[21] leading to excessive muscle fat accumulation and increased liver concentration of TG and cholesterol esters. High blood TG concentration in the form of VLDL tends to accompany this condition and induces cholesterol ester transfer protein activity, resulting in an increased transfer of TG from VLDL to high density lipoprotein (HDL) and a subsequent increase in HDL clearance and decreased HDL concentration^[21].

Insulin resistance in fatty liver

Peripheral insulin resistance affects carbohydrate and fat metabolism causing TG accumulation in the liver. Resistance to insulin stimulation of glucose uptake *via* glucose transporter-4 by skeletal muscle and adipose tissue, in conjunction with the inhibition of lipolysis in adipose tissue by insulin, diverts glucose to the liver where the insulin continues to stimulate de novo lipogenesis and increase the flux of fatty acids from adipose tissue to the liver^[6,22]. As a result, the liver TG concentration increases. It is unclear how impairment in lipid export *via* VLDL secretion, β -oxidation of FFAs, or other metabolic pathways result in an inability to maintain fat balance, which leads to the development of fatty liver^[22].

Fat induces hepatic insulin resistance

The mechanism underlying fat-induced hepatic insulin resistance is not understood. Recent evidence points to an accumulation of fat metabolites (IL-6, TNF- α) that activate various signal transduction pathways, such as serine/threonine kinases, i.e. protein kinase-C (PKC), c-JUN NH2-terminal kinase-1, (JNK) and inhibitor of kappa B kinase, as a key event in the pathway of fat-induced hepatic insulin resistance. Downstream effects include: nuclear factor-kappaB (NF- κ B) and activator protein-1 translocation to the nucleus resulting in increased production of inflammatory cytokines which inhibit insulin action^[6,23] (Figure 1). Under conditions of insulin resistance, excess lipid metabolites such as diacylglycerol can cause insulin resistance by activating PKC which binds to the insulin receptor and inhibits its tyrosine kinase activity. The activation of PKC may also interfere with the ability of insulin to phosphorylate insulin receptor substrate-2^[24].

Relationships among dietary habits, insulin resistance, postprandial lipemia and fatty liver

In the setting of excessive dietary fat intake, high levels of FFAs are delivered to the liver. Hepatocyte stimulation by FFAs leads to the intracellular translocation of Bax to the lysosome. Lysosomal permeability is increased, leading to release of cathepsin B. The presence of cathepsin B in the cytosol causes NF- κ B translocation into the nucleus with increased production and release of TNF- α which inhibits insulin action. Cathepsin B also induces mitochondrial dysfunction leading to hepatocyte apoptosis and progression from fatty liver to

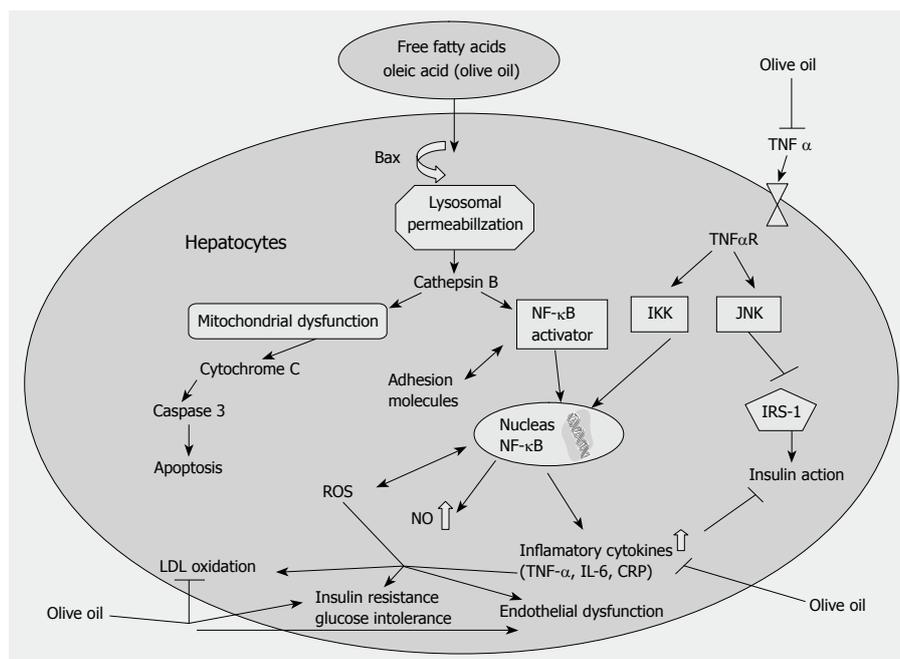


Figure 1 Molecular mechanism of the benefit of oleic acid in NAFLD. Increased levels of TNF- α leads to activation of stress-related protein kinases (IKK β , JNK) which induce NF- κ B translocation to the nucleus, resulting in increased production of inflammatory cytokines and reduced insulin sensitivity. Insulin sensitivity is further impaired by JNK-mediated phosphorylation of IRS-1. In the postprandial setting or after excessive inappropriate dietary intake, free fatty acids are delivered to the liver, taken up and accumulated in hepatocytes. This leads to the intracellular translocation of Bax to the lysosomes which leads to increased cathepsin B. This causes NF- κ B translocation to the nucleus with increased production and release of TNF- α and increased insulin resistance. Cathepsin B also causes mitochondrial dysfunction leading to hepatocyte apoptosis and progression to NASH. The role of olive oil in decreasing NF- κ B activation, decreasing LDL oxidation and in improving insulin resistance is illustrated.

steatohepatitis (Figure 1)^[25].

An impaired postprandial TG response has been reported in patients with NASH. This may promote the infiltration of fat into the liver by increasing TG uptake in the postprandial period^[26]. Enhanced lipogenesis appears as a prominent abnormality of hepatic fatty metabolism in subjects with NASH; the contribution of hepatic lipogenesis to TG secretion was 3 \times higher in patients with NAFLD as compared to healthy controls^[27]. NASH patients had significantly higher overnight fasting glucose or FFAs than controls, as well as higher saturated and monounsaturated levels in both studied lipid fractions, mainly due to an increase in palmitate, palmitoleate and oleic acids^[28]. NASH patients showed depletion of PUFAs (n-3 and n-6) in liver triglycerols. This results from defective PUFA desaturation or from a higher lipid peroxidation^[28]. The diet of NAFLD patients who were free of hyperlipidemia, diabetes and obesity was richer in saturated fat and poorer in PUFAs^[29]. Finally, a MUFA-rich diet improves postprandial glucose, lipid and glyp-1 responses in insulin-resistant subjects. Ingestion of an olive oil-based breakfast decreased postprandial glucose and insulin levels^[30].

DIETARY MUFAs AND NAFLD: HUMAN STUDIES

The beneficial effect of MUFAs such as those found in olive oil, nuts and avocados, on risk of cardiovascular disease and on lipid profile has been studied^[31]. Dietary MUFA (oleic acid) decreased oxidized LDL^[32,33], LDL cholesterol and TG concentration without the concomitant decrease in HDL^[34,35]. Additionally, replacement of carbohydrate and saturated fat with MUFAs led to a reduction in glucose and blood pressure and to an increase in HDL in patients with diabetes^[36]. A MUFA-rich diet (40% of energy as

fat), also decreased VLDL cholesterol and VLDL triglycerol and was more acceptable to patients with non insulin-dependent diabetes mellitus (NIDDM) than was a higher carbohydrate diet (28% of energy as fat)^[37]. A meta-analysis of studies in individuals with diabetes showed that a high fat diet with 22%-33% of the energy from MUFAs resulted in lower plasma total cholesterol, VLDL, and TG levels than did a low fat, high carbohydrate (49%-60% energy) diet^[38]. Therefore, an increase in intake of MUFAs, particularly as a replacement for SFA and as a higher proportion in the diet, instead of carbohydrate, may be beneficial for NAFLD patients. It has been demonstrated that consumption of MUFAs decreases blood TGs by increasing fatty acid oxidation through activation of peroxisome proliferator-activated receptor (PPAR) α or by reducing the activation of sterol regulatory element binding protein (SREBP) and inhibiting lipogenesis. Dietary MUFAs activate PPAR α and PPAR γ , increasing lipid oxidation, and decreasing insulin resistance leading to a reduction in hepatic steatosis^[39].

NAFLD, hypertension and hypertriglyceridemia are major components of metabolic syndrome. Four clinical studies have documented the beneficial effect of MUFAs in decreasing blood pressure^[40-43]. Moreover, another 6 dietary trials assessing the effect of MUFA intake on blood pressure showed beneficial effects^[44]. Although there are some inconsistencies in these studies, MUFA from olive oil in the context of the Mediterranean diet, plays a role in the primary prevention of NAFLD.

DIETARY MUFAs AND NAFLD: ANIMAL STUDIES

Recently, the authors evaluated the effect of different types of dietary fats on the hepatic lipid content and oxidative stress parameters in the livers of rats with

Table 1 Effect of olive oil on percentage of fatty acids in rat liver

Fatty acid components	Control	MCDD	MCDD + olive oil
C14:0 myristic	0.2 ± 0.2	0.5 ± 0.1	0.3 ± 0.2
C16:0 palmitic	19 ± 1	17 ± 1.1	15.3 ± 1.4
C16:1 palmitoleic	0.1 ± 0.2	0.7 ± 0.1	0.4 ± 0.2
C18:0 Stearic	21.8 ± 1.6	5.8 ± 0.6	5.7 ± 0.7
C18:1n9t elaidic	2.1 ± 0.1	1.5 ± 1.3	22.8 ± 1.3
C18:1n9c oleic	4.9 ± 0.8	20.7 ± 0.7	25.9 ± 3.3
C18:2n6c linolelaidic	18.6 ± 1.8	32.4 ± 0.7	30.5 ± 1.7
C18:3n3 linolenic	0.5 ± 0.3	0.3 ± 0.1	0.2 ± 0.2
C23:0 tricosanoic	22.0 ± 2.4	10.0 ± 1.1	7.8 ± 2.1
C20:4n6 arachidonic	0.1 ± 0.2	0	9.2 ± 0.6
C22:6n3 docosahexaenoic	4.5 ± 0.9	1.3 ± 0.5	1.2 ± 0.4
C20:5n3 eicosapentaenoic	0	0.1 ± 0.1	0

Enrichment of a MCDD by olive oil increases the oleic acid, long chain PUFA n6:n3 ratio, and arachidonic acid percentages in the rat livers. Data from reference 39.

experimental NAFLD^[45]. The study demonstrated that olive oil decreases the accumulation of TGs in the liver of rats. Severe fatty liver was seen in methionine-choline deficient diet (MCDD), MCDD + fish oil and in MCDD + butter fat groups, but not in the MCDD + olive oil group. The hepatic TG increase in the MCDD + olive oil group was blunted by 30% compared with the MCDD group. The serum TG increase was 10% lower in the MCDD + olive oil group compared with the MCDD group. In comparison with the control group, the long chain PUFA n6:n3 ratio increased in the MCDD + olive oil group by 345-fold (Table 1). Olive oil improved insulin resistance, increased the release of TG from the liver and decreased the flux of FFAs from peripheral adipose tissue back to the liver^[45]. A study from Spain showed that treatment with a balanced diet rich in olive oil contributed to the recovery of the liver from hepatic steatosis^[46]. This was achieved by decreasing activation of hepatic stellate cells by MUFAs, which are less susceptible to lipid peroxidation compared to PUFAs. Moreover, previous studies carried out in fibrotic rats showed that olive oil, in contrast to polyunsaturated oils, could protect against the development of fibrosis^[47]. In animal studies, saturated fat significantly increased insulin resistance, long and short chain omega-3 fatty acids improved it, and the effect of MUFAs and omega-6 polyunsaturated acids ranged somewhere between the two^[48]. In humans, shifting from a diet rich in SFA to one rich in MUFAs improved insulin sensitivity in healthy people^[49]. A MUFA-rich diet prevented central body fat distribution, improved insulin sensitivity and increased postprandial adiponectin expression compared to a carbohydrate-rich diet (with similar caloric intake) in insulin-resistant subjects^[50]. Furthermore, fasting plasma leptin fell during a MUFA-rich diet and has been associated with improved insulin action^[51]. Weight maintenance with a MUFA-rich diet improved homeostasis model assessment (HOMA) and fasting pro-insulin levels in insulin-resistant subjects. Ingestion of a virgin olive

Table 2 Mechanisms of action of olive oil on fatty liver

Mechanism	Component involved
Anti-inflammatory and immunomodulatory effects	Oleic acid
Anti-oxidants:	Phenolic compounds
Decrease lipid peroxidation	Oleic acid
Decrease oxidative DNA damage	Phenolic compounds: hydroxytyrosol, oleuropein, caffeic acid, o-coumaric acid, vanillic acid, and 3,4-dihydroxyphenylethanol (3,4-DHPEA).
Modulation of transduction pathways:	Oleic acid
Decreases arachidonic acid	Phenolic compounds: protocatechuic acid
Inhibits lipooxygenase	Hydroxytyrosol
Inhibits HMG-CoA reductase	Squalene
Decreases RAS activation	Squalene
Regulation of gene expression in liver regeneration:	Oleic acid
(Oleic acid inhibits δ 6-desaturase which decreases PGE2 and inhibits liver regeneration)	Minor compounds
Change in membrane fluidity and membrane peroxidation (estrogen modulator, regulates G protein)	Oleic acid Lignans

oil-based breakfast decreased postprandial glucose, triacylglycerol, and insulin concentrations, and increased HDL cholesterol and glucagon-like peptide-1 (GLP-1) concentrations as compared with a carbohydrate-rich diet^[50,52]. The Mediterranean diet elicits a less prothrombotic environment by modifying different hemostatic components, such as platelet aggregation, fibrinogen, Von Willebrand factor, plasma factor VII, tissue factor and plasminogen activator inhibitor type 1 plasma levels. The postprandial increase in activated factor VII is reduced by the intake of virgin olive oil in comparison with saturated fat^[53].

THE SPECIAL MECHANISM OF OLIVE OIL

Olive oil has traditionally been the principal oil of the Mediterranean diet. The MUFA diet prevents central body fat accumulation and decreases postprandial adiponectin expression induced by a carbohydrate-rich diet in insulin-resistant subjects^[50,54]. Mechanistic studies show a direct beneficial role for olive oil in improving plasma lipids in the treatment of metabolic syndrome^[44]. Unrefined or virgin olive oil has bioactive compounds with beneficial antioxidants action (Table 2)^[48]. Oleocanthal, a component found in extra virgin olive oil, is a natural anti-inflammatory compound that has a potency and profile strikingly similar to that of ibuprofen^[55]. The exact mechanism through which MUFAs and olive oil could modify hepatic TG content is not clear. Oleic acid from cooking oil was associated with lower insulin resistance in the general population^[56]. An olive oil-enriched diet contributes to redistribution of body fat and modifies the lipolytic efficiency of fat cells^[57]. Furthermore, n-9 fatty acids may regulate gene expression related to peripheral insulin sensitivity^[58],

increased endothelial vasoreactivity^[59], up-regulation of uncoupling protein mRNA in adipose tissue and muscle^[60], and expression of upregulates glucose transporter-2 in the liver^[61]. Oleic acid decreases the expression of genes involved in hepatic gluconeogenesis and lipogenesis and SREBP in Zucker fatty rats^[62]. Additional effects of olive oil beyond its MUFA composition relate to its polyphenols. Polyphenols present in olive oil, such as oleuropin, hydroxytyrosol, tyrosol and caffeic acid, have an important antioxidant and anti-inflammatory effect^[63,64]. In rat leukocytes, these molecules have been shown to inhibit leukotriene B4 generation at the 5-lipoxygenase level and to reduce the generation of reactive oxygen species^[65]. Moreover, a diet rich in olive oil improves endothelial function compared with a high carbohydrate diet or a high linoleic acid diet^[66,67]. Finally, the consumption of olive oil as a single item or within a Mediterranean diet showed a significant inverse association with TNF- α and vascular cell adhesion molecule-1 serum levels^[68], and improved glycemic tolerance through increased secretion of GLP-1^[69]. Moreover, an oleic acid-rich Mediterranean type diet may reduce the risk of atherosclerosis by decreasing the number of chylomicron remnant particles as compared to a linoleic acid-enriched diet^[70]. The principal mechanisms of action of olive oil are a decrease in NF- κ B activation, a decrease in LDL oxidation and an improvement in insulin resistance (Figure 1).

CONCLUSION

Dietary fat content modifies liver fat in overweight non diabetic subjects^[71]. NAFLD patients have a higher postprandial TG response and an increased production of large VLDL after an oral fat load compared with controls, despite normal fasting blood lipid concentration, which suggests that the metabolism of dietary fat is impaired in these individuals^[72]. Decreasing total fat consumption and shifting to MUFAs found in olive oil (20%-40% of total energy) or n-3 PUFAs found in fish oil (2 g/d) could lead to a decrease in postprandial lipidemia and steatosis^[73]. Further studies in humans are needed to ascertain whether the consumption of olive oil may be helpful in NAFLD patients.

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Inhibitory effect of acetylshikonin on human gastric carcinoma cell line SGC-7901 *in vitro* and *in vivo*

Yun Zeng, Gang Liu, Li-Ming Zhou

Yun Zeng, Gang Liu, Li-Ming Zhou, Department of Pharmacology, West China Medical Center of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041, Sichuan Province, China

Yun Zeng, Gang Liu, Institute of Materia Medica and Department of Pharmacology, North Sichuan Medical College, Nanchong 637007, Sichuan Province, China

Author contributions: Zeng Y and Liu G contributed equally to this work; Zeng Y, Liu G and Zhou LM designed the research; Zeng Y, Liu G and Zhou LM performed the research; Zeng Y, Liu G and Zhou LM analyzed the data; Zeng Y, Liu G and Zhou LM wrote the paper.

Correspondence to: Li-Ming Zhou, Professor, Department of Pharmacology, West China Medical Center of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041, Sichuan Province, China. zhou108@163.com

Telephone: +86-28-85503767 Fax: +86-28-85503767

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Abstract

AIM: To investigate the inhibitory effect of acetylshikonin on human gastric carcinoma cell line SGC-7901 and its mechanism.

METHODS: MTT assay was used to assess the inhibitory effect of acetylshikonin on proliferation of SGC-7901 cells. Apoptosis-inducing effect was determined by flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling with Hoechst staining. Expression of mRNA and protein in Bcl-2 and Bax was analyzed by reverse transcription-polymerase chain reaction and Western blot. Antitumor effect of acetylshikonin on a mouse SGC-7901 model was also determined.

RESULTS: Forty-eight hours after treatment with acetylshikonin, MTT assay showed that acetylshikonin inhibited the proliferation of SGC-7901 cells in a dose-dependent manner. The half maximal inhibitory concentration of acetylshikonin to SGC-7901 cells was 0.428 ± 0.07 mg/L. Cell shrinkage, nuclear pyknosis and chromatin condensation, which are the characteristics of cell apoptosis, were observed in treated SGC-7901 cells and the percentage of apoptosis increased in a dose-dependent manner. Acetylshikonin down-regulated the expression of Bcl-2 and up-regulated the expression of Bax in the treated SGC-7901 cells compared with the controls. The experiment *in vivo*

showed that 0.5, 1, and 2 mg/kg of acetylshikonin significantly inhibited the growth of tumor in the mouse SGC-7901 model, with an inhibitory rate of 25.00%-55.76%.

CONCLUSION: Acetylshikonin inhibits the growth of SGC-7901 cells *in vitro* and *in vivo* by inducing cell apoptosis.

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Key words: Acetylshikonin; Antitumor effect; SGC-7901 cells; Apoptosis

Peer reviewer: Meenakshisundaram Ananthanarayanan, Associated Professor, Department of Pediatrics, Annenberg Bldg, Rm.14-24A, Box 1664, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY, 10029, United States

Zeng Y, Liu G, Zhou LM. Inhibitory effect of acetylshikonin on human gastric carcinoma cell line SGC-7901 *in vitro* and *in vivo*. *World J Gastroenterol* 2009; 15(15): 1816-1820 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1816.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1816>

INTRODUCTION

Although advances have been made in clinical medicine, no effective treatment modalities are available for patients with advanced or metastatic tumors^[1]. Gastric cancer is one of the most common malignant tumors worldwide, and its incidence and mortality rank first in China^[2,3]. At present, gastric cancer patients have certain clinical responses to chemotherapy or radiation therapy, but they cannot tolerate it well^[4,5]. Therefore, it is absolutely necessary to explore drugs capable of preventing and treating gastric cancer and other malignancies.

Chemotherapy is one of the main treatment modalities for advanced cancer^[6]. It has been shown that acetylshikonin, one of the shikonin derivatives, is effective against sterilized and infected wounds^[7,8]. It has been demonstrated that acetylshikonin can inhibit tumor-induced cutaneous angiogenesis^[9] and has obvious antitumor effects on S180 sarcoma model^[10]. However, little is known about the effect of acetylshikonin on gastric cancer *in vitro*, especially *in vivo*. This study was to evaluate the ability of acetylshikonin to inhibit cell proliferation and induce apoptosis of human gastric cancer SGC-7901 cells. Our results demonstrate

that acetylshikonin could induce apoptosis by down-regulating Bcl-2 and up-regulating Bax in SGC-7901 cells. Significant antitumor effects of acetylshikonin could also be observed in a SGC-7901 tumor xenograft model of nude mice.

MATERIALS AND METHODS

Cell line and reagents

Human gastric carcinoma cell line SGC-7901 was obtained from the Institute of Materia Medica, North Sichuan Medical College (Nanchong, China). The cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Minhai Bioengineering Co., Lanzhou, China), and maintained at 37°C in a humidified atmosphere containing 50 mL/L CO₂. Acetylshikonin was obtained from Huakang Pharmaceutical Company (Deyang, China). Cyclophosphamide was provided by Hengrui Pharmaceutical Company (Jiangsu, China). MTT was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Cell viability assay

Effect of acetylshikonin on the viability of SGC-7901 cells was detected by MTT assay^[11]. Cells were plated in a 96-well plate (5 × 10³ cells/well) for 24 h, and then treated with different concentrations of acetylshikonin (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 mg/L). Tetrazolium dye, 3-(4, 5-dimethylthiazol-2)-2, 5-diphenyl-2H-tetrazolium bromide (MTT, Sigma; 5 mg/mL in PBS) was added to the medium for 4 h, and measured by spectrophotometry after 15 min at room temperature. Optical density (OD) which reflects the viable cell population in each well was determined.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay and cell morphology assessment

For apoptosis studies, cells were plated on glass cover slips. Samples were assessed 24 h after plated for the detection of TUNEL-positivity (brown cellular nuclei appeared) using an *in situ* cell death detection kit (Roche Diagnostic Corp., USA). Apoptotic rates of cells were also determined with Hoechst staining and cells showing condensed chromatin were defined as apoptotic^[12]. Percentage of apoptotic cells in each sample was counted under microscope and averaged over 10 fields (80-100 cells each).

Flow cytometry

Cells were harvested and pelleted at 1000 × *g* for 5 min, then fixed in 70% ethanol and washed with cold PBS. After suspended in 1 mL propidium iodide solution (50 µg/mL), 0.1% (w/v) sodium citrate, and 0.1% Triton X-100. Cells were incubated at 4°C in the dark for at least 15 min and analyzed with a flow cytometer (FACS, Beckman Coulter, USA) to determine the number of cells at each cell cycle and cell apoptosis rate as previously described^[11].

Western blot analysis

Expression of apoptosis-related proteins (Bcl-2 and

Bax) was analyzed by Western blotting as previously described^[11]. In brief, the cells were lysed in a lysis buffer at 4°C by sonication. Lysates were centrifuged at 15000 r/min for 15 min at 4°C. Proteins were separated on SDS-PAGE, transferred to nitrocellulose filters and incubated with antibodies against Bcl-2, Bax and actin, respectively. The membrane, were incubated with peroxidase-conjugated secondary antibodies, and detected with an enhanced chemiluminescence reagent.

Expression of Bcl-2 and Bax mRNA

Expression of Bcl-2 and Bax was semi-quantitatively detected by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the cells using the TRIzol reagent (Gibco) and reverse transcribed with the SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Sequences of the primers used for RT-PCR are listed in Table 1. PCR for Bcl-2 was performed at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, at 70°C for 30 s, at 72°C for 45 s and a final extension at 72°C for 7 min. PCR for Bax was performed at 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, at 57°C for 30 s, at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were separated on 10 g/L agarose gel stained with ethidium bromide and observed under ultraviolet light.

In vivo anti-tumor activity

Seven-week old male nude mice (supplied by Experimental Animal Center, West China Center of Medical Sciences, Sichuan University, China) were inoculated subcutaneously with transplanted human SGC-7901 gastric carcinoma cell line (5 × 10⁶ cells per mouse). Tumors were allowed to develop to about 75 mm × 75 mm × 75 mm. Thirty mice were randomized into 6 groups (*n* = 5). Mice in 3 acetylshikonin treatment groups received 0.5, 1 and 2 mg/kg acetylshikonin suspended in castor oil; mice in the castor oil group received an equal volume of castor oil; mice in control group received an equal volume of normal saline (NS), once a day for 15 consecutive days. Mice in the positive control group received cyclophosphamide (60 mg/kg) only on the first day. The animals were sacrificed and their tumors removed and weighed immediately. Tumor inhibitory rate was calculated according to the following formula: tumor inhibitory rate (%) = 1 - (W_{treated}/W_{control}) × 100%^[11].

Immunohistochemical staining

Thirty tumor tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Tumor tissue was cut into 4-µm thick sections. The sections were stained as previously described^[13]. Expressions of Bax, Bcl-2 and Caspase 3 were observed under an inverted phase contrast microscope. Photos were taken and analyzed by Image pro plus, and the integrated optical density (IOD) was calculated.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using Student's *t* test and chi-square test. *P* < 0.05 was considered statistically significant.

Table 1 Sequence of primers used in our study

	Product size	Sense primer	Anti-sense primer
Bax	311 bp	5'GCGTCCACCCAAGAAGCTGAG3'	5'ACCACCCTGGTCTTGGATCC3'
Bcl-2	280 bp	5'TGTGGCCTTCTTTGAGTTCG3'	5'TCACTTGTGGCTCAGATAGG3'
β -actin	300 bp	5'TCACCCACACTGTGCCATCTACGA3'	5'CAGCGGAACCGCTCATTGCCAATGG3'

Table 2 Effect of acetylshikonin on cell-cycle phase distribution and apoptotic ratios of SGC-7901 cells *in vitro* (mean \pm SD)

Concentration (mg/L)	Cell cycle phase distribution (%)			Apoptotic index (%)		
	G ₀ /G ₁	S	G ₂ /M	Hoechst	TUNEL	FACScan
0	53.73 \pm 4.61	36.29 \pm 7.32	9.98 \pm 2.35	5.76 \pm 0.58	5.34 \pm 0.64	6.15 \pm 0.75
0.2	54.82 \pm 4.12	33.45 \pm 6.05	11.73 \pm 3.90	11.05 \pm 2.10 ^a	11.56 \pm 1.62 ^a	10.13 \pm 1.69 ^a
0.4	56.92 \pm 5.04	30.15 \pm 5.84	12.93 \pm 5.32	28.76 \pm 3.55 ^b	30.89 \pm 2.35 ^b	25.40 \pm 2.46 ^b
0.8	57.12 \pm 2.94	28.58 \pm 5.21	14.30 \pm 4.79 ^a	52.25 \pm 6.16 ^b	56.33 \pm 5.78 ^b	48.73 \pm 6.55 ^b

Different concentrations of acetylshikonin were added to SGC-7901 cell culture for 24 h, cell-cycle phase distribution and apoptotic ratios were then analyzed by FACScan flow cytometry and TUNEL assay with Hoechst staining. Each experiment was independently performed three times. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

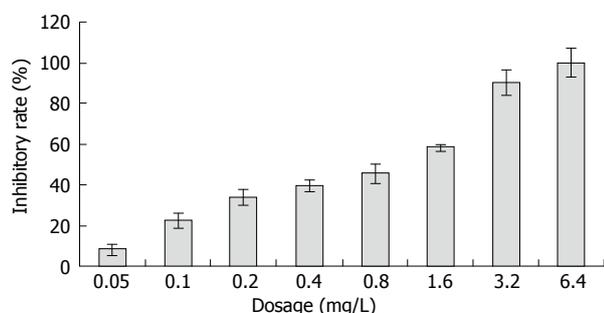


Figure 1 Effect of acetylshikonin on proliferation of SGC-7901 cells *in vitro*. SGC-7901 cells were treated with various concentrations of acetylshikonin for 48 h. Tumor growth inhibitory rate was determined by MTT assay.

RESULTS

Effect of acetylshikonin on the growth of SGC-7901 cells *in vitro*

The SGC-7901 cells were treated with various concentrations of acetylshikonin for 48 h and cell viability was determined by MTT assay. Acetylshikonin inhibited the growth of gastric cancer cells in a dose-dependent manner (Figure 1). Cell growth was suppressed by 33.7%, 39.3% and 45.5% 48 h after treatment with 0.2, 0.4 and 0.8 mg/L acetylshikonin, respectively. The inhibitory rate of 3.2 mg/L acetylshikonin for tumor cell growth was over 90%. The half maximal inhibitory concentration (IC₅₀) of acetylshikonin for SGC-7901 cells was 0.428 mg/L.

Acetylshikonin induced apoptosis of SGC-7901 cells

Twenty-four hours after exposure to acetylshikonin, SGC-7901 cells began to show morphologic features of apoptosis and some cells rounded up off the plate, exhibiting a smaller and circular shape. Cell cycle analysis revealed that the number of cells was different at the G₂/M phase after exposure to 0.2 and 0.4 mg/L acetylshikonin, but significantly decreased at the G₂/M phase after exposure to 0.8 mg/L acetylshikonin. Hoechst assay showed nuclear condensation, DNA

fragmentation and perinuclear apoptotic bodies after treatment with 0.2 mg/L acetylshikonin, indicating that acetylshikonin induces apoptosis in a dose-dependent manner (Table 2).

Acetylshikonin regulated the expression of Bcl-2 and Bax proteins

Western blot assay showed that acetylshikonin could induce or inhibit the expression of Bcl-2 and Bax. Twenty-four hours after treatment with indicated concentrations of acetylshikonin, the expression of Bax was markedly increased, while that of Bcl-2 had a trend to decrease. The Bax/Bcl-2 protein ratio was also elevated remarkably. The expression of actin remained unchanged (Figure 2).

Acetylshikonin regulated the expression of Bcl-2 and Bax mRNA

The expression of Bcl-2 and Bax was semi-quantitatively detected with β -actin as an internal standard. Acetylshikonin inhibited the expression of Bcl-2 in SGC-7901 cells in a dose-dependent manner. However, the expression of Bax mRNA increased with the concentration of acetylshikonin used (Figure 3).

Effect of acetylshikonin on inoculated tumors in mice

To determine whether acetylshikonin inhibits tumor growth *in vivo*, an equal number of SGC-7901 cells were injected into the right flanks of nude mice. Tumor growth was notably inhibited in mice after treatment with acetylshikonin (Figure 4). The inhibitory rate of tumor growth in 3 acetylshikonin groups and cyclophosphamide group was 55.76%, 39.42%, 25.00% and 58.65%, respectively. In addition, no toxicity judged by parallel monitoring of body weight was observed in acetylshikonin-treated mice. Immunohistochemistry data showed that the IOD value for Bcl-2 was 19.26 \pm 2.15 in the negative control group and 11.59 \pm 1.04 in the 2 mg/kg acetylshikonin group (Figure 5). However, the expression of Bax and Caspase 3 was markedly higher in 3 acetylshikonin groups than in negative control group.

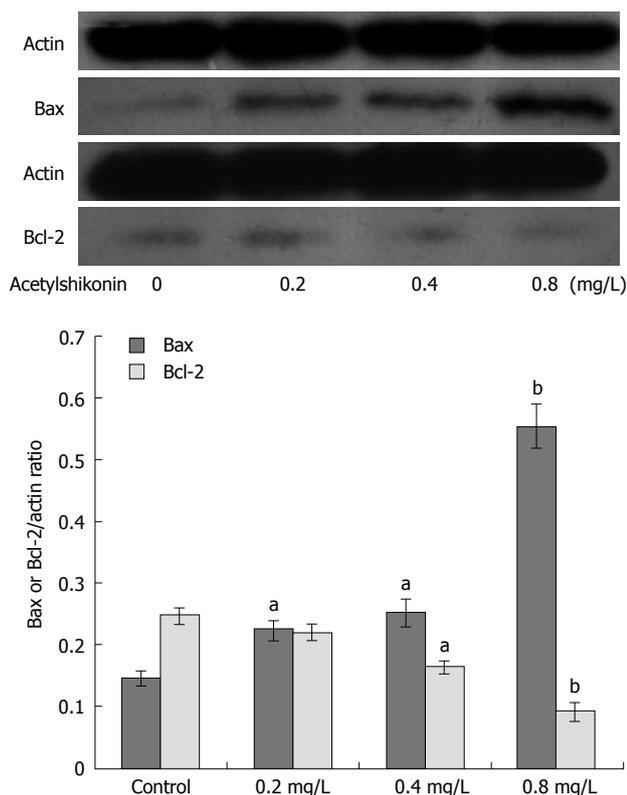


Figure 2 Effect of acetylshikonin on expression of Bcl-2 and Bax in SGC-7901 cells. Lower panel: Ratios of protein quantitation of Bcl-2 or Bax to actin. Each experiment was independently performed three times. ^a*P* < 0.05, ^b*P* < 0.01 vs control group.

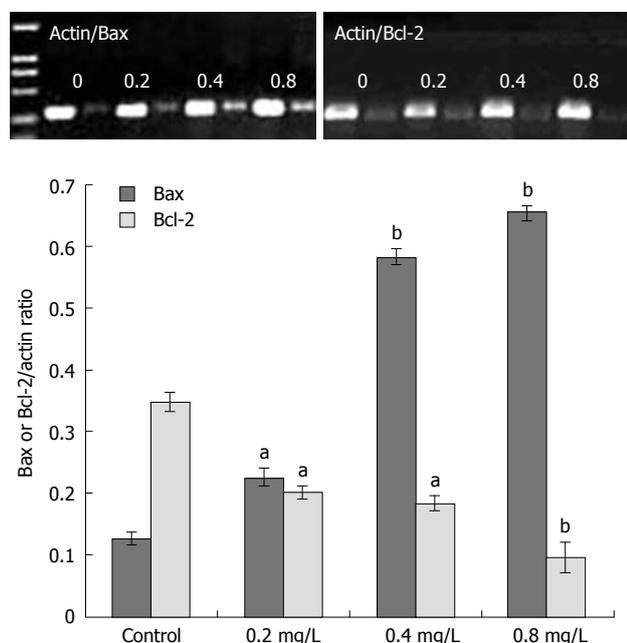


Figure 3 Effect of acetylshikonin on Bcl-2 and Bax mRNA expression levels in SGC-7901 cells. Lower panel: Ratios of mRNA quantitation of Bcl-2 or Bax to actin. Each experiment was independently performed three times. ^a*P* < 0.05, ^b*P* < 0.01 vs control group.

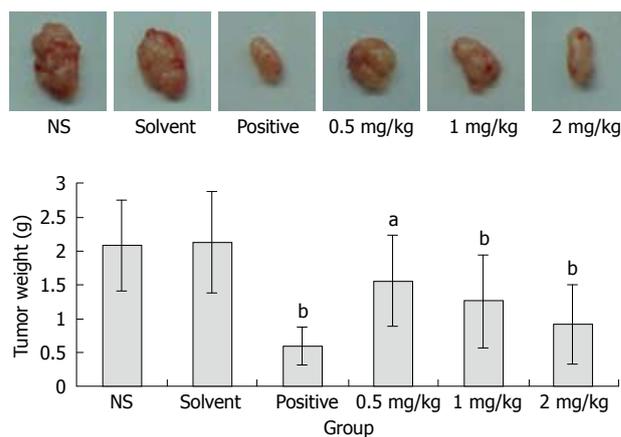


Figure 4 Anti-tumor effects of acetylshikonin *in vivo*. Representative tumors from each group were shown. Lower panel: Average tumor weight of sacrificed animals. ^a*P* < 0.05, ^b*P* < 0.01 vs control group.

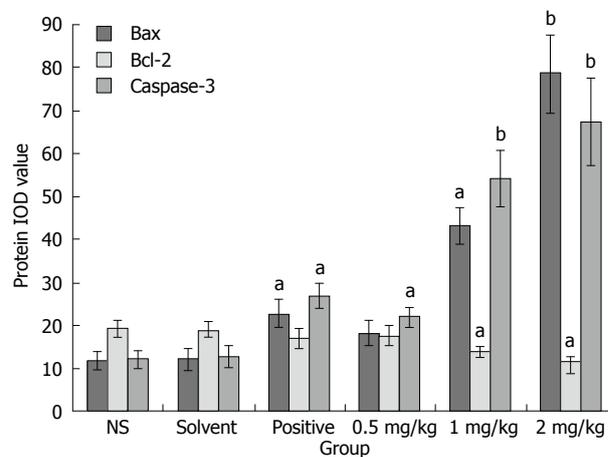


Figure 5 Effect of acetylshikonin on expression of Bax, Bcl-2 and Caspase-3 in SGC-7901 tumor tissues. Tumor tissue sections showed strong Bax/Caspase-3 cytoplasmic staining and weak Bcl-2 cytoplasmic staining of tumor cells in acetylshikonin treatment groups, as well as the integrated optical density (IOD) for the expression of Bax, Bcl-2 and Caspase-3 in SGC-7901 cells. ^a*P* < 0.05, ^b*P* < 0.01 vs control group.

DISCUSSION

Shikonin, isolated from roots of the medicinal herb *Lithospermum erythrorhizon* Siebold Zucc^[14], is

a Chinese herbal medicine with various biological activities, including anti-microbial, anti-fungal, anti-HIV, anti-inflammatory, anti-tumor, and wound-healing activities^[15-17]. However, its toxicity has limited it as a therapeutic agent^[14,17]. Acetylshikonin, one of the shikonin derivatives, has been shown to possess anti-cancer activity^[9,10] with less toxicity^[18], which has made acetylshikonin a promising anti-tumor agent. However, its effects on gastric cancer cells have not been reported.

In the present study, acetylshikonin suppressed the proliferation of SGC-7901 cells *in vitro*, and inhibited tumor growth in a SGC-7901 tumor xenograft model of nude mice. Generally, tumorigenesis and tumor progression are strongly associated with abnormal apoptosis. It has been shown that shikonin and its derivatives induce apoptosis of tumor cells^[16,19,20]. Our data demonstrate that acetylshikonin could also induce apoptosis of SGC-7901 cells with typical apoptotic alterations, including morphological changes and positive TUNEL assay, DNA fragmentation and apoptotic

sub-G1 peak.

Apoptosis is a complex process regulated by a variety of factors^[21,22]. Members of the Bcl-2 family are the important regulators in the apoptotic pathway with individual members that promote or suppress apoptosis. As an anti-apoptotic protein, Bcl-2 confers a negative control in the pathway of cellular suicide machinery, whereas Bax, a Bcl-2-homologous protein, promotes cell death by competing with Bcl-2^[23,24]. To explore whether apoptosis-related genes contribute to the inhibitory effect of acetylshikonin on SGC-7901 cells, the relative Bcl-2 and Bax expression levels induced by acetylshikonin were determined in this study. We found that acetylshikonin decreased the Bcl-2 expression while increased the Bax expression in a dose-dependent manner. Moreover, acetylshikonin up-regulated the expression level of Caspase 3 *in vivo*. Taken together, the reduced Bcl-2/Bax ratio, activating Caspases and cell apoptosis ultimately^[24], might serve as a mechanism of acetylshikonin underlying apoptosis of SGC-7901 cells.

In conclusion, acetylshikonin has significant antitumor effects both *in vitro* and *in vivo* by inducing apoptosis of SGC-7901 cells involved in down-regulation of Bcl-2 expression and up-regulation of Bax expression. Acetylshikonin can be used as a potent and selective agent in the treatment of human gastric adenocarcinoma.

COMMENTS

Background

Patients with gastric cancer have certain clinical responses to chemotherapy or radiation therapy, but they cannot tolerate it. Therefore, it is absolutely necessary to explore drugs capable of preventing and treating gastric cancer and other malignancies.

Research frontiers

Previous studies on shikonin, a chemical derived from a Chinese medicinal herb, have shown that shikonin has anti-tumor effects although it is toxic. Acetylshikonin, an acetyl derivative, has less toxicity and suppresses the growth of sarcomas. However, little is known about its effect on gastric cancer *in vitro*, especially *in vivo*.

Innovations and breakthroughs

The study demonstrated that acetylshikonin could exert significant effects on SGC-7901 cells both *in vitro* and *in vivo* by inducing cell apoptosis.

Applications

Acetylshikonin can be used as a potent and selective agent in treatment of human gastric adenocarcinoma.

Terminology

Apoptosis: A type of cell death, in which cells use a specialized cellular machinery to kill themselves, as well as a cell suicide mechanism enabling metazoans to control the cell number and eliminate them.

Peer review

The study analyzed the effect of acetylshikonin, derived from a Chinese medicinal herb, on gastric cancer cell line SGC-7901 *in vivo*, which is an interesting finding.

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Therapeutic effects of *Clostridium butyricum* on experimental colitis induced by oxazolone in rats

Hai-Qiang Zhang, Tomas T Ding, Jun-Sheng Zhao, Xin Yang, Hai-Xia Zhang, Juan-Juan Zhang, Yun-Long Cui

Hai-Qiang Zhang, Xin Yang, Hai-Xia Zhang, Juan-Juan Zhang, Qingdao Research Center of Microecology-Engineering Technology, Jiaonan 266400, Shandong Province, China

Tomas T Ding, BRITE/Department of Pharmaceutical Sciences, North Carolina Central University, Durham, NC 27707, United States

Jun-Sheng Zhao, Pathology Department of People's Hospital, Jiaonan 266400, Shandong Province, China

Yun-Long Cui, Qingdao East Sea Pharmaceutical Co. Ltd, Jiaonan 266400, Shandong Province, China

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Correspondence to: Yun-Long Cui, Qingdao East Sea Pharmaceutical Co. Ltd, 8 Shanghai Middle Road, Jiaonan 266400, Shandong Province, China. donghaicuiyunlong@yahoo.com.cn
Telephone: +86-532-87199876 Fax: +86-532-87199788

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Abstract

AIM: To evaluate the therapeutic effects of a probiotic supplement (*Clostridium butyricum*, CGMCC0313) in a chemically-induced rat model of experimental colitis.

METHODS: An experimental ulcerative colitis model was established by rectal injection of oxazolone into the colon of 40 Wistar rats randomly divided into four groups. The positive control group was sacrificed 3 d after colitis onset. The remaining groups were fed daily with either 2 mL of *C. butyricum* (2.3×10^{11} CFU/L), 2 mL of mesalamine (100 g/L), or 1 mL of sodium butyrate (50 mmol/L) for 21 d. The animals' body weight, behavior, and bowel movements were recorded weekly. After sacrifice, visual and microscopic observations of pathological changes of colon tissue were made, body weight and wet colon mass index were measured and recorded, and serum levels of interleukin-23 (IL-23) and TNF- α were measured using ELISA. Expression of calcitonin gene-related peptide in colon tissue was measured by RT-PCR. Finally, changes in rat intestinal microflora status were measured in all groups.

RESULTS: We found that treatment with *C. butyricum*

lowered the serum levels of both IL-23 and tumor necrosis factor- α (TNF- α) with similar or even better efficiency than that of mesalamine or sodium butyrate. The rat intestinal flora appeared to recover more quickly in the group treated with *C. butyricum* than in the mesalamine and sodium butyrate groups. Finally, we found that the expression level of calcitonin gene related peptide was elevated in colon tissue in the sodium butyrate treated group but not in the *C. butyricum* or mesalamine treated groups, indicating a sensitization of colon following sodium butyrate treatment.

CONCLUSION: In our experimental colitis model, treatment with *C. butyricum* CGMCC0313, a probiotic supplement, is at least as efficient as treatment with mesalamine.

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Key words: *Clostridium butyricum*; Interleukin-23; Tumor necrosis factor- α ; Calcitonin gene related peptide; Colitis

Peer reviewers: Jay Pravda, MD, Inflammatory Disease Research Center, Gainesville, Florida, 32614-2181, United States; Bettaieb Ali, PhD, Professor, Immunology and Immunotherapy of Cancer, INSERM U517, 7 Bd Jeanne d'Arc, Dijon 21079, France; Mohammad Abdollahi, Professor, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 1417614411, Iran

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INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD). The incidence rate of ulcerative colitis increases annually in China^[1,2]. A number of recent studies have been published that focus on the role of cellular inflammatory factors and related immune mechanisms during the onset and progression of IBD. Furthermore, animal experiments show that inflammation of the bowel can be induced by dysbiosis

of, or immune tolerance deficiencies related to, the intestinal microbiological flora^[3,4].

Steroid hormones, immunosuppressive agents or salicylic acid derivatives are used to treat IBD with modest results and often with serious side effects. Several recent studies, however, on the microecological therapy of UC using *Clostridium butyricum* preparations show promising results and have been drawing some attention in the biomedical research community^[5-9]. Sodium butyrate, although it is a confirmed anti-inflammatory agent for the treatment of experimental colitis^[10-14], can cause non-inflammatory colonic hypersensitivity^[15]. Calcitonin gene related peptide (CGRP) may increase organ sensitivity, and sensory afferents are implicated in peritoneal irritation of organs involved in inflammation^[16-18].

Based on animal experiments and clinical applications, we found that treatment of ulcerative colitis with *C. butyricum*, CGMCC0313.1 live bacterium, gives good results both in animal and human ulcerative colitis^[19-22]; but, the mechanisms are not yet fully understood. As a part of our effort to elucidate the therapeutic mechanisms of *C. butyricum* CGMCC0313.1, we used an oxazolone induced rat model of experimental colitis to measure the effect of intrarectally administered *C. butyricum* CGMCC0313.1 and two treatment controls on a set of UC relevant parameters.

Oxazolone is a chemical allergen and a sensitizing agent. Using oxazolone to induce colitis in rat constitutes a more satisfactory animal model of UC with a high degree of similarity to the histopathological characteristics and distribution of inflammation described in human UC^[23,24].

Thus, we compared *C. butyricum* CGMCC0313.1 with sodium butyrate and mesalamine (5-aminosalicylic acid, one of the standard prescriptions for ulcerative colitis) to measure each compound's effects on the repair of intestinal walls, on serum concentrations of interleukin-23 (IL-23) and tumor necrosis factor- α (TNF- α), on the level of CGRP-mRNA in rat colon tissue, and restoration of the balance of the intestinal microflora.

MATERIALS AND METHODS

Wistar SPF (specific-pathogen free) rats with body weight of 160-180 g were purchased from the Experimental Animal Center of Qingdao Institute for Drug Control, China. Oxazolone was purchased from Alfa Aesar (Great Britain). Mesalamine was purchased from Adepha Drug Group (France). Sodium butyrate was purchased from Sigma-Aldrich Chemical Company (St. Louis, Missouri, USA). *C. butyricum*, CGMCC0313.1 powder or capsules, were kindly provided by East Sea Pharmaceutical Co. Ltd, Shandong, China. Trizol was purchased from Invitrogen (USA). The transcription kit was purchased from Promega (USA). Primers were synthesized by Shanghai Biological Engineering Co., China. The EasyTek PCR Amplifier Kit was obtained from Shanghai Biological Engineering Co., China. The IL-23 ELISA Kit and TNF- α Kit was purchased from Wuhan Boster Biological Engineering Co. Ltd, China.

Establishing an animal model of experimental colitis

Our model was based on a method previously described by Lamprecht *et al*^[25] and modified by us. Briefly, we used 40 animals for testing. A 2 cm \times 2 cm area on the back of each animal was shaved to expose the skin. Using a cotton ball, 300 μ L Oxazolone (5% in absolute alcohol) was applied topically on the exposed area to induce an allergic reaction. After 5 and 7 d, 450 μ L of 5% oxazolone in a 50% ethanol solution, was injected using 1 mm diameter rubber tubing inserted into the colon through the rectum to about 8 cm proximal to the anal verge. To ensure even distribution of the oxazolone solution throughout the entire colon and cecum, the animals were kept in a vertical position for 45 s by holding them up by their tails after injections.

Groups and treatment

After the induction of colitis, the 40 treated animals were randomly divided into four groups. Thus, 10 animals belonged to the positive control (PC) group and received no treatment (three of these animals died during the study). Ten animals were assigned to the positive drug control (mesalamine, MA) group and received mesalamine treatment (two animals died during the study). Ten animals receiving probiotic treatment with *C. butyricum* CGMCC0313.1 (*C. butyricum*), were part of the probiotic (PB) group. Finally, 10 animals received treatment with sodium butyrate (two of these animals died during the study). A group of eight animals were kept as negative (NC) controls and received no oxazolone, nor any drug treatments. All animals in the mesalamine, probiotic and sodium butyrate groups were treated for 21 d, once per day, by feeding with either 2 mL of *C. butyricum* (2.3×10^{11} CFU/L), 2 mL of mesalamine (100 g/L), or 1 mL of sodium butyrate (50 mmol/L) *via* an orogastric tube. During the test period, animal behavior, bowel movements, and body weight was observed and recorded once per week. After 21 d of treatment, all animals were sacrificed by decapitation. The colons were cut longitudinally then cleaned with physiological saline. Excess water was removed with filtration paper before measuring the colon wet mass. Index of wet colon mass = colon wet mass (g)/body weight (kg). The colon tissue was subsequently perfused with a 10% formalin solution, gradually dehydrated with ethanol, embedded in paraffin and sliced into 5 μ m sections. The sections were wet mounted on glass slides and subjected to hematoxylin-eosin staining. Pathological changes in the animal tissues were identified under light microscope.

Measurement of IL-23 and TNF- α in rat serum

Blood was collected after decapitation and incubated overnight at 4°C. Serum was recovered after centrifugation and aliquoted. The aliquots were stored at -20°C for later tests. The serum levels IL-23 and TNF- α were measured using ELISA kits strictly following the manufacturer's instructions.

Extraction of total RNA from colon tissue

Colon tissue (100 mg) was ground to powder in liquid

nitrogen and 1 mL Trizol was added. After thorough mixing, the suspension was transferred into 1.5 mL Eppendorf tubes, kept in a -20°C freezer for 1-2 h, then stored in liquid nitrogen for later use.

Frozen 1.5 mL Eppendorf tubes were taken out from the liquid nitrogen and allowed to warm to room temperature for 5 min. Chloroform (200 µL) was added and the mixture was shaken for 20 s, incubated for 5 min at room temperature and spun for 15 min at 4°C and 10 000 r/min. Isopropanol (500 µL) was thoroughly mixed into the supernatant recovered after centrifugation. The solution was incubated for 10 min at room temperature and spun for 10 min at 4°C 10 000 r/min. The supernatant was discarded and the pellet was washed once in 75% ethanol and allowed to air dry for 10 min at room temperature. Finally, the pellet was dissolved in 30 µL DEPC-water. Three 5 µL samples were taken from the solution; one for OD measurement (20 × dilution), one for RNA content analysis, and one for agarose gel electrophoresis. The rest of the solution was stored in liquid nitrogen.

Confirmation of CGRP expression using RT-PCR

cDNA was prepared using the AMV reverse transcriptase reaction. Reverse transcription solution contained 4 µL 25 mmol/L MgCl₂, 2 µL 10 × PCR buffer, 2 µL dNTP, 0.5 µL recombinant RNasin, 0.7 µL AMV reverse transcriptase, and 1 µL dT oligo solution. RNA (3 µg) was added in each 20 µL reaction mixture. Reaction parameters were as follows: pre-denaturation for at 70°C 10 min; elongation at 42°C for 15 min; denaturation at 95°C for 5 min, and hold at 4°C for 5 min. Products were stored at -20°C.

PCR reaction mixtures (50 µL) were prepared containing 15 µL ddH₂O, 25 µL 2 × PCR buffer, 1 µL MgCl₂, 3 µL cDNA, and 3 µL of each primer (final concentration: 1 µmol/L) before mixing and spinning briefly.

For CGRP, the PCR reaction conditions were as follows: initialization at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 45 s at 56°C, and extension for 45 s at 72°C. The final extension was 10 min at 72°C. For β-actin, the same process was used, except the annealing temperature was adjusted to 50°C. After completion of the PCR-protocols, 5 µL of the reaction mixture was analyzed with agarose gel electrophoresis (1.5%). The bands were visualized under UV light.

The sequences of the primers were CGRP (sense AGGTCGGGAGGTGTGGTGAA and anti sense ATCCGCTTGAGGTTTAGCAGAG) and β-actin (sense ATCATGTTTGAGACCTTCAAC and antisense CATCTCTTGCTCGAAGTCCA).

Analysis of intestinal flora

Feces samples were collected under sterile conditions directly from the rat's rectum before and after induction of colitis and after the treatments were given. After weighing, the freshly collected feces was suspended in physiological saline (10 × w/v) and mixed well. Aliquots (100 µL) of the mixtures were spread evenly on selective

medium surfaces. After incubating 48 h at 37°C (aerobic bacteria) or 72 h at 37°C (anaerobic bacteria) live bacteria was counted (CFU/g). Intestinal content was cultured separately on EMB, BBL, MRS, LEVY, and FS media.

Data and statistical analysis

All data were given as mean ± SD. Data were analyzed in SPSS11.5, using one-way ANOVA to perform the comparisons among groups, then using least significant difference test (LSD-*t*) to perform the multi-comparisons among means, *P* < 0.05 was considered a significant difference.

RESULTS

Lower body weight, loose or bloody stool, and higher index of wet colon mass in rat colitis induced with oxazolone

The body weight decreased dramatically in the positive control group compared to the negative control group. The treatment groups also showed body weight loss; but, it was not as dramatic as the positive control group (Table 1). During the initiation phase of the UC model, most rats in the experimental group had loose, greasy or watery, sometimes bloody stools. Stools became normal in all treatment groups. Thus, the clinical symptoms of UC obviously improved upon treatment in this animal model. The wet colon mass index in the mesalamine group, the *C. butyricum* group and sodium butyrate group was clearly lower than the positive control wet colon mass index (*P* < 0.05) (Table 1).

Pathological changes in oxazolone-induced experimental colitis in rats

Animals in the positive control group were sacrificed three days after the experimental model was successfully established. Most pathological changes were found in the middle and lower part of colon with hyperemia and dropsy in the distal colon. Pathological changes were continuously distributed throughout the affected parts of the colon. Microscopic inspection of the tissues under light revealed abscessions of the mucous membrane epithelial cells, erosion of the mucous membrane and ulcer formation. We also noticed a decrease in the number of goblet cells and a disappearance or atrophy of the intestinal glands. Inflammation was located in or beneath the mucous membrane, although, in some cases, the muscle layers also appeared to be affected. Tissues were infiltrated mainly by lymphocytes, oxyphilic cells and plasmocytes; and less with neutrophil granulocytes. After treatment, the hyperemia-like characteristic of the mucous membrane improved, the swelling receded and the erosion healed. A few small ulcers were still discernible under microscopy, but the inflammatory cell infiltration, when apparent, consisted mainly of lymphocytes and acidophilic granular cells (Figure 1).

Serum levels of IL-23 and TNF-α in rat experimental colitis induced with oxazolone

The serum levels of IL-23 and TNF-α in the positive

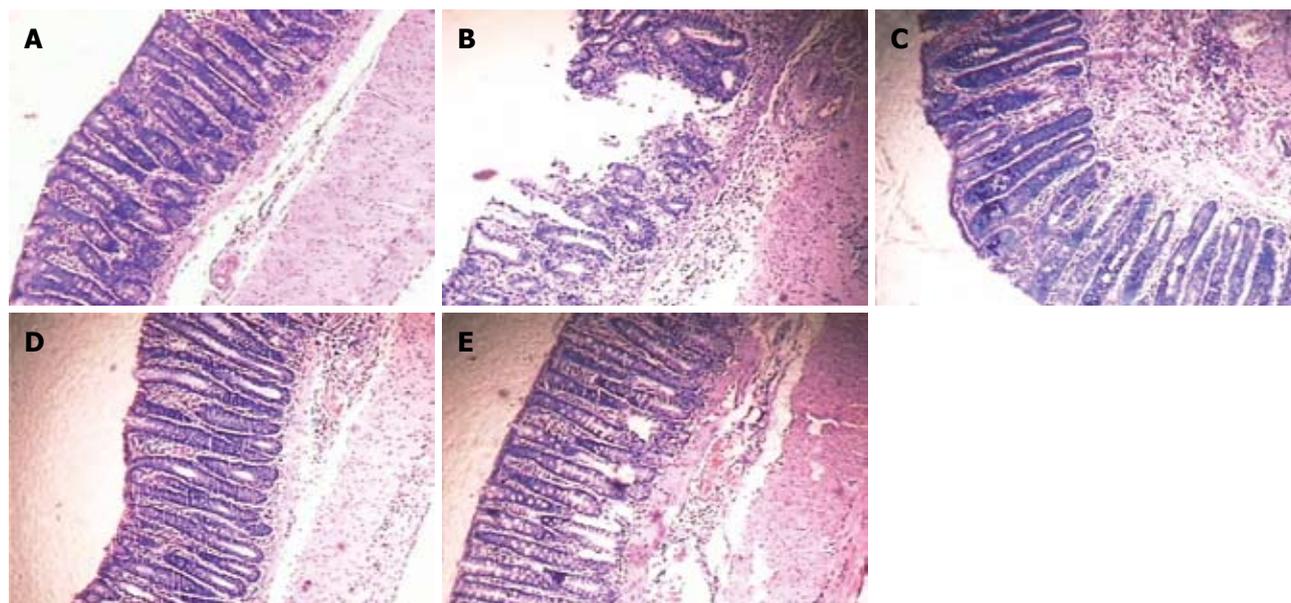


Figure 1 Histopathological sections of colons from oxazolone-induced rat colitis. Panel A, C, D, and E: Twenty-one days after establishment of model; B: Day 3 after establishment of model (HE \times 50). A: Negative control; B: Positive control; C: Probiotic (*C. butyricum*) group; D: Mesalamine group; E: Sodium butyrate group.

Table 1 Effects on body weight and wet colon mass index in rat colitis induced with oxazolone (mean \pm SD)

Group	n	Dose	Body weight (g)	Wet colon mass index (g/kg)
NC	8	N/A	191.3 \pm 24.2	5.3 \pm 1.2
PC	7	N/A	149.0 \pm 15.9 ^d	9.1 \pm 2.5 ^d
PB	10	2.3 \times 10 ¹¹ CFU/L	175.9 \pm 43.2	6.7 \pm 1.3 ^a
MA	8	100 g/L	179.8 \pm 18.4	6.9 \pm 2.3
SB	8	0.05 mol/L	175.8 \pm 23.0	6.5 \pm 1.6 ^a

^a $P < 0.05$ vs positive control; ^d $P < 0.01$ vs negative control; NC: Negative control; PC: Positive control; PB: Probiotic (*C. butyricum*); MA: Mesalamine; SB: Sodium butyrate.

control group was remarkably higher than the negative control group ($P < 0.05$, Table 2). After treatment, rat serum levels of IL-23 in *C. butyricum*, mesalamine and sodium butyrate groups became much lower than the positive control ($P < 0.01$). The levels of TNF- α in the *C. butyricum* group was clearly lower than the positive control, while there was no significant difference in the mesalamine and sodium butyrate groups (Table 2).

Sodium butyrate treatment increases CGRP expression levels

CGRP expression levels in the negative control, positive control, *C. butyricum* and mesalamine groups were weak, whereas the expression of CGRP in the sodium butyrate group was remarkably enhanced (Figure 2).

Effect on intestinal flora of rats with oxazolone-induced experimental colitis

In normal rats, intestinal *Colibacter*, *Bifidobacterium*, *Acidobacterium*, *Fusobacterium*, and *Clostridium* grew well (Table 3). After UC was established in this animal model, the number of intestinal *Bifidobacterium* and *Acidobacterium* in the positive control group decreased in

Table 2 IL-23 and TNF- α levels in rat serum in oxazolone-induced experimental colitis

Group (n = 7)	Dose	IL-23 (ng/L)	TNF- α (ng/L)
NC	N/A	5.75 \pm 2.51	15.93 \pm 11.36
PC	N/A	43.94 \pm 20.36 ^d	28.17 \pm 6.10 ^e
PB	2.3 \times 10 ¹¹ CFU/L	5.99 \pm 1.88 ^b	16.05 \pm 10.54 ^a
MA	100 g/L	8.81 \pm 3.78 ^b	23.54 \pm 11.03
SB	0.05 mol/L	8.38 \pm 4.48 ^b	23.18 \pm 6.48

^a $P < 0.05$, ^b $P < 0.01$ vs PC; ^c $P < 0.05$, ^d $P < 0.01$ vs NC. PC: Positive control; NC: Negative control; PB: Probiotic (*C. butyricum*); MA: Mesalamine; SB: Sodium butyrate.

comparison to the negative control ($P < 0.01$), whereas the number of *Colibacter*, *Fusobacterium* and *Clostridium* increased ($P < 0.01$). After treatment with *C. butyricum*, mesalamine or sodium butyrate, the amount of intestinal *Bifidobacterium* and *Acidobacterium* increased relative to the positive control, whereas the number of *Colibacter*, *Fusobacterium* and *Clostridium* decreased ($P < 0.01$ and $P < 0.05$). Compared with the *C. butyricum* group, the amount of *Colibacter* increased ($P < 0.01$ and $P < 0.05$), and the number of *Acidobacterium* clearly decreased (all $P < 0.01$) in the mesalamine and sodium butyrate groups. The amount of *Clostridium* in the mesalamine group was significantly lower than in the other groups ($P < 0.01$). The remaining bacterial groups showed no significant differences between the treatment groups (Table 3).

DISCUSSION

In our rat model of experimental colitis, the inflammatory disease is induced by intrarectal administration of oxazolone. Symptoms of inflammation of the distal rat colon included reddening and swelling of the mucous membrane. The continuously distributed pathological

Table 3 Effect on rat intestinal bacterial balance of oxazolone-induced experimental colitis ($n = 6$, \log_{10}^n CFU/g)

Group	<i>Colibacter</i>	<i>Bifidobacterium</i>	<i>Acidobacterium</i>	<i>Fusobacterium</i>	<i>Clostridium</i>
NC	6.87±0.4	9.42 ± 0.25	9.61 ± 0.12	2.92 ± 0.42	5.10 ± 0.19
PC	7.54 ± 0.13 ^d	8.88 ± 0.17 ^d	9.01 ± 0.15 ^d	4.37 ± 0.09 ^d	5.69 ± 0.11 ^d
PB	6.56 ± 0.35 ^b	9.62 ± 0.24 ^b	9.92 ± 0.05 ^b	3.57 ± 0.23 ^b	4.85 ± 0.17 ^b
MA	7.16 ± 0.20 ^{a,f}	9.54 ± 0.16 ^b	9.48 ± 0.13 ^{b,f}	3.79 ± 0.02 ^b	4.39 ± 0.29 ^b
SB	6.72 ± 0.18 ^{b,e}	9.46 ± 0.59 ^b	9.51 ± 0.09 ^{b,f}	3.72 ± 0.02 ^b	4.79 ± 0.18 ^{b,e}

^a $P < 0.05$, ^b $P < 0.01$ vs PC; ^d $P < 0.01$ vs NC; ^e $P < 0.05$, ^f $P < 0.01$ vs PB. NC: Negative control; PC: Positive control; PB: Probiotic (*C. butyricum*); MA: Mesalamine; SB: Sodium butyrate.

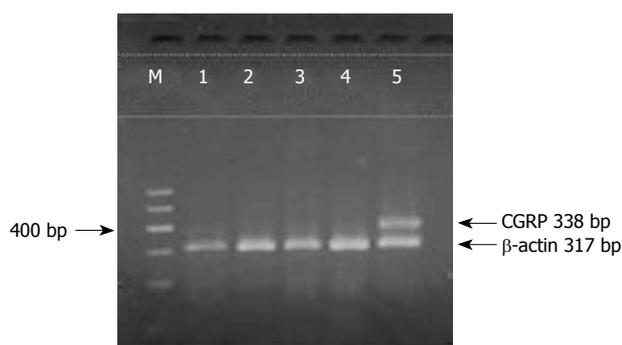


Figure 2 Effect on CGRP expression in treated or untreated rat experimental colitis induced with oxazolone. M: MW marker; 1: Negative control; 2: Positive control; 3: *C. butyricum*; 4: Mesalamine; 5: Sodium butyrate.

changes included the loss of epithelia cells, erosion of the epithelial mucous layer, ulcers, a decrease in the number of goblet cells and a decrease in gland density. The inflammation appeared to be located in or beneath the epithelial mucous layer, although in some cases the muscle layer showed infiltration of inflammatory cells. The wet colon mass index increased in the positive control group, as did the serum concentrations of cellular inflammation markers IL-23 and TNF- α .

After treatment with *C. butyricum*, mesalamine or sodium butyrate, the swelling and reddening of the colonic mucous membrane improved. The mucous membrane was almost healed, and the wet colon mass index decreased significantly. The serum content of IL-23 and TNF- α was remarkably decreased, almost back to the normal levels, and the balance of intestinal flora was restored.

IL-23 is a cellular factor, a new member of the IL-12 family. Similar to IL-12, it is a heterodimer, sharing the p40 subunit with IL-12. The p40 and p19 subunits form a covalently linked heterodimer *via* a disulfide bond. In addition to the interleukin receptor subunit 12R β 1 shared with IL-12, IL-23 has its own special receptor subunit IL23R. Activated dendritic cells, macrophages, T cells and blast cells all generate large amounts of p19 mRNA with Th1 cells expressing more p19 than Th2 cells. Among these cell types, only activated dendritic cells and macrophages produce both the p40^[26] and p19 required for the formation of IL-23, which is then secreted by these cells. Thus, human and rat IL-23 is mainly produced by activated dendritic cells^[27]. IL-23 can induce mononuclear cells and macrophages to express

inflammatory factors IL-1, IL-6 and TNF- α ^[28,29]. A recent study indicated that IL-23 is a necessary factor for the induction of chronic congenital or immune-modulated bowel diseases^[30]. Hue *et al*^[31] demonstrated that Th17 cells play a key role in mediating chronic spontaneous inflammation reactions, and that IL-23, but not IL-12, is essential for the induction of chronic bowel diseases. Together with IL-1, IL-23 can directly stimulate T helper cells to form Th17 competent cells that secrete IL-17. Interleukin 17 can enhance tissue inflammation reactions with the associated immune responses.

Several recent studies confirm a link between the IL-23 receptor IL23R and inflammatory bowel disease, both in Crohn's disease and ulcerative colitis patients^[32-37]. In our model, the serum levels of IL-23 rose significantly in the oxazolone treated animals, while the IL-23 levels dropped after treatment with mesalamine, *C. butyricum* or sodium butyrate. The decrease in serum IL-23 was greater in the group treated with *C. butyricum* than in both the mesalamine group and the sodium butyrate group.

TNF- α is mainly a product of macrophages that can induce widespread immune responses in many cell types. TNF- α can induce production of IEC prostaglandin and increase the expression of inner epithelial adherent molecule-1 further by promoting inflammation. It can also stimulate production of extracellular proteases and matrix metalloproteinases from fiber cell promotion. These proteases can degrade the matrix of the mucous membrane causing epithelial cell abscissions^[38]. Furthermore, TNF- α can increase the permeability of the intestinal epithelium by decreasing the expression of transmembrane core proteins associated with tight junctions^[39]. This is an important early pathological change in the mucous membrane in both IBS and IBD. Araki *et al*^[9] found that feeding *C. butyricum* can reduce intestinal mucous membrane wounds and the frequency of bloody diarrhea in rat-UC induced with dextran sodium sulfate (DSS). Also, Lu *et al*^[40] found a positive correlation between the severity of disease and the levels of IL-6 and TNF- α in patients with active ulcerative colitis during their clinical trials. Wan *et al*^[21] demonstrated that the expression of TNF- α rose remarkably before and clearly dropped after *C. butyricum* treatment in a rat-UC model induced by immunological challenge using colonic mucosal membrane protein. They also found that the treatment effects were better when *C. butyricum* was combined with mesalamine than

mesalamine alone. Our data indicate that the serum concentrations of TNF- α were significantly increased in the oxazolone-treated animals when compared with the negative control group. After treatment with a *C. butyricum* preparation, the TNF- α level decreased. Mesalamine and sodium butyrate had similar, but lesser effects on the TNF- α levels in our study.

Calcitonin Gene-Related Peptide (CGRP) consists of 37 amino acids and is widely distributed throughout the central nervous system (CNS), particularly in the accessory nerves, with a high concentration in the dorsal root ganglia (DRG) of the spinal cord. Retrograde labeling and IP Western blots confirmed that primary spinal afferent innervations of the mouse colon wall to a high degree are CGRP containing neuritic fibers^[41]. Some studies indicate that CGRP is involved in the induction of peritoneal irritation by promoting release and suppressing the degradation of substance P, thus enhancing the prevalence of substance P^[18,19]. About 50% of the CGRP containing dendritic cells also contain neurokinin, and CGRP can increase organ hypersensitivity by adjusting the expression of neurokinin 1 receptors in the primary synaptic cell bodies of the spinal cord^[20]. In our study, we found no increase in the CGRP expression levels in the groups treated with mesalamine or *C. butyricum*, whereas we found a significant increase of the CGRP expression level in the sodium butyrate group. This indicates that *C. butyricum* or mesalamine treatment did not increase organ hypersensitivity, whereas sodium butyrate treatment, although it appears to be a treatment for colitis induced with oxazolone, might also cause organ hypersensitivity. This is in agreement with data published by Xing *et al*^[42] indicating that sodium butyrate can increase the CGRP expression in spinal cord neurons in a dose dependent manner.

According to our data, treatment with *C. butyricum* leads to the recovery of the balance of the intestinal microflora of the experimental animals (Table 3). Daron Zhang *et al*^[43] gave *C. butyricum* to patients suffering from irritable bowel syndrome. They found that *C. butyricum* suppresses the proliferation of putrefactive and pathogenic bacteria, while it promotes the proliferation of intestinal *Bifidobacterium* and *Acidobacterium* and other beneficial microbes. The amount of beneficial microbes was significantly increased in mouse feces after treatment with *C. butyricum*^[44]. *C. butyricum* has been shown to suppress intestinal enterohemorrhagic *Escherichia coli*, *Shigella dysenteriae*, *Cholera salmonella*, and *Cholera bacillus in vitro*^[45]. Our data shows that the counts of intestinal *Bifidobacterium* and *Acidobacterium* rose dramatically and almost returned to normal after the treatment with live *C. butyricum*. The prevalence of the conditional disease germs *Colibacter*, *Fusobacterium* and *Clostridium* increased after the induction of colitis, and dropped significantly after treatment. Our data also indicate that the *C. butyricum* preparation used in our study had a better effect than mesalamine and sodium butyrate on the restoration of intestinal microbe balances, especially at decreasing intestinal bacillus counts and increasing *Acidobacterium* and

Clostridium (Table 3).

Thus, in our experimental model of UC, treatment with live *C. butyricum* CGMCC313.1 had a similar or better effect than both mesalamine and sodium butyrate, both on the levels of the inflammatory effectors monitored in this study (IL-23 and TNF- α) and on restoring the balance of the intestinal microflora. The general idea that probiotics based on carefully selected microbes constitute a treatment for UC worthy of consideration is supported by results from clinical trials. Thus, a recent meta-analysis of the results from six published clinical trials concluded that probiotic treatment may reduce UC relapses better than placebo and equivalently to mesalamine treatment^[46]. During our study on experimental colitis in rats, we found that treatment with a probiotic containing one well-characterized microorganism promoted the repair of the colon mucosa and recovery of intestinal flora. Thus, probiotics must continue to be a target for investigation both as a potential treatment for active UC and for the management of UC to prevent relapse.

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COMMENTS

Background

Ulcerative colitis is a chronic condition characterized by recurring episodes of intestinal inflammation that affect individuals throughout life. Ulcerative colitis and Crohn's disease, a related disorder, are together called Inflammatory Bowel Disease.

Research frontiers

No effective cure is known for ulcerative colitis. Patients are usually treated with anti-inflammatory drugs, sometimes in combination with antibiotics to relieve symptoms. Treatment with food containing live microorganisms (probiotics) may, according to some recent clinical trials, improve symptoms, possibly with fewer side effects than conventional treatment.

Innovations and breakthroughs

This study showed that treatment with a microorganism, *Clostridium butyricum* CGMCC0313.1, can facilitate healing and repair of the intestinal wall in rats suffering from experimental colitis induced with a haptening agent. Interestingly, both *C. butyricum* and mesalamine, the control drug, lowered the serum levels of the inflammatory cytokines IL-23 and TNF- α , but the rat intestinal flora appeared to recover faster in the animals treated with the microorganism than in those treated with mesalamine.

Applications

By understanding how probiotic treatment alleviates the symptoms of experimental (ulcerative) colitis, new avenues for research into treatment of inflammatory bowel diseases will open. Our study demonstrates that, in our experimental model of ulcerative colitis, *C. butyricum* acts as an anti-inflammatory agent drug rather than just assisting in the recovery of the intestinal flora. Identifying bacterial strains with these properties might lead to novel treatments of ulcerative colitis and other inflammatory disorders of the colon and rectum.

Peer review

This is a fine study comparing the anti-inflammatory efficacy of *C. butyricum*, mesalamine and sodium butyrate on oxazolone-induced colitis in rats.

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Protective effect of tea polyphenols against paracetamol-induced hepatotoxicity in mice is significantly correlated with cytochrome P450 suppression

Xia Chen, Chang-Kai Sun, Guo-Zhu Han, Jin-Yong Peng, Ying Li, Yan-Xia Liu, Yuan-Yuan Lv, Ke-Xin Liu, Qin Zhou, Hui-Jun Sun

Xia Chen, Chang-Kai Sun, Guo-Zhu Han, Jin-Yong Peng, Ying Li, Yan-Xia Liu, Yuan-Yuan Lv, Ke-Xin Liu, Qin Zhou, Hui-Jun Sun, Department of Pharmacology, Dalian Medical University, Dalian 116044, Liaoning Province, China

Author contributions: Chen X, Sun CK contributed equally to this work; Han GZ, Sun HJ designed the research; Chen X, Liu YX, Lv YY performed the research; Peng JY, Li Y analyzed the data; Sun HJ, Liu KX wrote the paper; Zhou Q provided the vital reagents and analytical tools.

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Correspondence to: Hui-Jun Sun, Professor, Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, 9 west section, Lvshun South Road, Lvshunkou District, Dalian 116044, Liaoning Province, China. sunhuijun@hotmail.com

Telephone: +86-411-86110413 Fax: +86-411-86110407

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Abstract

AIM: To investigate the hepatoprotective activity of tea polyphenols (TP) and its relation with cytochrome P450 (CYP450) expression in mice.

METHODS: Hepatic CYP450 and CYPb₅ levels were measured by UV-spectrophotometry in mice 2 d after intraperitoneal TP (25, 50 and 100 mg/kg per day). Then the mice were intragastrically pre-treated with TP (100, 200 and 400 mg/kg per day) for six days before paracetamol (1000 mg/kg) was given. Their acute mortality was compared with that of control mice. The mice were pre-treated with TP (100, 200, and 400 mg/kg per day) for five days before paracetamol (500 mg/kg) was given. Hepatic CYP2E1 and CYP1A2 protein and mRNA expression levels were evaluated by Western blotting, immunohistochemical staining and transcriptase-polymerase chain reaction.

RESULTS: The hepatic CYP450 and CYPb₅ levels in mice of TP-treated groups (100, 200 and 400 mg/kg per day) were decreased in a dose-dependent manner compared with those in the negative control mice.

TP significantly attenuated the paracetamol-induced hepatic injury and dramatically reduced the mortality of paracetamol-treated mice. Furthermore, TP reduced CYP2E1 and CYP1A2 expression at both protein and mRNA levels in a dose-dependent manner.

CONCLUSION: TP possess potential hepatoprotective properties and can suppress CYP450 expression.

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Key words: Tea polyphenols; Cytochrome P450; Paracetamol-induced hepatotoxicity

Peer reviewer: Vincent Lai, PhD, Derby NHS Foundation Trust, Utooxeter Road, Derby DE22 3NE, United Kingdom

Chen X, Sun CK, Han GZ, Peng JY, Li Y, Liu YX, Lv YY, Liu KX, Zhou Q, Sun HJ. Protective effect of tea polyphenols against paracetamol-induced hepatotoxicity in mice is significantly correlated with cytochrome P450 suppression. *World J Gastroenterol* 2009; 15(15): 1829-1835 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1829.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1829>

INTRODUCTION

Tea has been consumed in China to promote health and longevity since 3000 B.C. It is now a popular beverage all over the world. Tea polyphenols (TP) are a large and diverse class of compounds extracted from tea. The major compounds of TP are picatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin-3-gallate (EGCG). Recent studies indicate that TP can prevent oxidative stress-related diseases, including cancer, cardiovascular and degenerative diseases and have other bioactive properties^[1-4].

In recent years, the interest in understanding the metabolic benefits of TP has been increasing. Liver is the main organ responsible for the metabolism of TP. Liu *et al*^[5] reported that TP can markedly increase cytochrome P450 (CYP450) activity in rats. However, the effect of TP on CYP450 activity remains controversial^[6,7].

Until now, no one could give a clear explanation of the different results. CYP450 enzymes play a pivotal role not only in the metabolism of xenobiotics, but also in the biosynthesis and catabolism of endogenous substrates, such as vitamins, fatty acids, hormones and prostaglandins^[8]. Alteration in hepatic CYP450 enzyme expression would affect the pharmacokinetic profiles of clinically used drugs. Furthermore, both induction and suppression of several CYP450s may lead to cellular oxidative stress and tissue injury in response to xenobiotics^[9].

Paracetamol, one of the most widely used hepatotoxic drugs, is safe at therapeutic doses, but causes liver failure in overdoses^[10,11]. When a normal dose is used, paracetamol is extensively metabolized by conjugation with sulphate and glucuronic acid. A small fraction of the drug is subjected to oxidation reactions catalyzed by CYP450 enzymes in the liver, resulting in generation of N-acetyl-p-benzo-quinoneimine (NAPQI), a highly electrophilic metabolite that triggers ensuing liver damage. Exposure to high doses of paracetamol increases the NAPQI level. Normally, toxic oxidation metabolites generated in the liver are converted into non-toxic metabolites excreted in urine via conjugation with glutathione (GSH) containing sulphhydryl groups. However, high doses of paracetamol limit the ability of GSH to detoxify NAPQI, and result in the consumption of liver GSH stores^[12,13]. It was reported that oxidative stress constitutes a major mechanism underlying the pathogenesis of paracetamol-induced liver damage^[14,15].

There are three families of CYP450 isoforms, CYP1, CYP2, and CYP3, which are mainly involved in the biotransformation of xenobiotics^[16]. It has been shown that CYP2E1, CYP1A2, and intracellular GSH play an important role in the hepatotoxicity induced by paracetamol^[17-19]. The present study was to investigate the hepatoprotective activity of TP and its relation with CYP450 expression in mice.

MATERIALS AND METHODS

Animal treatment

TP obtained from Wuyuan Tea Plantation (Jiangxi Province, China) with a purity of > 98% containing 63.84% of EGCG and 7.30% of ECG were decaffeinated. Kunming male mice, weighing 18-22 g, provided by Experimental Animal Center of Dalian Medical University, were used in the study. The mice were randomly divided into high, medium, low TP dose groups, positive and negative control groups ($n = 10$). The mice in low, medium and high dose TP groups were intraperitoneally injected with 25, 50 and 100 mg/kg of TP per day for two days. The mice in positive control group were given 50 mg/kg chloramphenicol one hour before they were killed. The mice in the negative control group were given the same volume of saline solution for two days. On day 3, all the mice were killed by decapitation, with their livers removed immediately and cleaned with a cold saline solution. Specimens were stored at -80°C for preparation of liver microsomes.

Male mice in each group received intragastric TP at the dose of 100, 200, and 400 mg/kg, once a day for six days. The mice in positive control group were given 900 mg/kg N-acetylcysteine, once a day. The mice in negative control group were given the same volume of a saline solution. All the mice were given 1000 mg/kg paracetamol one hour later and the acute mortality of mice in both groups was recorded 72 h after paracetamol was given.

Male mice in each group received intragastric TP at the dose of 100, 200, and 400 mg/kg, once a day for five days. The mice in model and negative control groups were given the same volume of a saline solution. The mice were given 500 mg/kg paracetamol one hour later, except for the mice in negative control group. All the mice were killed by decapitation 24 h later with their livers removed immediately and cleaned with a cold saline solution. Specimens were fixed in formalin for histological examination or snap-frozen in liquid nitrogen and stored at -80°C for protein and RNA extraction.

Preparation of liver microsomes

Liver microsomes extracted from frozen samples were prepared as previously described^[20]. Microsomal protein level was measured as previously described^[21] with bovine serum albumin as a standard.

Measurement of microsomal enzyme levels

The levels of CYP450 and CYPb₅ were measured as previously described^[22].

Western blot assay

Aliquots (80 μg) of liver microsomes from each sample were loaded onto each lane of 10% SDS-PAGE gel electrophoresis and electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were probed with rabbit antibody against mice CYP2E1 (Boster Biological Technology Co., Ltd, Wuhan, China) at a dilution of 1:400 or CYP1A2 (Santa Cruz Biotechnology, Inc., California, USA) at a dilution of 1:500 and peroxidase-conjugated affinity-pure goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) according to the manufacturer's constructions. The signals were visualized with a DAB assay kit (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) and analyzed with Quantity One software.

Histology and immunohistochemical assay

All specimens embedded in paraffin were cut into 4- μm thick serial sections. The specimens were mounted on slides and deparaffinized with graded concentrations of xylene and ethanol, and stained with hematoxylin and eosin (HE). For immunohistochemical staining, all sections were immersed in 3% H_2O_2 for 15 min at room temperature to block the endogenous peroxidase activity. To stain with CYP2E1 and CYP1A2, the sections were predigested with 0.4% proteinase K for 5 min at 37°C . The sections were then incubated with 10% normal goat serum for 15 min to reduce the nonspecific background

Table 1 Sequences of oligonucleotide primers

Primer	Sequence
CYP2E1 F	5'-CACCGTTGCCTTGCTTGCTCG-3'
CYP2E1 R	5'-CTCATGAGCTCCAGACACTTC-3'
CYP1A2 F	5'-AGTGTTCGGATGGTCAGAGC-3'
CYP1A2 R	5'-GCAAGAGGATGCTGACGTCG-3'
GAPDH F	5'-ATGGTGAAGGTCGGTGTGAAC-3'
GAPDH R	5'-GTCCTCTGGGTGGCAGTGATG-3'

Table 2 Effects of TP on CYP450 and CYPb₅ levels (mean \pm SD)

Group (<i>n</i> = 10)	CYP450 (nmol/g)	CYPb ₅ (nmol/g)
Negative control	0.354 \pm 0.024 ^b	0.160 \pm 0.011 ^b
Low dose	0.279 \pm 0.014 ^{a,b}	0.108 \pm 0.004 ^{a,b}
Medium dose	0.204 \pm 0.016 ^{a,b}	0.077 \pm 0.006 ^a
High dose	0.130 \pm 0.016 ^a	0.048 \pm 0.006 ^a
Positive control	0.147 \pm 0.019 ^a	0.061 \pm 0.009 ^a

^a*P* < 0.01 vs negative control group (NS group); ^b*P* < 0.01 vs positive control group (chloramphenicol group).

staining and reacted with polyclonal rabbit anti-mouse antibody (Ab) at 4°C overnight. The working dilution of Abs used for examining mouse CYP2E1 and CYP1A2 in the liver was diluted at 1:100 and 1:50. After rinsing with phosphate-buffered saline (pH 7.4), the sections were incubated with biotinylated anti-rabbit immunoglobulin secondary Abs at room temperature for 30 min. Horseradish peroxidase labeled streptomycin-avidin complex was used to detect the second antibody. Finally, the sections were counterstained with hematoxylin before examination under a light microscope. The brown or dark brown stained cells were considered positive cells.

Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from liver tissue with the TRIquick reagent (Solarbio Science & Technology CO., Ltd, Beijing, China). RNA purity was assessed by the optical densities at 260 nm and 280 nm, and the integrity was verified by electrophoresis on a 1% agarose gel containing 0.5 μ g/mL of ethidium bromide. Reverse transcription was conducted for 30 min at 42°C from 500 ng of purified RNA in 10 μ L of reverse transcriptions system reaction mixture using AMV reverse transcriptase (TaKaRa, Japan), followed by 30 cycles of PCR (denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min). The amplified products were electrophoresed on a 1.5% agarose gel using 100 bp DNA ladder markers (TaKaRa, Japan) as a standard to determine the molecular size and visualized with ethidium bromide staining. Images of the ethidium bromide-stained agarose gel were acquired with a Gel Doc EQ system and quantified using Quantity One software. The sequences of oligonucleotide primers are provided in Table 1.

Statistical analysis

Data were calculated separately and expressed as mean

Table 3 Effect of TP on mortality induced by paracetamol in mice

Group (<i>n</i> = 10)	Dose (mg/kg)	Death (<i>n</i>)	Death rate (%)	Protection rate (%)
Negative control		9	90	10
Positive control		2	20	80
TP				
Low	100	7	70	30
Medium	200	4	40	60
High	400	2	20	80

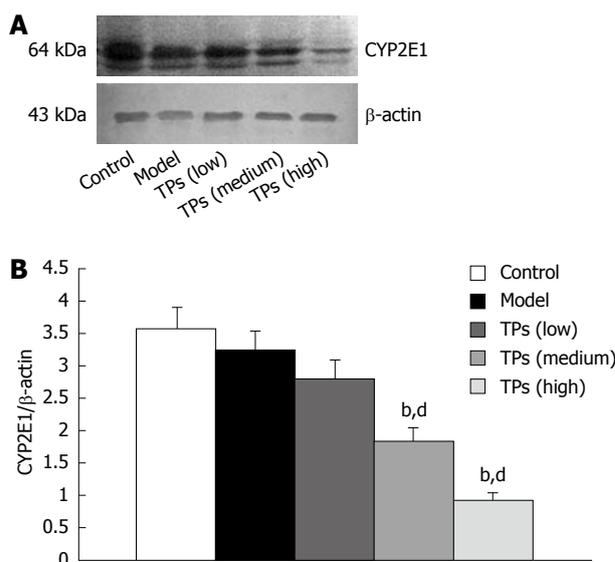


Figure 1 Western blot analysis of protein for CYP2E1 in liver tissue of mice showing representative bands of each group (A) and normalized densitometric ratios of CYP2E1 to β -actin (B). ^b*P* < 0.01 vs control group; ^d*P* < 0.01 vs model group.

\pm SD. Statistical significances were analyzed by one-way ANOVA followed by Student Newman-Keuls test using SPSS Version 11.5. The difference was considered significant at two-tailed. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of TP on CYP450 and CYPb₅ levels

Two days after peritoneal injection of TP, the CYP450 and CYPb₅ levels in livers of mice were measured. The CYP450 and CYPb₅ levels were significantly lower in the high, medium and low dose TP groups than in the negative control group (*P* < 0.01), indicating that TP can reduce the CYP450 and CYPb₅ levels in liver of mice. The CYP450 level was higher in the medium and low dose groups than in the positive control group (*P* < 0.01); but, it was not significantly different between the high dose and positive control groups (*P* > 0.05). The CYPb₅ level was markedly different in the low dose group (*P* < 0.01); but it, was not significantly different in the high and medium dose groups (*P* > 0.05) compared the positive control group, indicating that TP can suppress both CYP450 and CYPb₅ in mice in a dose-dependent manner (Table 2).

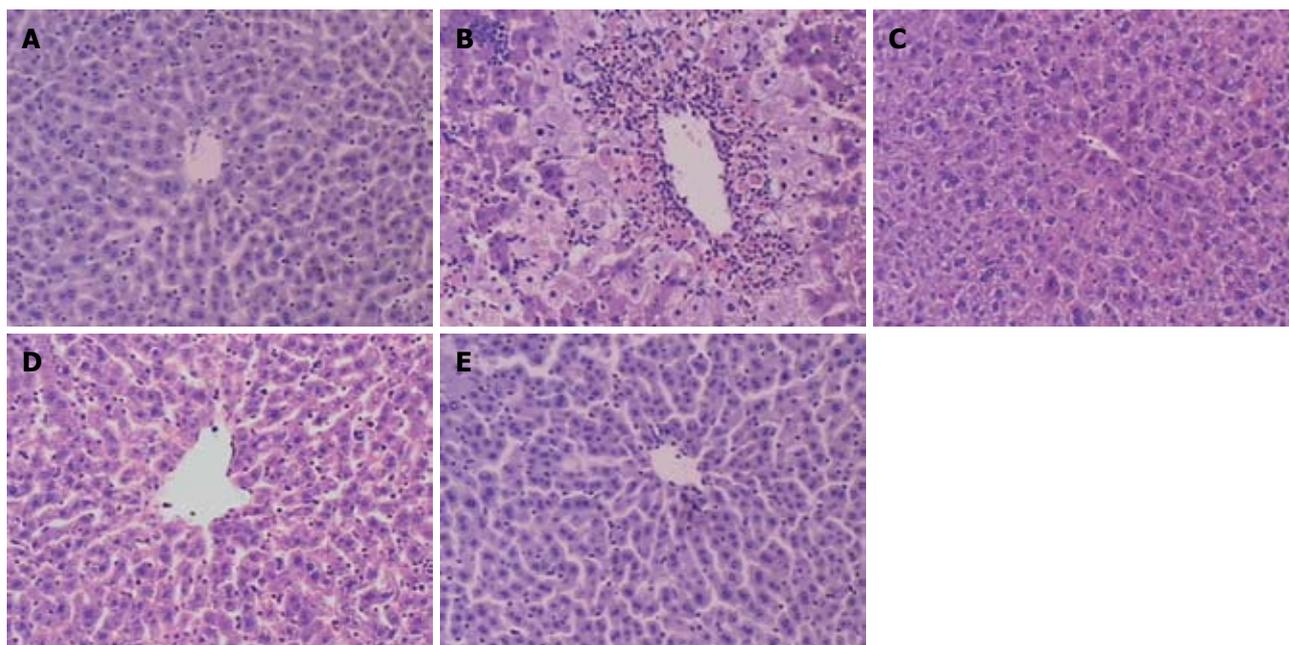


Figure 2 Changes of HE staining in liver tissue of mice 24 h after paracetamol administration ($\times 100$) in control group with a normal central lobular region (A), model group with some central lobular hepatocyte necrosis and microvesicular fatty change (B), low TP dose group (C), medium TP dose group (D) and high TP dose group (E). The pathological change of liver was much milder in different TP dose groups than in model group.

Effect of TP on mortality of paracetamol-treated mice

We examined the effect of TP on mortality of paracetamol-treated mice. The mortality rate of mice in the negative control group, low, medium and high dose TP groups was 90%, 70%, 40% and 20%, respectively, indicating that TP can significantly reduce the mortality of paracetamol-treated mice. The effect of high dose TP on mortality of paracetamol-treated mice was not different from that of mice in the positive control group (Table 3).

Western blot analysis

Western blot showed strong positive CYP2E1 signals in the control and model groups. The expression of CYP2E1 in the low dose group was not significantly different from that in the control and model groups. However, TP significantly decreased the CYP2E1 expression level in the medium and high dose groups in a dose-dependent manner (Figure 1). The expression of CYP1A2 and CYP2E1 protein was similar in the liver microsomes (Data not shown).

Histology analysis

Liver tissues from the control group showed a normal lobular architecture with central veins and radiating hepatic cords (Figure 2A). However, liver tissues from the model group showed formation of more fibrous tissues extending into the hepatic lobules, leading to their complete separation. A large number of inflammatory cells infiltrated the intralobular and interlobular regions. The liver structure was in disorder and there were more necrotic and fatty degenerated liver cells than in the controls (Figure 2B). In the three TP treatment groups, however, hepatocyte degeneration, necrosis and infiltration of inflammatory cells were all apparently ameliorated (Figure 2C-E). Compared with the model

group, the liver condition in TP treatment groups was significantly improved in a dose-dependent manner.

Immunohistochemical analysis of liver CYP2E1 and CYP1A2

The expression of CYP2E1 showed a deep brown immunostaining. No difference was found in the expression of CYP2E1 between the control and model groups. However, in medium and high dose groups, TP significantly suppressed the CYP2E1 expression in a dose-dependent manner (Figure 3A).

The expression level of CYP1A2 (showing a deep brown immunostaining) was markedly lower in the low, medium and high dose TP groups than in the control and model groups (Figure 3B).

Expression levels of CYP2E1 and CYP1A2 mRNA

The expression levels of CYP2E1, CYP1A2 and GAPDH cDNAs in the mouse liver were assayed by RT-PCR (Figure 4A), and the ratio of CYP2E1 and CYP1A2/ GAPDH cDNAs was calculated (Figure 4B). TP significantly inhibited the CYP2E1 mRNA expression in medium and high dose groups ($P < 0.01$), compared to the control and model groups ($P > 0.05$). The expression of CYP1A2 mRNA was reduced in all TP groups compared with the control and model groups ($P < 0.05$), indicating that TP can suppress the expression of CYP2E1 and CYP1A2 mRNA in a dose-dependent manner.

DISCUSSION

Hepatic drug metabolizing enzyme is called mixed-function oxidase or monooxygenase containing many enzymes including phase I enzymes such as

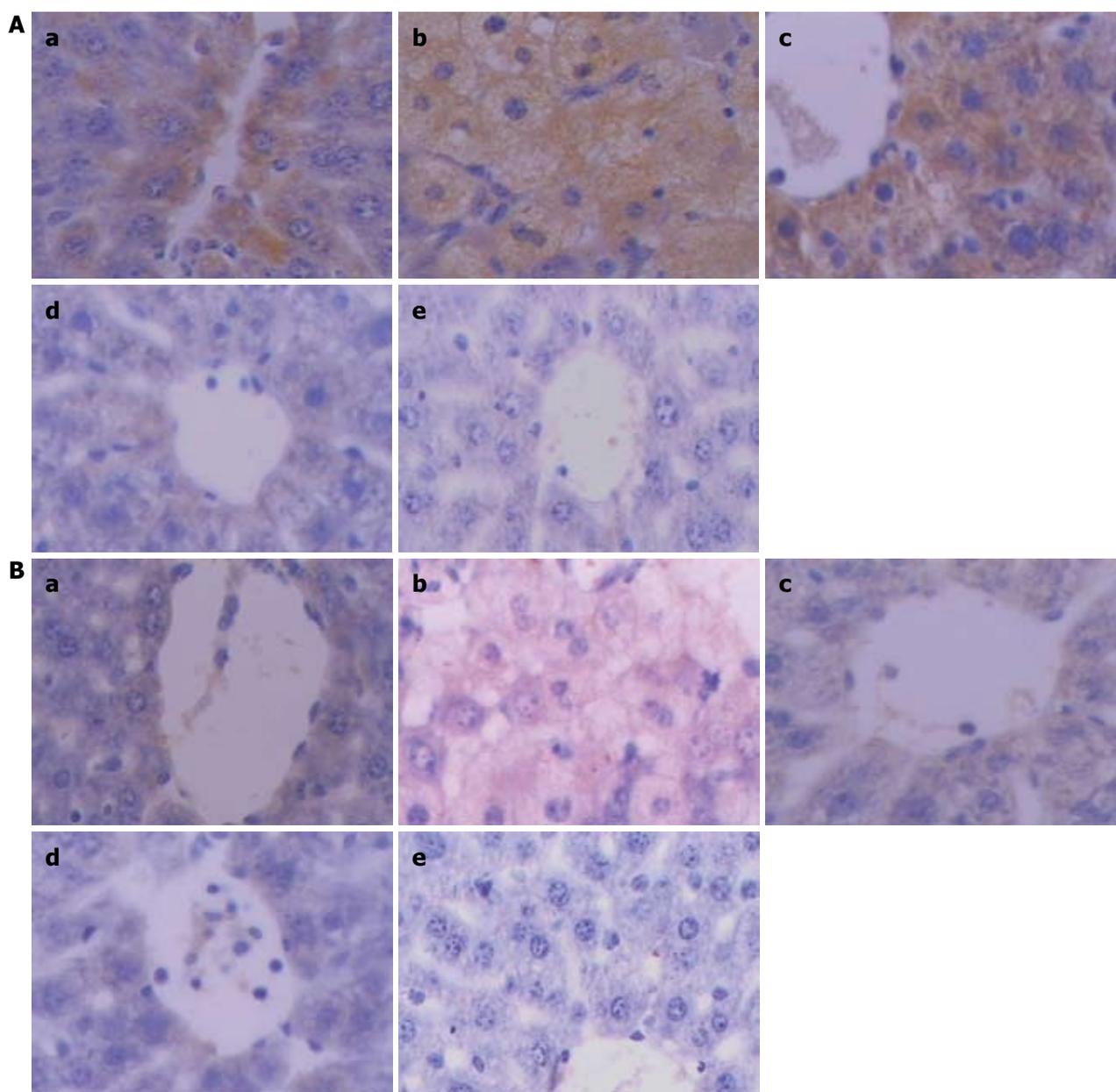


Figure 3 Immunohistochemical staining showing expression of CYP2E1 (A) and CYP1A2 (B) in liver tissue of mice 24 h after paracetamol administration ($\times 400$) in control group (a), model group (b), low TP dose group (c), medium TP dose group (d) and high TP dose group (e). The brown or dark brown stained cells were considered positive.

CYP450 and CYPb_s, *etc.*, and plays a prominent role in the metabolism of many pharmaceutical agents and activation or deactivation of potential carcinogens. Acquiring metabolic information and determining the effect of chemicals on hepatic drug metabolizing enzymes are important in developing clinically safe and efficient medications^[23].

TP have various pharmacological effects on cancer, cardiovascular and degenerative diseases and have other bioactive properties^[1-4]. In this experiment, TP significantly suppressed the CYP450 and CYPb_s levels in liver of mice, suggesting that TP can restrain the activity of drug metabolizing enzymes.

In the present study, 100, 200 and 400 mg/kg TP could reduce the mortality of mice after treatment with paracetamol (Table 2). The immunohistochemical study

showed that TP markedly alleviated paracetamol- induced hepatotoxicity, thus improving alterations in liver tissue pathology. Especially marked hepatoprotective effects of TP were observed in the high dose TP group with its diffuse hemorrhage and necrosis changed into focal hemorrhage and necrosis (Figure 2) compared to the model group that received a single dose of 500 mg/kg paracetamol, indicating that TP can protect liver of mice against paracetamol injury, which is consistent with the reported data^[24].

It was reported that many mechanisms are involved in paracetamol hepatotoxicity^[25], showing that the toxicity is mediated by CYP450 metabolism of paracetamol to NAPQI which covalently binds to critical proteins leading to inactivation of these proteins, especially after GSH depletion. CYP2E1 is usually assumed to be the

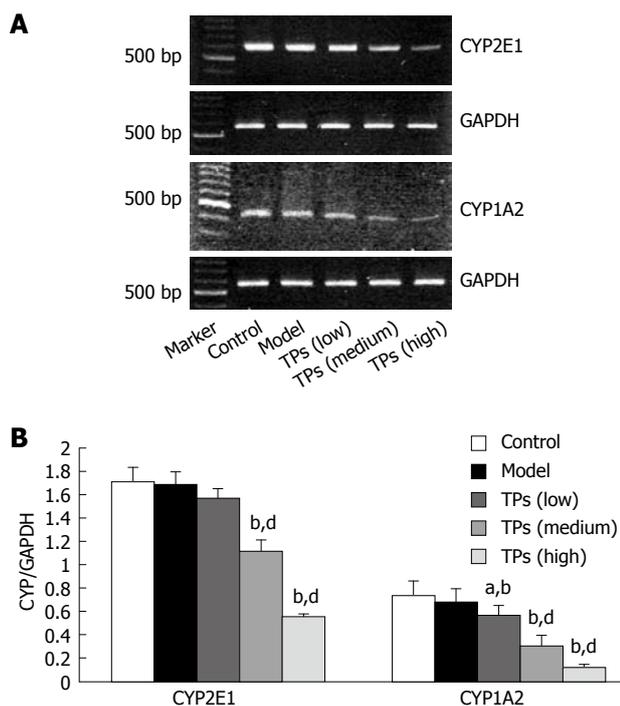


Figure 4 RT-PCR analysis of mRNA for CYP2E1 and CYP1A2 expression in liver tissue of mice showing representative bands of each group (A) and normalized densitometric ratio of CYP2E and CYP1A2 to GAPDH (B). ^a $P < 0.05$ vs model group; ^b $P < 0.01$ vs control group; ^d $P < 0.01$ vs model group.

most active CYP450 in catalyzing the metabolism of paracetamol to hepatotoxic NAPQI^[15,18]. Studies with knockout mice showed that CYP2E1 and CYP1A2 play an important role in the metabolism of paracetamol^[26,27]. To confirm whether the protective effects of TP on liver are correlated with the inhibition of CYP2E1 and CYP1A2, we investigated the effect of TP on the expression of CYP2E1 and CYP1A2 mRNA and protein.

In the present study, 200 mg/kg and 400 mg/kg TP significantly down-regulated the CYP2E1 expression either at gene level (Figure 4) or at protein level (Figures 1 and 3) as shown by RT-PCR, Western blot and immunohistochemistry. The effects of TP on the expression of CYP1A2 and CYP2E1 gene and protein were similar. It was reported that the CYP1A2 level in increased in rats after drinking a 2% solution of green or black tea^[28]. Caffeine, a component of tea, is responsible for the induction of CYP1A2^[29]. In our study, however, different doses of TP (100, 200 and 400 mg/kg) significantly suppressed the expression of CYP1A2 protein and mRNA, partly because TP used in our experiment did not contain caffeine.

The above findings indicate that the dose-dependent liver-protective effects of TP are likely related to the suppression of CYP2E1 and CYP1A2 expression, which reduces NAPQI associated with the paracetamol hepatotoxicity, and effectively protects liver against paracetamol hepatotoxicity.

In conclusion, TP restrain the activity of drug metabolizing enzymes, CYP450 and CYPb_s, and protect liver against paracetamol-induced injury. The protective

effects of TP are associated with the expression of CYP2E1 and CYP1A2 gene and protein.

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COMMENTS

Background

Paracetamol is one of the most widely used hepatotoxic drugs, which is safe at therapeutic doses, but causes liver failure at overdoses. High doses of paracetamol limit the ability of glutathione (GSH) to detoxify N-acetyl-p-benzoquinonimine (NAPQI), and results in consumption of liver GSH stores. There are three families of cytochrome P450 (CYP450) isoforms, CYP1, CYP2, and CYP3, which are mainly involved in the biotransformation of xenobiotics. It has been shown that CYP2E1, CYP1A2 and intracellular GSH play an important role in paracetamol-induced hepatotoxicity. Tea polyphenols (TP) are a large and diverse class of compounds extracted from tea. Liver is the main organ responsible for the metabolism of TP. Some work has been done in the field of TP modulation or interaction with drug metabolizing enzymes. However, until now, no one could give a clear explanation for this.

Research frontiers

TP are a large and diverse class of compounds extracted from tea. Liver is the main organ responsible for the metabolism of TP. Some work has been done in the field of TP modulation or interaction with drug metabolizing enzymes. However, until now, no one could give a clear explanation for this. The research hotspot is to investigate the effects of TP on CYP450 and CYPb_s expression in livers of male mice. Whether the protective effect of TP on paracetamol-induced hepatotoxicity in mice is related with their effect on CYP450 was studied.

Innovations and breakthroughs

The protective effect of TP on paracetamol-induced hepatotoxicity was found to be related with their effect on CYP450.

Applications

TP possess potential hepatoprotective properties, which are closely related with their suppressive effect on CYP450 expression.

Terminology

Paracetamol-induced hepatotoxicity: When a normal dose is used, paracetamol is metabolized extensively by conjugation with sulphate and glucuronic acid. A small fraction of paracetamol is subjected to oxidation reactions catalyzed by CYP450 enzymes in the liver, resulting in generation of NAPQI, a highly electrophilic metabolite that triggers the ensuing liver damage.

Peer review

This is a good descriptive study in which the authors gave a clear explanation of the effect of TP on the expression of CYP450, CYP2E1 and CYP1A2 in liver of mice at both protein and mRNA levels. The interesting results suggest that TP can significantly attenuate paracetamol-induced hepatic injury, which is closely related with their suppressive effect on CYP450 expression.

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ORIGINAL ARTICLES

Clinical usefulness of ^{18}F -FDG PET/CT in the restaging of esophageal cancer after surgical resection and radiotherapy

Long Sun, Xin-Hui Su, Yong-Song Guan, Wei-Ming Pan, Zuo-Ming Luo, Ji-Hong Wei, Long Zhao, Hua Wu

Long Sun, Xin-Hui Su, Wei-Ming Pan, Zuo-Ming Luo, Ji-Hong Wei, Long Zhao, Hua Wu, Minnan PET Center and Department of Nuclear Medicine, the First Hospital of Xiamen, Fujian Medical University, Xiamen 316003, Fujian Province, China

Yong-Song Guan, State Key Laboratory of Biotherapy, West China Medical School, Sichuan University, Gaopeng Street, Keyuan Road 4, Chengdu 610041, Sichuan Province, China

Author contributions: Sun L wrote the paper and organized the figures and patient data; Su XH, Pan WM, Luo ZM, Zhao L, and Wei JH completed patient follow-ups and data collection; Guan YS helped to write, organize, and correct the paper; Wu H supervised the writing and organization process.

Correspondence to: Hua Wu, Minnan PET Center and Department of Nuclear Medicine, the First Hospital of Xiamen, Fujian Medical University, Xiamen 316003, Fujian Province, China. wuhua1025@163.com

Telephone: +86-592-2139527 Fax: +86-592-2139527

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The three false positive PET/CT findings comprised chronic inflammation of mediastinal lymph nodes ($n = 2$) and anastomosis inflammation ($n = 1$). PET/CT demonstrated distant metastasis in 10 patients. ^{18}F -FDG PET/CT imaging-guided salvage treatment in nine patients was performed. Treatment regimens were changed in 12 (60%) patients after introducing ^{18}F -FDG PET/CT into their conventional post-treatment follow-up program.

CONCLUSION: Whole body ^{18}F -FDG PET/CT is effective in detecting relapse of esophageal cancer after surgical resection and radiotherapy. It could also have important clinical impact on the management of esophageal cancer, influencing both clinical restaging and salvage treatment of patients.

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Key words: ^{18}F -fluorodeoxyglucose; Positron emission tomography/computed tomography; Esophageal cancer; Surgical resection; Radiotherapy radiation; Restaging

Peer reviewer: Dr. Serdar Karakose, Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

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Abstract

AIM: To evaluate the clinical usefulness of ^{18}F -fluorodeoxyglucose positron emission and computed tomography (^{18}F -FDG PET/CT) in restaging of esophageal cancer after surgical resection and radiotherapy.

METHODS: Between January 2007 and Aug 2008, twenty histopathologically diagnosed esophageal cancer patients underwent 25 PET/CT scans (three patients had two scans and one patient had three scans) for restaging after surgical resection and radiotherapy. The standard reference for tumor recurrence was histopathologic confirmation or clinical follow-up for at least ten months after ^{18}F -FDG PET/CT examinations.

RESULTS: Tumor recurrence was confirmed histopathologically in seven of the 20 patients (35%) and by clinical and radiological follow-up in 13 (65%). ^{18}F -FDG PET/CT was positive in 14 patients (68.4%) and negative in six (31.6%). ^{18}F -FDG PET/CT was true positive in 11 patients, false positive in three and true negative in six. Overall, the accuracy of ^{18}F -FDG PET/CT was 85%, negative predictive value (NPV) was 100%, and positive predictive value (PPV) was 78.6%.

INTRODUCTION

Esophageal cancer is one of the least studied and deadliest cancers worldwide and one of the 10 most prevalent cancers worldwide and has an unfavorable prognosis among digestive tract malignancies^[1,2]. The management of esophageal cancer has evolved from surgery alone to definitive and preoperative chemotherapy-radiation therapy. The best option for curative treatment for patients with esophageal cancer is radical surgery; however, the long-term survival is only 25%^[3]. Postoperative tumor recurrence is not uncommon

in patients undergoing curative resection for esophageal cancer and can be categorized as either locoregional (locoregional lymph node metastases, anastomotic recurrence) or distant (hematogenous metastases, pleural or peritoneal seeding). Lymph node recurrence and hematogenous metastasis to solid organs (commonly to the lung) are the usual patterns of recurrence^[4,5].

¹⁸F-fluorodeoxyglucose positron emission tomography (¹⁸F-FDG PET) and, particularly, ¹⁸F-FDG positron emission and computed tomography (¹⁸F-FDG PET/CT) are widely accepted imaging methods in the management of a wide variety of cancers^[6]. In the initial staging of esophageal cancer, a preoperative PET scan may be useful in detecting additional cases of metastatic disease before costly and toxic definitive therapy^[7]. Currently, ¹⁸F-FDG PET and PET/CT also seem to be the best available tool for neoadjuvant therapy response assessment in esophageal cancer^[8,9]. However, the utility and limitation of ¹⁸F-FDG PET/CT in patients with esophageal cancer treated by surgical resection and post operation radiation is not clear. In this study, we aimed to analyze the value of ¹⁸F-FDG PET/CT scans in the follow up of patients with esophageal cancer treated with combined surgical treatment and radiotherapy management.

MATERIALS AND METHODS

Patients

A retrospective review of our electronic database of 20 patients with esophageal cancer after surgical resection and following radiotherapy (15 males and 5 female; age range, 39-68 years; mean age, 55.1 years) imaged by ¹⁸F-FDG PET/CT between January 2007 and Aug 2008 was performed to select and analyze ¹⁸F-FDG PET/CT scans findings of patients with or without clinically and/or radiologically suspicious findings for restaging. Twenty patients had undergone 25 PET/CT scans (three patients had two scans and one patient had three scans). The standard reference for tumor recurrence consisted of histopathological confirmation or clinical follow-up for at least ten months after ¹⁸F-FDG PET/CT.

¹⁸F-FDG PET/CT technique

The patients were asked to fast for at least 4 h before undergoing ¹⁸F-FDG PET/CT. Their blood glucose levels were within the normal range (70-120 mg/dL) prior to intravenous injection of ¹⁸F-FDG. The patients received an intravenous injection of 370-666 MBq (10-18 mCi) of ¹⁸F-FDG. Data acquisition by an integrated PET/CT system (Discovery STE; GE Medical Systems, Milwaukee, WI, USA) was performed within 60 min after injection. The procedure for data acquisition was as follows: CT scanning was performed first, from the head to the pelvic floor, with 110 kV, 110 mA, a tube rotation time of 0.5 s, a 3.3-mm section thickness, which was matched to the PET section thickness. Immediately after CT scanning, a PET emission scan that covered the identical transverse field of view was obtained. Acquisition time was three minutes per table position. PET image data sets were reconstructed iteratively by

Table 1 Patient characteristics

Clinical characteristics	Data
Mean age (yr)	55.1 (39-68)
Gender	
Males	15
Females	5
Mean time after treatment to PET/CT exam	1 mo-5 yr; mean 20 mo
Mean follow-up time after PET/CT exam	1 mo-18 mo; mean 11 mo

applying the CT data for attenuation correction, and coregistered images were displayed on a workstation.

Definitive diagnoses of positive and negative findings

Reviewer 1 and reviewer 2, who were aware of other clinical or imaging information, read the ¹⁸F-FDG PET/CT images on a high-resolution computer screen. The reviewers reached a consensus in cases of discrepancy. Reviewer 1 had 20 years of experience in both nuclear medicine and radiology, and reviewer 2 had 5 years of experience in both nuclear medicine and radiology. On the basis of knowledge of the normal biodistribution of FDG, lesions were identified as foci with increased tracer accumulation relative to that in comparable normal contralateral structures and surrounding soft tissues. The lesions were qualitatively graded as definitely or probably abnormal (i.e. representing tumors) if the accumulation of FDG was markedly to moderately increased. Diffuse mildly increased activity or no increased activity (in the case of an abnormality identified on CT for which no corresponding abnormality was present on PET) was considered normal or as benign disease. Quantification of the tumor metabolic activity was obtained using the Standardized Uptake Value (SUV) normalized to body weight. mean \pm SD of maximum-pixel SUV (SUVmax) of the lesions were calculated.

RESULTS

The characteristics of the patients are summarized in Table 1. For suspected recurrent esophageal cancer, the mean patient age was 55.1 years with a tendency of male gender distribution (75%). ¹⁸F-FDG PET/CT was positive in 14 patients (68.4%) and negative in six (31.6%). When correlated with final diagnosis, which was confirmed by histopathological evidence of tumor recurrence in seven of the 20 patients (35%) and by clinical and radiological follow-up in 13 (65%), ¹⁸F-FDG PET/CT was true positive in 11 patients (Table 2, Figures 1 and 2), false positive in three and true negative in six. The three false positive PET/CT findings were chronic inflammation of mediastinal lymph nodes ($n = 2$) and anastomosis inflammation ($n = 1$) (Figure 3), which were confirmed by at least 11 mo of clinical and radiological follow-up. There were no false negatives in our group. Overall, the accuracy of ¹⁸F-FDG PET/CT was 85%, negative predictive value (NPV) was 100%, and positive predictive value (PPV) was 78.6%.

Notably, ¹⁸F-FDG PET/CT demonstrated true positive distant metastasis in 90.9% (10/11) patients.

Table 2 PET/CT findings in 11 patients with true positive PET/CT scans

No.	PET/CT findings	SUV max	Further treatment plan	Interval time
1	Retroperitoneal lymph nodes	4.5	Surgical resection of metastases	5 yr
2	Retroperitoneal lymph nodes	6.5	Local radiotherapy	1yr
	Lung metastasis	1.6	Chemotherapy	
3	Mediastinal lymph nodes	5.2	Chemotherapy	13 mo
4	Supraclavicular lymph nodes	3.3	Local radiotherapy	11 mo
5	Retroperitoneal lymph nodes	6.0	Local radiotherapy	2 mo
6	Supraclavicular lymph nodes	8.4	Chemotherapy	11 mo
	Retroperitoneal lymph nodes	3.6		
	Liver metastasis	10.6	RF-ablation	
7	Retroperitoneal lymph nodes	12.1	Chemotherapy	14 mo
	Liver metastasis	14.0	RF-ablation	
8	Liver metastasis	8.4	RF-ablation	16 mo
	Supraclavicular lymph nodes	3.6	Chemotherapy	
	Retroperitoneal lymph nodes	10.6		
9	Supraclavicular lymph nodes	3.2	Local radiotherapy	1 mo
10	Supraclavicular lymph nodes	6.7		1 mo
	Osseous metastasis	5.3		
	Peritoneal carcinomatosis	7.9		
11	Anastomosis recurrence	7.1	Stent implant	5 mo
	Retroperitoneal lymph nodes	8.5	Chemotherapy	

RF-ablation: Radiofrequency-ablation.

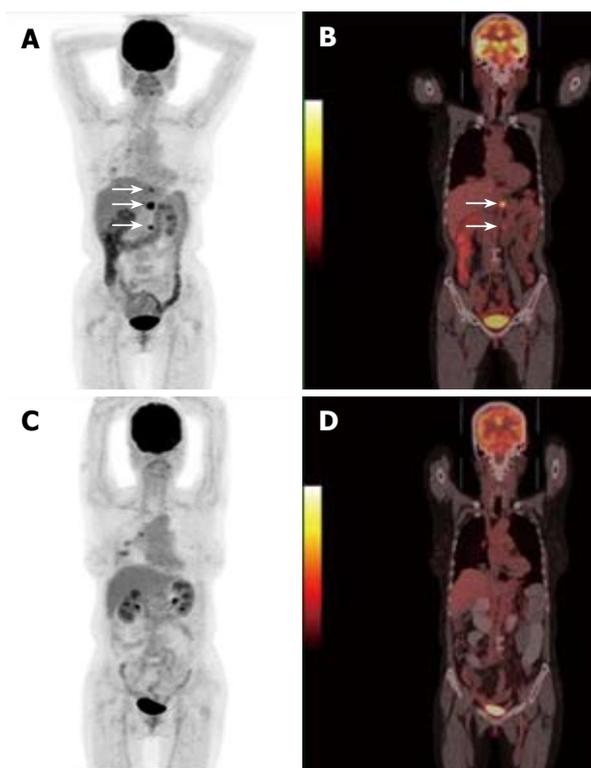


Figure 1 A 56-year-woman who had esophageal cancer resection 5 years ago. PET/CT displayed multi-retroperitoneal lymph node recurrence (arrows in A and B). The patient accepted retroperitoneal lymph node resection, which was later verified as esophageal cancer metastasis by histopathology. PET/CT Follow-up 10 mo after her second operation demonstrated no new recurrent lesions (C, D).

Except for 45.5% (5/11) patients who suffered from supraclavicular lymph nodes metastasis, the remaining 54.5% (6/11) patients were asymptomatic, with no evidence of relapse disease. These remaining patients underwent further PET/CT scans as part of routine post-operative surveillance. ^{18}F -FDG PET/CT imaging-guided salvage treatment (surgical resections of

metastasis lesions, additional radiotherapy, chemotherapy, and radiofrequency ablation of hepatic metastatic tumors) in 10 patients was performed within two weeks after the ^{18}F -FDG PET/CT scan. Clinical decisions of treatment were changed in 12 (60%) patients after introducing ^{18}F -FDG PET/CT into their conventional post-treatment follow-up program.

In our study, 90.9% (10/11) cases of recurrence after curative resection occurred within 16 mo and 9.1% (1/11) occurred within 5 years, respectively, after the initial resection. A high percentage of first failures presented as supraclavicular lymph nodes and retroperitoneal lymph nodes metastases.

DISCUSSION

Surgery is still the main treatment option for esophageal cancer; however, long-term survival has remained poor, even when a curative operation is performed^[10]. Despite increasingly extended radical esophagectomy for esophageal cancer, many patients continue to experience relapse of the disease^[11]. About one half of the patients develop recurrent disease within three years after the operation, and most of them develop mediastinal lymph node, liver, bone, or lung metastasis^[12]. Anastomotic recurrence is a major reason of late mortality following esophago-gastrectomy for carcinoma of the esophagus and esophago-gastric junction, using either the Ivor Lewis approach with intra-thoracic anastomosis^[13]. However, neoplastic recurrence was most common at the supraclavicular lymph nodes and retroperitoneal lymph nodes (81.8%; 9/11); only one patient suffered from mediastinal lymph nodes recurrence and one patient suffered from anastomotic recurrence in our study results. Cooper *et al*^[14] reported that concurrent postoperative chemotherapy and radiotherapy significantly improved the rates of local and regional

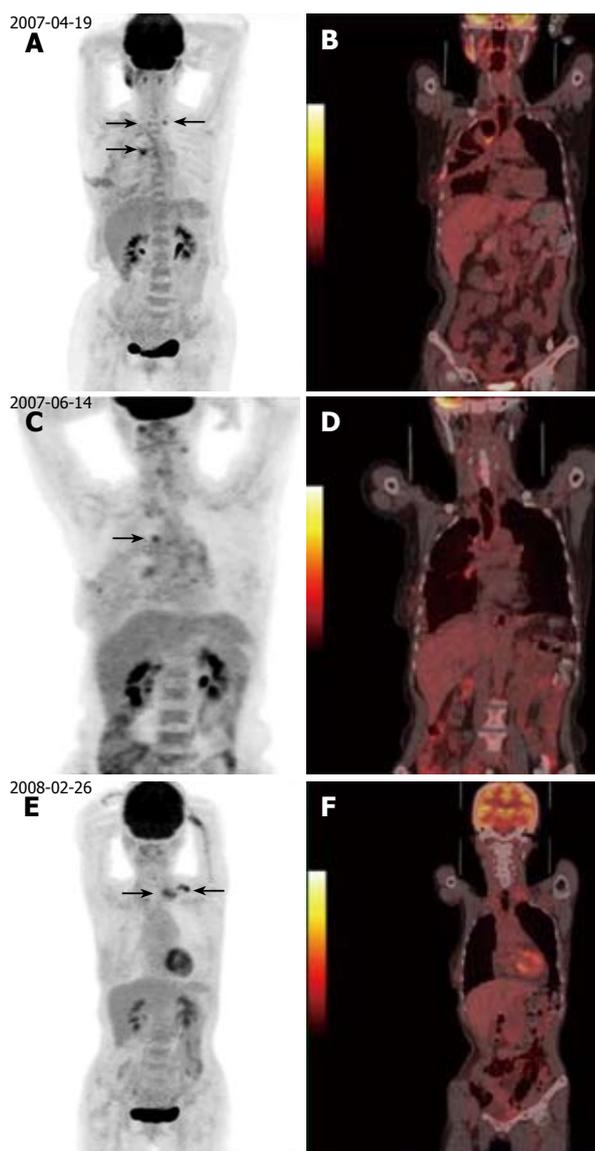


Figure 2 A 62-year-old woman had esophageal cancer resection. She underwent three PET/CT scans (1.5, 4.5 and 11 mo after treatment, respectively) as part of routine post-operative surveillance. The first PET/CT imaging (1.5 mo) revealed hypermetabolic activity in the anastomosis and supraclavicular lymph nodes (arrows, A, B). The second PET/CT imaging (4.5 mo) showed decreasing of hypermetabolic activity at anastomosis and supraclavicular lymph nodes (arrows, C, D). The third PET/CT imaging (11 mo) showed no abnormal FDG uptake at anastomosis, but revealed new focal hypermetabolic activity at left supraclavicular lymph nodes (arrows, E, F). Inflammation of lymph nodes and anastomosis at the first and second PET/CT scan were confirmed by the third PET/CT examination. New relapse at the left supraclavicular lymph nodes was later verified by biopsy.

control and disease-free survival among high-risk patients with resected head and neck cancer. All of our patients accepted postoperative radiotherapy, which may be beneficial in the control of mediastinum recurrence.

It was reported that PET/CT using the radiolabeled glucose analog, ^{18}F -FDG, was valuable in detecting recurrence of esophageal cancer, particularly when anatomic imaging modalities have presented equivocal interpretations^[15]. ^{18}F -FDG PET/CT is a metabolic imaging technique where the scope covers the whole body (from the skull to the lower limbs). Major advantages

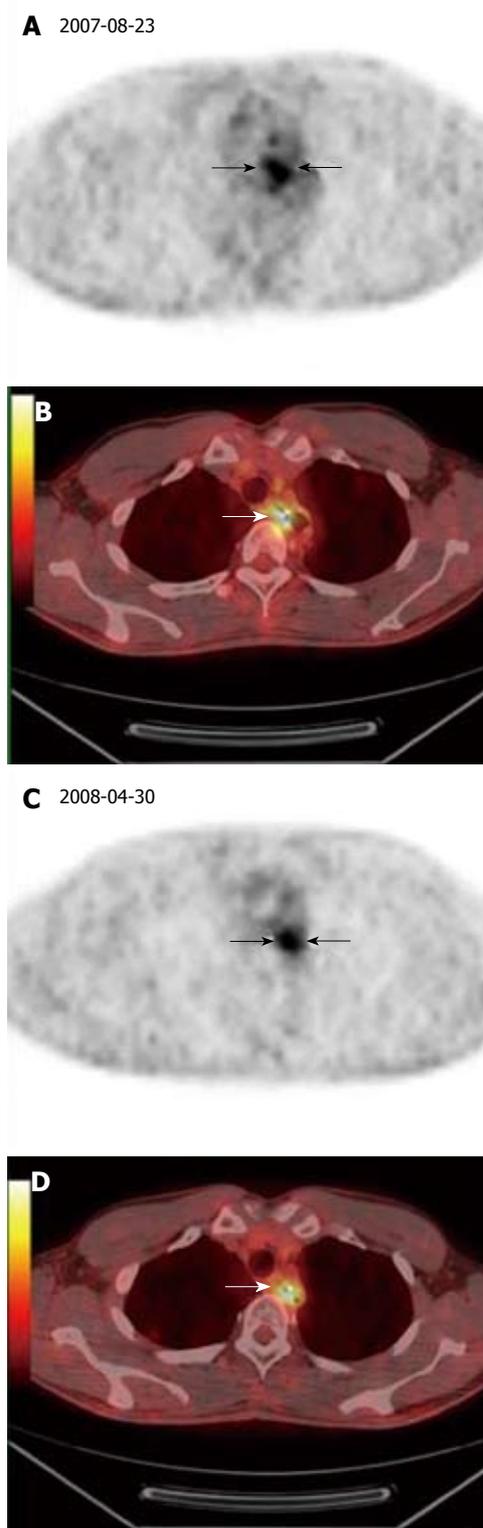


Figure 3 A 40-year-old asymptomatic man who had esophageal cancer resection 30 mo ago underwent PET/CT as part of routine post-operative surveillance. The first PET/CT revealed hypermetabolic activity at the anastomosis (arrows, A, B) 5 mo after treatment. The second PET/CT showed hypermetabolic activity at the anastomosis (arrows, C, D) 30 mo after treatment. The final diagnosis by endoscopic biopsy was anastomotic inflammation.

of ^{18}F -FDG PET/CT are the ability to perform full body examinations, the potential to detect locoregionally recurrence and distant metastatic lesions in one single examination, and the possibility of distinguishing new

active disease from scar or necrotic tissue^[16,17]. However, conventional CT and magnetic resonance imaging (MRI) examinations only cover local regions of the body. The current study is the first that specifically focused on the utility of ¹⁸F-FDG PET/CT imaging for whole body diagnosis and staging of recurrent esophageal cancer after curative resection and postoperative radiotherapy. Our preliminary results suggest that ¹⁸F-FDG PET/CT provides a highly sensitive diagnosis of recurrent disease, both for locoregionally recurrence and distant metastasis.

Whether earlier diagnosis of recurrent esophageal cancer would improve patient survival has not yet been reported^[18,19]. The possibility of a survival benefit, however, can be deduced indirectly from results obtained in our previous study with early detection of recurrence in asymptomatic post-operation patients with gastric cancer, indicating a survival benefit of nine months^[20]. The available therapeutic modalities in recurrent esophageal cancer are radical re-resection, palliative resection and bypass, laser thermocoagulation, stenting, chemotherapy, brachytherapy, and radiotherapy, alone or in combination^[21,22]. The choice of a specific therapeutic modality depends on the extent of the recurrence. Patients with metastatic esophageal cancer have a median survival time of six months^[23,24]. However, one patient who suffered from retroperitoneal lymph node recurrence around the pancreas accepted retroperitoneal lymph node resection and partial excision of the pancreas. The patient remained in a good condition over a 10-mo follow-up period.

The prognosis of patients with post-operative loco-regional recurrence of esophageal cancer is poor. However, long-term survival might be expected by definitive radiotherapy for the patients with small-size tumors and with a good performance status^[25]. Raoul *et al*^[26] reported the survival rates of patients with postoperative recurrence treated with chemoradiotherapy to be 47.1% at one year, 17.1% at two years and 4.3% at three years. Although the average follow-up time of this group of patients is only 11 mo, our study results of nine patients also indicated that ¹⁸F-FDG PET/CT-guided salvage therapy and earlier diagnosis of recurrent esophageal cancer might improve patient survival. PET/CT also has advantages in individualized treatment for patients. Morphological imaging with CT or MRI after radiofrequency ablation of malignant liver tumors is hampered by rim-like enhancement in the ablation margin, making the identification of residual or local recurrent tumors in the ablation zone difficult^[27,28]. In our study, three patients with hepatic metastatic tumors from esophageal cancer accepted radiofrequency-ablation of metastatic lesions. ¹⁸F-FDG PET/CT proved to be more accurate in morphological imaging with CT or MRI when making radiofrequency-ablation plans and assessing the liver for residual tumors after radiofrequency-ablation.

A noteworthy finding in the current study is the high incidence [21.4%; (3/14)] of false-positive ¹⁸F-FDG PET/CT findings at the mediastinal lymph nodes ($n = 2$) and anastomotic inflammation ($n = 1$). False-

positive FDG uptake at inflammatory lesions is widely known and remains a major problem in the diagnosis of oncologic patients^[29,30]. Three other patients had high-moderate uptake at the mediastinal lymph nodes. The FDG uptake in the three patients probably reflected a normal inflammatory healing process after operation and radiation. In our experience, the presence of the stomach in the thoracic cavity after the operation induces focal atelectasis and lung inflammation in many of patients after treatment, which might be also contribute to the increasing FDG uptake in mediastinal lymph nodes.

PET scans for response assessment should also be timed correctly^[31,32]. Treatment with surgical resection and radiation often results in temporary inflammatory changes that may show up positive on a PET/CT scan when there is no real disease. These changes take some time to subside^[33,34]. In our experience, it is recommended that PET/CT scans be performed only after at least two weeks of chemotherapy and five-six weeks of radiation for the true picture to emerge. Thus, the sophisticated surgical procedures used in esophagectomy and radiation treatment after operation can result in anatomical and functional changes. Clinicians at PET/CT centers must understand how these procedures can affect imaging data and be familiar with the appearances of postoperative anatomical changes, complications, and tumor recurrence, to ensure accurate evaluation of affected patients.

In conclusion, ¹⁸F-FDG PET/CT has the potential to be a highly sensitive diagnostic tool for accurate whole-body staging of asymptomatic and symptomatic recurrent esophageal cancer. ¹⁸F-FDG PET/CT-guided salvage treatment to the early recurrent lesion might improve patient survival in a considerable proportion of patients. Due to the false-positive findings based on ¹⁸F-FDG accumulation in areas of inflammation, interpretation of ¹⁸F-FDG PET/CT results is optimized by understanding the diagnostic limitations and pitfalls that may be encountered, together with knowledge of the natural history of esophageal cancer and the staging and treatment options available.

COMMENTS

Background

The incidence of esophageal cancer has steadily increased over the past three decades. The best option for curative treatment for patients with esophageal cancer is radical surgery, with a long-term survival of only 25%. Postoperative tumor recurrence is not uncommon in patients undergoing curative resection for esophageal cancer. The outcome of esophagectomy could be improved by optimal diagnostic strategies leading to adequate guided salvage treatment. In this study, we aimed to analyze the value of ¹⁸F-fluorodeoxyglucose positron emission and computed tomography (¹⁸F-FDG PET/CT) scans in the follow up of patients with esophageal cancer treated with combined surgical treatment and radiotherapy management.

Research frontiers

¹⁸F-FDG PET and, particularly, ¹⁸F-FDG PET/CT are widely accepted imaging methods in the management of a wide variety of cancers. However, the utility and limitation of ¹⁸F-FDG PET/CT in patients with esophageal cancer treated by surgical resection and post operation radiation is not clear.

Innovations and breakthroughs

Whole body ¹⁸F-FDG PET/CT was effective in detecting relapse in esophageal

cancer after surgical resection and radiotherapy and also had important clinical impacts on the management of esophageal cancer; influencing both clinical restaging and salvage treatment of patients.

Applications

This has the potential to be a powerful technology for restaging of esophageal cancer after surgical resection and radiotherapy.

Peer review

This manuscript can be presented as an initial report and is an interesting article about the application of PET-CT to restaging of esophageal cancer after surgical resection and radiotherapy.

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Clinical impact of selective transarterial chemoembolization on hepatocellular carcinoma: A cohort study

Rodolfo Sacco, Marco Bertini, Pasquale Petruzzi, Michele Bertoni, Irene Bargellini, Giampaolo Bresci, Graziana Federici, Luigi Gambardella, Salvatore Metrangolo, Giuseppe Parisi, Antonio Romano, Antonio Scaramuzzino, Emanuele Tumino, Alessandro Silvestri, Emanuele Altomare, Claudio Vignali, Alfonso Capria

Rodolfo Sacco, Marco Bertini, Michele Bertoni, Giampaolo Bresci, Graziana Federici, Luigi Gambardella, Salvatore Metrangolo, Giuseppe Parisi, Antonio Romano, Antonio Scaramuzzino, Emanuele Tumino, Alessandro Silvestri, Alfonso Capria, Gastroenterology Department, Pisa University Hospital, Via Paradisa 2, 56124 Pisa, Italy

Pasquale Petruzzi, Irene Bargellini, Claudio Vignali, Department of Diagnostic and Interventional Radiology, Pisa University Hospital, Via Paradisa 2, 56124 Pisa, Italy

Rodolfo Sacco, Emanuele Altomare, Institute of Internal Medicine, University of Foggia, Viale Pinto, 71100 Foggia, Italy

Author contributions: Sacco R took care of patients, designed the study, collected and analyzed data, and wrote the manuscript; Bertini M took care of patients, collected and analyzed data, performed statistical analysis and was involved in editing the manuscript; Petruzzi P, Bargellini I and Vignali C performed TACE treatments and contributed to discussion; Bertoni M, Bresci G, Federici G, Gambardella L, Metrangolo S, Parisi G, Romano A, Tumino E and Scaramuzzino A took care of patients and collected data; Silvestri A coordinated the nursing staff; Altomare E and Capria A coordinated the work group and contributed to discussion.

Correspondence to: Rodolfo Sacco, MD, PhD, Gastroenterology Department, Pisa University Hospital, Via Paradisa 2, 56124 Pisa, Italy. r.sacco@ao-pisa.toscana.it

Telephone: +39-50-997411 Fax: +39-50-997412

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Abstract

AIM: To prospectively evaluate the short and long term clinical impact of selective transarterial chemoembolization (TACE) on liver function in patients with hepatocellular carcinoma (HCC). To assess side effects in relation to treatments. To analyze the overall survival and HCC progression free survival probability.

METHODS: One hundred and seventeen cirrhotic patients with HCC were enrolled. Baseline liver function included Child-Pugh score and serum levels of alanine-aminotransferase (ALT), prothrombin time (PT) and bilirubin. According to Cancer Liver Italian Program (CLIP) and Barcelona Clinic Liver Cancer (BCLC)

staging systems, 71 patients were eligible for TACE; 32 had previously received treatment for HCC. No significant differences in liver function were observed between previously treated and not treated patients. TACE was performed by selective catheterization of the arteries nourishing the lesions. While hospitalized, patients underwent clinical, hematologic and ultrasonographic assessments. One month after TACE a CT scan was performed to assess tumor response. A second TACE was performed "on demand". Liver function tests were checked in all patients every four months.

RESULTS: After first TACE, the mean Child-Pugh score increased from a mean baseline 5.62 ± 1.12 to 6.11 ± 1.57 at discharge time ($P < 0.0001$), decreasing after four months to 5.81 ± 0.73 (not significant). ALT, PT and bilirubin significantly ($P < 0.0001$) increased 24 h after TACE and progressively decreased until discharge. After the second TACE, variations in Child-Pugh score, ALT, PT and bilirubin were comparable to that described after the first TACE. No major complications were observed. The mean follow-up was 14.7 ± 6.3 mo (median: 16 mo). Only one patient died. No other patient experienced important long term worsening of clinical status. The overall survival probability at twenty-four months was 98.18% with a correspondent HCC progression free survival probability of 69%.

CONCLUSION: Selective TACE may produce significant, but transitory increases in ALT values, with no major impact on liver function and Child-Pugh score. Preservation of liver function is achievable also in patients previously treated with other therapeutic modalities and in patients undergoing multiple TACE cycles. Liver function can remain stable in the long-term, with optimal medium term survival. This result can be achieved through rigorous patient selection on the basis of tumour characteristics and clinical conditions.

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Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Liver function; Liver cirrhosis; Child-Pugh score

Peer reviewers: R Todd Stravitz, MD, FACP, FACP, Associate Professor of Medicine, Section of Hepatology, Hume-Lee Transplant Center, Virginia Commonwealth University, Richmond, VA 23298-0341, United States; Tsianos Epameinondas, MD, PhD, Professor, 1st Division Of Internal Medicine & Hepato-Gastroenterology Unit, Medical school University of Ioannina, PO Box 1186 Ioannina 45110, Greece

Sacco R, Bertini M, Petrucci P, Bertoni M, Bargellini I, Bresci G, Federici G, Gambardella L, Mentrangolo S, Parisi G, Romano A, Scaramuzzino A, Tumino E, Silvestri A, Altomare E, Vignali C, Capria A. Clinical impact of selective transarterial chemoembolization on hepatocellular carcinoma: A cohort study. *World J Gastroenterol* 2009; 15(15): 1843-1848 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1843.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1843>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm in the world^[1] and its incidence is increasing worldwide^[2]. Overall, HCC is associated with liver cirrhosis in 80% of cases and it is the leading cause of death among cirrhotic patients^[3].

The treatment of patients with HCC has evolved in the last few years. However, curative treatments such as liver resection, liver transplantation or percutaneous ablation [percutaneous ethanol injection (PEI) and radiofrequency ablation (RF)] are applicable in only 30%-40% of cases^[4]. Since transarterial chemoembolization (TACE) was introduced as a palliative treatment in patients with unresectable HCC, it has become one of the most common forms of interventional therapy^[5]. Recently, it has been demonstrated that TACE improves survival compared with best supportive care in meta-analyses of randomized trials^[6,7] and in two individual clinical trials^[8,9].

Nowadays TACE is often performed by selective catheterization of the hepatic segmental arteries nourishing the HCC lesions, to limit as much as possible the injury to the surrounding non tumorous liver as reported in previous studies^[10-12]. However, although selective TACE is currently widely used, to our knowledge there are no reported extensive data from large series on both short and long term effects of this treatment on liver function. Because the optimal number of sessions is not known^[13], it is debatable if repeated courses of selective TACE may progressively impair liver function and if they are well tolerated or are limited by major side effects.

On this basis, in the present study, we prospectively evaluated the short and long term impact of selective TACE on liver function in a consecutive series of patients with liver cirrhosis and HCC. Side effects in relation to treatments were also assessed. Furthermore, we analyzed the overall survival and HCC progression free survival probability.

MATERIALS AND METHODS

Patients and HCC diagnosis

From September 1st 2006 to August 31st 2008, at a

Table 1 Patients' demographic characteristics and etiology of liver cirrhosis *n* (%)

	Patients
Patients number	117
Gender	
Male	81 (69.2)
Female	36 (30.8)
Age (yr)	
mean \pm SD	69 \pm 7.8
Range	43-85
Causes of cirrhosis	
HBV (hepatitis B)	9 (7.7)
HBV and NAFLD	5 (4.3)
HBV and alcohol abuse	1 (0.09)
HCV (hepatitis C)	59 (50.4)
HCV and NAFLD	16 (13.7)
HCV and alcohol abuse	10 (8.5)
Alcohol abuse	8 (6.8)
Alcohol abuse and overweight	8 (6.8)
Sarcoidosis	1 (0.09)

NAFLD: Non-alcoholic fatty liver disease.

single center, we prospectively evaluated 117 consecutive patients with liver cirrhosis and HCC. Patients' demographic characteristics and etiology of liver cirrhosis are reported in Table 1.

HCC diagnosis was established by means of alpha-fetoprotein (AFP) assay, abdominal ultrasound examination and cross-sectional imaging, including at least one multiphase contrast-enhanced spiral or multidetector CT (CT; Hi Speed CT/I or light speed plus, GE Medical Systems, Milwaukee, USA). Extrahepatic metastases were ruled out by chest X-ray and bone scintigraphy. The HCC Tumor-Node-Metastasis (TNM) stages for our patients were: 44 (37.6%) patients T1 N0 M0, 40 (34.1%) patients T2 N0 M0, 32 (27.3%) patients T4 N0 M0, 1 (0.8%) patient T4 N0 M1.

Baseline evaluation of liver function included determination of the Child-Pugh score and serum levels of alanine-aminotransferase (ALT), prothrombin time (PT) and bilirubin.

The Child-Pugh class of disease was A (score 5-6) in 71 patients, B (score 7-9) in 26, and C (score 11-15) in 20.

We also stratified patients according to the staging system for HCC of Cancer Liver Italian Program (CLIP)^[14]: it combines Child-Pugh staging with tumor criteria (tumor morphology, portal invasion and AFP levels). The CLIP score stratification of our patients is described in Figure 1.

According to the Barcelona Clinic Liver Cancer (BCLC) staging system^[15], which assesses tumor characteristics and liver function to generate a treatment algorithm, 13 patients were suitable for curative treatments (resection, liver transplant, PEI/RF) and 33 patients had advanced (i.e. portal invasion) or terminal stage HCC suitable only for symptomatic treatments. The remaining 71 patients were considered eligible for TACE. In this group mean HCC size was 39.8 \pm 14.1 mm.

We further stratified the patients suitable for TACE in two subgroups according to whether or not they had positive anamnesis for previous treatments for

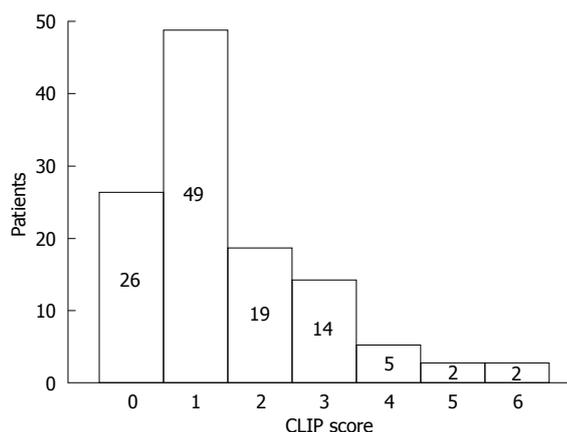


Figure 1 Stratification of HCC patients according to CLIP score.

HCC. Thirty-two out of 71 patients (45%) had received treatment for HCC (TACE in 15, liver resection in 4, PEI/RF in 3 cases) before coming to our center. In these pretreated patients, the mean time between the first diagnosis of HCC and inclusion in our study was 16.7 ± 22.4 mo. No significant differences in Child-Pugh and CLIP scores were observed between previously and not previously treated patients (Table 2).

TACE protocol

Informed consent was obtained from all patients after the nature and the purpose of TACE had been fully explained. Digital subtraction angiography (DSA; Multistar, Siemens, Erlangen, Germany) was performed in all patients immediately before TACE.

TACE was performed by selective catheterization of the hepatic segmental arteries nourishing the lesions, using either 5-F catheters (Simmons 1 and Cobra; Mallinckrodt, St Louis, USA or Hydrophilic Simmons 1 and Cobra; Terumo, Tokyo, Japan) or 3-F coaxial microcatheters (Tracker 18; Vascular Access System, Target, St José, USA; SP Catheter; Terumo). A mixture of iodised oil (Lipiodol UltraFluid; Laboratories Guerbet, Aulnay-sous-Bois, France) and epirubicin hydrochloride (Farmorubicina; Farmitalia Carlo Erba, Milan, Italy) was injected, followed by selective arterial embolization using gelatin sponge particles (Spongostan Standard; Johnson and Johnson Medical Limited, Gargrave, Skipton, UK).

The amount of administered Lipiodol (10-30 mL) and anticancer drug (40-100 mg) was decided on the basis of number, location and diameter of lesions.

Follow-up

After TACE procedure, the patients recovered with about 20 h bed rest. After this time, compression of the femoral artery, used as an access for administration of medication, was removed. Every 2 h during the first 6 h patients underwent clinical examination (abdominal evaluation and measurements of pulse rate, arterial blood pressure and body temperature).

A routine hematologic check was performed in all patients the morning after TACE and at discharge time,

Table 2 Child-Pugh and CLIP score in 71 patients eligible for TACE at enrollment (mean \pm SD)

	Not pretreated patients	Previously treated patients
Number of patients	39	32
Child-Pugh score	5.82 ± 0.82	5.4 ± 1.2
CLIP score	1.12 ± 0.82	0.86 ± 0.83

usually 5-7 d after the procedure. If severe abdominal pain or fever persisted during hospitalization, patients underwent abdominal ultrasonography to help exclude complications. One month after TACE a CT scan of the liver was performed to assess tumor response. TACE was repeated "on demand", if there was no tumor response, insufficient tumor response or progressive disease.

Tumor response to TACE was evaluated according to World Health Organization (WHO) criteria (complete response (CR): complete disappearance of all known disease and no new lesions; partial response (PR): at least 50% reduction in total tumor load of all measurable lesions; stable disease (ST): does not qualify for CR/PR or progressive disease; progressive disease (PD): at least 25% increase in size of one or more measurable lesions or the appearance of new lesions) modified according to the EASL amendments that take into account the reduction in viable tumor volume due to TACE-induced necrosis^[16].

Liver function tests were checked in all patients every four months in order to evaluate hepatic functional reserve.

Statistical analysis

Descriptive statistics (mean \pm SD) were provided when appropriate. Parametric data were tested with Student's paired *t*-test. Overall survival and HCC progression free survival curves were determined by the Kaplan-Meier method. A two sided *P* value of less 0.05 was considered statistically significant. Statistical analysis was performed with SAS software (SAS Institute, Cary, NC).

RESULTS

Ninety-eight TACE procedures were performed in 71 patients (mean number of treatments per patient 1.4 ± 0.61). After first TACE, the mean Child-Pugh score increased from a mean baseline 5.62 ± 1.12 to 6.11 ± 1.57 at discharge time ($P < 0.0001$), decreasing after four months to 5.81 ± 0.73 (not significant).

ALT, PT and bilirubin significantly ($P < 0.0001$) increased 24 h after TACE and then progressively decreased until discharge (Figure 2). After the second TACE cycle, variation of Child-Pugh scores (mean procedural value of 5.71 ± 0.78 , discharge value to 5.9 ± 1.07 , $P = 0.056$ not significant) and of ALT, PT and bilirubin were comparable to that described after the first TACE cycle.

No major complications were observed in our series of patients after TACE. After the first TACE cycle, post chemoembolization syndrome occurred in 23 (32.4%) patients, whereas 4 (5.6%) patients developed

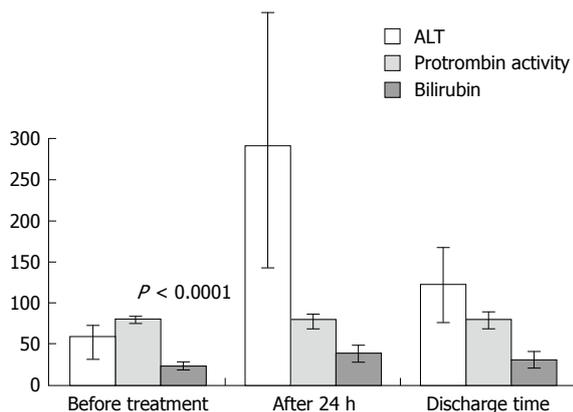


Figure 2 Variations of ALT, PT and bilirubin after the first TACE (71 patients).

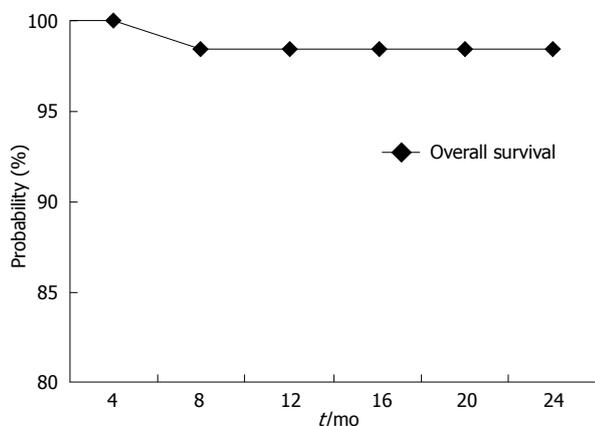


Figure 3 Overall survival probability according to Kaplan-Meier analysis.

acute cholecystitis and 2 (2.8%) patients presented with hematomas at the site of the femoral artery used as an access for administration of medication.

Following a second TACE cycle (22 patients), 5 (22.7%) patients experienced post chemoembolization syndrome, 1 (4.5%) patient presented with acute cholecystitis and 1 (4.5%) case showed mild ascites. After both the first or second TACE, side effects were successfully managed with medical therapy. When comparing patients according to whether or not they had positive anamnesis for previous treatments for HCC, we did not find significant differences in the modifications of pre- and post-TACE Child-Pugh scores.

Six months after chemoembolization in 57 (80%) out of 71 patients, the mean tumor size had not changed. In the remaining 14 (20%) patients, mean tumor size was 44.8 ± 17.7 mm. The mean follow-up was 14.7 ± 6.3 mo (median 16 mo). At the end of the follow-up, 49 (69%) of the treated patients presented with a CR; 21 (29.57%) patients had a PR; only 1 (1.4%) patient had progression of the tumor that involved more than 50% of the liver, with rapidly progressive worsening of clinical condition and appearance of bone metastasis. No other patient with class A disease was reclassified as having a class B disease, and no other patient with class B disease was reclassified as having class C disease after either the first

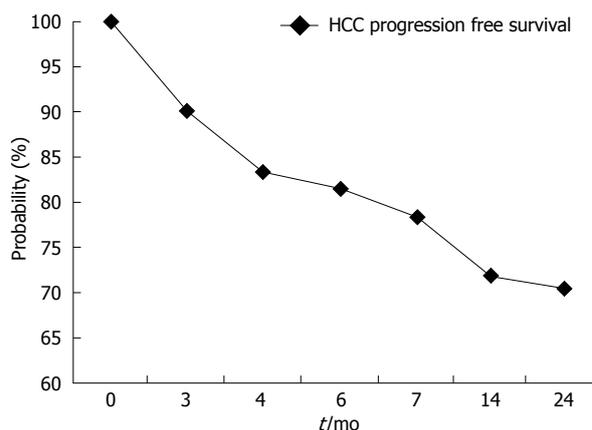


Figure 4 HCC progression free survival probability according to Kaplan-Meier analysis.

or the second TACE.

On follow-up, despite slight fluctuations in Child-Pugh score, no other patient experienced important long term worsening of clinical status and there was no progression of HCC that could modify CLIP or BCLC.

The overall survival probability at 24 mo was 98.18% (Figure 3) with a correspondent HCC progression free survival probability of 69% (Figure 4).

DISCUSSION

Our single center study prospectively evaluated a large cohort of patients with liver cirrhosis and HCC to assess the short and long term clinical impact of selective TACE on liver function.

TACE is the most widely used treatment in patients with HCC who are considered unsuitable candidates for surgery and/or ablative therapies^[17,18] and, recently, it has been shown to improve survival compared with best supportive care. The proper selection of candidates for TACE appears to be a key point. Even if a consensus has not yet been reached, the best candidates for TACE seem to be asymptomatic patients with preserved liver functions without vascular invasion or extrahepatic tumor spread^[19]. In fact, the benefits of the procedure should not be offset by treatment-induced liver damage. Thus, to minimize the injury to nontumoral liver tissue, TACE is often performed by selective (or superselective) catheterization of the hepatic segmental or subsegmental arteries nourishing the tumor.

Although the possible impairment of liver function is a critical point when assessing TACE feasibility, only a few studies (which are not recent) were adequately reported, evaluating the effects of non-selective TACE on hepatic function^[20-23]. Furthermore, to the best of our knowledge, there are no extensive published data on the short and long term clinical impact of selective TACE in HCC patients.

Attempts to improve the classification and prognostic prediction of HCC are still evolving and there is no agreement on the best staging system that can be recommended worldwide^[24]. For this reason, in our study patients suitable for TACE were selected using Child-

Pugh, TNM, CLIP and BCLC staging systems. In this series, we had not only naive HCC patients: at the time of enrollment, about half of our patients had previously been treated for HCC. In our patients, after either first or second TACE, at discharge time, the increase in Child-Pugh score approached significance in comparison with baseline scores, without important variations in the patients' clinical status.

We observed a statistically significant increase in ALT, PT and bilirubin 24 h after treatment. Interestingly, ALT values increased the most, when compared to PT and bilirubin. This phenomenon could be the expression of selective treatment that enables sufficient tumor necrosis without impairing liver function.

TACE was well tolerated by all patients, with no major complication observed in this series. As already known, the most frequent side effects were post-chemoembolization syndrome often associated with temporarily increased liver enzymes. The post-chemoembolization syndrome, which can have widely variable manifestations, consists usually of fever, abdominal pain, nausea, vomiting and leucocytosis: often it requires only patient monitoring and pain control, but it can prolong the hospitalization period^[25].

As an institutional policy, we usually discharged patients 5-7 d after the procedure even if no major side effects occurred. This is in contrast with other authors who discharged patients 48-72 h after TACE^[26,27]. Indeed we believe that this approach enables early diagnosis and treatment of possible clinical complications that could become symptomatic even a few days after treatment.

Considering the long-term follow-up, only slight and clinically negligible impairment of liver function was observed in our patients, with only one case of rapid HCC progression.

Liver function remained substantially stable independent of whether patients had undergone previous treatments or were treated by repeated TACE. In our study TACE was repeated "on demand", when there was evidence of insufficient tumor response, tumor recurrence or disease progression. According to our data, this type of treatment schedule may help preserving liver function and can be well tolerated in elderly patients, often suffering from co-morbidities, as well as in patients previously treated with other therapeutic techniques. TACE repeated at a fixed time, until the planned number of courses has been reached, may cause progressive liver atrophy and vascular damage^[28]. The decision to repeat TACE should be based not only on tumor response or progression, but also on patients' clinical conditions and tolerance, which have to be assessed before each new course.

Recently, it has been demonstrated that TACE improves survival compared with best supportive care in patients with unresectable HCC. Indeed, our 2 years cumulative survival probability was 98%, which compares favorably with previous studies. We believe that this good clinical result may be related to the strict criteria applied in the selection of patients who may benefit from TACE. To this purpose, different staging systems should be used, to determine tumor characteristics and underlying liver disease, as these factors impact patient survival and

treatment options.

In conclusion, our prospective study demonstrates that TACE may produce a significant, but transitory increase in ALT values, with no major impact on liver function and Child-Pugh scores, as an expression of treatment selectivity and tolerability. Preservation of liver function is achievable in patients previously treated with other therapeutic modalities as well as in patients undergoing multiple TACE cycles. Moreover, liver function can remain stable in the long-term, with optimal medium term survival. However, this result can be achieved only through rigorous patient selection, on the basis of tumor characteristics and clinical conditions.

At this time, further studies are warranted to consider the clinical impact of new methods of chemoembolization using drug-eluting particles, which could allow larger tumor necrosis with reduced systemic effects.

COMMENTS

Background

Since transarterial chemoembolization (TACE) was introduced as a palliative treatment in patients with unresectable hepatocellular carcinoma (HCC), it has become one of the most common forms of interventional therapy. However, although selective TACE is currently widely used, up to now there are no reported extensive data from large series on both short and long term effects of this treatment on liver function. It is debatable whether repeated courses of selective TACE progressively impair liver function and if they are well tolerated or are limited by major side effects.

Research frontiers

In the area of treatment of HCC, selective TACE is one of the most common forms of interventional therapy. In this field, the research hotspot is how to reduce TACE-related morbidity and how to improve overall survival and HCC progression free survival.

Applications

The study results suggest the proper selection of candidates for TACE using different staging systems and careful clinical management after the procedure. TACE repeated "on demand" may help preserving liver function and can be well tolerated in elderly patients as well as in patients previously treated with other therapeutic techniques.

Terminology

Selective TACE: it is a transarterial chemoembolization performed by a selective catheterization of the hepatic segmental arteries nourishing the HCC lesions, to limit as far as possible the injury to the surrounding non tumorous liver; TACE "on demand": it is a transarterial chemoembolization repeated not at a fixed time but in cases of absent or insufficient tumor response or progressive disease.

Peer review

This is a very interesting manuscript that describes the efficacy of selective transarterial chemoembolization in patients with non-operable hepatocellular carcinoma. The procedure was associated with minimal complications or alteration of liver function. The excellent long-term results, when considering tumor progression and survival, could be achieved only through rigorous patient selection, on the basis of tumor characteristics and clinical conditions.

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Prevalence of lactase persistent/non-persistent genotypes and milk consumption in a young population in north-west Russia

Yulia Khabarova, Suvi Torniainen, Hanna Nurmi, Irma Järvelä, Mauri Isokoski, Kari Mattila

Yulia Khabarova, Department of Family Medicine, Northern State Medical University, Arkhangelsk 163000, Russia

Suvi Torniainen, Hanna Nurmi, Irma Järvelä, Department of Medical Genetics, University of Helsinki, Helsinki 00014, Finland

Mauri Isokoski, Tampere School of Public Health, University of Tampere, Tampere 33014, Finland

Kari Mattila, Medical School, University of Tampere, 33014 and Centre of General Practice, Health District of Pirkanmaa, Tampere 33521, Finland

Author contributions: Khabarova Y designed the research, collected the material, processed the data, and wrote the paper; Torniainen S performed genetic analyses of samples, and wrote the Methods section; Nurmi H provided assistance and technical support for laboratory work and participated in analyses of genotypes; Järvelä I designed the research, supervised the research, and wrote the paper; Isokoski M proposed the study idea, supervised, supported and designed the study, and wrote the paper; Mattila K supervised, supported and designed the study, processed the data, and wrote the paper.

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Correspondence to: Yulia Khabarova, Department of Family Medicine, Northern State Medical University, Troitsky pr.51, Arkhangelsk, 163000, Russia. yuliakaterina@rambler.ru

Telephone: +7-8182-655194 Fax: +7-8182-655194

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Abstract

AIM: To estimate the prevalence of the lactase non-persistent genotype (C/C-13910) in a northern Russian population in accordance with ethnicity, and to evaluate self-reported milk consumption depending on lactase activity.

METHODS: Blood samples for genotyping lactase activity, defining the C/T-13910 variant by polymerase chain reaction, and direct sequencing were taken from 231 medical students of Russian origin aged 17-26 years. We analyzed milk product consumption by questionnaire which was specially designed for the estimation of milk consumption and abdominal complaints.

RESULTS: We found that the prevalence of the C/C-13190 genotype in the northern Russian population

was 35.6%. The other genotypes nearby C/T-13910 and associated with lactase activity were not present in the study population. The consumption of milk among people with the non-persistent genotype tended to be lower than among the lactose tolerant subjects, but was not statistically significant.

CONCLUSION: An investigation of the lactase persistent genotype in a northern Russian population has not been performed before. The genotype did not affect the consumption of milk products in this population which could be explained by low consumption of milk products among the entire study population.

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Key words: C/C-13910 genotype; Hypolactasia; Lactase persistence/non-persistence; Lactose malabsorption; Milk consumption; North-west Russia

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INTRODUCTION

The prevalence of adult-type hypolactasia (primary lactose malabsorption, lactase non-persistence) varies considerably between different races and populations^[1,2]. Inheritance of primary lactose malabsorption is controlled by a single recessive autosomal gene^[3].

Simoons^[4] and McCracken^[5] suggested the cultural historical hypothesis to explain the differences in prevalence of hypolactasia. Hence, people with lactase persistence could survive better because they could use all nutrients in milk without having diarrhea. It is possible that they had more children than subjects

with hypolactasia. The idea of the calcium absorption hypothesis was proposed by Flatz^[6], who suggested that as lactose is a stimulant of calcium absorption, people who consume milk have less rickets, pelvic deformities and more children. This was genetic selection to the benefit of lactase persistence.

Several laboratory methods are used in the diagnosis of hypolactasia. Previously the prevalence of hypolactasia was detected by a lactose tolerance test (LTT) or a lactose tolerance test with ethanol (LTTE)^[7,8]. The "gold standard" which can be used as a reference method is the direct determination of lactase activity in the small intestinal mucosa, taken by biopsy; however, the invasiveness of the test does not allow its use in everyday practice and screening^[7]. In 2002 the genetic variant associated with adult-type hypolactasia, a one base polymorphism C/T-13910 (rs 4988234) upstream of the lactase coding gene on chromosome 2 was identified. The C/T-13910 variant is located in the OCT-1 binding site and acts as an enhancer^[9]. The variant is inherited recessively so that the C-13910 allele in a homozygous form (the C/C-13910 genotype) is always associated with adult-type hypolactasia and the T-13910 allele (C/T and T/T-13910 genotypes) with persistent lactase activity^[10]. The specificity of the genetic test is 100% with a sensitivity of 93% in subjects older than 12 years^[11]. In addition, several other suggested variants (C/G-13907, T/C-13913, T/G-13915 and G/C-14010) nearby the C/T-13910 variant were identified in African and Middle Eastern populations^[12-14]. Of these variants, 13915 T/G and 14010 G/C are associated with lactase persistence^[12,14].

Data on the prevalence of hypolactasia from studies performed in the territories of Russia are available. The prevalence of lactase malabsorption among Russians has been found to vary from 13% to 57% according to different authors and the different methods used^[15-17]. Many studies were carried out in other populations living in the territories of Russia and the prevalence of lactose malabsorption among them was between 11% and 94%^[16-20].

Genotyping has been used in only two Russian studies^[21,22] which showed that the prevalence of hypolactasia among Russians living in the central part ranged from 36% to 50% and depended on the area of residence^[22].

The main hypothesis of the current study was that people with the C/C-13910 genotype will avoid using milk or their milk consumption will be less compared to those with the lactase persistent genotype.

The objective was to estimate the prevalence of the lactase non-persistent genotype in a northern Russian population of students from the Medical University of Archangelsk. We determined the frequency of the C/T-13910 variants in accordance with ethnicity and estimated self-reported milk consumption and its relationship to the C/T-13910 genotypes.

MATERIALS AND METHODS

Study group

The study was performed in collaboration with the

Department of Family Medicine, Northern State Medical University (NSMU), Archangelsk, Russia, the Department of Medical Genetics, University of Helsinki and the Department of General Practice, University of Tampere. The study was approved by the Ethics Committee of NSMU (No. 08/06 from 29.11.2006). Medical students from different faculties aged 17 to 26 years were enrolled into the investigation. All subjects gave written informed consent and completed a questionnaire on their personal data, self-reported health status, milk consumption habits, ethnicity and their place of birth as well as their parents' and grandparents' place of birth. Blood samples were taken from 241 students. Of these, 231 reported their origin as Russian; but, according to the origins of grandparents (at least three out of four grandparents) only 149 were considered to have Russian ethnicity. Of the others, 61 did not report the data on grandparents and these subjects were classified as "missing information" on ethnicity. Twenty one students who reported their origin as Russian had at least 2 grandparents with another origin mostly Ukrainian, but also Byelorussians, Komi, Mordvinian and others. Final analysis of the prevalence of the C/C-13910 genotype was based on one of three subgroups: Russians, the group with missing data on grandparents and the mixed origin group (Table 1). We included students who were chosen by random methods from four main faculties of the university in the investigation. Randomization in our study was done according to standard rules. The NSMU has 16 faculties and each of them has 10 to 12 groups of students. We took every third group of students according to an official list from every fourth faculty. As a result we received 176 students chosen by a random method. The remaining 65 were taken at an annual medical review. Although that sample was not random, there was no selection because every student who attended medical review during a certain time (while we were collecting the material) was taken. Age and sex do not affect gene frequency.

The number of students and the selection process are presented in Figure 1. The majority of students (155 of 231) who participated in the study were born in the Archangelsk region, the others were born in different regions of north-west Russia (Figure 2).

Questionnaire

We used the questionnaire designed by Sahi^[3] with some modifications in the present study. Milk consumption was estimated using questions about milk and sour milk consumption. For comparison, we divided all subjects into two groups (Table 2). The first group consisted of students who were consumers of milk regardless of amount (answer "Yes" in Table 2) and the second group consisted of those who never consumed milk (answer "No" in Table 2).

Analyses of genotype

DNA was amplified by polymerase chain reaction (PCR). We used Taq polymerase (Dynazyme, Finnzymes, Espoo, Finland) with the conditions described elsewhere. The

Table 1 The distribution of the lactase-persistent/non-persistent genotypes by ethnicity *n* (%)

	CC-13910 ¹	CT-13910 ²	TT-13910 ²	Total
Russian	53 (35.6)	76 (51.0)	20 (13.4)	149 (100)
Others	9 (42.8)	11 (52.4)	1 (4.8)	21 (100)
Information missing	22 (36.1)	26 (42.6)	13 (21.3)	61 (100)
Total	84 (36.5)	112 (48.7)	34 (14.8)	231 (100)

¹Lactase non-persistent genotype; ²Lactase persistent genotype.

Table 2 Consumption of milk products by different C/T-13910 genotype variants *n* (%)

Consumption	CC-13910 ¹	CT-13910 and TT-13910 ²	Total
Milk			
Yes	31 (36.9)	71 (48.3)	102 (44.2)
No	53 (63.1)	76 (51.7)	129 (55.8)
Total	84 (100)	147 (100)	231 (100)
Sour milk			
Yes	41 (48.8)	89 (60.5)	130 (56.3)
No	43 (51.2)	58 (39.5)	101 (43.7)
Total	84 (100)	147 (100)	231 (100)

¹Lactase non-persistent genotype; ²Lactase persistent genotype.

used forward primer was 5'-CCTCGTTAATACCCACT GACCTA-3' and the reverse primer was 5'-GTCACCTT GATATGATGAGAGCA-3' which covered about 400 bp regions on both sides of the C/T-13910 variant. The PCR product was verified by 1.5% agarose gel electrophoresis (with ethidium bromide). The PCR products were purified using shrimp alkaline phosphatase (USB) and exonuclease I (New England Biolabs) at 37°C for 60 min and at 80°C for 15 min.

During sequencing, BigDye 3.1 terminator (Applied Biosystems) was used according to the manufacturer's instructions. Sequencing conditions were as follows: 96°C for 1 min, then 25 cycles at 96°C for 10 s, 55°C for 5 s and 60°C for 4 min. The sequencing reaction followed purification by Millipore Multiscreen plates (Millipore, USA) with Sephadex G-50 Superfine sepharose (Amersham Biosciences, Sweden), electrophoresis using the ABI 3730 DNA Analyzer (Applied Biosystems) and base calling by Sequencing Analysis 5.2 software (Applied Biosystems). The obtained sequence was analyzed using Sequencher 4.6. software (Gene Codes, USA)^[23].

Statistical analyses

The Chi-square test was used to test the difference between groups of genotype in milk consumption. *P* < 0.05 was considered statistically significant.

RESULTS

The prevalence of the C/C-genotype was found to be 35.6% among young Russian people who were born and lived in north-west Russia (Table 1). None of the previously identified other variants (C/G-13907, T/C-13913, T/G-13915 and G/C-14010) were present in the study population.

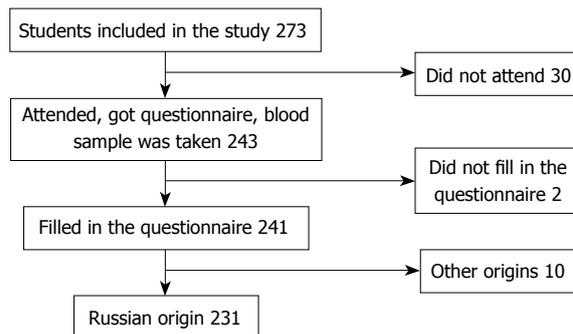


Figure 1 Steps in the data collection process.



Figure 2 Study group by region of birth place in north-west Russia.

The frequency of milk consumption varied among the milk consumers; however, the majority of subjects consumed 1-2 glasses per week or less and only 5 persons of 231 consumed 3 or more glasses per week. The same pattern was found for the consumption of sour milk. Only 3 persons in the entire study population consumed 3 or more glasses of sour milk per week.

Among subjects with lactase non-persistence (C/C-13910 genotype) more than half (63.1%) did not consume milk at all compared with those with the C/T-13910 variant (49.6%). The differences in milk consumption between lactose persistent subjects (C/T and T/T variants together) and those with hypolactasia (C/C-13910 genotype) was not statistically significant (*P* = 0.094).

The consumption of sour milk did not have a significant effect within the group of “non-consumers”; but, subjects with the C/T- and the T/T -variants consumed sour milk relatively more often than subjects with the C/C-13910 genotype (59.3%, 64.7% and 48.8%, respectively). The sour milk consumption among lactose non-persistent subjects (C/C-variant) and lactase persistent subjects (C/T- and T/T- variant together) was not statistically significant (*P* = 0.084).

All 5 persons who consumed milk products in

relatively greater amounts (3 and more glasses per week) had the lactase persistent genotype.

We estimated the gastrointestinal symptoms among subjects with lactase persistent and non-persistent genotypes. However, differences in the frequencies of symptoms were not statistically significant.

DISCUSSION

The prevalence of adult-type hypolactasia among Russians is in accordance with previous investigations and varies from 13% to at least 50%^[15-17,21,22]. The majority of previous studies were performed using the lactose tolerance test, and only two of the recent studies performed in Russia^[21,22], used the same direct genotyping we used in the present investigation. However, these researchers studied the frequency of the lactase persistent/non-persistent genotype among other populations living in the territories of Russia, but did not estimate the milk consumption.

Genetic testing is a direct method of diagnosing adult-type hypolactasia, and is not confounded by environmental factors which may affect the results of the other tests. Therefore the results of this study specify the actual prevalence of adult-type hypolactasia in Russia. An investigation into the lactase persistent genotype among a northern Russian population has not been performed before.

Since the variants (C/G-13907, T/C-13913, T/G-13915 and G/C-14010) nearby C/T-13910 were previously associated with lactase activity in southern parts of the world and were not present in this study, it is possible that these variants are not responsible for the regulation of lactase activity in north-west Russia. This is in agreement with results obtained from other countries in northern Europe^[23,24].

More than half of the study subjects did not drink milk and almost 45% did not drink sour milk at all. The great majority of "milk-consumers" drank very small quantities of milk, 1-2 glasses or less per week. Apparently, this is a habitual feature of the subjects in the study population and it did not depend on genotype. However, those five persons who drank the most milk had the lactase persistent genotype. Our data on the consumption of sour milk confirmed the results of previous studies which showed that people with lactose intolerance are more able to tolerate fermented milk because lactose in these products is reduced by 30%-40%^[25].

It is well-known that people with lactase non-persistence can consume small quantities of milk without any problems^[26]. The results of our study strengthen this theory, even though we did not reach statistical significance in milk product consumption between the lactase persistent and non-persistent subjects. In summary, genotype does not affect the consumption of milk products in a northern Russian population which can be explained by low consumption of milk products among the entire study population.

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COMMENTS

Background

The prevalence of adult-type hypolactasia (primary lactose malabsorption, lactase non-persistence) varies considerably between different races and populations. Data on the prevalence of hypolactasia from previous studies performed in the territories of Russia are limited especially for northern Russian populations. Moreover, the majority of these studies were performed using other methods. The inheritance of primary lactose malabsorption is controlled by a single recessive autosomal gene and genotyping allows us to determine the true prevalence of the lactase persistent/non-persistent gene. Thus, the prevalence of adult-type hypolactasia among the current population becomes obvious. The hypothesis that the lactase non-persistent genotype can affect milk and sour milk consumption has not been tested before in a northern Russian population.

Research frontiers

This was the first time that genotyping of the lactase persistent gene was performed in a population from north-west Russia and an estimation of milk products consumption according to genotype was performed.

Innovations and breakthroughs

In previous studies, the prevalence of lactase malabsorption among Russians has been found to vary between 13% and 57% according to different authors and the different methods used. Many studies were carried out in other populations living in the territories of Russia where there was great variation in the prevalence of lactose malabsorption, from 11% to 94%. Genotyping has a specificity of 100% and a sensitivity of 93%. Genetic methods have been used before in only two Russian studies which showed the prevalence of hypolactasia among Russians living in the central part to range from 36% to 50% depending on the area of residence. An estimation of the lactase non-persistent genotype affecting milk and dairy milk product consumption in a northern Russian population has not been done before.

Applications

The study results suggest that the prevalence of the lactase non-persistent genotype in a northern Russian population is 35.6% which is more than that in northern Europe and less than that in southern Europe. This prevalence confirms the historical hypothesis of lactase malabsorption. However, the genotype does not affect milk and sour milk consumption among northern Russians which could be a result of relatively low milk product consumption among the entire study population.

Terminology

Hypolactasia means very low activity of lactase in the jejunal mucosa; Lactose malabsorption describes poor lactose hydrolyzing capacity; Adult-type hypolactasia (primary lactose malabsorption, lactase non-persistence) - primary type of hypolactasia which is controlled by a single recessive autosomal gene; CC-13910 genotype defines lactase non-persistence; CT-13910, TT-13910 genotypes define lactase persistence.

Peer review

This manuscript has potential for contribution regarding genetic subtypes and milk intolerance.

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BRIEF ARTICLES

Incidence and survival of stomach cancer in a high-risk population of Chile

Katy Heise, Enriqueta Bertran, Marcelo E Andia, Catterina Ferreccio

Katy Heise, Enriqueta Bertran, Regional Office of the Chilean Ministry of Health, Region de Los Rios, Valdivia, 5110422, Chile

Marcelo E Andia, Radiology Department, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, 8330024, Chile

Catterina Ferreccio, Public Health Department, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, 8330073, Chile

Author contributions: Heise K designed the study, directed and conducted the data collection, processing and analysis, and wrote the first draft of the article; Bertran E collaborated in data collection and analysis; Andia ME collaborated in data analysis and review of the drafts of the manuscript; Ferreccio C participated in the study design, data analysis, and reviewed, edited and translated the final manuscript.

Correspondence to: Catterina Ferreccio, Public Health Department, School of Medicine, Pontificia Universidad Católica de Chile, Marcoleta 434, Santiago, 8330073, Chile. cferrec@med.puc.cl

Telephone: +56-2-3543038 Fax: +56-2-6331840

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associated with survival were later included in a Cox multivariate model.

RESULTS: Between 1998 and 2002, 529 primary gastric cancers occurred in Valdivia (crude incidence rate 29.2 per 100000 inhabitants). Most cases were male (69.0%), residents of urban areas (57.5%) and Hispanic (83.2%), with a low education level (84.5% < 8 school years). SC SIR was higher in men than women (40.8 and 14.8 respectively, $P < 0.001$), risk factors were low education RR 4.4 (95% CI: 2.9-6.8) and 1.6, (95% CI: 1.1-2.1) for women and men respectively and Mapuche ethnicity only significant for women (RR 2.2, 95% CI: 1.2-3.7). Of all cases, 76.4% were histologically confirmed, 11.5% had a death certificate only (DCO), 56.1% were TNM stage IV; 445 cases (84.1%) were eligible for survival analysis, all completed five years follow-up; 42 remained alive, 392 died of SC and 11 died from other causes. Specific 5-year survival, excluding cases with DCO, was 10.6% (95% CI: 7.7-13.5); 5-year relative survival rate was 12.3% (95% CI: 9.1-16.1), men 10.9% (95% CI: 7.4-15.2) and women 16.1% (95% CI: 9.5-24.5). Five-year specific survival was higher for patients aged < 55 years (17.3%), with intestinal type of cancer (14.6%), without metastasis (22.2%), tumor size < 4 cm (60.0%), without lymphatic invasion (77.1%), only involvement of the mucous membrane (100%). Statistically significant independent prognostic factors were: TNM staging, diffuse type, metastasis, supraclavicular adenopathy, palpable tumor, and hepatitis or ascites.

CONCLUSION: Social determinants are the main risk factors for SC, but not for survival. An advanced clinical stage at consultation is the main cause of poor SC survival.

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Key words: Survival analysis; Stomach neoplasms; Survival rate; Incidence; Risk factors; Gastrectomy

Peer reviewer: Dr. Mark S Pearce, Paediatric and Lifecourse Epidemiology Research Group, School of Clinical Medical Sciences, University of Newcastle, Sir James Spence Institute, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, United Kingdom

Heise K, Bertran E, Andia ME, Ferreccio C. Incidence and survival of stomach cancer in a high-risk population of Chile.

Abstract

AIM: To study the incidence and survival rate of stomach cancer (SC) and its associated factors in a high risk population in Chile.

METHODS: The population-based cancer registry of Valdivia, included in the International Agency for Research on Cancer system, covers 356396 residents of Valdivia Province, Southern Chile. We studied all SC cases entered in this Registry during 1998-2002 (529 cases). Population data came from the Chilean census (2002). Standardized incidence rates per 100000 inhabitants (SIR) using the world population, cumulative risk of developing cancer before age 75, and rate ratios by sex, age, ethnicity and social factors were estimated. Relative survival (Ederer II method) and age-standardized estimates (Brenner method) were calculated. Specific survival rates (Kaplan-Meier) were measured at 3 and 5 years and survival curves were analyzed with the Logrank and Breslow tests. Survival was studied in relation to demographics, clinical presentation, laboratory results and medical management of the cases. Those variables significantly

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INTRODUCTION

In 2002, stomach cancer (SC) was the fourth most common cancer in the world, with 900 937 cases and 700 349 deaths. Two-thirds of these occurred in developing countries^[1]. The high risk zones encompass Asia, Eastern Europe, and the Andean region of South America, all of diverse geographical characteristics^[2]. Chile, a representative of the Andean Region, presents an SC average mortality rate per 100 000 in 1990-2005 of 25.1 for men and 13.2 for women^[1]. This high SC mortality has not changed in the last 20 years^[3]. The existence of a population-based Cancer Registry in Valdivia^[4] gave us the opportunity to measure SC incidence and survival in a middle developing country. The Cancer Registry of Valdivia was initiated in 1982 and encompasses the inhabitants of an area of 18 429 square kilometres in Southern Chile; the data are included in the International Agency for Research on Cancer (IARC) cancer reports in 5 continents^[4]. The aims of this study were to measure SC incidence and risk factors and to assess SC survival.

MATERIALS AND METHODS

Cancer registry of Valdivia

The cancer registry of Valdivia covers a population of 356 396 people, and encompasses a systematic review of records in private and public clinical centers, hospitals and laboratories, and death certificates to identify every cancer case or cancer death occurring among residents of the Valdivia area. To guarantee complete case-ascertainment, it includes various national databases of particular cancers, death certificates and hospital records to identify residents of Valdivia who may have been diagnosed in other regions. This allows near 100% case ascertainment verification in the catchment area.

Cases

The subjects in the study were the 535 SC cases residents of Valdivia, identified in the Cancer Registry of Valdivia between the years 1998 and 2002. Of these, 6 cases were deemed non-eligible due to lack of information, leaving 529 subjects eligible for the study (98.9% of the total cases). In calculating the survival rate, 18 cases were excluded because they had a history of a previous cancer, apart from non-melanoma skin cancer; and 66 were excluded because the only information came from a death certificate or autopsy, leaving 445 cases (84.1%) to estimate SC survival. Deaths occurring within 30 d of a surgery with curative intention (3 cases) were considered deaths from other causes^[5]. Deaths from other causes were included, but censored.

Incidence and risk factors of stomach cancer

Standardized incidence rates, using the world population,

cumulative risk of developing cancer before the age of 75 and rate ratios were estimated according to the methodology proposed by the IARC^[6]. Population data were obtained from the National Institute of Statistics and the Chilean census of 2002^[7-9]. The risk of developing SC was estimated by sex, age, ethnicity (considering anyone with at least one Mapuche surname to be Mapuche, and the rest of the population to be Hispanic/European), education level (0-8 years and more than 8 years of education), urban or rural residency.

Survival analysis

Relative survival was calculated following the Ederer II method^[10] and age-standardized estimates were calculated using the approach proposed by Brenner *et al*^[11]. Specific survival rates were measured at 3 and 5 years using the Kaplan-Maier method and survival curves for selected characteristics were compared using the Logrank and Breslow tests^[12]. Those variables significantly associated with survival were later included in a Cox multivariate model^[12]. Survival was studied in relation to demographics, clinical presentation, laboratory results and medical management of the cases. The characteristics of the tumor registered were: morphology according to the Lauren classification^[13], size measured at its largest diameter, location in the stomach (fundus, body or antrum or all the stomach) and invasion of the gastric wall. Stage of the tumor was based on TNM classification system^[14]. The intervals between the onset of symptoms, the diagnosis and definitive treatment were determined.

RESULTS

Incidence of gastric cancer

Between 1998 and 2002, 529 primary gastric cancers were detected amongst the residents of Valdivia (crude incidence rate 29.2 per 100 000 inhabitants). The majority of cases were men (69.0%), residents of urban areas (57.5%), had a low education level (84.5% had 8 or less years of schooling) and were predominantly of Hispanic origin (83.2%) (Table 1). Age of cases ranged from 27 to 94 years, being significantly lower in men than women with mean age of 66.8 years [95% confidence intervals (CI): 65.5-68.1] and 70.4 years (95% CI: 68.2-72.6), respectively. Relative risk (RR) of SC was higher among men, particularly at age 55-64 years. Mapuche ethnicity was a significant risk factor only for women (RR 2.2, 95% CI: 1.2-3.7), and low education status was a stronger risk factor for women than for men: RR 4.4, (95% CI: 2.9-6.8) and 1.6, (95% CI: 1.1-2.1), respectively; the highest differential of risk between men and women was found among cases with more than 8 years of education (RR = 7.5, $P < 0.001$) (Table 1).

Clinical characteristics of the cases

Weight loss and epigastric pain were the most common symptoms in patients at diagnosis, and both symptoms were significantly more common in males than females (Table 2). Signs considered to indicate poor prognosis:

Table 1 SIR of stomach cancer by selected characteristics, Valdivia, Chile, 1998-2002

Characteristic	Cases	Males: 365			Females: 164			Male vs female	
		%	SIR	95% CI	%	SIR	95% CI	RR	P-value
All	529	69	40.8	36.7-45.2	31	14.8	12.1-16.7	2.8	< 0.001
Age (yr)									
< 35	8	0.8	0.5	0.0-1.2	3.0	0.9	0.1-1.8	0.6	0.45
35-44	22	4.4	11.7	5.9-17.4	3.6	4.6	0.9-8.2	2.6	0.04
45-54	55	11.2	44.6	30.9-58.2	8.4	15.4	7.3-23.4	2.9	< 0.001
55-64	94	21.4	125.7	97.8-153.6	9.6	23.9	12.2-35.6	5.3	< 0.001
65-74	164	32.1	291.3	238.6-344.0	28.7	100.5	72.1-128.9	2.9	< 0.001
75-84	86	14.8	448.7	329.3-568.1	19.8	214.3	141.3-287.4	2.1	0.001
≥ 85	100	15.3	501.4	370.4-632.4	26.9	292.2	206.9-377.4	1.7	0.006
Ethnicity Mapuche	89	15	47.2	34.5-59.9	21.0	28.1 ^a	18.5-37.7	1.7	0.016
Hispanic/European	440	85	40.0	35.5-44.5	79.0	12.7 ^a	10.5-15.0	3.1	< 0.001
School years									
≤ 8	420	81	43.3 ^a	38.1-48.5	92.0	16.2 ^a	13.3-19.1	2.7	< 0.001
> 8	77	19	27.6 ^a	20.5-34.7	8.0	3.7 ^a	1.5-5.9	7.5	< 0.001
Residence rural	220	48	48.0 ^a	40.7-55.3	30.0	14.5	10.4-18.7	3.3	< 0.001
Urban	297	52	34.5 ^a	29.5-39.6	70.0	13.9	11.2-16.6	2.5	< 0.001

^aSignificant risk factors between characteristics groups; RR: Relative risk.

palpable epigastric mass, ascites or supraclavicular adenopathy, each were present in less than 15% of cases; only a palpable epigastric mass was significantly more common among females (Table 2). The main detection source was histology; only 11.5% of cases were identified by their death certificate only. The latter cases were significantly older: in those over 80, 45.5% and 15.1% were confirmed by death certificate only and histology, respectively ($P < 0.001$); there was also a higher proportion of women (22.6% versus 6.6%, $P < 0.001$) in this group.

In the majority of cases, the time interval between the beginning of symptoms and diagnosis was more than 3 mo. Early diagnoses were more frequent among men (Table 2). The main diagnostic procedures were gastric endoscopy and gastric biopsy (Table 2). At diagnosis only 5.5% of cases were in Stage I or II of the TNM classification; these earlier stages were more frequent among women (7.3%) than men (4.7%) (Table 2). The three most common histological types of SC both in men and women were: tubular adenocarcinoma (36%), undefined adenocarcinoma (20.2%) and signet ring cell carcinoma (18.7%) (Table 2). The fundus was the most frequent localization of the SC (29.9%) and the tumor location was not determined in a third of the cases. Information about tobacco or alcohol consumption was available for 65.4% and 63.9% of cases, respectively; tobacco use was declared by 51.4% of men and 29.1% of women ($P < 0.001$), and alcohol consumption was declared by 73.6% of men and 31.3% of women ($P < 0.001$) (Table 2).

Five years survival

Follow-up of the 445 cases included in the survival analysis was concluded on December 31st 2007, with 100% of the cases included in the 5-year analysis; only 42 cases remained alive, 392 cases had died of SC and 11 died from other causes. Specific survival at 5 years, including cases with a death certificate only, was

9.6% (95% CI: 6.9-12.3), excluding cases with a death certificate only, the survival was 10.6% (95% CI: 7.7-13.5); lower in men 9.8%, (95% CI: 6.5-13.1) than in women 12.9% (95% CI: 6.7-19.1). The 5-year relative survival rate, adjusted for the life expectancy of the population, was 12.3% (95% CI: 9.1-16.1), increasing the difference in survival of men 10.9% (95% CI: 7.4-15.2) and women 16.1% (95% CI: 9.5-24.5).

In univariate analysis, the significant factors for greater than 5 years survival were: younger age; Hispanic/European ethnicity; urban residency; lack of some clinical markers (supraclavicular adenopathy, palpable mass, ascites, or vomiting); gastrectomy; intestinal histological type; localized tumor (Figure 1A); size less than 4 cm, limited to the mucosa, without lymphatic invasion or metastasis; TNM stage 1 (Table 3). Tumors with proximal and distal localization had the same TNM distribution. However, tumors localized in the body had better 5-year survival than those localized to the fundus or in all the stomach, 23.0% vs 11.4% ($P = 0.049$) and 0% ($P < 0.001$), respectively; there was no statistical difference in survival between tumor location in the body and antrum (23.0% and 14.8%, $P = 0.16$, respectively).

The cumulative survival curve of Stage I SC was significantly higher than Stage III, Stage IV or unknown stage (Figure 1B), and was similar in men and women (Figure 1C and D).

Multivariate survival analysis

The multivariate models only included cases with sufficient clinical data (271 cases, 69%). All cases (445) had "unknown staging" more frequently than the cases included in the multivariate models (17.8% and 8.9% respectively, $P = 0.001$) while their 5-year survival was lower (10.6% vs 14.1% $P = 0.002$). Nevertheless, all cases were similar to those included in the multivariate analysis with regard to socio-demographics and to the variables associated with survival in the univariate analysis (listed in Table 3).

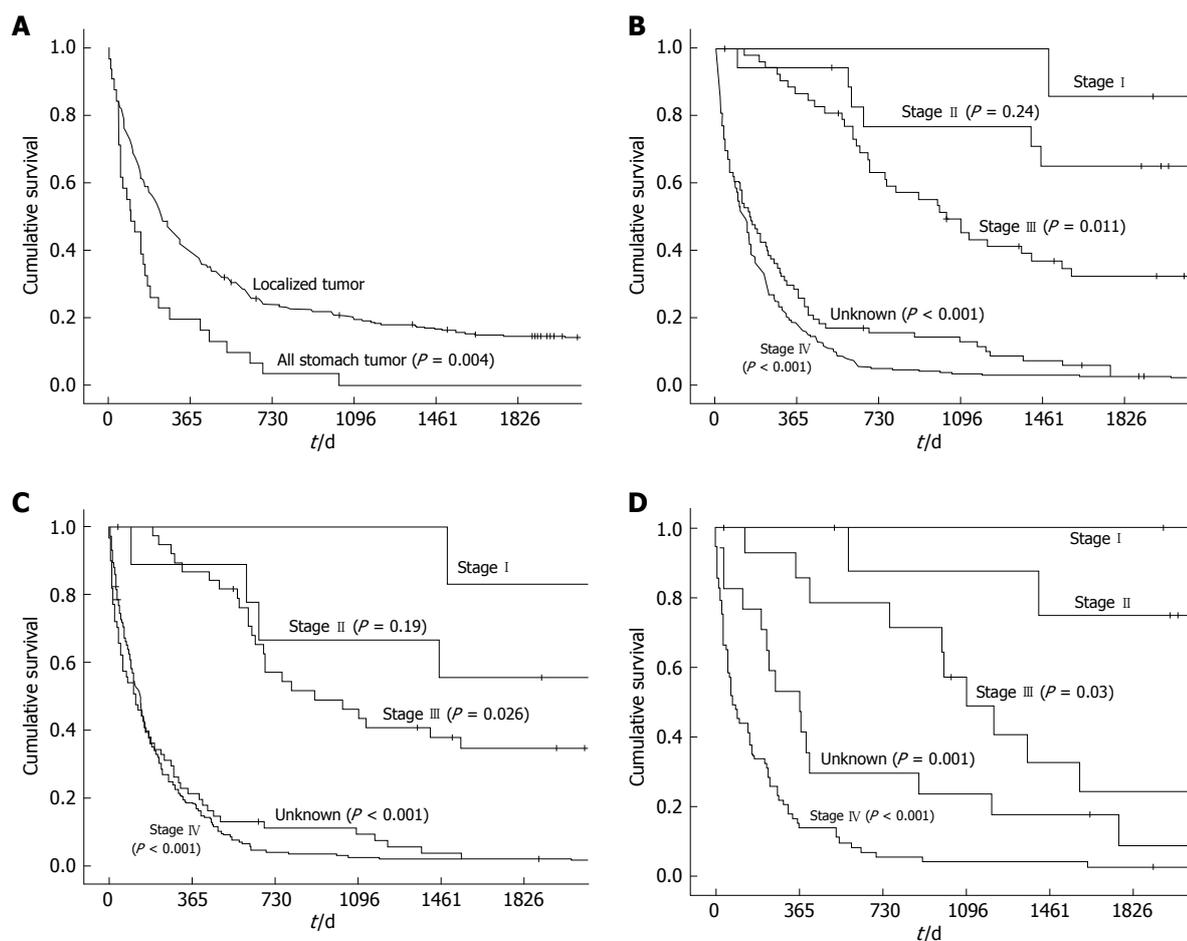


Figure 1 Stomach cancer-specific survival in Valdivia 1998-2005. A: Tumor location; B: TNM stage classification; C: Men by TNM; D: Women by TNM.

In the multivariate analysis, only 6 variables maintained their statistical significance as independent prognostic factors (Table 4). TNM staging was the strongest prognostic variable, the risk of dying in the following year was 23 times higher with stage IV compared with stage I. Other factors of poor prognosis were: diffuse type, presence of metastasis, supraclavicular adenopathy, a palpable tumor and hepatitis or ascites (Table 4).

DISCUSSION

We confirmed that Chile, in particular southern Chile, where Valdivia is located, has one of the highest risks of stomach cancer reported, particularly among men (SIR 40.8 per 100 000 inhabitants). This population has many factors that have been associated with SC such as poverty, a high rural population (32%), poor sanitary conditions in 43% of houses^[15], high prevalence of smokers (37.4%), high alcohol consumption (alcohol dependence in the Chilean population varies from 7.2% to 14.1% among low and high socioeconomic groups and from 5.0% to 17.6% among females and males respectively)^[16]; low daily consumption of fruits and vegetables (32.1%)^[17]; and high prevalence of *Helicobacter pylori* (*H pylori*) infection, especially among young people^[18].

We could not demonstrate an independent effect of race in the incidence of SC among men; but, for women,

Mapuche ancestry was a significant risk factor (RR: 2.2, 95% CI: 1.2-3.7). Similarly low schooling was a stronger risk factor among women (RR: 4.4 and 1.6 for women and men, respectively) and the highest male/female ratio of 7.5 occurred among people with greater schooling, suggesting that behavioral or environmental factors preferentially protect women of Hispanic origin and with more years of education. In the 2003 National Health Survey of Chile, more educated women had significantly better nutritional indicators than men of high or low educational level and women of low educational level, with body mass index of 25.0 *vs* 27.8, 26.1 and 28.0, respectively^[19]. This better nutritional status is associated with a higher intake of fruits and vegetables among these women, a protective factor for gastric cancer^[20]. Also, the 2006 Quality of Life National Survey showed that women added salt to the meals before eating less frequently than men (8.5% and 10.9%, respectively)^[17]. In the population, as well as in our series, smoking and alcohol consumption were less frequent in women than men. Interaction of smoking and drinking has been demonstrated to play a role in SC^[21]. Plus, in Chile, *H pylori* infection was also 10% lower among women than men^[18]. These environmental factors may interact with susceptibility factors, such as alcohol dehydrogenase polymorphisms^[22-24], to explain the predominance among men and also the higher frequency of this cancer in the

Table 2 Clinical characteristics of the stomach cancer cases entered in the population-based cancer registry of Valdivia 1998-2002 (*n* = 529)

Characteristic	Cases	Total (%)	Males (%)	Females (%)	<i>P</i> -value sex diff.
Sign and symptoms at diagnosis					
Weight loss	294	55.6	61.1	43.3	< 0.001
Epigastric pain	285	53.9	57.0	47.0	0.03
Gastrointestinal bleeding	108	20.4	21.4	18.3	0.42
Vomiting	106	20.0	20.8	18.3	0.50
Abdominal distension	83	15.7	14.8	17.7	0.40
Palpable epigastric mass	56	14.7	12.9	19.2	0.016
Ascites	51	13.4	13.3	13.5	0.97
Supraclavicular adenopathy	31	8.5	9.7	5.1	0.16
Main detection source					
Histological studies ¹	404	76.4	81.3	65.2	< 0.001
Radiological diagnosis	25	4.7	4.9	4.3	0.74
Clinical or autopsy	39	7.3	7.2	7.9	0.75
Only death certificate	61	11.5	6.6	22.6	< 0.001
Duration of symptoms ²					
< 3 mo	126	32.9	35.9	24.2	0.033
3 to 6 mo	98	25.6	25.4	26.3	0.86
> 6 mo	159	41.5	38.7	49.5	0.06
Main diagnostic workout					
Gastric endoscopy	412	79.4	84.6	67.7	< 0.001
Gastric biopsy	401	75.8	80.8	64.6	< 0.001
Ecography	191	36.1	37.8	32.3	0.22
Computed tomography	114	21.6	22.7	18.9	< 0.001
Staging TNM					
Stage I	9	1.7	2.2	0.6	0.19
Stage II	20	3.8	2.5	6.7	0.02
Stage III	53	10.0	10.4	9.1	0.65
Stage IV	297	56.1	58.6	50.6	0.09
Not determined	150	28.4	26.3	32.9	0.12
Histological type					
Tubular adenocarcinoma	148	36.0	35.2	38.3	0.56
Tubulopapillary adenocarcinoma	35	8.5	7.6	11.2	0.25
Papillary adenocarcinoma	11	2.7	2.0	4.7	0.14
Mucinous adenocarcinoma	11	2.7	2.3	3.7	0.43
Undefined adenocarcinoma	83	20.2	22.4	14.0	0.06
Signet ring cell carcinoma	77	18.7	18.1	20.6	0.57
Non Hodgkin lymphoma	6	1.5	1.6	0.9	0.60
Neuroendocrine carcinoma	4	1.0	0.7	1.9	0.27
Other carcinoma	36	8.8	9.5	6.5	0.35
Tumor location					
Body	57	10.8	11.5	9.1	0.42
Fundus	158	29.9	33.7	21.3	0.004
Antrum	122	23.1	21.9	25.6	0.35
All stomach	33	6.2	6.8	4.9	0.39
Unknown	159	30.1	26.0	39.0	0.003
Habits					
Tobacco use	346	44.8	51.4	29.1	< 0.001
Alcohol use	338	61.2	73.6	31.3	< 0.001

¹Includes 3 cases diagnosed based on a biopsy of metastasis; ²Time between first symptoms and diagnosis.

Mapuche areas of southern Chile^[18].

In this population-based study only 10.6% of SC survived the 5-year period following the detection of SC. When cases detected by death certificate only were included, the 5-year survival dropped to 9.6% (95% CI: 6.9-12.3). Previous studies based on cases detected at the hospital, reported higher survival rates of 12% to 48.9%^[25-27], probably due to a selection bias created at hospital admission, when cases in the advanced stages of the disease who will not benefit from medical care are referred to palliative care units. The mean age of SC in our series was similar to that reported by European population-based cancer registries^[28,29], and was 10 years

greater than the mean age reported in hospital-based survival studies. The 5-year relative survival rate, adjusted for the life expectancy of the population, was 12.3%, half that reported in population registries in North America^[30] and Europe^[31], where the survival rates are between 20.1% and 25.6%. Main risk factors of this poor survival were either clinical (palpable tumor, ascites, hepatitis) or related to the tumor characteristics (stage, histological type, metastasis, nodes); there were no significant social determinants of survival. This underlines the relevance of the advanced stage at consultation of most cases. When a gastrectomy with the intention to cure was undertaken the 5-year specific

Table 3 Factors associated with SC survival. Valdivia 1998-2002. Kaplan-Meier (*n* = 445 cases). Univariate analyses

Variable	<i>n</i> (%)	3-yr survival		5-yr survival		<i>P</i> -value 5-yr survival
		%	95% CI	%	95% CI	
Sex						
Female	120 (27.0)	20.3	13.0-27.6	12.9	6.7-19.1	-
Male	325 (73.0)	13.4	9.7-17.1	9.8	6.5-13.1	0.56
Age (yr)						
< 55	81 (18.2)	21.0	12.1-29.9	17.3	9.1-25.5	0.037
55-79	300 (67.4)	15.5	11.3-19.7	9.9	6.4-13.4	0.006
> 80	64 (14.4)	6.3	0-12.8	4.2	0-9.7	-
Ethnic group						
Mapuche ¹	76 (17.1)	7.3	1.2-13.4	4.4	0-9.2	-
Hispanic	369 (82.9)	16.9	13.0-20.8	11.9	8.5-15.3	0.02
School years						
0 to 8	348 (83.6)	9.1	6.0-12.2	6.4	3.7-9.1	-
> 8	68 (16.4)	19.7	10.2-29.3	8.9	2.0-15.8	0.08
Home						
Urban	248 (57.1)	18.7	13.8-23.6	12.9	8.6-17.2	0.006
Rural	186 (42.9)	10.6	6.1-15.1	8.2	4.1-12.3	-
Sign and symptoms at diagnosis						
Supraclavicular adenopathy						
Yes	28 (8.0)	0	-	0	-	-
No	321 (92.0)	18.6	14.3-22.9	13.8	9.9-17.7	0.00018
Palpable mass						
Yes	52 (14.4)	8.7	1.0-16.4	6.2	0-13.1	-
No	310 (85.6)	17.8	13.5-22.1	13.0	9.2-16.8	0.00001
Ascitis						
Yes	47 (13.0)	0	-	0	-	-
No	315 (87.0)	18.9	14.5-23.3	13.8	9.9-17.0	0.000002
GI bleeding						
Yes	101 (22.7)	20.1	12.2-28.0	13.7	6.9-20.5	0.87
No	344 (77.3)	13.8	10.1-17.5	9.7	6.5-12.9	-
Vomiting						
Yes	100 (22.5)	10.3	4.3-16.3	6.2	1.4-11.0	-
No	345 (77.5)	16.8	12.8-20.8	11.9	8.4-15.4	0.046
Weight loss						
Yes	277 (62.2)	13.6	9.5-17.7	10.0	6.4-13.6	0.45
No	168 (37.8)	18.1	12.2-24.0	11.5	6.5-16.5	-
Abdominal distension						
Yes	78 (17.5)	11.5	4.4-18.6	7.7	1.8-13.6	0.13
No	367 (82.5)	16.1	12.3-19.9	11.2	7.9-14.5	-
Pain						
Yes	271 (60.9)	19.0	14.2-23.8	14.5	10.2-18.8	0.14
No	174 (39.1)	9.6	5.2-14.0	4.6	1.4-7.8	-
Months with symptoms						
< 3	117 (32.3)	14.9	8.3-21.5	9.3	3.9-14.7	-
3 to 6	95 (26.2)	17.6	9.9-25.3	14.2	7.1-21.3	0.32
> 6	150 (41.4)	16.6	10.5-22.7	13.7	8.1-19.4	0.30
Gastrectomy						
Yes	128 (28.8)	44.8	36.0-53.6	33.6	25.1-42.1	0.0000001
No	317 (71.2)	3.5	1.4-5.6	1.3	0-2.6	-
Surgical intention						
Curative	89 (42.4)	50.4	39.9-60.9	38.6	28.3-48.9	0.0000001
Palliative	121 (57.6)	14.4	5.8-23.0	10.4	2.6-18.2	-
Lauren class						
Intestinal	220 (59.1)	21.0	15.5-26.5	14.6	9.8-19.4	0.0000003
Diffuse	152 (40.9)	11.2	6.1-16.3	7.0	2.9-11.1	-
Tumor						
Localized	325 (91.0)	20.0	15.6-24.4	14.6	10.6-18.6	0.004
All stomach	32 (9.0)	0	-	0	-	-
Size						
< 4 cm	16 (12.8)	67.0	43.3-90.7	60.0	35.1-84.9	-
4 to 9.9 cm	71 (56.8)	48.3	36.3-60.3	35.5	23.9-47.1	0.161
> 10 cm	38 (30.4)	29.0	15.6-43.4	21.1	8.1-34.1	0.006
Depth						
Mucosa	9 (7.1)	100	-	100	-	-
Muscle/subserosa	7 (5.6)	85.7	59.8-100	71.4	37.9-100	0.14
Serosa	110 (87.3)	38.2	29.0-47.5	27.2	18.6-35.8	0.003

Lymphatic invasion stage						
0	21 (17.8)	94.9	85.1-100	77.1	57.3-96.9	-
I	36 (30.5)	55.4	39.1-71.7	40.2	23.8-56.6	0.008
II	33 (28.0)	38.3	21.5-55.1	25.3	10.1-40.5	0.0005
III	28 (23.7)	10.7	0-22.2	10.7	0-22.2	0.00001
Metastasis						
0	185 (47.0)	30.6	23.5-37.4	22.2	16.0-28.4	-
1	209 (53.0)	2.5	0.3-4.7	1.5	0-3.2	0.0000001
TNM						
I	9 (2.1)	100	-	85.7	59.8-100	-
II	18 (4.1)	77.3	57.7-96.9	65.4	42.9-87.9	0.24
III	53 (12.1)	49.2	35.5-62.9	32.2	19.1-45.3	0.011
IV	281 (64.0)	3.0	1.0-5.0	2.2	0.4-4.0	0.00003
Unknown	78 (17.8)	12.3	4.9-19.7	3.8	0-8.3	0.00007

¹Native American Indian residents of southern Chile.

Table 4 Multivariate analysis of prognostic factors of stomach cancer survival population-based registry of Valdivia (Kaplan-Meier)

	OR	95% CI	P-value
Staging TNM			
Stage I	1.0		
Stage II	2.77	0.3-23.8	0.35
Stage III	6.87	0.9-50.6	0.06
Stage IV	22.53	3.1-165.2	0.002
Unknown	23.35	3.1-173.6	0.002
Lauren classification			
Intestinal	1.0		
Diffuse	1.68	1.3-2.2	< 0.001
Metastasis			
No	1.0		
Yes	1.58	1.1-2.3	0.019
Supraclavicular adenopathy			
No	1.0		
Yes	1.69	1.0-2.8	0.037
Palpable tumor			
No	1.0		
Yes	2.04	1.4-3.1	0.001
Hepatitis or ascites			
No	1.0		
Yes	2.51	1.6-3.9	< 0.001

survival was 38.6%, which is comparable to reports from Europe and North America in either population-based or hospital-based registries: 45% in Spain^[32], 46.1% in Japan^[33], 29.7% in Florence^[29] and 32.6% in Côte d'Or^[34]. We found that the stage of the disease was the main prognostic factor for survival as has been reported by others^[26,27,32,33]. Thus, in our population the high incidence of SC was aggravated by a very late diagnosis of the disease. 11.5% of cases were never recognized by the medical system and were discovered based on death certificates, while another 20% of cases were inoperable at their first medical consultation. Only 5.5% of cases were found in stages I or II at the moment of diagnosis, lower than what has been reported in other population-based cancer registries in places with no screening programs such as Florence (36.7% of cases in stages I or II)^[29] and Changle, China (30.9% in stages I or II)^[35]; in Florence, where methods are the same as those in our study, a higher 5-year relative survival was reported than in our series (22.7%, 95% CI: 20.5%-24.9% and 12.3%, 95% CI: 9.1-16.1, respectively). Most cases presenting at

earlier stages, stage I or II, were under age 54 in men and under age 68 in women, suggesting that in men the disease is initiated earlier or evolves faster (data not presented in the tables). Time elapsed between the onset of symptoms and medical attention, 8.4 mo, was twice as high as that reported in La Coruña, Spain^[32]. Most cases (55%), exceeded the current Chilean standard of less than 45 d between first consultation and diagnosis, and 44% of operated cases exceeded the 30 d interval between diagnosis and treatment. Thus late consultation and slow medical management contributed to the high mortality of SC in this series.

In our series, there were 0.7 proximal tumors for each distal tumor, half of that described in industrialized countries such as the USA 1.46^[36] or Canada 1.5^[37]. Distal cancers, the majority in our series, are mostly associated with *H pylori* and poverty, and have a somewhat better prognosis than proximal cancers, so that the biological characteristics of the tumor did not provide a plausible explanation for our poor survival rates.

Screening of SC based on endoscopy and radiography has been shown to reduce the mortality rate in Japan^[38] but not in Chile^[39,40]. In mid 2006, a nationwide screening program was initiated in Chile, offering endoscopic examination to patients over 40 years with dyspeptic symptoms. The usefulness of symptoms as selection criteria for endoscopy has recently been discussed by Maconi^[41] because symptoms of early stage SC are highly nonspecific while warning symptoms, such as a palpable abdominal mass, represent an advanced stage of the disease. Others are proposing a combination of serological tests to screen for gastric atrophy, *Helicobacter*^[42] plus alcohol dehydrogenase^[24], followed by gastroscopy of cases that screen positive. Such a strategy would allow screening of a broad population in a short time to be followed by more invasive techniques in a much smaller group, estimated as 12% in Chile^[42].

SC has a high incidence rate and a poor prognosis in the province of Valdivia. Main factors associated with poor survival were delays in obtaining medical care: time taken to seek medical care, time to be diagnosed and time to receive medical treatment; once medical care was obtained survival was comparable to other series. Current efforts to shorten these times with greater use of gastric endoscopy may improve this situation.

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COMMENTS

Background

Stomach cancer (SC) has been the main cancer killer in the Chilean population since the 1950s. Despite important socioeconomic development of the country and improvements in health indicators, SC mortality has not decreased. To date, there are no population-based studies of SC incidence and survival in Chile which would illuminate the causes behind the high SC mortality, particularly among men. The cancer registry of Valdivia is currently the only population-based cancer registry in Chile included in the International Agency for Research on Cancer reports and is one of 10 cancer registries in Latin America. This is the first report of SC survival in the Chilean population.

Research frontiers

There have been few studies of the real magnitude of the SC problem and the characteristics of the patients who survive or die from this cancer.

Innovations and breakthroughs

The most innovative product is the exact measurement of the incidence and survival of SC using the most up-to-date statistical methods which provide data which is easily comparable among populations.

Applications

This report represents a baseline of the SC situation in Valdivia and will permit evaluation of future interventions aimed to control SC. The methods presented here can be used to analyze any other cancer covered by a cancer registry.

Terminology

Incidence rate: calculated from the new occurrences of primary SC in the whole area divided by its population in the study period. Standardized incidence rate: the incidence rate adjusted by the age structure of a theoretical population, the world population, to permit direct comparisons between populations of diverse age structure. Specific survival rate: the number of cancer cases that are alive at the end of the study period divided by the person-time of cases over the period at risk of dying of SC. Relative survival rate: the specific survival rate adjusted by the life expectancy of the baseline population.

Peer review

This report presents invaluable data on SC incidence and SC survival in an area at high risk of SC in a middle developing country of Latin America. It provides information about the clinical presentation of SC cases, their socio-demographic characteristics and risk factors and prognostic factors of survival. The results indicate that a late stage at diagnoses is by far the principal explanatory factor of poor survival of SC in the area.

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Non-alcoholic steatohepatitis with normal aminotransferase values

Hüseyin Saadettin Uslusoy, Selim Giray Nak, Macit Gülten, Zeynep Biyıklı

Hüseyin Saadettin Uslusoy, Selim Giray Nak, Macit Gülten, Department of Gastroenterology, Uludag University Medical School, Bursa 16059, Turkey

Zeynep Biyıklı, Department of Biostatistics, Ankara University School of Medicine, Ankara 06100, Turkey

Author contributions: Uslusoy HS and Nak SG performed the majority of the study; Gülten M provided design of the study, the sources and references in the literature and translations; Biyıklı Z performed the statistical studies, including data acquisition, analysis and interpretation.

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Correspondence to: Hüseyin Saadettin Uslusoy, MD, Department of Gastroenterology, Yozgat State Hospital, Yozgat 66200, Turkey. huslusoy.23@hotmail.com

Telephone: +90-354-2121070 Fax: +90-354-2120923

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Abstract

AIM: To investigate the aspects of liver histology in patients with non-alcoholic steatohepatitis (NASH) who had normal aminotransferase levels.

METHODS: Thirty-four patients diagnosed with liver steatosis by ultrasonographic examination participated in the study. We compared all non-alcoholic fatty liver disease and NASH cases, according to aminotransferase level, aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio and presence of metabolic syndrome.

RESULTS: Sixteen of 25 patients with high aminotransferase levels were diagnosed with NASH and nine with simple fatty liver according to liver histology. Among the nine patients with normal aminotransferase levels, seven had NASH and two had simple fatty liver. The patients with normal and high liver enzyme levels had almost the same prevalence of NASH and metabolic syndrome. Liver histology did not reveal any difference according to aminotransferase levels and AST/ALT ratio.

CONCLUSION: Aminotransferase levels and AST/ALT ratio do not seem to be reliable predictors for NASH. Despite numerous non-invasive biomarkers, all patients with fatty liver should undergo liver biopsy.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common liver pathology that begins from simple steatosis and then progresses to steatohepatitis and ends with cirrhosis or liver failure^[1,2]. While simple steatosis is accepted as a benign state, steatohepatitis, because of elevated liver enzymes [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and certain metabolic abnormalities such as diabetes, obesity, dyslipidemia, hypertension and insulin resistance is assessed as a serious condition^[3-6]. Nevertheless, according to the literature, some non-alcoholic steatohepatitis (NASH) patients can have normal aminotransferase levels or conversely some patients with high liver enzymes may not have steatohepatitis^[7]. It has emerged that some NAFLD patients may not fulfill all criteria concerned with metabolic syndrome and do not have insulin resistance^[8]. Some studies have revealed that NAFLD can progress in the absence of metabolic disorders, as defined also in lean and non-diabetic patients, males and children^[9]. We aimed to show that NASH can occur without an increase in liver enzyme levels and that there may not be progression of NASH in fatty liver patients with high liver enzymes. More attention has been paid to these issues in recent years; but, further research especially related to large populations should be performed.

MATERIALS AND METHODS

Study design and patients

In the present retrospective study, we aimed to establish

the presence of patients with normal liver enzymes who were diagnosed histologically with NASH, to compare NAFLD and NASH patients according to liver enzyme levels, and to establish whether histological aspects of NAFLD differ with liver enzyme levels. This study included 34 patients who attended the Gastroenterology Division of Uludag University. The patients were diagnosed with liver steatosis by ultrasonography and complete clinical, anthropometric and laboratory evaluations and liver biopsies were performed. Exclusion criteria were as follows: alcohol consumption of ≥ 20 g/d, pregnancy, presence of hepatitis B and C infection, autoimmune liver diseases, hemochromatosis, Wilson's disease, α -1 antitrypsin deficiency, toxic liver diseases, primary biliary cirrhosis, primary sclerosing cholangitis, respectively.

Laboratory studies

Anthropometric, clinical and laboratory features of all NAFLD cases with both high and normal liver enzyme levels were recorded. Anthropometric parameters consisted of height, weight, body mass index (BMI), waist and hip circumferences and waist/hip ratio. Overweight and obesity were defined as BMI ≥ 25 kg/m² and ≥ 30 kg/m², respectively. Biochemical assessments included ALT, AST, γ -glutamyltransferase (GGT), alkaline phosphatase (ALP), bilirubin, albumin, high-density lipoprotein (HDL)-cholesterol, triglycerides, glucose, insulin levels and oral glucose tolerance test (OGTT). The diagnosis of type 2 diabetes, impaired glucose intolerance (IGT), impaired fasting glycemia were dependent on American Diabetes Association (ADA) criteria and patients taking oral antidiabetics or insulin therapy were accepted as having diabetes. The cut-off values of ALT and AST were 0-43 and 0-40 IU/L, respectively. Resting blood pressure $\geq 140/90$ mmHg or treatment with antihypertensive drugs indicated hypertension. Cut-off levels of hypertriglyceridemia and low HDL-cholesterol status were ≥ 150 mg/dL and ≤ 40 mg/dL, respectively. Lipid-lowering drug therapy indicated dyslipidemia. Fasting insulin level was 6-27 μ U/mL and presence of insulin resistance was defined when homeostasis model assessment of insulin resistance (HOMA-IR) value was ≥ 2.70 . Description of metabolic syndrome was made according to World Health Organization criteria (BMI ≥ 30 kg/m², increased waist/hip ratio of > 0.90 in men and > 0.85 in women, fasting blood glucose ≥ 110 mg/dL, presence of IGT, HOMA-IR ≥ 2.70 , triglycerides ≥ 150 mg/dL, HDL-cholesterol ≤ 40 mg/dL in men and ≤ 50 mg/dL in women, arterial blood pressure $\geq 140/90$ mmHg and microalbuminuria^[10]). At least three of these criteria were required to diagnose the metabolic syndrome. Liver biopsy was performed according to the severity of the clinical features and permission from the patients. The study was approved by the hospital ethics committee.

Histological assessment

All liver biopsy specimens were evaluated by a liver pathologist according to the criteria of Brunt *et al*^[11]. NASH was diagnosed according to the criteria as

presented below: steatosis (mild, moderate and severe) and two of the three features: (1) necro-inflammation with mononuclear cells and/or polymorphonuclear leukocytes, (2) ballooning degeneration of hepatocytes and (3) perisinusoidal and/or bridging fibrosis.

Statistical analysis

Statistical analysis was performed by using the SPSS statistical software version 15.0 for Windows. $P < 0.05$ was considered to indicate statistical significance. According to the data distribution, independent *t*-test and Mann-Whitney test were used to compare continuous variables related to the case groups. Pearson's Chi-square test and Fischer's absolute Chi-square test were used to compare categorical variables.

RESULTS

The features of 34 patients (12 male, 22 female) with liver steatosis who attended the Gastroenterology Division of Uludag University were investigated. Slight and blunt abdominal pain were present in 77.7% of patients with elevated liver enzymes and in 64.0% of patients with normal liver enzymes. The prevalence of hepatomegaly was 44.4% and 28% in the high liver enzyme and normal liver enzyme group, respectively. All 34 patients underwent liver biopsy according to their clinical and laboratory aspects. Twenty-five patients had elevated and the remaining nine had normal liver enzyme levels. Sixteen of 25 patients with high liver enzymes were diagnosed with NASH and nine of 25 as simple fatty liver with respect to liver histology. When we analyzed the nine patients with normal ALT levels, seven were diagnosed with NASH and two with simple fatty liver according to liver histology.

Anthropometric, clinical and laboratory results of all NAFLD patients according to high and normal liver enzyme groups are presented in Table 1. The patients with high and normal aminotransferase levels had almost same prevalence of NASH (77.7% *versus* 64.0%). Only the proportion of patients with hypertriglyceridemia in the normal liver enzyme group was significantly higher than in the group with elevated liver enzyme. However according to the Table 1, the number and proportions of the other metabolic risk factors in high and normal liver enzyme levels groups did not differ. Moreover, a comparison was performed between the elevated and normal aminotransferase level groups in NASH patients and there were no significant differences (Table 2). The prevalence of metabolic syndrome was similar in NASH patients both with elevated or normal aminotransferase levels.

There were seven patients with normal aminotransferase levels who were diagnosed with NASH. Six of these were female (mean age: 52.8 years) and one was male. Five of the female patients were obese (average BMI: 31 kg/m²) and one was morbidly obese (BMI: 49.3 kg/m²). All six female patients had metabolic syndrome (five of whom had three risk factors and one had four risk factors). Two of the six female

Table 1 Anthropometric, clinical and laboratory features of all NAFLD cases according to liver enzyme levels *n* (%)

	According to liver enzyme levels (<i>n</i> = 34)		<i>P</i>
	Normal liver enzyme (<i>n</i> = 9)	Elevated liver enzyme (<i>n</i> = 25)	
Age (yr)	52.0 ± 6.16	49.8 ± 6.77	> 0.05
Gender (male/female)	1/8 (11.2/88.8)	10/15 (40/60)	> 0.05
Hepatomegaly	4 (44.4)	7 (28.0)	> 0.05
BMI (kg/m ²)			
Normal weight	0 (0)	1 (4)	> 0.05
Overweight	0 (0)	2 (8)	> 0.05
Obese	7 (77.8)	20 (80.0)	> 0.05
Morbid obese	2 (22.2)	2 (8.0)	> 0.05
Waist/hip ratio	6 (66.7)	19 (76.0)	> 0.05
Systolic blood pressure (mmHg) ¹	130 (110-150)	120 (110-180)	> 0.05
Diastolic blood pressure (mmHg) ¹	76 (60-90)	70 (60-120)	> 0.05
Hypertension	4 (44.4)	12 (48.0)	> 0.05
HDL-Cholesterol (mg/dL)	43.56 ± 7.99	45.52 ± 7.04	> 0.05
Low-HDL level	6 (66.7)	11 (44.0)	> 0.05
Triglycerides (mg/dL) ¹	187 (43-360)	125 (39-304)	> 0.05
Hypertriglyceridemia	7 (77.8)	8 (32.0)	< 0.05
Fasting glucose (mg/dL) ¹	101 (73-164)	101 (87-195)	> 0.05
Diabetes	2 (22.2)	6 (24.0)	> 0.05
Impaired glucose tolerance	3 (33.3)	7 (28.0)	> 0.05
Fasting insulin (μU/mL) ¹	11.8 (6-23.66)	15.23 (4.76-47.3)	> 0.05
Insulin resistance-HOMA-IR	2 (22.2)	14 (56.0)	> 0.05
AST (IU/L) ¹	17 (14-29)	49 (33-92)	< 0.05
ALT (IU/L) ¹	13 (13-33)	81 (32-166)	< 0.05
GGT (IU/L) ¹	19 (15-33)	42 (15-426)	< 0.05
ALP (IU/L) ¹	96 (52-113)	81 (51-154)	> 0.05
NASH	7 (77.7)	16 (64.0)	> 0.05
Metabolic syndrome	8 (88.8)	19 (76)	> 0.05

¹Data expressed as median (minimum-maximum); BMI: Body mass index; HDL: High-density lipoprotein; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase. Only the prevalence of hyperglyceridemia in NAFLD patients with normal liver enzyme levels was higher than in those with elevated liver enzyme.

patients had overt diabetes, two had impaired glucose tolerance and the remaining two had normal OGTT results. Two female patients who were non-diabetic had insulin resistance determined by HOMA test. Three female patients had hypertension and five had hypertriglyceridemia. The male patient with NASH was 46 years old with central obesity (BMI: 30.5 kg/m²). He also had insulin resistance (HOMA-IR:3.46), but had no other risk factors such as diabetes, hypertriglyceridemia, hypertension and microalbuminuria; therefore he was not diagnosed with metabolic syndrome.

In Table 3, the findings of all NASH patients according to the presence of metabolic syndrome are presented and evaluated. According to Table 3, average age, the prevalences of female gender, hypertension, low-HDL level and hypertriglyceridemia were significantly higher in patients with metabolic syndrome than those in patients without metabolic syndrome. Interestingly, among the 18 NASH patients with metabolic syndrome twelve had elevated and six had normal liver enzyme levels as stated at Table 2. Similarly, among the 5 NASH patients without metabolic syndrome, there were four

Table 2 Features of NASH patients according to high and normal aminotransferase levels *n* (%)

	NASH cases (<i>n</i> = 23)		<i>P</i>
	Normal aminotransferase levels (<i>n</i> = 7)	Elevated aminotransferase levels (<i>n</i> = 16)	
Age (yr)	52.8 ± 5.04	50.44 ± 5.12	> 0.05
Gender (male/female)	1/6 (14.2/85.8)	6/10 (37.5/62.5)	> 0.05
Hepatomegaly	2 (28.5)	5 (31.2)	> 0.05
BMI (kg/m ²)			
Normal weight	-	-	
Overweight	-	-	
Obese	6 (85.7)	14 (87.5)	> 0.05
Morbid obese	1 (14.3)	2 (12.5)	> 0.05
Waist/hip ratio	5 (71.4)	12 (75.0)	> 0.05
Systolic blood pressure (mmHg) ¹	130.0 (110-150)	120 (110-180)	> 0.05
Diastolic blood pressure (mmHg) ¹	80 (60-90)	70 (60-120)	> 0.05
Hypertension	3 (42.9)	7 (43.8)	> 0.05
HDL-Cholesterol (mg/dL)	43.86 ± 8.97	44.19 ± 4.07	> 0.05
Low-HDL level	4 (57.1)	9 (56.3)	> 0.05
Triglycerides (mg/dL)	183.57 ± 95.82	130.44 ± 59.46	> 0.05
Hypertriglyceridemia	5 (71.4)	5 (31.3)	> 0.05
Fasting glucose (mg/dL) ¹	101 (73-164)	102.83 (87-195)	> 0.05
Diabetes	2 (28.6)	4 (25.0)	> 0.05
Impaired glucose tolerance	3 (42.9)	6 (37.5)	> 0.05
Fasting insulin (μU/mL)	11.8	13.96	> 0.05
Insulin resistance-HOMA-IR	1 (14.3)	9 (56.3)	> 0.05
AST (IU/L) ¹	18 (14-29)	46 (33-92)	< 0.05
ALT (IU/L) ¹	21 (15-39)	77 (32-158)	< 0.05
GGT (IU/L) ¹	21 (15-33)	40 (16-119)	< 0.05
ALP (IU/L) ¹	96 (52-111)	78.5 (55-152)	> 0.05
Metabolic syndrome	6 (85.7)	12 (75.0)	> 0.05

¹Data expressed as median (minimum-maximum); BMI: Body mass index; HDL: High-density lipoprotein; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase. There was no difference between the features of NASH patients with high and normal aminotransferase levels.

patients with elevated and one with normal liver enzyme levels. But these results were not statistically significant. In addition, the proportion of patients with obesity was similar in both the elevated and normal liver enzyme groups with metabolic syndrome.

Table 4 represents the features of liver histology in all NAFLD patients according to liver enzyme levels. In Table 4, patients with elevated liver enzyme levels seemed to have more severe liver histology than those with normal liver enzyme levels, but this was not statistically significant.

Liver histology in patients with normal liver enzyme levels (1 male and 8 female patients) was described in detail below. Histologically defined NASH was present in one male and six female patients. Among six female patients, four had mild, one had moderate and one had severe necroinflammation, and fibrosis (stage 1) was detected only in two patients. In one male patient with NASH, the features of liver histology consisted of moderate steatosis and necroinflammation, but no fibrosis. Simple fatty liver was detected in two female patients in the normal liver enzyme group and one had mild and the other had moderate steatosis.

Table 5 shows the features of liver histology in all NAFLD patients according to AST/ALT ratio

Table 3 Features of NASH patients with high and normal aminotransferase levels according to the presence of metabolic syndrome *n* (%)

	NASH cases (<i>n</i> = 23)		<i>P</i>
	With metabolic syndrome (<i>n</i> = 18)	Without metabolic syndrome (<i>n</i> = 5)	
Average age (yr)	52.7 ± 4.34	45.4 ± 3.20	< 0.05
Gender (male/female)	3/15 (16.7/83.3)	4/1 (80.0/20.0)	< 0.05
Hepatomegaly	5 (29.4)	2 (50)	> 0.05
BMI (kg/m ²)			
Normal weight	-	-	
Overweight	-	-	
Obese	15 (83.3)	5 (100)	> 0.05
Morbid obese	3 (16.7)	0 (0)	> 0.05
Waist/hip ratio	13/ (72.2)	4 (80.0)	> 0.05
Hypertension	10 (55.6)	0 (0)	< 0.05
Low HDL level	13 (72.2)	0 (0)	< 0.05
Hypertriglyceridemia	10 (55.6)	0 (0)	< 0.05
Diabetes	5 (27.8)	1 (20.0)	> 0.05
Impaired glucose tolerance	8 (44.4)	1 (20.0)	> 0.05
Insulin resistance-	6 (75)	4 (80.0)	> 0.05
HOMA-IR			
AST (IU/L)	38.06 ± 16.63	53.60 ± 27.04	> 0.05
ALT (IU/L)	57.39 ± 32.127	84.8 ± 49.37	> 0.05
AST/ALT > 1	3 (16.6)	1 (20.0)	> 0.05
GGT (IU/L) ¹	29.5 (15-83)	33 (16-119)	> 0.05
ALP (IU/L)	83.44 ± 18.19	98.20 ± 38.78	> 0.05

¹Data expressed as median (minimum-maximum); BMI: Body mass index; HDL: High-density lipoprotein; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase. There were significant differences between NASH cases with or without metabolic syndrome according to average age, gender and the presence of hypertension, low-HDL level and hypertriglyceridemia.

values. However, there were no statistically significant differences between patient groups according to AST/ALT ratio. Table 6 compares patients with NASH and without NASH according to anthropometric, clinical and laboratory features; and only the prevalence of obesity was significantly different between these two groups.

DISCUSSION

An increase in ALT value is regarded as a parameter for the diagnosis of NASH. The criteria for establishing a diagnosis of NASH are elevated aminotransferases (ALT and AST), histological features resembling to those in alcoholic steatohepatitis and exclusion of other liver diseases^[2-4]. However, anthropometric, clinical and histological aspects of NASH with normal ALT levels have not been investigated extensively. In this retrospective study, we aimed to describe the anthropometric, clinical and histological features of NASH patients with normal ALT values and to compare these with NASH cases that had elevated ALT levels.

There have been many studies on normal ranges for serum ALT levels. The new cut-off values for upper limits of serum ALT levels are now proposed as 30 U/L for men and 19 U/L for women^[12]. Amarpurkar *et al*^[13] have expressed that after excluding other liver disorders, normal ALT may not exclude severe liver diseases and, hence, liver biopsy may be necessary

Table 4 Liver histology according to the level of liver enzymes in all NAFLD cases *n* (%)

Liver histology	All NAFLD patients	
	Patients with normal aminotransferase levels (<i>n</i> = 9)	Patients with elevated aminotransferase levels (<i>n</i> = 25)
Steatosis		
Mild	5 (55.5)	12 (48.0)
Moderate	3 (33.3)	7 (28.0)
Severe	1 (11.2)	6 (24.0)
Necroinflammation		
Absent	2 (22.2)	9 (36.0)
Mild	4 (44.4)	3 (12.0)
Moderate	2 (22.2)	10 (40.0)
Severe	1 (11.2)	3 (12.0)
Fibrosis		
Absent	7 (77.7)	16 (64.0)
Perisinusoidal/pericellular	2 (22.3)	6 (24.0)
Periportal	0 (0)	2 (8.0)
Bridging	0 (0)	1 (4.0)

Due to the small number of patients, statistical evaluation and *P* values were not available. Patients with elevated liver enzyme levels seemed to have more severe liver histology than those with normal liver enzyme levels, but this was not statistically significant.

Table 5 Liver histology according to AST/ALT ratio values in all NAFLD cases *n* (%)

Liver histology	All NAFLD patients	
	Patients with AST/ALT > 1 (<i>n</i> = 6)	Patients with AST/ALT < 1 (<i>n</i> = 28)
Steatosis		
Mild	2 (33.3)	15 (53.5)
Moderate	2 (33.3)	7 (25.0)
Severe	2 (33.4)	6 (21.5)
Necroinflammation		
Absent	3 (50.0)	3 (10.7)
Mild	0 (0)	12 (42.9)
Moderate	1 (16.7)	11 (39.2)
Severe	2 (33.3)	2 (7.2)
Fibrosis		
Absent	4 (66.6)	18 (64.3)
Perisinusoidal/pericellular	1 (16.6)	7 (25.0)
Periportal	0 (0)	3 (10.7)
Bridging	1 (16.6)	0 (0)

Due to the small number of patients statistical evaluation and *P* values were not available. There were no significant differences between patient groups according to AST/ALT ratio.

to detect the seriousness of liver diseases. Likewise, Kunde *et al*^[14] have stated that the diagnostic use for ALT to determine NASH was insufficient in their study. Fracanzani *et al*^[15] have expressed the view that normal ALT is not a reliable criterion to exclude patients from liver biopsy. Mofrad *et al* have found that histological features of NAFLD may progress in persons with normal ALT values and the liver histology in these persons is not very different from that in patients with high ALT levels and in addition having a low or normal ALT level is not a reliable indicator against the presence of steatohepatitis^[7]. Sorrentino *et al*^[16] have detected that

Table 6 Comparison between patients with NASH and without NASH according to anthropometric, clinical and laboratory features *n* (%)

	All NAFLD patients		<i>P</i>
	Patients with NASH (<i>n</i> = 23)	Patients without NASH (<i>n</i> = 11)	
Age (yr)	51.17 ± 5.11	48.82 ± 9.05	> 0.05
Gender: male/female	7/16 (30.4/69.6)	4/7 (36.4/63.6)	> 0.05
Hepatomegaly	7 (29.2)	4 (36.4)	> 0.05
Obesity (BMI ≥ 30)	23 (100)	8 (72.7)	< 0.05
Waist/hip ratio	17 (63.6)	7 (73.9)	> 0.05
Systolic blood pressure (mmHg) ¹	120 (110-180)	130 (110-180)	> 0.05
Diastolic blood pressure (mmHg) ¹	70 (65-110)	70 (60-120)	> 0.05
Hypertension	10 (45.5)	6 (60)	> 0.05
HDL-Cholesterol (mg/dL)	44.09 ± 5.77	46.91 ± 9.66	> 0.05
Low-HDL level	13 (56.5)	4 (36.4)	> 0.05
Triglycerides (mg/dL)	146.61 ± 74.43	174.18 ± 83.85	> 0.05
Hypertriglyceridemia	10 (43.5)	5 (45.5)	> 0.05
Fasting glucose (mg/dL) ¹	102.33 (73-195)	100 (83-125)	> 0.05
Diabetes	6 (26.1)	2 (18.2)	> 0.05
Impaired glucose tolerance	9 (39.1)	1 (9.1)	> 0.05
Fasting insulin (μU/mL) ¹	13.44 (4.76-47.3)	16.60 (6-21.80)	> 0.05
Insulin resistance-HOMA-IR	13 (81.3)	6 (66.7)	> 0.05
AST (U/L) ¹	40 (14-92)	51 (14-78)	> 0.05
ALT (U/L) ¹	67 (15-158)	82 (13-166)	> 0.05
GGT (U/L) ¹	33 (15-119)	43 (15-426)	> 0.05
ALP (U/L) ¹	81 (52-152)	81 (51-154)	> 0.05
AST/ALT > 1	4 (17.3)	2 (18.1)	> 0.05
Metabolic syndrome	18 (78.3)	9 (81.8)	> 0.05

¹Data expressed as median (minimum-maximum); BMI: Body mass index; HOMA: Homeostasis model assessment; HDL: High-density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase. There was a significant difference between patients with or without NASH according to the presence of obesity only.

liver enzyme levels are not reliable markers of NASH. However, Chen *et al.*¹⁷¹ have determined that elevated serum ALT level may be associated with NAFLD but it is not a reliable parameter for metabolic risk factors. Furthermore, Amarapurka *et al.*¹⁸¹ have added that high levels of transaminases are non-specific and may not indicate the progression of NASH. Therefore, liver biopsy is necessary in grading and staging of NASH to predict disease progression. Lizardi-Cervera *et al.*¹⁹¹ have also revealed that the number of patients with NASH was not increased with high levels of AST. Sebastiani *et al.*²⁰¹ have revealed that the usefulness of non-invasive biomarkers is very restricted in general practice.

In our study, the patients were comparable with respect to age, gender, anthropometric features, imaging aspects and laboratory findings. Interestingly, in NAFLD patients only the prevalence of hypertriglyceridemia in normal liver enzyme group was higher than those with elevated liver enzyme. All other clinical, histological and laboratory findings of patients with normal ALT were no different from those with elevated ALT in the NAFLD group. Moreover, the prevalence of NASH in NAFLD patients with elevated and normal aminotransferase (ALT) levels was similar. These findings suppose that NASH can progress without an increase in ALT levels and this

raises the suspicion that this parameter may not be a reliable marker of NASH. In the present study patients with elevated and normal ALT levels in the NASH group had no significant differences, including the prevalence of metabolic syndrome and insulin resistance. These results suggest that cases with normal aminotransferase levels can also have steatohepatitis and metabolic syndrome and not only simple fatty liver and, hence, a liver biopsy is not an incorrect approach for these patients. We found that the histopathological features of the liver were more severe in NASH patients with high liver enzyme levels than in those with normal liver enzyme levels; but, the difference was not statistically significant.

We compared the NASH patients according to the presence of metabolic syndrome; we did not detect any difference according to liver enzyme levels. Consequently, in our opinion, liver biopsy for cases diagnosed with fatty liver by imaging methods may be beneficial even if they have normal aminotransferase levels.

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COMMENTS

Background

Nowadays certain non-alcoholic steatohepatitis (NASH) cases are detected without an elevation in liver enzyme levels. To make a distinction between NASH and simple fatty liver the approach for all non-alcoholic fatty liver disease (NAFLD) patients should be assessed again regarding the utility and reliability of noninvasive biochemical and imaging modalities as opposed to liver biopsy which still remains the gold standard.

Research frontiers

It was almost impossible formerly to recognize the prognosis of fatty liver, and certain fatty liver patients with elevated liver enzymes may not have NASH. To avoid serious outcomes of NASH such as cirrhosis, hepatocellular carcinoma and liver failure, and to detect these complications early, the accurate and definitive diagnosis of NASH, and protective measures, life style modification and appropriate treatment are essential.

Innovations and breakthroughs

The authors demonstrated that anthropometric, clinical, laboratory and imaging features of NAFLD patients including certain noninvasive markers may not reflect or always be in agreement with the histological aspects of liver biopsy in NASH. The importance and accuracy of liver histopathology should not be disregarded.

Applications

After initial evaluation and discrimination of NAFLD patients by anthropometrical, clinical, laboratory and imaging studies, liver biopsy which is the gold standard should be considered and performed when available.

Terminology

While simple fatty liver is only an accumulation of triglycerides in liver and is accepted to be a benign condition, it is not definitely known which patients will keep their stationary state or which patient and when will progress to steatohepatitis. NASH indicates necroinflammation and fibrosis in liver and may progress to cirrhosis, hepatocellular carcinoma and liver failure.

Peer review

This article is useful for the purpose of evaluating and establishing principles for the best approach in patients with NAFLD.

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Clinical implications of fatty pancreas: Correlations between fatty pancreas and metabolic syndrome

Jun Seok Lee, Sang Heum Kim, Dae Won Jun, Jee Hye Han, Eun Chul Jang, Ji Young Park, Byung Kwan Son, Seong Hwan Kim, Yoon Ju Jo, Young Sook Park, Yong Soo Kim

Jun Seok Lee, Dae Won Jun, Ji Young Park, Byung Kwan Son, Seong Hwan Kim, Yoon Ju Jo, Young Sook Park, Department of Internal Medicine, Eulji University School of Medicine, Seoul 139-711, South Korea

Sang Heum Kim, Department of Radiology, Eulji University School of Medicine, Seoul 139-711, South Korea

Jee Hye Han, Department of Family Medicine, Eulji University School of Medicine, Seoul 139-711, South Korea

Eun Chul Jang, Department of Occupational and Environmental Medicine, College of Medicine, Soonchunhyang University, Chunan 145-511, South Korea

Yong Soo Kim, Department of Radiology, Hanyang University School of Medicine, Guri 231-574, South Korea

Author contributions: Lee JS and Kim SH performed the majority of study and wrote the manuscript; Han JH and Jang EC performed the statistical analysis; Kim YS, Park YS, and Park JY reviewed the radiological data; Son BK, Kim SH, and Jo YJ made the data set and edited the manuscript; and Jun DW supervised the study and edited the manuscript.

Correspondence to: Dr. Dae Won Jun, Department of Department of Internal Medicine, Eulji University School of Medicine, 280-1 Hageye 1-dong, Nowon-gu, Seoul 139-711, South Korea. noshin1004@yahoo.co.kr

Telephone: +82-2-9708494 Fax: +82-2-9720068

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Abstract

AIM: To investigate the clinical implications of lipid deposition in the pancreas (fatty pancreas).

METHODS: The subjects of this study were 293 patients who had undergone abdominal computed tomography (CT) and sonography. Fatty pancreas was diagnosed by sonographic findings and subdivided into mild, moderate, and severe fatty pancreas groups comparing to the retroperitoneal fat echogenicity.

RESULTS: Fatty pancreas was associated with higher levels for visceral fat, waist circumference, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglyceride, high density lipoprotein, free fatty acid, γ -GTP, insulin, and the homeostasis model assessment of insulin resistance (HOMA-IR) than the control group ($P < 0.05$). HOMA-IR, visceral fat, triglyceride, and ALT also tended

to increase with the degree of fat deposition in the pancreas on sonography. In a multivariate logistic regression analysis, HOMA-IR, visceral fat, and ALT level were independently related to fatty pancreas after adjustment for age, body mass index, and lipid profile. The incidence of metabolic syndrome in the fatty pancreas group was significantly higher than in the control group, and the numbers of metabolic syndrome parameters were significantly higher in the fatty pancreas group ($P < 0.05$).

CONCLUSION: Sonographic fatty pancreas showed higher insulin resistance, visceral fat area, triglyceride, and ALT levels than normal pancreases. Fatty pancreas also showed a strong correlation with metabolic syndrome.

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Key words: Fatty pancreas; Metabolic syndrome; Insulin resistance

Peer reviewer: Dr. Massimo Raimondo, Division of Gastroenterology and Hepatology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States

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INTRODUCTION

It has been reported that increases in triglyceride and free fatty acids causes ectopic fat deposition in the liver, heart, muscles, and pancreas. This is called steatosis and is known to be related to obesity and/or insulin resistance^[1]. In particular, steatosis in the liver (or fatty liver) refers to fat deposition in hepatocytes, and its pathophysiology, diagnostic criteria, clinical implications are already well known. However, few studies have been done on lipid deposition in the pancreas (fatty pancreas) and its clinical implications.

A recent animal study reported that lipid deposition in pancreas islet cells due to a high fat/high glucose diet could damage pancreatic beta cells and make them hyperglycemic^[1]. However, another study reported that there was no histological evidence of the existence of steatosis in the human pancreas and no reports have clearly demonstrated that the increase of pancreas echogenicity implied steatosis of the pancreas or fat deposition^[2]. Autopsy studies reported pathologic findings of pancreatic steatosis in interlobular septa rather than in pancreas acinar cells^[3-5], and fat deposition related to aging^[6,7]. According to an animal study, pancreatic steatosis caused anomalies of pancreas islet cells leading to hyperglycemia^[8-10]. Some authors suggested that pancreas islet cell damage that occurs in pancreatic steatosis is accompanied by hyperlipidemia and this plays an important part in the generation of type 2 diabetes^[11]. Gullo *et al*^[12] suggested that hypercholesterolemia causes fat deposition in the pancreas and this change is related to hyperamylasemia. In this study we investigated the clinical implications of fatty pancreas and the correlation between insulin resistance and metabolic syndrome.

MATERIALS AND METHODS

Patients

The subjects of this study were adults who visited an obesity clinic and for whom doctors performed physical examinations and took their medical histories. Those who had diabetes, pancreatic disease, thyroid disease, renal disease, liver disease, and who drank alcohol over 40 g daily for male and 20 g daily for female were excluded from the subjects. Among them, 293 people who underwent abdomen sonography and/or fat measurement CT for evaluation of abdominal fat distribution were chosen as the subjects. Daily alcohol intake, past history, and other general characteristics of the subjects were collected through questionnaires.

Clinical and biochemical parameters

We measured the height and body weight of subjects in light clothing to calculate the Body Mass Index ($BMI = \text{kg}/\text{m}^2$). For waist circumference, we used a tape measure and measured in the middle part between the lowest rib and the iliac crest of the pelvis, horizontal to the ground with the subject in an upright position. Blood samples were test for liver functions, fasting glucose, HbA1c, insulin, total cholesterol, triglyceride, high density lipoprotein, and low density lipoprotein, after fasting for 12 h.

Abdomen sonography and diagnostic criteria of fatty pancreas

All subjects received tests with the same sonograph (Envisor HD, Phillips, Bothell USA) using a convex 5-2 MHz transducer by one radiologist to minimize biases by different testers. The increase of echogenicity of the pancreatic body over the kidney echogenicity was

classified as fatty pancreas, and in other cases as non-fatty pancreas. As the pancreas could not be compared with the kidney in the same window, the radiologist compared pancreatic echogenicity with kidney echogenicity using the difference between liver and kidney echogenicity and liver and pancreas echogenicity. Fatty pancreas was subdivided into four stages: control group (non-fatty pancreas), where the pancreas echogenicity was similar to the kidney parenchymal echogenicity; light fatty pancreas, where the pancreas echogenicity was higher than the kidney echogenicity, but very much lower than the retroperitoneal fat echogenicity; severely fatty pancreas, where the pancreas echogenicity was higher than the kidney echogenicity, but a little lower than the retroperitoneal fat echogenicity; and highly fatty pancreas, where the pancreas echogenicity was similar to the retroperitoneal fat echogenicity.

Fat distribution and computed tomography

Abdomen visceral fat, subcutaneous fat, total fat, and thigh muscle fat areas were measured. A 16-channel multiplex abdominal CT system (General Electric Medical Systems, Milwaukee, USA) was used and measurements were made at the location of the umbilicus and middle part of the thigh. The embedded computer was used for calculations. Hounsfield units (HU) were measured at five different parts of the pancreas (head, neck, body, tail, and uncinata process) and three different parts of the spleen. The differences between the mean values of them were determined. If this difference was -5 or lower, it was classified into the fatty pancreas group and the rest were classified into the non-fatty pancreas group.

Evaluation of insulin resistance

Insulin concentration was measured using a chemiluminescent immunoassay (Immulite 2000, Diagnostic Products Corp., Los Angeles, CA; CV, < 7%). The measure of insulin resistance was obtained using the HOMA-IR (Homeostasis model assessment-insulin resistance), and the calculation formula shown below: $HOMA-IR: [\text{Fasting blood sugar (mmol/L)} \times \text{Fasting insulin } (\mu\text{U/mL})/22.5]$ ^[13].

Definition of metabolic syndrome

The criteria for metabolic syndrome diagnosis followed the NCEP-Adult Treatment Panel III (ATP III), and the visceral obesity was defined by substituting it with the standard waist circumference in the Asia-Pacific Region. Diagnostic criteria were defined as when three or more items of the following were met: visceral obesity (waist circumference ≥ 90 cm for males, or waist circumference ≥ 80 cm for females), increased triglyceride (≥ 150 mg/dL), decreased HDL (< 40 mg/dL for males, < 50 mg/dL for females), hypertension ($\geq 130/85$ mmHg), and fasting glucose (≥ 110 mg/dL).

Statistical analysis

For statistical analysis, the SPSS for Windows (version

11.0; SPSS, Chicago, Ill) was used. The Student's *t*-test was used for comparison between the two groups according to the existence or non-existence of fatty pancreas; the χ^2 test for relationship between fatty pancreas and metabolic syndrome; the multiple logistic analysis was used for analysis of independent correlation factors related to fatty pancreas; and the ANOVA test was used to compare the four groups according to the severity of fatty pancreas.

RESULTS

Clinical characteristics of fatty pancreas

The mean age of 293 subjects was 44.9 ± 9.5 years. There were 133 males (45.4%) and 160 females (54.6%). Among them, 180 (61.4%) were diagnosed as having fatty pancreas from the abdomen sonography; 93 males (51.7%) and 87 females (48.3%). The subjects were divided into the fatty pancreas group and the non-fatty pancreas group for comparison of clinical characteristics. In the fatty pancreas group to compared non-fatty pancreas group, the mean body mass index (26.5 ± 3.1 kg/m² *vs* 24.4 ± 3.2 kg/m², $P < 0.001$), waist circumference (88.9 ± 8.5 *vs* 82.1 ± 9.3 , $P < 0.001$), and visceral fat (10767 ± 4260 *vs* 7462 ± 3244 , $P < 0.001$) were statistically higher. The aspartate aminotransferase (AST) (30.2 ± 20.7 *vs* 23.8 ± 11.1 , $P = 0.001$), alanine aminotransferase (ALT) (40.3 ± 32.2 *vs* 25.0 ± 23.0 , $P < 0.001$), total cholesterol (205.2 ± 35.2 *vs* 192.9 ± 36.4 , $P = 0.005$), triglyceride (159.8 ± 92.5 *vs* 119.6 ± 70.3 , $P < 0.001$), high density lipoprotein cholesterol (47.7 ± 10.6 *vs* 51.9 ± 10.1 , $P = 0.001$), γ -glutamyl transpeptidase (γ -GGT) (46.5 ± 43.0 *vs* 29.9 ± 29.9 , $P < 0.001$), fasting insulin concentration (6.8 ± 3.6 *vs* 5.4 ± 2.1 , $P < 0.001$), and homeostasis model assessment of insulin resistance (HOMA-IR) (3.2 ± 2.1 *vs* 2.3 ± 1.0 , $P < 0.001$), free fatty acid (767.9 ± 324.9 *vs* 639.3 ± 287.6 , $P = 0.001$) also showed significant differences between the two groups ($P < 0.05$). However, age, fasting glucose, low-density lipoprotein, and cholesterol concentration did not show any differences (Table 1).

Factors related to fatty pancreas

To analyze the factors related to fatty pancreas, we conducted a multiple logistic analysis for variables, which were found to have significant relationships with fatty pancreas from the univariate analysis. The multiple logistic analysis was conducted by adding variables in three steps. In the first stage, Model I, age, sex, HOMA-IR, and fasting glucose were used as the independent variables and fatty pancreas as the dependent variable. In Model II, triglyceride and free fatty acid, which were already known to be related to fatty liver, were added to Model I. In Model III, abdominal visceral fat, subcutaneous fat area, and thigh muscle fat area were added to Model II to evaluate the body fat distribution. The multiple logistic analysis found that HOMA-IR had a strong correlation with fatty pancreas, even after correction of age and sex, and this did not change even after correction for triglycerides, cholesterol and free

Table 1 Clinical and laboratory characteristics of study groups

	Non-fatty pancreas (n = 113)	Fatty pancreas (n = 180)	P-value
Age (yr)	44.4 ± 9.7	45.4 ± 8.5	NS
Sex (male/female)	34/79	93/87	< 0.001
BMI (kg/m ²)	24.4 ± 3.2	26.5 ± 3.1	< 0.001
WC (cm)	82.1 ± 9.3	88.9 ± 8.5	< 0.001
Visceral Fat(mm ²)	7462 ± 3244	10767 ± 4260	< 0.001
AST (IU/L)	23.8 ± 11.1	30.2 ± 20.7	0.001
ALT (IU/L)	25.0 ± 23.0	40.3 ± 32.2	< 0.001
FBS (mg/dL)	99.7 ± 24.0	102.9 ± 26.2	NS
TC (mg/dL)	192.9 ± 36.4	205.2 ± 35.2	0.005
TG (mg/dL)	119.6 ± 70.3	159.8 ± 92.5	< 0.001
LDL (mg/dL)	118.6 ± 36.3	124.8 ± 27.4	NS
HDL (mg/dL)	51.9 ± 10.1	47.7 ± 10.6	0.001
γ -GT (mg/dL)	29.9 ± 29.9	46.5 ± 43.0	< 0.001
Fasting insulin (μ U/mL)	5.4 ± 2.1	6.8 ± 3.6	< 0.001
HOMR-IR	2.3 ± 1.0	3.2 ± 2.1	< 0.001
FFA (mg/dL)	639.3 ± 287.6	767.9 ± 324.9	0.001

$P < 0.05$ by *t*-test; NS: Not significant; BMI: Body mass index; WC: Waist circumference; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; FBS: Fasting blood sugar; TC: Total cholesterol; TG: Triglyceride; HDL: High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; γ -GT: Gamma glutamyl transpeptidase; FFA: Free fatty acid.

Table 2 Multivariate logistic regression analysis for the fatty pancreas

Model		Odds ratio (95% CI)	P-value
Model 1	Age (yr)	1.025 (0.999-1.051)	0.059
	Sex (male/female)	0.397 (1.474-4.311)	0.001
	Fasting glucose (mg/dL)	0.998 (0.987-1.010)	0.747
	HOMA-IR	2.319 (1.431-3.757)	0.001
Model 2	Model 1 (HOMA-IR)	1.761 (1.041-2.978)	0.035
	+		
	TC (mg/dL)	1.005 (0.998-1.013)	0.184
	TG (mg/dL)	1.004 (1.000-1.008)	0.060
	FFA (mg/dL)	1.000 (0.999-1.001)	0.409
Model 3	Model 2 (HOMA-IR)	0.990 (0.533-1.839)	0.973
	+		
	BMI (kg/m ²)	1.137 (0.965-1.339)	0.125
	Visceral fat	1.000 (1.000-1.000)	0.006
	Subcutaneous fat	1.000 (1.000-1.000)	0.932
	Thigh fat	1.000 (1.000-1.000)	0.214

Model 1: HOMA-IR and fasting glucose were adjusted for age, sex; Model 2: In addition to Model I, HOMA-IR and fasting glucose were adjusted for lipid profiles (TC, TG, FFA); Model 3: In addition to Model II, HOMA-IR and fasting glucose were adjusted for BMI and fat distribution factors (abdominal visceral fat area, abdominal subcutaneous fat area, thigh fat area). $P < 0.05$ was considered to be significant throughout the analysis.

fatty acid. However, after correction with the visceral fat area, the visceral fat showed the strongest correlation with fatty pancreas; but, HOMA-IR did not show a significant correlation (Table 2).

Relationship between fatty pancreas and metabolic syndrome

An analysis of 104 subjects who showed metabolic syndrome based on ATP III criteria found fatty pancreas in the sonography of 80 (76.9%) patients, which was

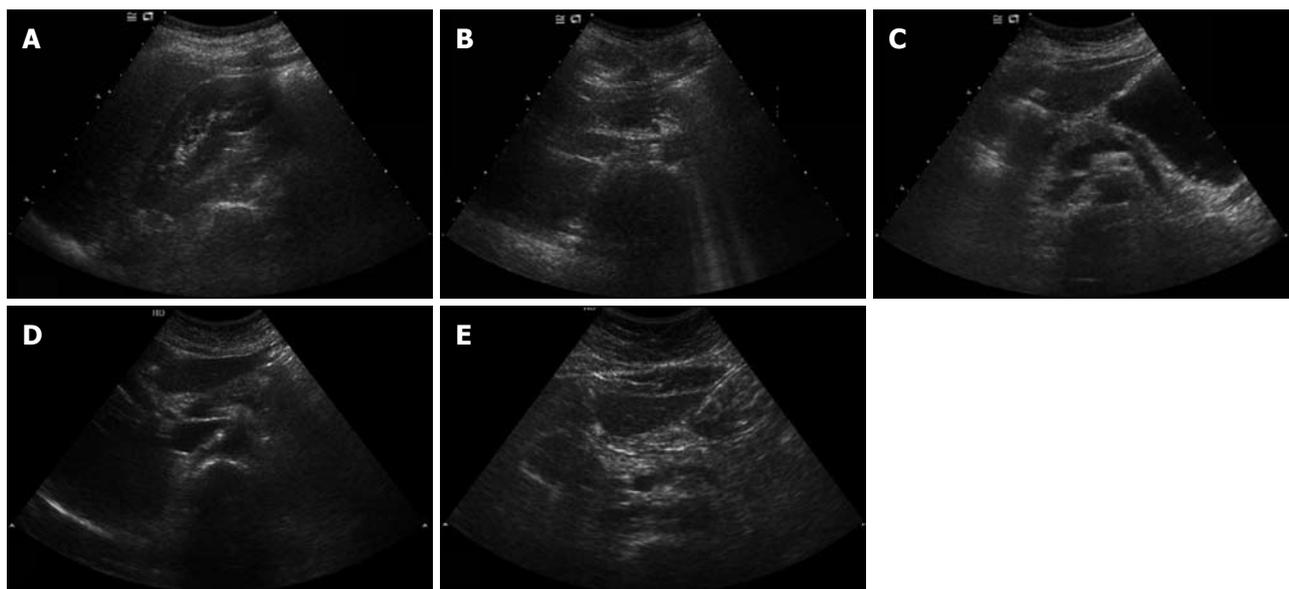


Figure 1 Four echogenicity grades of fatty pancreas. A and B: Non-fatty pancreas, pancreatic echogenicity is equal to renal cortical echogenicity; C: Mild fatty pancreas, pancreatic echogenicity is definitely lower than retroperitoneal fat; D: Moderate fatty pancreas, pancreatic echogenicity is slightly lower than retroperitoneal fat; E: Severe fatty pancreas, pancreatic echogenicity is equal to retroperitoneal fat.

Table 3 Prevalence of metabolic syndrome and metabolic components in the fatty pancreas

	Non-fatty pancreas	Fatty pancreas	¹ P-value
Metabolic syndrome	24 (23.1%)	80 (76.9%)	< 0.001
Number ²	1.4 ± 1.2	2.3 ± 1.4	< 0.001

¹P < 0.05 by chi-square (χ^2) test; ²Number: Number of metabolic components.

a significantly higher proportion of the fatty pancreas group ($P < 0.001$). Furthermore, the number of metabolic syndrome parameters (waist circumference, HDL, triglyceride, fasting glucose, blood pressure) in the fatty pancreas group (2.3 ± 1.4) was statistically significantly higher than that of the non-fatty pancreas group (1.4 ± 1.2) ($P < 0.001$) (Table 3).

Comparison of characteristics according to fatty pancreas by sonography

Among the 180 subjects who showed fatty pancreas, 90 patients were found to have mild fatty pancreas, 68 moderate fatty pancreas, and 22 severe fatty pancreas. The four groups, including the control group of non-fatty pancreas, were compared. We analyzed the correlation between HOMA-IR, visceral fat, triglyceride, and ALT, which were found to be related to fatty pancreas by multivariate multiple logistic analysis and the degree of fatty pancreas. HOMA-IR, visceral fat, triglyceride, and ALT tended to increase with the degree of fat deposition in the pancreas on sonography (Figure 1; Table 4).

Comparison of fatty pancreas by sonography and abdominal CT

To determine correlations between metabolic parameters and fatty pancreas appearing on CT finding pancreas

Table 4 Univariate correlation between ultrasonographic severity of fatty pancreas and several metabolic parameters

	Ultrasonographic severity of fatty pancreas (γ)	P-value
HOMA-IR	0.250	< 0.001
Visceral fat	0.396	< 0.001
TG (mg/dL)	0.245	< 0.001
ALT (IU/L)	0.276	< 0.001

γ : Correlation coefficient.

and those found by sonography, the difference between the average Hounsfield Units (HU) from mean pancreas HU to mean spleen HU was calculated. If the difference was -5 or lower the subjects were classified into the fatty pancreas group on CT finding, and others were classified into the non-fatty pancreas group. A comparison of metabolic syndrome factors and body measurement factors found no difference in visceral fat, lipid profile, and liver chemistry between the two groups, based on CT findings.

Frequency of concurrent fatty liver and fatty pancreas

The total number of fatty pancreas or fatty liver patients was 184. Concurrence of fatty pancreas and fatty liver on sonography was found in 125 subjects (67.9%); fatty pancreas with normal liver was found in 55 (29.9%) and fatty liver without fatty pancreas was only found in four (2.2%) patients. In other words, among 180 fatty liver patients, 125 subjects also had fatty pancreases (96.8%), and the negative predictive value of fatty liver in normal pancreas was 96.4%

DISCUSSION

The pathophysiology and diagnostic criteria for steatosis

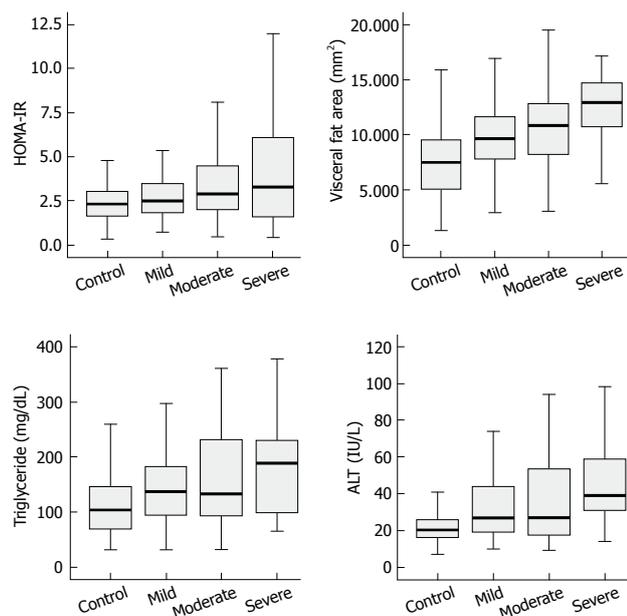


Figure 2 Values of HOMA-IR, visceral fat, triglyceride and ALT in four echogenicity grades of fatty pancreas. $P < 0.05$, by ANOVA based on Tukey's multiple comparison test.

in liver or fatty liver are already established. Pancreas echogenicity in abdomen sonography is known to be determined by peripancreatic fat deposition rather than pancreatic parenchymal deposition^[6], and it was reported that this correlated with age and subcutaneous fat^[14]. However, there have been few studies on lipid deposition in the pancreas and its clinical implications.

Sonography has been widely used as a tool for evaluation of fat deposition in the pancreas, and the pancreas echogenicity has been traditionally compared with liver echogenicity^[7,14]. However, this does not seem to be a good method, because the liver is metabolically very active and its echogenicity exhibits high variance^[15,16]. The concurrence of fatty pancreas and fatty liver on sonography was very high in our data (67.9%). Therefore, liver echogenicity was not a good reference value for diagnosis of fatty pancreas. In contrast, spleen and kidney are known to be metabolically less variable than liver. Therefore, the authors compared the pancreas echogenicity with kidney parenchymal echogenicity, unlike prior studies. This seems to be a reasonable method because the kidney is metabolically more stable than the liver, although it is difficult to compare both the pancreas and the kidney in the same window.

We performed a multiple logistic regression analysis that corrected for age, sex, and serum lipid and found that insulin resistance was independently related to the existence of fatty pancreas in various models. Subjects with fatty pancreas showed strong association with frequency of metabolic syndrome, and fatty pancreas correlated with a number of the parameters of metabolic syndrome, including insulin resistance. However, after adjusting for factors related to body fat distribution, particularly visceral fat, the strong association with insulin resistance disappeared. The reason for this seems to be that visceral fat is a much stronger relational

factor and influenced the relationship between fatty pancreas and insulin resistance. This suggests that fatty pancreas is a risk factor for metabolic syndrome or another manifestation of metabolic syndrome, such as the previously reported correlations of nonalcoholic fatty liver disease with obesity, insulin resistance, and metabolic syndrome^[17,18].

Furthermore, this study subdivided fatty pancreas by the degree of pancreas echogenicity, and compared it with HOMA-IR, visceral fat, triglyceride, and ALT. The correlation coefficients were 0.250, 0.396, 0.245, and 0.276, respectively ($P < 0.001$), demonstrating statistically significant correlation with fatty pancreas severity (Figure 2). A comparative analysis with various other factors is needed with a greater number of subjects.

This study found that HOMA-IR, an insulin resistance marker, had an independently significant correlation with fatty pancreas; but, there were no differences in fasting blood sugar between the two groups. The reason for this seems to be that diabetes was excluded from the selection conditions for subjects.

To compare the echogenicity on sonography with objective Hounsfield units on CT, this study conducted mean pancreas and spleen HU, and analyzed the clinical meaning of fatty pancreas using by CT imaging. However, unlike sonography, no statistical difference in clinical and biochemical parameters could be found. It seems that fat deposition in the pancreas shows different patterns from that in the liver. There is a report that fat deposition was markedly increased in pancreas islet cells of people aged over 60 years^[19], and an animal study demonstrated lipid deposition in pancreas islet cells induced by a high fat/high sucrose diet^[1]. On the other hand, another study claimed that fat deposition in the human pancreas does not occur in the parenchymal cells of the pancreas, but is only limited to interstitial stroma^[20]. Some researchers insist that steatosis in the pancreas or fat deposition in pancreatic cells does not exist in humans^[12]. However, according to some studies that contained pathological observations on the human pancreas^[3-5], fat deposition in the human pancreas appears to occur mainly in interlobular septa, rather than in cells. However, it is difficult to arrive at clear conclusions due to the difficulty of tissue collection from the pancreas, which limits histological proof. The results of the subgroup analysis, which investigated the relationship of degree of fatty pancreas with CT, seems to be due to such characteristics of fat deposition in the pancreas. In other words, because pancreas fat deposition mainly occurs in interlobular septa, the CT images of pancreas show severe non-homogenous patterns with big differences in Hounsfield Units depending on the part measured. Consequently, for CT judgment of pancreas fat deposition it is more appropriate to evaluate the degree of irregular lobulated contour by fat deposition in interlobular septa rather than by Hounsfield Units^[6,20,21]. Therefore, sonography seems to be the more useful imaging technique for judgment of pancreas fat deposition, and a comparison with MRI, which is known to be excellent in judgment of fat deposition, is needed.

Evaluation of pancreas by abdominal sonography is introduced as a screening tool for diagnosis of pancreatic disease by many authors because of its significant accuracy, cost-effectiveness, and few side effects^[22]. Therefore, evaluating pancreas echogenicity during abdominal sonography, which is frequently used in health examinations, is expected to play a part as another indicator for screening for metabolic syndrome and insulin resistance.

This study does suffer from a few limitations. Firstly, the fatty pancreas group ($n = 180$) was larger than the non-fatty pancreas group ($n = 113$), because it was a retrospective study of limited subjects of a single medical center. Secondly, the hyperinsulinemic euglycemic clamp technique, which is known to be the most accurate evaluation method of insulin resistance, was not used and this needs to be incorporated into future studies. Thirdly, the degree of fatty pancreas was not well defined, and there might be problems with inter-observer agreement among ultrasonographers when grading different levels of pancreas echogenicity.

An interesting finding of this study is that in a majority of cases (67.9%), fatty pancreas and fatty liver were found concurrently on sonography, and most fatty liver patients (96.9%) also showed fatty pancreas. Although the positive predictive value of fatty liver in fatty pancreas was 69.4%, the negative predictive value of fatty liver in normal pancreas was 96.4%. This implies that fatty pancreas could be used as the initial indicator of 'ectopic fat deposition' and as an early marker of insulin resistance, which is a key element of fatty liver and/or metabolic syndrome. More studies will be necessary on the role of fatty pancreas as an early marker of ectopic fat deposition or insulin resistance.

This is the first study that evaluated pancreatic steatosis using sonography and the clinical factors related to it, including its relations with insulin resistance and metabolic syndrome. Additional studies are needed to investigate the actual progress of fatty pancreas into metabolic diseases.

COMMENTS

Background

Pathophysiology, diagnostic criteria, and clinical implications of steatosis in liver or fatty liver are already well known. However, there are few studies on the lipid deposition in pancreas (fatty pancreas) and its clinical implications. The authors evaluated the clinical implications of fatty pancreas.

Research frontiers

In particular, steatosis in liver or fatty liver refers to fat deposition in hepatocyte, and its pathophysiology, diagnostic criteria, clinical implications are already well known. However, researches on lipid deposition in pancreas (fatty pancreas) and its clinical implications are still insufficient.

Innovations and breakthroughs

Insulin resistance, visceral fat, triglyceride, and alanine aminotransferase (ALT) tended to increase with the degree of fat deposition in the pancreas on sonography. In a multivariate logistic regression analysis, insulin resistance, visceral fat, and ALT level were independently related to fatty pancreas after adjustment for age, body mass index, and lipid profile. Incidence of metabolic syndrome in the fatty pancreas group was significantly higher than in the control group.

Applications

A majority of cases (67.9%) of fatty pancreas and fatty liver were found concurrently on sonography. Although positive predictive value of fatty liver in fatty pancreas was 69.4%, the negative predictive value of fatty liver in normal pancreas was 96.4%. This implies the possibility of fatty pancreas as the initial indicator of 'ectopic fat deposition' and as an early marker of insulin resistance, which is a key element of fatty liver and/or metabolic syndrome.

Terminology

The increase in echogenicity of pancreatic body over the kidney echogenicity was classified as fatty pancreas. Fatty pancreas was subdivided into four stages: control group (non-fatty pancreas), where pancreas echogenicity was similar to kidney parenchymal echogenicity; light fatty pancreas, where the pancreas echogenicity was higher than kidney echogenicity, but very much lower than the retroperitoneal fat echogenicity; severely fatty pancreas, where the pancreas echogenicity was higher than kidney echogenicity, but a little lower than the retroperitoneal fat echogenicity; and highly fatty pancreas, where the pancreas echogenicity was similar to the retroperitoneal fat echogenicity.

Peer review

This study contains useful information on a difficult topic to study; i.e. fatty pancreas. The authors also speculate on a possible relationship between metabolic syndrome and fatty pancreas and/or fatty liver.

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BRIEF ARTICLES

Surgical management of gallbladder sarcomatoid carcinoma

Keng-Hao Liu, Ta-Sen Yeh, Tsann-Long Hwang, Yi-Yin Jan, Miin-Fu Chen

Keng-Hao Liu, Ta-Sen Yeh, Tsann-Long Hwang, Yi-Yin Jan, Miin-Fu Chen, Department of General Surgery, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan 333, Taiwan, China

Author contributions: Liu KH collected patient data; Yeh TS, Hwang TL, Jan YY and Chen MF designed the research; Liu KH wrote the paper.

Correspondence to: Dr. Keng-Hao Liu, Department of General Surgery, Chang Gung Memorial Hospital, Chang Gung University, #5, Fu-Hsing Street, Kwei-Shan, Taoyuan 333, Taiwan, China. tcfs631c@yahoo.com.tw

Telephone: +886-3-3281200 Fax: +886-3-3285818

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Peer reviewer: Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

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Abstract

AIM: To study the behavior as well as optimal treatment of gallbladder sarcomatoid carcinoma, we reviewed the results of treatment of gallbladder sarcomatoid carcinoma from Chang Gung Memorial Hospital.

METHODS: From 1987 to 2005, six patients were diagnosed with gallbladder sarcomatoid carcinoma and treated at our institution. Tumor staging was based on 2002 revised tumor-node-metastasis (TNM) staging for gall bladder cancer from the American Joint Committee on Cancer. The clinical presentation, laboratory data and preoperative workup were reviewed retrospectively.

RESULTS: Five patients were female and one was male. The age ranged from 51 to 66 years (median, 58 years). Surgical procedures included three curative resections, two palliative resections and one biopsy. There were two surgical complications (33.3%) and one case of surgical mortality (16.7%). The follow-up time ranged from 30 d to 5 mo. The median survival was 2.5 mo. The prognosis was extremely poor, even after curative resection and postoperative chemotherapy.

CONCLUSION: The prognosis of gallbladder sarcomatoid carcinoma was not dependent on TNM stage and was always dismal. The clinicopathological features were different from those of gall bladder cancer.

INTRODUCTION

Primary gallbladder carcinoma is the fifth most common gastrointestinal tract malignancy and the most common malignancy in the biliary tract^[1,2]. Despite improvements in imaging modalities and therapeutic facilities, the prognosis of gallbladder carcinoma is still poor. Tumors that possess epithelial and mesenchymal components are so-called sarcomatoid carcinomas. Gallbladder sarcomatoid carcinoma is a rare and atypical subset of gallbladder carcinoma, and only 44 cases have been described in the English-language literature worldwide^[3-18]. All of these have been case reports and have little information about the clinical behavior and optimal treatment of these tumors. In order to define the behavior and prognosis of gallbladder sarcomatoid carcinoma, we reviewed retrospectively the data of six patients from Chang Gung Memorial Hospital (Taoyuan, Taiwan).

MATERIALS AND METHODS

From 1987 to 2005, six patients were diagnosed with gallbladder sarcomatoid carcinoma and treated at our institution. The histology was confirmed in all patients by tissues taken from either surgical or biopsy specimens. In total, there were 141 patients diagnosed with gallbladder cancer who received surgical treatment during this period. Among them were 124 with adenocarcinoma (87.9%), eight with adenosquamous carcinoma (5.7%), six with sarcomatoid carcinoma (4.3%), two with squamous cell carcinoma (1.4%), and

Table 1 Clinical features, operative methods and survival of six patients with gallbladder sarcomatoid carcinoma

	Age/sex	Jaundice	Operative method	Pathological staging	Chemotherapy	Survival
Case 1	51/F	No	Curative resection	T3N1M0 (II b)	Yes	3 mo
Case 2	66/M	Yes	Palliative	T4NxM0 (IV)	No	2 mo
Case 3	60/F	No	Palliative resection	T3N0M0 (II a)	No	2 mo
Case 4	65/F	No	Curative resection	T3N1M0 (II b)	No	20 d ¹
Case 5	56/F	No	Curative resection	T2N1M0 (II b)	Yes	5 mo
Case 6	53/F	Yes	Palliative resection	T3N1M1 (IV)	Yes	5 mo

F: Female; M: Male; ¹Surgical mortality.

one with neuroendocrine carcinoma (0.7%). Tumor staging was based on the 2002 revised tumor-node-metastasis (TNM) staging for gallbladder cancer from the American Joint Committee on Cancer (AJCC)^[19]. The clinical presentation, laboratory data and preoperative workup, including abdominal sonography, computerized tomography (CT), magnetic resonance imaging (MRI) and endoscopic retrograde cholangiopancreatography (ERCP) were reviewed retrospectively. Extensive surgery was defined as cholecystectomy combined with one or more of the following procedures: liver resection of the involved gallbladder fossa, common hepatic artery lymph nodes and hepatic proper artery lymph nodes, and resection of the extrahepatic bile duct, or other organs invaded by tumor directly. Cholecystectomy was defined as cholecystectomy alone without other extensive surgical procedures. Palliative surgery included cholecystectomy, drainage of biliary obstruction or biopsy of tumor only.

RESULTS

Five patients were female and one was male. The age ranged from 51 to 66 years (median, 58 years). Table 1 displays the clinical features, operative methods and survival of the six patients with gallbladder sarcomatoid carcinoma. Abdominal pain (83%) was the most frequent complaint in these patients, and two (33.3%) were found to have jaundice. No patient had anemia. Except in patients with jaundice, the liver function tests were all normal. Carcinoembryonic antigen (CEA) level was elevated in one patient and carbohydrate antigen 19-9 (CA19-9) level was elevated in two patients. Five patients were diagnosed with gallbladder cancer preoperatively and one was diagnosed with gallstones with chronic cholecystitis. All six patients underwent surgical procedures, including three curative resections and three palliative procedures. Four patients underwent extensive surgery, including three cholecystectomies with partial resection of the gallbladder bed of the liver, and one cholecystectomy with right hemicolectomy for direct tumor invasion of the hepatic flexure of the colon. One patient underwent laparoscopic cholecystectomy only, after preoperative diagnosis with gallstones and chronic cholecystitis. The remaining patient received laparotomy and biopsy of the tumor only as a result of peritoneal seeding during operation. There were two surgical complications (33.3%) including one

postoperative hematoma and one case of postoperative sepsis caused by bile leakage. One case of surgical mortality (16.7%) was encountered after bile leakage. Three patients received fluorouracil-based chemotherapy postoperatively. Pathological staging was one stage II a, three II b, and two stage IV.

The follow-up time ranged from 30 d to 5 mo. Except for the case of surgical mortality, all patients died within 5 mo from tumor recurrence or disease progression. The median survival was 2.5 mo. The prognosis was extremely poor even, after curative resection with or without postoperative chemotherapy.

DISCUSSION

The most common histological type of gallbladder cancer is adenocarcinoma, and sarcomatoid carcinoma is extremely rare^[2-18]. Chao *et al.*^[11] have reported the incidence of sarcomatoid carcinoma from gallbladder cancer as 4.1%. It has been described that sarcomatoid carcinoma arises from totipotent stromal stem cells^[8] and is composed of epithelial and mesenchymal components that contain undifferentiated spindle or stellate cells^[3,4,6,8,9,11,14-18]. The cancer was first reported by Landsteiner in 1907, and until now, only 44 cases have been reported in the English-language literature, with an age range of 45-91 years^[2-18]. However, in our review, patient age was limited to the sixth and seventh decades of life. At our institution, the female-to-male ratio for gallbladder cancer was about 2:1, which was similar to the ratio for gallbladder sarcomatoid carcinoma reported in the literature^[1,2,4]. In the present study, the female-to-male ratio was 5:1.

Patients with gallbladder sarcomatoid carcinoma usually present with abdominal pain, jaundice, nausea, and poor oral intake, and some may present with a palpable abdominal mass and weight loss. Symptoms may persist from several days to years. Liver function tests are normal, except in patients with obstructive jaundice caused by tumor invasion of the biliary tract^[3-18]. Most cases have been reported to have normal CEA and CA19-9 levels, and in our series, the CEA level was elevated in one case and CA19-9 level was elevated in two cases. Mass lesions can be identified by abdominal sonography and CT. Sonographic studies have shown an echogenic mass, with or without areas of necrosis occupying the gallbladder lumen, and the wall may be diffuse, localized or irregular. The

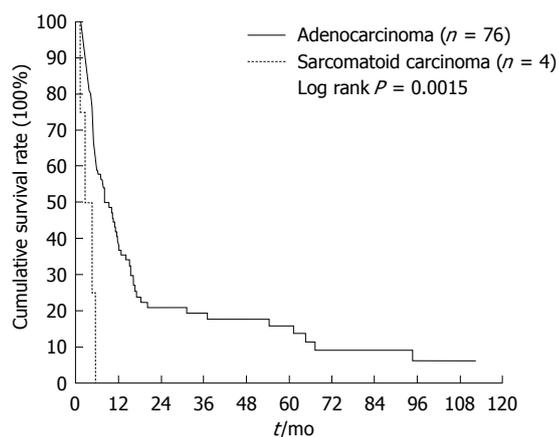


Figure 1 Survival curve of adenocarcinoma and sarcomatoid carcinoma of the gallbladder (excluding stage I, biopsy only and surgical mortality patients).

characteristics of gallbladder sarcomatoid carcinoma are similar to those of adenocarcinoma of the gallbladder, and it is difficult to distinguish between these two tumors. However, if there is speckled calcification within the tumor upon CT, gallbladder carcinoma with calcification, calcified gallstones, porcelain gallbladder and ossifying sarcomatoid carcinoma should be the in differential diagnosis of the tumor^[6,13,16]. Other associated findings, including gallstones, liver invasion or metastasis, retroperitoneal organ invasion or lymph node enlargement along the hepatoduodenal ligament, can be identified by CT. In our series, five patients (83.3%) were diagnosed preoperatively with gallbladder cancer, and the remaining one was diagnosed with a 1.5-cm gallstone.

T and N stages are important prognostic factors in gallbladder cancer^[20]. However, there are no prognostic factors determined in gallbladder sarcomatoid carcinoma. Surgery is suggested as the only recognized treatment for gallbladder sarcomatoid carcinoma; either radiotherapy or chemotherapy has no benefit on survival^[8]. However, even after curative extensive surgery with combined gallbladder/liver bed resection or combined resection of involved organs, many patients die shortly after surgery from recurrence or metastasis. After excluding stage I gallbladder adenocarcinoma, biopsy only and surgical mortality patients, the survival rate for gallbladder sarcomatoid carcinoma and stage II-IV gallbladder adenocarcinoma is shown in Figure 1. One-year survival rate for gallbladder sarcomatoid carcinoma *vs* adenocarcinoma was 0% *vs* 38.2% \pm 5.57% (χ^2 , $P < 0.05$). Among the 44 cases reviewed in the literature, the mean survival time was 2 mo and only two patients have survived for > 1 year^[11,12]. In our series, the median survival time was 2.5 mo and the longest survival was only 5 mo. Apart from the one case of surgical mortality, all patients died from cancer recurrence or disease progression.

In conclusion, better survival of gallbladder cancer was seen in patients with early-stage disease. However, the prognosis of gallbladder sarcomatoid carcinoma was not dependent on TNM stage, and was always dismal. The

clinicopathological features were different from those of gallbladder cancer, and will be necessary to accumulate additional reports of such patients to clarify these issues.

COMMENTS

Background

Gallbladder cancer is the most common malignancy in the biliary tract. Sarcomatoid carcinoma of the gallbladder is a rare and atypical subset of gallbladder carcinoma, and only 44 cases have been described in the English-language literature worldwide.

Research frontiers

Little information about the clinical behavior and optimal treatment of these tumors has been reported. In this study, the authors retrospectively reviewed clinical presentation and treatment results of six patients from Chang Gung Memorial Hospital Taoyuan, Taiwan.

Innovations and breakthroughs

Only case reports have been described before. This is believed to be the first series of patients diagnosed with gallbladder sarcomatoid carcinoma in a single institution.

Applications

Tumor staging was based on the 2002 revised tumor-node-metastasis (TNM) staging for gallbladder cancer from the American Joint Committee on Cancer. The clinical presentation, laboratory data and preoperative imaging workup were reviewed. Surgical procedures, postoperative chemotherapy and survival rate after treatment were discussed by the authors.

Terminology

Sarcomatoid carcinoma arises from totipotent stromal stem cells, and is composed of epithelial and mesenchymal components that contain undifferentiated spindle or stellate cells.

Peer review

The authors emphasized that the prognosis of gallbladder sarcomatoid carcinoma was not dependent on TNM stage, and was always dismal. The clinicopathological features are different from those of gallbladder adenocarcinoma.

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Percutaneous portal venoplasty and stenting for anastomotic stenosis after liver transplantation

Bao-Jie Wei, Ren-You Zhai, Jian-Feng Wang, Ding-Ke Dai, Ping Yu

Bao-Jie Wei, Ren-You Zhai, Jian-Feng Wang, Ding-Ke Dai, Ping Yu, Department of Radiology, Beijing Chaoyang Hospital, Capital Medical University, Beijing 100020, China

Author contributions: Wei BJ and Zhai RY designed the research; Wei BJ, Zhai RY, Wang JF, Dai DK and Yu P performed the research; Wei BJ, Zhai RY, Dai DK and Yu P analyzed the data; Wei BJ wrote the paper with a contribution from Wang JF; Zhai RY revised the paper.

Correspondence to: Ren-You Zhai, MD, Chief, Department of Radiology, Beijing Chaoyang Hospital, Capital Medical University, No. 8, Gongren Tiyuchang South Road, Chaoyang District, Beijing 100020, China. ryzhai219@hotmail.com

Telephone: +86-10-85231908 Fax: +86-10-65935214

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Abstract

AIM: To review percutaneous transhepatic portal venoplasty and stenting (PTPVS) for portal vein anastomotic stenosis (PVAS) after liver transplantation (LT).

METHODS: From April 2004 to June 2008, 16 of 18 consecutive patients (11 male and 5 female; aged 17-66 years, mean age 40.4 years) underwent PTPVS for PVAS. PVAS occurred 2-10 mo after LT (mean 5.0 mo). Three asymptomatic patients were detected on routine screening color Doppler ultrasonography (CDUS). Fifteen patients who also had typical clinical signs of portal hypertension (PHT) were identified by contrast-enhanced computerized tomography (CT) or magnetic resonance imaging. All procedures were performed under local anesthesia. If there was a PVAS < 75%, the portal pressure was measured. Portal venoplasty was performed with an undersized balloon and slowly inflated. All stents were deployed immediately following the predilation. Follow-ups, including clinical course, stenosis recurrence and stent patency which were evaluated by CDUS and CT, were performed.

RESULTS: Technical success was achieved in all patients. No procedure-related complications occurred. Liver function was normalized gradually and the symptoms of PHT also improved following PTPVS. In 2 of 3 asymptomatic patients, portal venoplasty and stenting were not performed because of pressure gradients < 5 mmHg. They were observed with

periodic CDUS or CT. PTPVS was performed in 16 patients. In 2 patients, the mean pressure gradients decreased from 15.5 mmHg to 3.0 mmHg. In the remaining 14 patients, a pressure gradient was not obtained because of > 75% stenosis and typical clinical signs of PHT. In a 51-year-old woman, who suffered from massive ascites and severe bilateral lower limb edema after secondary LT, PVAS complicated hepatic vein stenosis and inferior vena cava (IVC) stenosis. Before PTPVS, a self-expandable and a balloon-expandable metallic stent were deployed in the IVC and right hepatic vein respectively. The ascites and edema resolved gradually after treatment. The portosystemic collateral vessels resulting from PHT were visualized in 14 patients. Gastroesophageal varices became invisible on poststenting portography in 9 patients. In a 28-year-old man with hepatic encephalopathy, a pre-existing meso-caval shunt was detected due to visualization of IVC on portography. After stenting, contrast agents flowed mainly into IVC *via* the shunt and little flowed into the portal vein. A covered stent was deployed into the superior mesenteric vein to occlude the shunt. Portal hepatopetal flow was restored and the IVC became invisible. The patient recovered from hepatic encephalopathy. A balloon-expandable Palmaz stent was deployed into hepatic artery for anastomotic stenosis before PTPVS. Percutaneous transhepatic internal-external biliary drainage was performed in 2 patients with obstructive jaundice. Portal venous patency was maintained for 3.3-56.6 mo (mean 33.0 mo) and all patients remained asymptomatic.

CONCLUSION: With technical refinements, early detection and prompt treatment of complications, and advances in immunotherapy, excellent results can be achieved in LT.

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Key words: Portal vein; Anastomotic stenosis; Venoplasty; Stent; Liver transplantation

Peer reviewer: Peter L Moses, MD, FACC, AGAF, Professor, University of Vermont College of Medicine Section of Gastroenterology & Hepatology, 111 Colchester Avenue, Smith 237B, MCHV, Burlington, VT 05401, United States

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INTRODUCTION

Liver transplantation is an important option in the management of end-stage liver disease, severe acute liver failure and some metabolic liver disorders. Postoperative vascular complications have been well documented. The incidence of portal venous complications following liver transplantation is considered to be relatively uncommon in comparison with hepatic arterial complications, yet they can be potentially devastating and lead to graft loss. In the past, portal venous complications were managed with surgical treatments such as thrombectomy, anastomosis revision or retransplantation. However, surgical management of these complications has been limited by technical difficulties due to postsurgical fibrosis and limitations in the length of the involved venous structures^[1]. Percutaneous interventional procedures have gained worldwide acceptance for alleviating the symptoms of portal hypertension and preserving the graft, due to their minimal invasiveness as well as low complication and high success rates^[2]. In this study, we retrospectively reviewed 16 cases that received percutaneous transhepatic portal venoplasty and stenting (PTPVS).

MATERIALS AND METHODS

Patients

From April 2004 to June 2008, 16 of 18 consecutive patients (11 male and 5 female; aged 17-66 years, mean age 40.4 years) underwent PTPVS for portal vein anastomotic stenosis (PVAS) after liver transplantation (LT). Nine patients were from other hospitals. One patient had a living donor LT; another patient received a second graft. The right branch of the portal vein of the donor was anastomosed to the main portal vein of the recipient by end-to-end anastomosis in the living donor LT; all other reconstructions of the portal vein were performed by standard end-to-end anastomosis of the recipient and donor portal vein. PVAS occurred 2-10 mo after LT (mean 5.0 mo).

Three of 18 patients were asymptomatic and were detected on routine screening color Doppler ultrasonography (CDUS). Seven patients presented with increased liver function tests; two patients complicated with obstructive jaundice. Fifteen patients presented the typical clinical signs of portal hypertension (PHT), which included variceal bleeding ($n = 7$), ascites ($n = 5$), and splenomegaly ($n = 4$) with or without thrombocytopenia. The 15 symptomatic patients also were identified by other noninvasive imaging examinations.

The initial diagnosis of PVAS was based on CDUS in all 18 patients, and 15 of 18 patients also underwent

contrast-enhanced computerized tomography (CT) or magnetic resonance imaging (MRI) to confirm the stenosis. The criteria of CDUS for the detection of PVAS were as follows: > 50% narrowing of the stenotic segment compared with the main portal venous diameter in adults or to a diameter < 2.5 mm in children on gray-scale imaging; the presence of an acceleration of flow at the stenosis or a poststenotic jet flow or scarcity flow of the intrahepatic portal vein on Doppler US^[1,3,4]. The criterion for PVAS on CT or MRI was > 50% narrowing of the main portal venous diameter with or without poststenotic dilatation^[5].

Procedures

Informed consent was obtained from each patient. Our institutional review committee did not have to give approval for this retrospective study. All procedures were performed *via* a right-sided intercostal approach under local anesthesia. The transplanted liver was punctured with a 22-gauge Chiba needle (Neff Percutaneous Access Set, NPAS-100-RH-NT; COOK Co., USA) under fluoroscopic guidance, and the needle was targeted to the peripheral branch of the portal vein. After confirming puncture of the intrahepatic portal vein with a test dose injection of contrast media, a 0.018-inch nitinol guidewire (NPAS; COOK, USA) was advanced into the main portal vein. The needle was exchanged for a 4.0-French coaxial dilator and 6.0-French sheath (NPAS; COOK, USA) combination included in the introducer system over the guidewire, then the 0.018-inch guidewire was exchanged for a 0.035-inch hydrophilic coating guidewire (Terumo Co., Japan), and then a 7.0-French vascular sheath (Terumo, Japan) was inserted over the guidewire into the portal vein. Initial portography was obtained with a 5.0-French catheter (KMP; COOK, USA or Cobra; Terumo, Japan). If there was an anastomotic stenosis < 75%, the portal pressure was measured at the postanastomotic main portal vein or at the level of the hepatic hilar portal bifurcation. A disposable pressure transducer system (Utah Medical Products, USA) was used for measuring portal pressure. The catheter was guided by the 0.035-inch guidewire through the stenotic segment. Portography including the splenic vein and superior mesenteric vein was obtained (Figure 1A) and the preanastomotic portal pressure was also measured. The criteria for definite diagnosis of PVAS were as follows: stenosis > 50% of the main portal venous diameter^[6] and a pressure gradient across the stenosis > 5 mmHg^[3,7,8].

Portal vein anastomotic venoplasty was performed with a percutaneous transluminal angioplasty balloon dilatation catheter (Powerflex P3; Cordis, Johnson & Johnson Co., USA or Synergy; Boston Scientific Co., USA). The balloon had a smaller diameter than that of the allograft portal vein. Balloon pressure was slowly and gradually elevated using an inflation syringe (Basix Compak; Merit Medical Systems Co., USA) within a pressure limit of 10-atm until the balloon's waist was effaced (Figure 1B). Postvenoplasty portography was repeated to assess the results (Figure 1C). A self-

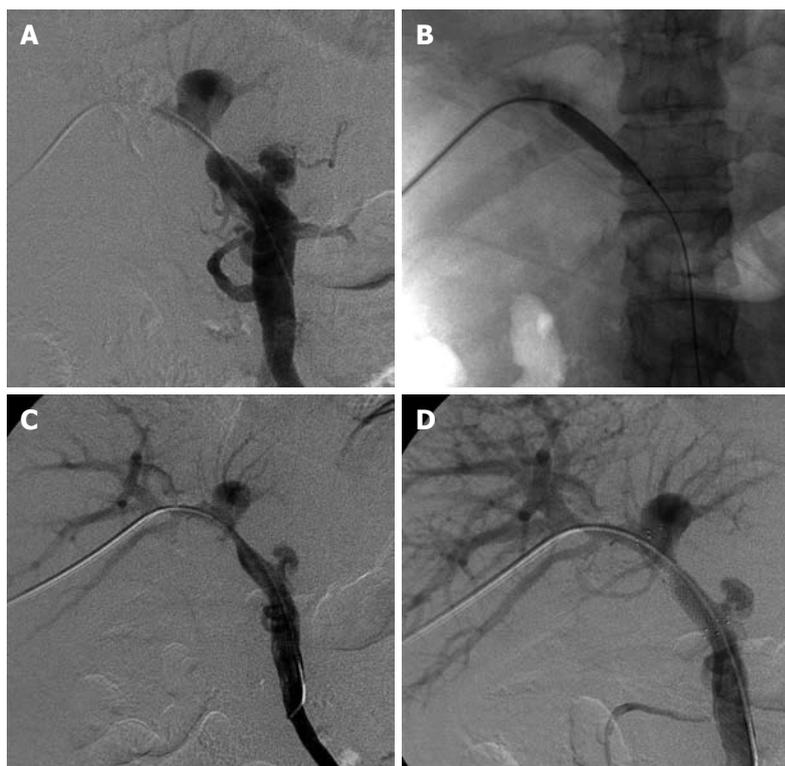


Figure 1 Procedure of percutaneous transhepatic portal venoplasty and stenting. A: Percutaneous transhepatic portography revealed a severe anastomotic stenosis and gastroesophageal varices; B: Portal venoplasty was performed with an undersized balloon and the balloon was slowly inflated; C: Postvenoplasty portography showed a residual stenosis, but gastroesophageal varices became almost invisible; D: Poststenting portography visualized portal venous patency and inflow had recovered completely.

expandable metallic stent (SMART Control; Cordis, USA) was deployed to cover the stenosis with minimal angulation between the portal vein and the stent. The stent had the same or 1-2 mm larger diameter than that of the allograft portal vein. Poststenting portography (Figure 1D) and the pressure gradient were obtained repeatedly to assess the results. If the deployed stent showed an hourglass deformity of $> 50\%$ of its normal diameter, balloon postdilatation was performed. If a satisfactory result had been achieved, the catheter was removed and the puncture tract was embolized with compressed gelfoam bars through the cut vascular sheath.

No intravenous or systemic heparinization was used. Poststenting anticoagulation was achieved by oral administration of aspirin enteric-coated tablets (Bayaspirin, Bayer S.p.A., Italy) 100 mg/d for at least 6 mo.

Technical success, complications, clinical signs and symptoms, laboratory values including the liver function test and the imaging surveillance results after PTPVS were documented. Technical success of the procedure was defined as $< 30\%$ residual stenosis being observed on portography with the absence of varices or collateral circulation^[5]. Follow-up, including clinical course, stenosis recurrence and stent patency which were evaluated by CDUS and CT, were performed.

RESULTS

Technical success of PTPVS was achieved in all 16 patients. No procedure-related complications occurred. In these patients, liver function was normalized gradually and clinical manifestations related to PHT were improved following PTPVS.

In three asymptomatic patients, a Yashiro type catheter (Terumo, Japan) was introduced into the splenic artery or superior mesenteric artery *via* a right femoral artery approach and indirect portography was obtained to confirm the diagnosis of PVAS. More than 50% stenosis of portal vein anastomosis was further demonstrated using percutaneous transhepatic portography, but portal venoplasty and stenting were not performed in two patients because pressure gradients across the stenosis were < 5 mmHg. They were observed with periodic CDUS or CT. Portal venoplasty (using 8-10 mm diameter and 40 mm length balloons) and stenting (using 10-12 mm diameter and 40-60 mm length stents) was performed in 16 patients. In 2 patients, the mean initial pressure gradient across the stenosis was 15.5 mmHg and then it decreased to 3.0 mmHg after PTPVS. In the remaining 14 patients, a pressure gradient was not obtained because of $> 75\%$ stenosis and typical clinical signs of PHT.

In a 51-year-old woman, who suffered from massive ascites and severe bilateral lower limb edema after secondary LT, CDUS and CT detected PVAS complicated by hepatic vein stenosis and inferior vena cava stenosis. These venous stenoses were identified by indirect portography, percutaneous transhepatic hepatic venography and inferior vena cavography. First, a self-expandable metallic stent (COOK-Z, GZV-30-75; COOK, USA) was placed in the inferior vena cava *via* a right femoral vein approach. Second, a balloon-expandable metallic stent (IntraStent, SPM16-26-08-B; ev3 Co., USA) was deployed in the right hepatic vein *via* an intercostal transhepatic route. Last, PTPVS was performed. The ascites and edema resolved gradually after treatment.



Figure 2 A 28-year-old man with a pre-existing meso-caval shunt. A: Initial portography revealed an almost occlusive anastomosis; B: Portography detected a pre-existing meso-caval shunt due to visualization of the inferior vena cava; C: After stenting, the contrast agents flowed mainly into the inferior vena cava via the shunt and little flowed into the portal vein; D: A covered stent was deployed into the superior mesenteric vein to occlude the shunt. Portal hepatopetal flow was restored and the inferior vena cava became invisible.

The portosystemic collateral vessels resulting from PHT were visualized by initial portography in 14 patients. Gastroesophageal varices became invisible on poststenting portography in 9 patients. To avoid variceal bleeding, the residual gastroesophageal flow was obstructed with platinum embolization coils (Tornado, MWCE-35-8/4-5-Tornado; COOK, USA) in 2 remaining patients. No further procedure was performed for anorectal varices and pre-existing splenorenal shunt because the abnormal flow became reduced after stenting.

In a 28-year-old man with hepatic encephalopathy, a pre-existing meso-caval shunt was detected due to visualization of the IVC on initial portography (Figure 2A and B). A meso-caval shunt was performed due to refractory massive ascites and recurrent variceal bleeding before LT. After stenting, it was seen that the contrast agents flowed mainly into the IVC *via* the shunt and little flowed into the portal vein (Figure 2C). To maintain adequate hepatoportal perfusion pressure and to avoid thrombosis in the portal stent or liver failure, a covered stent (Wallgraft Endoprosthesis; Boston Scientific, USA) with a 10 mm diameter and a 50 mm length was deployed into the superior mesenteric vein to occlude the shunt. Once more portography revealed that portal hepatopetal flow was restored and the IVC became invisible (Figure 2D). The patient felt abdominal pain after the procedure, but the symptom subsided a week later. The patient recovered from hepatic encephalopathy.

A balloon-expandable Palmaz stent (Genesis PG1840PMW; Cordis, USA) was deployed into the hepatic artery *via* a right femoral artery route for anastomotic stenosis 2 mo before PTPVS. Percutaneous transhepatic internal-external biliary drainages were performed with a biliary drainage catheter (Ultrathane MAC-LOC, ULT8.5-38-40-P-32S-CLB-RH; COOK, USA) in 2 patients complicated with obstructive jaundice.

The follow-up results of CDUS in all 16 patients and CT scan in 6 patients revealed portal venous patency was maintained for 3.3-56.6 mo (mean 33.0 mo).

These patients remained asymptomatic at the time this manuscript was completed.

DISCUSSION

Portal venoplasty and stenting

The rate of portal venous complications after LT, which include primary portal vein anastomotic stenosis or portal vein thrombosis, has been reported to be below 3%^[9-11]. However, in children with reduced-size LT and living donor LT, the incidence of portal venous stenosis or thrombosis is higher than in adults with deceased donor transplantation, because the donor portal segment is relatively short, and due to interposition grafts and size mismatch of the portal vein diameter between donors and recipients. Factors that increased the risk of portal venous complications were pre-existing vein thrombosis or hypoplasia and large portocaval collaterals^[12].

Since the first report of portal venous angioplasty and stent placement through a transhepatic approach after LT by Olcott *et al*^[13] in 1990, percutaneous transhepatic interventional procedures have gained worldwide acceptance for treatment of these complications following LT, due to their minimal invasiveness as well as low complication and high success rates^[2,14]. Nonetheless, the reported recurrence rate has been relatively high, i.e. 28.6%-36.8%, following balloon angioplasty alone^[3,6,7].

Stents have usually been used to treat recurrent and elastic portal venous stenoses following balloon angioplasty, as this procedure has several potential complications^[3,6,7]. Nevertheless, Ko *et al*^[4] preferred to perform primary stent placement rather than balloon angioplasty in the early posttransplantation period (less than 1 mo). In our study, all stents were deployed immediately following balloon angioplasty for two reasons. First, balloon predilation can reduce the incidence of stent displacement (most jump forward) during stent deployment, especially when the stenosis is severe. Second, direct venoplasty and stenting can recover the normal portal flow once and for all, because repeat percutaneous transhepatic portal venoplasty may lead to puncture injuries in the transplanted

liver and increase the incidence of procedure-related complications, such as intrahepatic pseudoaneurysm, arteriovenous fistulas, subcapsular hematoma, bleeding through the puncture tract of the graft, or portal venous thrombosis^[2-4,6,7].

The luminal area is proportional to the square of the radius; flow is proportional to the fourth power of the radius. Thus, any small improvement is magnified. Intimal damage can lead to platelet aggregation that can in turn lead to short-term occlusion or long-term restenosis. Therefore, any intimal tears should be avoided at all costs, even to the extent of accepting a less visually appealing angiographic result. Slow and gradual inflation of the balloon during angioplasty results in fewer large intimal tears, flaps, and dissections than the more commonly used method of “blow it up, let it down”^[15]. In our study, portal venoplasty was performed with an undersized balloon and the balloon was slowly and gradually inflated. Although portal vein thrombosis or stent-edge stenosis may occur, the follow-up results revealed portal venous patency was maintained for a mean 33.0 mo.

Although the etiology of anastomotic stenosis was unclear in our patients, it suggested fibrosis or intimal hyperplasia, which retained an hourglass deformity on the deployed stent.

Role of imaging examination

It is important to detect vascular complications after liver transplantation, because most stenoses or thromboses are frequently treatable with interventional procedures; but, if left untreated, many vascular complications may progress to graft failure. However, compared with biliary obstructions, early portal venous stenosis is difficult to detect from clinical signs and symptoms alone. Furthermore, sometimes the portal venous anastomotic site cannot be seen with ultrasonography because of intestinal artifacts. In such a case, other noninvasive cross-sectional imaging modalities, CT and MRI, may be valuable.

Ultrasonography is often used to screen for vascular abnormalities, including hepatic arterial stenosis and thrombosis, and the less common stenoses or thromboses of the portal vein, hepatic veins, and inferior vena cava. Precise anatomy of the vascular abnormalities is often better determined on CT or MRI, especially when a focal stenosis occurs in the distal IVC, or in the hepatic artery proximal to the porta hepatis where it is difficult to image directly by ultrasonography. CT and MRI give detailed imaging, while ultrasonography tends to give more physiologic data. CT and MRI can provide a more comprehensive evaluation of the transplanted liver; reveal abnormalities of vascular structure; and depict bile ducts, liver parenchyma, and extrahepatic tissues. Moreover, CT angiography and MR angiography can be used to evaluate the extent and degree of the portosystemic collateral vessels resulting from PHT. Magnetic resonance cholangiopancreatography can be valuable to detect focal biliary abnormalities; however,

percutaneous transhepatic cholangiography remains the gold standard for biliary complications^[16].

When a patient is asymptomatic, indirect portography is a recommended option to identify a portal venous stenosis, as this procedure has a relatively lower incidence of procedure-related complications and no puncture injuries to the graft. When there is a requirement for measurement of portal pressure gradients across a stenosis or further interventional procedures, percutaneous transhepatic portography is necessary.

Portal venography with measurement of pressure gradient across the stenosis remains the most reliable examination^[16]; but, the procedure is an invasive one. Although some reports have considered a transstenotic pressure gradient of > 5 mmHg as abnormal^[7,8,17], no standard guidelines for a significant pressure gradient have yet been established. Park *et al*^[9] believed that the pressure gradient is not directly correlated with the clinical results, and mentioned that portal venoplasty might not be so helpful for patients whose clinical symptoms are possibly related with graft dysfunction and not with the stenosis. The treatment is valuable if patients have symptoms related to portal venous inflow abnormality or PHT even though the pressure gradient is not significant^[4]. In patients who do not have evidence of PHT, and have normal hepatic function, stenoses may be observed for progression with periodic ultrasound. Moreover, in patients with PHT, the potential contribution of underlying hepatic parenchymal disease (rejection or recurrent hepatitis) must be considered. However, if portal venous stenosis is suspected as being a significant contributor to PHT, therapeutic intervention is necessary.

Negative findings on serial CDUS and the absence of clinical symptoms during the follow-up period might prompt us to regard these patients as not having any hemodynamically significant vascular abnormalities.

In conclusion, percutaneous transhepatic portal venoplasty and stenting for anastomotic stenosis after liver transplantation is a safe and effective procedure for alleviating the signs and symptoms of portal hypertension and preserving the graft. With technical refinements, early detection and prompt treatment of complications, and advances in immunotherapy, excellent results can be achieved in liver transplantation.

COMMENTS

Background

Portal vein anastomotic stenosis after liver transplantation is an uncommon vascular complication that may result in graft loss if not promptly treated. In the past, portal venous complications were managed with surgical treatments such as thrombectomy, anastomosis revision or retransplantation. However, surgical management of these complications has been limited by technical difficulties due to postsurgical fibrosis and limitations in the length of the involved venous structures. Percutaneous interventional procedures have gained worldwide acceptance for alleviating the symptoms of portal hypertension and preserving the graft, due to their minimal invasiveness as well as low complication and high success rates.

Research frontiers

The reported recurrence rate has been relatively high, i.e. 28.6%-36.8%, following balloon angioplasty alone. Stents have usually been used to treat

recurrent and elastic portal venous stenoses following balloon angioplasty, as this procedure has several potential complications. Nevertheless, Ko *et al* preferred to perform primary stent placement rather than balloon angioplasty in the early posttransplantation period (< 1 mo).

Innovations and breakthroughs

In this study, all stents were deployed immediately following balloon angioplasty for two reasons. First, balloon predilation can reduce the incidence of stent displacement during its deployment. Second, direct venoplasty and stenting can recover normal portal flow once and for all, because repeat percutaneous transhepatic portal venoplasty may lead to puncture injuries to the transplanted liver and increase the incidence of procedure-related complications. Intimal damage can lead to platelet aggregation that can in turn lead to short-term occlusion or long-term restenosis. Slow and gradual inflation of the balloon during angioplasty results in fewer large intimal tears, flaps, and dissections than the more commonly used method of "blow it up, let it down." In this study, portal venoplasty was performed with an undersized balloon and the balloon was slowly and gradually inflated. Although portal vein thrombosis or stent-edge stenosis may occur, the follow-up results revealed portal venous patency was maintained for a mean of 33.0 mo. The treatment is valuable if patients have symptoms related to portal venous inflow abnormality or portal hypertension even though the pressure gradient is not significant.

Applications

Percutaneous transhepatic portal venoplasty and stenting (PTPVS) could be used for portal anastomotic stenosis after liver transplantation to alleviate the signs and symptoms of portal hypertension and preserve the graft.

Peer review

PTPVS for anastomotic stenosis after liver transplantation is a safe and effective procedure for alleviating the signs and symptoms of portal hypertension and preserving the graft. The results are encouraging and suggest that the method of undersized balloon and slow and gradual inflation can reduce intimal damage and keep the portal venous patency for a mean of 33.0 mo after stenting.

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Gender and metabolic differences of gallstone diseases

Hui Sun, Hong Tang, Shan Jiang, Li Zeng, En-Qiang Chen, Tao-You Zhou, You-Juan Wang

Hui Sun, Hong Tang, En-Qiang Chen, Tao-You Zhou, Center of Infectious Diseases, West China Hospital of Sichuan University; Division of Molecular Biology of Infectious Diseases, the State Key Laboratory of Biotherapy (Sichuan University), Chengdu 610041, Sichuan Province, China

Shan Jiang, First Department of Internal Medicine, Wusheng People's Hospital, Guangan 638400, Sichuan Province, China

Li Zeng, You-Juan Wang, Physical Examination Center, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

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Correspondence to: You-Juan Wang, Associate Professor, Physical Examination Center, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China. yjwang1963@163.com

Telephone: +86-28-85422866 Fax: +86-28-85422818

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a high level of fasting plasma glucose was obvious in gallstone disease ($P < 0.05$), and in women, hypertriglyceridemia or obesity were significant in gallstone disease ($P < 0.05$).

CONCLUSION: We assume that age and sex are profoundly associated with the incidence of gallstone disease; the metabolic risk factors for gallstone disease were different between men and women.

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Key words: Gallstone disease; Metabolic disorder; Risk factor; Sex; Age

Peer reviewer: Dr. Karel van Erpecum, Department of Gastroenterology and Hepatology, University Hospital Utrecht, PO Box 855003508 GA, Utrecht, The Netherlands

Sun H, Tang H, Jiang S, Zeng L, Chen EQ, Zhou TY, Wang YJ. Gender and metabolic differences of gallstone diseases. *World J Gastroenterol* 2009; 15(15): 1886-1891 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1886.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1886>

Abstract

AIM: To investigate the risk factors for gallstone disease in the general population of Chengdu, China.

METHODS: This study was conducted at the West China Hospital. Subjects who received a physical examination at this hospital between January and December 2007 were included. Body mass index, blood pressure, fasting plasma glucose, serum lipid and lipoproteins concentrations were analyzed. Gallstone disease was diagnosed by ultrasound or on the basis of a history of cholecystectomy because of gallstone disease. Unconditional logistic regression analysis was used to investigate the risk factors for gallstone disease, and the Chi-square test was used to analyze differences in the incidence of metabolic disorders between subjects with and without gallstone disease.

RESULTS: A total of 3573 people were included, 10.7% (384/3573) of whom had gallstone diseases. Multiple logistic regression analysis indicated that the incidence of gallstone disease in subjects aged 40-64 or ≥ 65 years was significantly different from that in those aged 18-39 years ($P < 0.05$); the incidence was higher in women than in men ($P < 0.05$). In men,

INTRODUCTION

Gallstone disease is prevalent worldwide; however, its prevalence varies by region. In Western countries, the prevalence of gallstone disease reportedly ranges from approximately 7.9% in men to 16.6% in women^[1]. In Asians it ranges from approximately 3% to 15%, is nearly non-existent (less than 5%) in Africans^[2,3], and ranges from 4.21% to 11% in China^[4]. The prevalence of gallstone disease is also high in some ethnic groups, e.g. 73% in Pima Indian women; 29.5% and 64.1% of American Indian men and women, respectively; and 8.9% and 26.7% of Mexican American men and women, respectively^[1,5,6]. From a medical economic perspective, gallstone disease is the most common reason for hospitalization and creates a high burden in the United States^[7] and other Western countries^[8]. Many recent studies have shown that gallstone disease is related to age, sex, and metabolic disorders, such as obesity, dyslipidemia (hypertriglyceridemia), and type 2 diabetes^[9-11]. The pathogenesis of gallstone disease is suggested to be multifactorial and probably develops from complex interactions between many genetic and

environmental factors^[12,13].

Because of an increase in the Westernization of dietary habits and a decrease in physical activity, the prevalence of gallstone disease has increased in the Chinese population in recent years. From a public health standpoint, it is not only important to study the background prevalence of gallstone disease regionally, but to also explore the demographic and biological markers related to the development of gallstone disease. Meanwhile, gallstone disease can result in serious outcomes, such as acute gallstone pancreatitis and gallbladder cancer. If we can predict which factors contribute to the development of gallstone disease, we can prevent it by controlling these factors. The present study was designed to explore the potential risk factors for gallstone disease and to improve the understanding of the overall pathogenesis of this disease.

MATERIALS AND METHODS

Data resource and data collection

This study was conducted at the physical examination center of West-China Hospital at Sichuan University. This hospital provides medical care mainly for middle- and high-income individuals from Chengdu City and the surrounding metropolitan areas. Our sample population consisted of consecutive subjects who were referred to the physical examination center by their companies as an annual requirement. Data collection, including age, sex, demographic data, history of systemic diseases and gastrointestinal surgery, and a complete physical examination were done by the doctors at the physical examination center. Ultrasonography of the abdomen was conducted by ultrasonographers using a scanner equipped with a 3.5-MHz transducer (Philips Medical Systems, Bothell, USA). Blood samples were drawn *via* venipuncture from the study participants, after they had fasted overnight, by clinical nurses for laboratory examination. Fasting plasma glucose (FPG), triglyceride, total cholesterol, high-density-lipoprotein cholesterol (HDL-C), and low-density-lipoprotein cholesterol (LDL-C) concentrations were measured using Hitachi Modular analyze system (Roche Modular DPP, Hitachi Ltd, Tokyo, Japan).

Diagnosis criteria

Gallstone disease was defined as the presence of strong intraluminal echoes that were gravity-dependent or that attenuated ultrasound transmission (acoustic shadowing) during abdominal ultrasonography or as a history of cholecystectomy because of gallstone disease.

Obesity was defined as a body mass index (BMI) ≥ 25 kg/m² in both men and women according to the redefined World Health Organization criteria for the Asia Pacific Region^[14]. High blood pressure was defined as a systolic blood pressure (SBP) ≥ 140 mmHg or a diastolic blood pressure (DBP) ≥ 90 mmHg or a history of hypertension. Subjects with an FPG ≥ 1260 mg/L and/or a history of diabetes were considered to have diabetes mellitus (DM). Hypertriglyceridemia was defined as a

Table 1 OR of individual risk factors and their association with gallstone disease

Risk factors	<i>n</i>	Gallstone (%)	OR	95% CI
Sex				
Men	181/1825	9.9	1.00	-
Women	203/1748	11.6	1.19	0.97-1.48
Age (yr)				
18-39	89/1622	5.5	1.00	-
40-64	226/1695	13.3	2.65	2.05-3.42
≥ 65	69/256	27.0	6.36	4.48-9.01
BMI				
< 25.0 kg/m ²	281/2841	9.9	1.00	-
≥ 25.0 kg/m ²	103/732	14.1	1.49	1.17-1.90
Hypertension				
No	269/2933	9.2	1.00	-
Yes	115/640	18.0	2.17	1.71-2.75
FPG				
< 1100 mg/L	341/3417	10.0	1.00	-
≥ 1100 mg/L and < 1260 mg/L	14/60	23.3	2.75	1.49-5.05
≥ 1260 mg/L	29/96	30.2	3.90	2.49-6.12
Triglyceride				
< 1500 mg/L	206/2445	8.4	1.00	-
≥ 1500 mg/L	178/1128	15.8	2.04	1.64-2.52
HDL				
< 350 mg/L (men); < 390 mg/L (women)	26/133	19.5	1.00	-
≥ 350 mg/L (men); ≥ 390 mg/L (women)	358/3440	10.4	0.48	0.31-0.74
Total cholesterol				
< 2200 mg/L	343/3323	10.3	1.00	-
≥ 2200 mg/L	41/250	16.4	1.70	1.20-2.43
LDL				
< 1550 mg/L	360/3429	10.5	1.00	-
≥ 1550 mg/L	24/144	16.7	1.71	1.09-2.68

triglyceride concentration ≥ 1500 mg/L. Low HDL-C was defined as an HDL-C level < 350 mg/L in men or < 390 mg/L in women. Hypercholesterolemia was defined as a total cholesterol level ≥ 2200 mg/L. High LDL-C was defined as an LDL-C level ≥ 1550 mg/L.

Statistical analysis

Categorical data are presented as the number of cases and percentages. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL). Odds ratios (ORs) were calculated with the variables coded in a multivariate form. Pearson's Chi-square or Fisher's exact tests were used for categorical variables. Multiple logistic regression analysis was performed to investigate the independent factors associated with gallstone disease. In all cases, tests of significance were 2-tailed, $P < 0.05$ indicated statistical significance.

RESULTS

A total of 3573 subjects undergoing an annual health examination from January to December 2007 were included: 1825 men and 1748 women. The prevalence of gallstone disease among the study subjects was 10.7% (384/3573): 9.9% in men and 11.6% in women. The results of univariate analysis of individual factors and their association with gallstone disease among the 3573 subjects are shown in Table 1. The factors significantly

Table 2 Multivariate logistic regression analysis for gallstone disease

Variables	OR	95% CI	P
Female sex	1.70	1.35-2.15	< 0.001
Age (yr)			
40 to 64	2.44	1.88-3.17	< 0.001
≥ 65	5.83	4.02-8.44	< 0.001
FBG ≥ 1260 mg/L	2.12	1.31-3.43	0.002
Triglycerides ≥ 1500 mg/L	1.67	1.31-2.13	< 0.001

The dependent variable was the presence or absence of gallstone disease. The covariates included sex, age of 40-64 and ≥ 65 years, BMI ≥ 25.0 kg/m², high blood pressure (SBP ≥ 40 mmHg or DBP ≥ 90 mmHg or a history of hypertension), an FPG level between 1100 and 1260 mg/L and ≥ 1260 mg/L, a triglyceride level ≥ 1500 mg/L, an HDL level ≥ 350 mg/L in men or ≥ 390 mg/L in women, a total cholesterol level ≥ 2200 mg/L, and an LDL level ≥ 1550 mg/L.

associated with gallstone disease were an age of 40-64 years and an age ≥ 65 years, a BMI ≥ 25.0 kg/m², high blood pressure, an FPG level between 1100 and 1260 mg/L, an FPG level ≥ 1260 mg/L, a triglyceride level ≥ 1500 mg/L, a total cholesterol level ≥ 2200 mg/L, and an LDL level ≥ 1550 mg/L ($P < 0.05$). In contrast, a low HDL-C level was inversely associated with gallstone disease ($P < 0.05$). As shown in Table 1, the prevalence of gallstone disease for each metabolic disorder was 14.1% for obesity, 18.0% for hypertension, 30.2% for DM, 15.8% for hypertriglyceridemia, and 10.4% for a low HDL-C level.

In order to identify the risk factors, we further performed a multivariate logistic regression analysis (backward stepping); the results are shown in Table 2. Women aged 40-64 years and ≥ 65 years, with an FPG level ≥ 1260 mg/L, and a triglyceride level ≥ 1500 mg/L were positively correlated with gallstone disease.

The incidence of metabolic disorders in the groups with and without gallstone disease is shown in Table 3. Obesity, hypertension, DM, hypertriglyceridemia, a low HDL-C level, and hypercholesterolemia were found in 26.8%, 29.9%, 7.6%, 46.4%, 6.8%, and 10.7% of subjects with gallstone disease, respectively. In the group without gallstone disease, the incidences of obesity, hypertension, DM, hypertriglyceridemia, a low HDL-C level, and hypercholesterolemia were 19.7%, 16.5%, 2.1%, 29.8%, 3.4%, and 6.6%, respectively. The incidences of all metabolic disorders were higher in the group with gallstone disease than in the group without gallstone disease ($P < 0.01$).

The results of univariate analysis of metabolic factors and their association with gallstone disease in different sexes are shown in Table 4. In men, the factors significantly associated with gallstone disease were high blood pressure, an FPG level between 1100 and 1260 mg/L, an FPG level ≥ 1260 mg/L, and a triglyceride level ≥ 1500 mg/L ($P < 0.05$). In women, the factors significantly associated with gallstone disease were a BMI ≥ 25.0 kg/m², high blood pressure, an FPG level between 1100 and 1260 mg/L, an FPG level ≥ 1260 mg/L, a triglyceride level ≥ 1500 mg/L, a total

cholesterol level ≥ 2200 mg/L, and an LDL-C level ≥ 1550 mg/L ($P < 0.05$). A low HDL-C level was inversely associated with gallstone disease only in women ($P < 0.05$).

To control the covariates simultaneously, multivariate logistic regression analysis (backward stepping) was performed (Table 5). The analysis revealed that an FPG level ≥ 1260 mg/L was a significant independent predictor of gallstone disease in men ($P = 0.005$) and a BMI ≥ 25.0 kg/m² and a triglyceride level ≥ 1500 mg/L were predictors of gallstone disease in women ($P < 0.05$).

DISCUSSION

One of the important benefits of early screening for gallstone disease is that ultrasonography can detect asymptomatic cases, which results in early treatment and the prevention of serious outcomes such as acute gallstone pancreatitis and gallbladder cancer^[15]. However, few reports on the prevalence and possible etiology of gallstone disease have been published in China. In the present study, gallstone disease appeared to be common in the test population, i.e. an estimated 10.7% of the test population in Chengdu, China, had gallstone disease. The reported prevalence of gallstone disease is approximately 3.6% in Japan and 4.3%-5.0% in Taiwan^[16-18]. The apparently higher prevalence rate in our study may have been due to the Westernized lifestyle of our patients, who were of middle-to-high income class. Another possible reason for such differences has been related to the fact that this was a hospital-based study which was unlikely the population study that could represent the general population.

The present study, in accordance with reports from Western countries and other regions of Asia, showed that an older age is a significant risk factor for gallstone disease^[16,18,19]. In contrast, gallstone disease is virtually absent in children and adolescents aged 8-19 years^[20]. Long-term exposure to many risk factors, as is true for the elderly, may increase the risk of gallstone disease. At the same time, sedentary activity, which is greater in the elderly than in younger populations, may also increase the risk of gallstone disease^[21,22]. Furthermore, gallstone disease is also an acquired disease influenced by chronic environmental factors plus an aging effect^[23].

In concordance with the findings of previous studies, female sex was also a major risk factor for gallstone disease in the present study. The commonly perceived opinion that women are at greater risk of developing gallstone disease than men may largely be due to extraneous risk factors, such as pregnancy and sex hormones. The number of pregnancies is the main one related to the high rates of gallstone disease in women. Sex hormones are most likely to be responsible for the increased risk. Estrogen increases biliary cholesterol secretion causing cholesterol super saturation of bile. Thus, hormone replacement therapy in postmenopausal women has been described to be associated with an increased risk for gallstone disease^[24,25]. Some studies have also shown a relation between oral contraceptive use and a high prevalence of gallstone disease^[26,27].

Table 3 Prevalence of metabolic disorders in the subjects with and without gallstone disease *n* (%)

Metabolic disorders	Gallstone disease	No gallstone disease	χ^2	<i>P</i>
Obesity	103/384 (26.8)	629/3189 (19.7)	10.603	0.001
Hypertension	115/384 (29.9)	525/3189 (16.5)	42.387	< 0.001
Diabetes mellitus	29/384 (7.6)	67/3189 (2.1)	40.550	< 0.001
Hypertriglyceridemia	178/384 (46.4)	950/3189 (29.8)	43.529	< 0.001
Low HDL-C	26/384 (6.8)	107/3189 (3.4)	11.157	0.001
Hypercholesterolemia	41/384 (10.7)	209/3189 (6.6)	8.954	0.003

Table 4 Univariate analysis of metabolic risk factors for gallstone disease in gender

Risk factors	Men				Women			
	<i>n</i>	Gallstone disease (%)	OR	95% CI	<i>n</i>	Gallstone disease (%)	OR	95% CI
BMI								
< 25 kg/m ²	113/1235	9.1	1.00	-	168/1606	10.5	1.00	-
≥ 25 kg/m ²	68/590	11.5	1.29	0.94-1.78	35/142	24.6	2.80	1.85-4.24
Hypertension								
No	120/1391	8.6	1.00	-	149/1542	9.7	1.00	-
Yes	61/434	14.4	1.73	1.25-2.41	54/206	26.2	3.32	2.33-4.73
FPG								
< 1100 mg/L	152/1710	8.9	1.00	-	189/1707	11.1	1.00	-
≥ 1100 mg/L and < 1260 mg/L	11/47	23.4	3.13	1.56-6.28	3/13	23.1	2.41	0.66-8.83
≥ 1260 mg/L	18/68	26.5	3.69	2.10-6.49	11/28	39.3	5.20	2.40-11.26
Triglyceride								
< 1500 mg/L	79/1012	7.8	1.00	-	127/1433	8.9	1.00	-
≥ 1500 mg/L	102/813	12.5	1.69	1.24-2.31	76/315	24.1	3.27	2.38-4.49
HDL								
< 350 mg/L (men); < 390 mg/L (women)	167/1738	9.6	1.00	-	191/1702	11.2	1.00	-
≥ 350 mg/L (men); ≥ 390 mg/L (women)	14/87	16.1	1.80	0.99-3.27	12/46	26.1	2.79	1.42-5.48
Total cholesterol								
< 2200 mg/L	163/1682	9.7	1.00	-	180/1641	11.0	1.00	-
≥ 2200 mg/L	18/143	12.6	1.34	0.80-2.26	23/107	21.5	2.22	1.37-3.62

Table 5 Multivariate logistic regression analysis for gallstone disease in gender

Variables	Men		Women	
	OR	95% CI	OR	95% CI
BMI ≥ 25.0 kg/m ²	-	-	1.59	1.01-2.50 (<i>P</i> = 0.046)
FBG ≥ 1260 mg/L	2.30	1.28-4.12 (<i>P</i> = 0.005)	-	-
Triglyceride ≥ 1500 mg/L	1.37	0.99-1.90 (<i>P</i> = 0.057)	2.17	1.54-3.07 (<i>P</i> < 0.001)

The dependent variable was the presence or absence of gallstone disease. The covariates included a BMI ≥ 25.0 kg/m², high blood pressure (SBP ≥ 140 mmHg or DBP ≥ 90 mmHg or a history of hypertension), an FPG level between 1100 and 1260 mg/L and ≥ 1260 mg/L, a triglyceride level ≥ 1500 mg/L, a low HDL level (men: ≥ 350 mg/L; women: ≥ 390 mg/L), and a total cholesterol level ≥ 2200 mg/L.

Previous population studies have reported inconsistent associations of DM with gallstone disease. A study in Rome showed that DM was associated with an increased risk of gallstone disease in men and women separately^[28]. A study of Hispanic Americans found a positive association between DM and self-reported gallstone disease in women, but not in men^[26]. A study in Italy failed to find any relation between DM and gallstone disease in men and women combined^[29]. The present analyses showed a positive association between DM

and gallstone disease in men, but not in women. The mechanism underlying the relation of DM with gallstone disease may be fasting hyperinsulinemia, which can overly activate the rate-limiting enzyme for cholesterol synthesis^[30] and finally leads to cholesterol saturation in the bile. Reduced motility of the gallbladder in persons with diabetes is another possible explanation^[31,32].

In our study, obesity only showed a positive association with gallstone disease in women. Previous studies have found disparate findings for BMI or relative weight in men with gallstone disease^[29,33,34]. However, three population screening surveys using ultrasonography failed to find a positive association between BMI and gallstone disease in men in Italy, Denmark, and the United States^[26,35,36], whereas all three showed a positive association in women. The discrepant findings for BMI in men with gallstone disease have not been fully explained. A possible reason for these findings may be that BMI is not a suitable standard of obesity in men. Waist-to-hip ratio may be a better measure of obesity. The mechanism responsible for the increased risk of gallstone disease in obese persons may be the increase in bile saturation that results from an increase in the biliary secretion of cholesterol, which likely depends on the higher synthesis rate of cholesterol in obese persons^[23].

The present study showed that hypertriglyceridemia was a risk factor for gallstone disease only in women.

However, high total cholesterol, low HDL-C, and high LDL-C levels were negatively associated with the risk of gallstone disease in both men and women. The present finding is different from that of previous studies, which noted a positive relation between hypertriglyceridemia and gallstone disease^[36]. However, a cross-sectional study in Denmark failed to find a significant association between gallstone disease and plasma lipid levels (including triglyceride, total cholesterol, HDL-C, and LDL-C)^[37]. Further studies are needed to clarify whether elevated levels of plasma lipids are independent risk factors for gallstone disease.

A major limitation of the present study was the potential self-selection bias due to the hospital-based study design, which resulted in a sample that was not representative of the general population in western China. However, we believe that our findings are useful as background data for future studies of the epidemiology of gallstone disease in China. Second, our measurements were inadequate. Some factors that might play an important role in gallstone disease development, such as oral contraceptive use and waist-to-hip ratio, were not collected in detail. Third, measurement error and different pathogenicities may have occurred, because the measurements were only made at one time point. Therefore, future studies need to determine whether these factors affect the results of our study.

In conclusion, older age, and female sex are associated with the prevalence of gallstone disease in both men and women. Obesity and hypertriglyceridemia were positively associated with gallstone disease in women, but not in men, whereas DM (FPG \geq 1260 mg/L) was positively associated with gallstone disease only in men.

COMMENTS

Background

Gallstone disease is one of the most prevalent gastrointestinal diseases with a substantial burden to health care systems. Because the pathogenesis of gallstone disease is still not well defined and strategies for prevention and efficient non-surgical therapies are missing, further studies are required. Many researchers have shown that gallstone disease is related to age, sex, and metabolic disorders, such as obesity, dyslipidemia (hypertriglyceridemia), and type 2 diabetes. However, the findings concerning metabolic disorders and gallstone disease are disparate in different regions and ethnicity.

Research frontiers

There are a cluster of metabolic syndromes which includes obesity, glucose intolerance, increased low-density-lipoprotein cholesterol, triacylglycerol, diminished high-density-lipoprotein cholesterol and hypertension. The number of gallstone patients is increasing with a high prevalence of metabolic syndrome.

Innovations and breakthroughs

This study confirmed that age and sex are positive risk factors for gallstone disease; but, the association between metabolic disorders and gallstone disease is different for men and women. Furthermore, the study complemented the background prevalence of gallstone disease in Chengdu, China.

Applications

The results of this paper can guide clinicians to target high-risk groups for related inspection and early treatment. Furthermore, preventive strategies can be identified and planned according to these results.

Terminology

Gallstone disease, formally known as cholelithiasis, occurs when gallstones formed in the bile duct, which are abnormal masses of a solid mixture of cholesterol crystals, mucin, and calcium bilirubinate proteins. It is asymptomatic

in most patients. Sometimes it can cause dyspepsia and other gastrointestinal symptoms or biliary colic or Mirizzi syndrome.

Peer review

This paper provides information about the incidence and risk factors of gallstone disease in China. The results of this study can give information for further research to explore the pathogenesis of gallstone disease and the role of metabolic syndrome in the process of gallstone formation.

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BRIEF ARTICLES

Addition of hepatectomy decreases liver recurrence and leads to long survival in hilar cholangiocarcinoma

Zheng Shi, Ming-Zhi Yang, Qing-Liang He, Rong-Wen Ou, You-Ting Chen

Zheng Shi, Ming-Zhi Yang, Qing-Liang He, Rong-Wen Ou, You-Ting Chen, Department of Hepatopancreatobiliary Surgery, First Affiliated Hospital, Fujian Medical University, Fuzhou 350005, Fujian Province, China

Author contributions: Shi Z and He QL performed the majority of diagnoses and treatment of the patients and also edited the manuscript; Yang MZ designed the study and wrote the manuscript, Ou RW and Chen YT collected all the patients' material.

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Correspondence to: Ming-Zhi Yang, PhD, Department of General Surgery, First Affiliated Hospital, Fujian Medical University, 20 Chazhong Road, Fuzhou 350005, Fujian Province, China. drhugomd@msn.com

Telephone: +86-591-87982563 Fax: +86-591-87982556

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CONCLUSION: Hepatectomy, especially including the caudate lobe combined with bile duct resection should be considered standard treatment to cure hilar cholangiocarcinoma.

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Key words: Curative resection; Hepatectomy; Hilar cholangiocarcinoma; Recurrence; Survival

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Abstract

AIM: To evaluate hepatic recurrence and prognostic factors for survival in patients with surgically resected hilar cholangiocarcinoma in a single institution over the last 13 years.

METHODS: From 1994 to 2007, all patients with hilar cholangiocarcinoma referred to a surgical clinic were evaluated. Demographic data, tumor characteristics, and outcome were analyzed retrospectively. Outcome was compared in patients who underwent additional liver resection with resection of the tumor.

RESULTS: Of the 69 patients submitted to laparotomy for tumor resection, curative resection (R_0 resection) was performed in 40 patients, and palliative resection in 29. Thirty-one patients had only duct resection, and 38 patients had combined duct resection with liver resection including 34 total or part caudate lobes. Curative rates with the combined hepatectomy were significantly improved compared with those without additional hepatectomy (27/38 vs 13/31; $\chi^2 = 5.94$, $P < 0.05$). Concomitant liver resection was associated with a decreased incidence of initial recurrence in liver one year after surgery (11/38 vs 23/31; $\chi^2 = 13.98$, $P < 0.01$). The 3-year survival rate after R_0 resection was 30.7% and was 10.5% for palliative resection. R_0 resection improved the 3-year survival rate (30.7% vs 10.5%; $\chi^2 = 12.47$, $P < 0.01$).

INTRODUCTION

The surgical treatment of hilar cholangiocarcinoma has changed completely in recent decades, before 1980 the majority of patients were not resected, and in a few cases local excision of the tumor was performed with low radicality and poor long-term outcome. Since 1980, indications for resection have progressively improved and liver resection has been associated with bile duct resection in order to increase radicality and achieve better survival results^[1-5]. In contrast to reports from 2 or 3 decades ago, today most patients with hilar cholangiocarcinoma are diagnosed pre-mortem. The most important factor affecting prognosis is resectability of the tumor. Patients who undergo resection with curative intent have 3-year survival rates as high as 50% and 5-year survival rates between 10% and 44%. Significant determinants of improved prognosis in patients undergoing curative resection include well-differentiated tumors, absence of lymph node metastases, absence of direct tumor extension into the liver, papillary histology, serum bilirubin at presentation of less than 9 mg/dL, and a near-normal or normal performance status. Palliative resection, surgical bypass procedures, and various types of intubation and drainage procedures are associated with 3-year survival rates from 0% to 4%^[6-8].

The prognosis of patients with hilar cholangiocarcinoma is poor, and the survival rate reported so far describes a very limited life expectancy, < 3 mo if no treatment is offered. Although radical hilar tumor is a formidable challenge for surgeons, endoscopic transpapillary and/or percutaneous transhepatic biliary drainage offers the best survival^[9-11].

Local resection seems to have a very narrow role because of its poor results compared with those of hepatectomy associated with bile duct resection. We have tried to address the above-mentioned issues in this systematic retrospective analysis of the literature.

MATERIALS AND METHODS

Data selection

Long-term follow-up of patients with hilar cholangiocarcinoma undergoing surgical resection was performed by retrospective analysis. The study included 69 unselected consecutive patients whose tumor resection was attainable (46 males and 23 females with a mean age of 58 years) during treatment for hilar cholangiocarcinoma from 1994 to 2007 in the Department of General Surgery, at First Affiliated Hospital, Fujian Medical University. Data acquisition was based on hospital records. Furthermore, follow-up data were obtained by telephone contact with relatives of the patients. In order to evaluate the life expectancy of patients with hilar cholangiocarcinoma, follow-up analysis was performed from the time the patient received his or her first treatment until death. Negative margins of bile duct at final pathologic reports had R₀ resection, otherwise positive margins had palliative resection.

Bismuth classification and treatment strategies

The biliary stricture location was classified in relation to the confluence of hepatic ducts as described by Bismuth-Corlette. Bismuth stage was assessed by endoscopic retrograde cholangiography, endoscopic retrograde cholangioscopy, percutaneous transhepatic cholangiography and/or percutaneous transhepatic cholangioscopy. 3D images provided accurate information on the relationship between hilar cholangiocarcinoma and adjacent vessels. This technique is a powerful new tool for improving the proportion of potentially curative resections^[12]. In addition, selected patients underwent computed tomography (CT-scan), magnetic resonance imaging (MRI), or magnetic resonance cholangiopancreatography (MRCP). The final diagnosis was made by surgical specimens in addition to resection of the extrahepatic bile ducts with complete porta hepatis and lymphadenectomy. An en bloc resection of the right or left hepatic lobe and caudate lobe was performed in many patients. Biliary-enteric construction was completed with a single hepaticojejunostomy to the bile duct using a Roux-en-Y limb.

Statistical analysis

All data were analyzed with SPSS 15.0 statistical package.

Table 1 Pathologic characteristics and serum bilirubin level in patients

Number of patients	Lymph node metastases	Extension in liver parenchyma	Invasion of nervous tissue	Serum bilirubin (μmol/L)
Curative resection	11	17	20	267 ± 158
Palliative resection	8	13	19	280 ± 161

There were no significant differences between the data of the two groups (Curative resection *vs* palliative resection, $P > 0.05$).

Cumulative overall survival rate was calculated by the Kaplan-Meier method using the log rank test. Intergroup comparisons of hepatic recurrence rate and survival rate were analyzed using the χ^2 test. Significance was accepted with 95% confidence.

RESULTS

Characteristics of patients

A total of 69 consecutive patients underwent surgical resection for malignant hilar bile duct tumors during 1994-2007. The characteristics of the patients entered into this study included a mean age of 58 ± 10.5 years. Of the 69 patients at the time of final diagnosis, 10 were diagnosed as Bismuth stage I, 23 were diagnosed as Bismuth stage II, 35 as Bismuth stage III, and 1 as Bismuth stage IV. All the patients presented with jaundice and almost complete obstruction of the common and left or right hepatic duct. Pathologic characteristics and serum bilirubin levels of the patients are shown in Table 1.

Surgical treatment

We performed 31 local resections including the extrahepatic bile duct, gallbladder, and regional node-bearing tissue. From 2004, a resection usually included the caudate lobe and was performed in 8 cases for Bismuth stage I and II. In patients who did not have prior extensive abdominal surgery, and who demonstrated evidence of direct tumor extension into the right or left lobes of the liver Bismuth stage III, surgery was performed in addition to liver resection in 22 patients, which encompassed an en bloc extended left or right hepatectomy including 15 caudate lobes. In all, 40 patients with negative margins of the bile duct at final pathologic reports had radical resection with complete tumor removal. The other 29 patients had palliative resection. Radical resection rates combined with hepatectomy significantly improved curative rates compared with those without additional hepatectomy ($27/38$ *vs* $13/31$; $\chi^2 = 5.94$, $P < 0.05$). The surgical treatment of the patients is shown in Table 2.

Liver tumor recurrence and survival rate of patients

Two patients with additional liver resection died within 30 d of surgery, and only one patient without liver resection died. Three patients had liver recurrence one year after operation in the 12 patients who had resection

Table 2 Surgical treatment of Bismuth stage

Number of patients	Without hepatectomy	Addition of hepatectomy	
		Caudate lobe	Without caudate lobe
Bismuth I	7	2	1
Bismuth II	11	10	2
Bismuth III	13	15	7
Bismuth IV	0	1	0

Radical resection rates combined with hepatectomy significantly improved curative rates compared with those without additional hepatectomy (27/38 vs 13/31; $\chi^2 = 5.94$, $P < 0.05$).

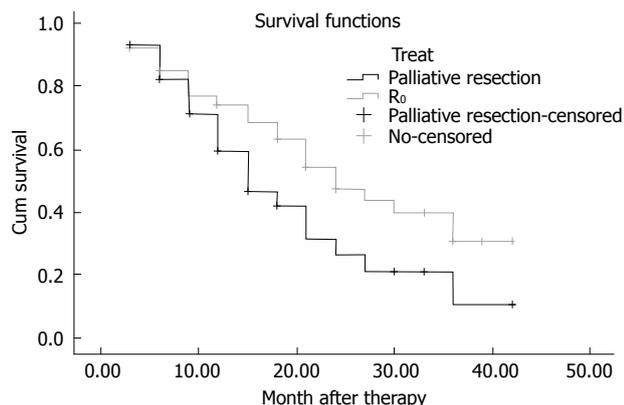


Figure 1 Kaplan-Meier estimate for survival depending on R₀/palliative resection. The three-year survival rate after R₀ resection was 30.7% and was 10.5% for palliative resection, R₀ resection improved the 3-year survival rate (30.7% vs 10.5%; $\chi^2 = 12.47$, $P < 0.01$).

including the caudate lobe for Bismuth stage I and II, otherwise, there were 11 liver recurrences within one year of surgery in 18 patients without resection of the liver lobe for Bismuth stage I and II. Concomitant liver resection was associated with a decreased incidence of initial recurrence in the liver one year after surgery (11/38 vs 23/31; $\chi^2 = 13.98$, $P < 0.01$) compared with those without liver resection. R₀ resection improved 3-year survival rates (30.7% vs 10.5%; $\chi^2 = 12.47$, $P < 0.01$). The Kaplan-Meier estimates for survival depending on R₀/palliative resection are shown in Figure 1.

DISCUSSION

Cholangiocarcinoma at the liver hilum, or Klatskin tumor, is the most common type of bile duct cancer. It is often unresectable owing to regional extension into the liver, surrounding lymphatics, and, most notably, hilar vascular structures^[13]. It can occur against the background of primary sclerosing cholangitis with its associated liver disease, and may elude detection until an advanced stage^[14]. Chemotherapy, as well as radiation or photodynamic therapy, yield negligible response rates, and do not favorably impact either local control or long-term survival. Effective adjuvant agents are still lacking. For unresectable cholangiocarcinoma, chemotherapy-impregnated biliary stents and photodynamic therapy have been used^[15,16].

The only effective treatment for hilar cholangio-

carcinoma is major surgery^[17]. Surgeons have pushed the technical envelope to achieve negative margins. In patients with hilar cholangiocarcinoma, concomitant hepatic resection is associated with improved median disease-specific and disease-free survival, and decreased hepatic recurrence^[18]. Today, extended biliary-hepatic resections together with vascular resections/reconstructions are being performed. Survival rates, however, have still not exceeded 40%^[19]. In our research the 3-year survival rates after R₀ resection were 30.7%, considerably more than the 10.5% achieved after palliative resection. After all, it is not uncommon to find skip lesions above and below the primary cancer. Despite negative frozen sections of margins at the time of index resection, surgeons will occasionally be left to deal with positive margins at final pathologic reports. This is an insidious cancer, prone to submucosal spread, and especially when it is well differentiated it can be difficult to realize^[20]. Frozen section analysis of the proximal bile duct margin is misleading in 9% of patients. Among patients who are determined to have negative duct margins intraoperatively, only 60% will have margins adequately wide enough to be associated with an improvement in disease-specific survival^[21]. Therefore in our research negative margins of bile duct at final pathologic reports had R₀ resection, otherwise positive margins had palliative resection, and then Bismuth stage II became Bismuth stage III for the final diagnosis. The resection must encompass the bile duct and areas of the liver at risk of involvement by direct tumor extension as well as the lymph nodes draining the region. Because cholangiocarcinoma is known to spread along the wall of the bile ducts and because the caudate lobe is a frequent site of tumor recurrence following extrahepatic duct resection, surgeons perform a resection that includes the caudate lobe. However, from 2004 in our research, a resection including the caudate lobe was carried out in 8 cases for Bismuth stage I and II, because of the small number of patients the results were not enough to show that liver recurrence within one year of surgery was related to resection of the liver lobe for Bismuth stage I and II. Major hepatectomy can improve the outcome of hilar cholangiocarcinoma. Compared with nonoperative treatment or R₀ hepatectomy, R₁ resection in patients with no other risk factors can offer long-term survival^[22]. If evidence of direct tumor extension into both the right and left lobes of the liver is demonstrated, no further surgery is performed. In contrast, tumor extension into only the right or the left lobe that can be encompassed by an en bloc extended left or right hepatectomy is not a contraindication to proceed with resection. In cases where the hilar cholangiocarcinoma extends directly into the right or left bile duct, we performed an en bloc extended right or left hepatectomy.

Combining extended right or left hepatectomy is recommended when preoperative imaging suggests extension above the hilum to either side. Furthermore, CT liver volume estimates can guide resection strategy, even to include preoperative selective portal vein embolization to induce remnant hypertrophy. Others describe how

radical vascular resections can be attempted to achieve wide tumor clearance. Resection increases survival, but carries the risk of significant morbidity and mortality^[23-26]. In our research of 69 patients submitted to laparotomy for tumor resection, R₀ resection was performed in 40 patients, and palliative resection in 29. 31 patients had only duct resection, 38 patients had combined duct resection with liver resection including 28 total or part caudate lobes. R₀ resection rates combined with hepatectomy significantly improved the recurrence rate compared with those without additional hepatectomy. Concomitant liver resection was associated with a decreased incidence of initial recurrence in the liver one year after surgery. The 3-year survival rate after R₀ resection was 30.7% and was 10.5% after palliative resection. R₀ resection, thus, improved the 3-year survival rate.

A transhepatic approach may be useful when performing extensive hilar bile duct resection for bile duct stricture of biliary disease at the hepatic hilus, especially in high-risk patients who are unfit for major hepatectomy as well as in those with benign bile duct stricture and low-grade malignancy^[27]. In highly selected patients with advanced hilar cholangiocellular carcinoma, a high hilar resection is technically safe and oncologically justifiable. In combination with our new technique of sheath-to-enteric anastomosis, patients benefit considerably from the preservation of liver parenchyma with low postoperative morbidity and very short in-hospital stay^[28]. Excellent survival rates without any in-hospital deaths have been demonstrated following right trisectionectomy with caudate lobectomy. This procedure may be an effective surgical technique which can be executed to achieve low mortality rate and high pathological curability for hilar cholangiocarcinomas, with the exception of Bismuth type III (b)^[29].

In patients with hilar cholangiocarcinoma, local resection is not an adequate treatment for hilar cholangiocarcinoma involving the bile duct confluence; associated liver resection should be recommended. In Bismuth-Corlette type I and II hilar cholangiocarcinoma, survival benefits with the association of biliary and liver resection have been reported. Concomitant hepatic resection is associated with improved rates of radical resection, decreased hepatic recurrence, and longer survival. Therefore, hepatectomy especially including the caudate lobe combined with bile duct resection should be considered standard treatment to cure hilar cholangiocarcinoma. The surgical approach to Bismuth type I and II hilar cholangiocarcinomas should be determined according to cholangiographic tumor type. For nodular and infiltrating tumors, right hepatectomy is essential; for papillary tumors, bile duct resection with or without limited hepatectomy is adequate^[30].

resection is good and has a high 5-year survival rate. In contrast to reports from 2 or 3 decades ago, today most patients with hilar cholangiocarcinoma are diagnosed preoperatively. The most important factor affecting prognosis is resectability of the tumor; however, the surgical prognosis is poor even when local tumor resection is performed. Local resection seems to have a very narrow role. Thus, finding a surgical approach for the treatment of hilar cholangiocarcinoma is essential.

Research frontiers

In patients with hilar cholangiocarcinoma, local resection is not an adequate treatment for hilar cholangiocarcinoma involving the bile duct confluence; associated liver resection should be recommended. In Bismuth-Corlette type I and II hilar cholangiocarcinoma, survival benefits with the association of biliary and liver resection have been reported. Concomitant hepatic resection is associated with improved rates of radical resection, and decreased hepatic recurrence. Therefore, the surgical approach to hilar cholangiocarcinomas should be determined according to cholangiographic tumor type.

Innovations and breakthroughs

Of the 69 patients submitted to laparotomy for tumor resection, curative resection was performed in 40 patients, and palliative resection in 29. Thirty-one patients had only duct resection, and 38 patients had duct resection combined with liver resection including 34 total or part caudate lobes. Curative rates with the combined hepatectomy significantly improved compared with those without additional hepatectomy. Concomitant liver resection was associated with a decreased incidence of initial recurrence in the liver one year after surgery, and curative resection improved 3-year survival rates.

Applications

The study results suggest that hepatectomy, especially including the caudate lobe combined with bile duct resection should be considered standard treatment to cure hilar cholangiocarcinoma.

Peer review

Concomitant liver resection was associated with improved curative and survival rates and decreased hepatic recurrence. The content of the paper has merit since this cancer has a poor prognosis and is still a challenge for surgeons and clinicians.

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COMMENTS

Background

Hilar cholangiocarcinoma was first reported by Gerald Klatskin in 1965. Many therapeutic methods have been established to treat this type of tumor, surgical

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***Pneumocystis jiroveci* pneumonia and pneumomediastinum in an anti-TNF α naive patient with ulcerative colitis**

James C Lee, Deborah C Bell, Richard M Guinness, Tariq Ahmad

James C Lee, Deborah C Bell, Richard M Guinness, Tariq Ahmad, Department of Gastroenterology, Royal Devon and Exeter Hospital, Barrack Road, Exeter, EX2 5DW, United Kingdom

Author contributions: Lee JC and Bell DC wrote the paper; Guinness RM reviewed and reported the radiological investigations; Lee JC and Ahmad T edited the paper.

Correspondence to: Dr. Tariq Ahmad, Department of Gastroenterology, Royal Devon and Exeter Hospital, Barrack Road, Exeter, EX2 5DW,

United Kingdom. tariq.ahmad@rdeft.nhs.uk

Telephone: +44-1392-406241 Fax: +44-1392-402810

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Peer reviewers: Steven D Wexner, MD, Professor of Surgery, The Cleveland Clinic Foundation Health Sciences Center of the Ohio State University, and Clinical Professor, Department of Surgery, Division of General Surgery, University of South Florida College of Medicine, 21st Century Oncology Chair in Colorectal Surgery, Chairman Department of Colorectal Surgery, Chief of Staff, Cleveland Clinic Florida, 2950 Cleveland Clinic Boulevard, Weston, Florida 33331, United States; Limas Kupcinskas, Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

Lee JC, Bell DC, Guinness RM, Ahmad T. *Pneumocystis jiroveci* pneumonia and pneumomediastinum in an anti-TNF α naive patient with ulcerative colitis. *World J Gastroenterol* 2009; 15(15): 1897-1900 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1897.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1897>

Abstract

We report the case of a 21-year-old man who was noted to have pneumomediastinum during an admission for an acute flare of ulcerative colitis. At that time, he was on maintenance treatment with azathioprine at a dose of 1.25 mg/kg per day, and had not received supplementary steroids for 9 mo. He had never received anti-tumor necrosis factor (TNF) α therapy. Shortly after apparently effective treatment with intravenous steroids and an increased dose of azathioprine, he developed worsening colitic and new respiratory symptoms, and was diagnosed with *Pneumocystis jiroveci* (*carinii*) pneumonia (PCP). Pneumomediastinum is rare in immunocompetent hosts, but is a recognized complication of PCP in human immunodeficiency virus (HIV) patients, although our patient's HIV test was negative. Treatment of PCP with co-trimoxazole resulted in resolution of both respiratory and gastrointestinal symptoms, without the need to increase the steroid dose. There is increasing vigilance for opportunistic infections in patients with inflammatory bowel disease following the advent of anti-TNF α therapy. This case emphasizes the importance of considering the possibility of such infections in all patients with inflammatory bowel disease, irrespective of the immunosuppressants they receive, and highlights the potential of steroid-responsive opportunistic infections to mimic worsening colitic symptoms in patients with ulcerative colitis.

INTRODUCTION

Pneumocystis jiroveci (*carinii*) is a unicellular fungus that is found in the respiratory tracts of many mammals, including humans. The organism was first described in 1909 by Chagas^[1] and later by Delanoë^[2] who ultimately named the organism. Years later, Jirovec's group isolated the organism from humans^[3], and the organism was subsequently renamed after him. *P. jiroveci* (*carinii*) pneumonia (PCP) is the most common opportunistic infection in human immunodeficiency virus (HIV)-infected patients and, by definition, constitutes an acquired immunodeficiency syndrome (AIDS)-defining illness. In our hospital, which serves a population of 450 000, the incidence of PCP is approximately 12 per year. The majority of these are in patients following either solid organ, or bone marrow transplantation. Recently, there has been increased awareness and reporting of PCP and other opportunistic infections in patients with inflammatory bowel disease and other inflammatory conditions. This has been attributed to the increased use of anti-tumor necrosis factor (TNF) α therapy^[4-8], but may reflect the increased surveillance for such infections in this group of patients. We report a case that highlights the importance of considering opportunistic infection in any immunosuppressed

patients with inflammatory bowel disease, who present with unusual symptoms and signs, including those of worsening disease activity.

CASE REPORT

A 21-year-old Caucasian man presented in June 2005 age 18 years with 7 d of bloody diarrhea and abdominal discomfort. He reported a similar, but less severe, self-limiting episode 2 mo earlier. He had never smoked tobacco. On admission he had a temperature of 38°C and a pulse of 90 bpm. Admission laboratory tests included hemoglobin 7.5 g/dL (normal range 13.0-18.0 g/dL), mean corpuscular volume 69.4 fl (normal range 82-98 fl), C-reactive protein (CRP) 188 mg/L (normal range < 3 mg/L), and negative stool cultures. Flexible sigmoidoscopy demonstrated erythematous mucosa with a mucopurulent exudate and confluent ulceration, which continued beyond the extent of the examination. A diagnosis of acute severe ulcerative colitis was made and he was commenced on intravenous and rectal steroids. Histology subsequently confirmed distorted crypt architecture with cryptitis, micro-abscesses and increased inflammatory cells in the lamina propria. He responded well to intensive therapy and by day 3 his stool frequency had fallen to 3/d and CRP to 34 mg/L. He was discharged on day 7 with a reducing course of oral prednisolone and mesalazine (Asacol™ 800 mg *bid*). He was reluctant to use topical therapy. His symptoms relapsed once the prednisolone was reduced below 15 mg/d, and thus azathioprine was introduced in September 2005. He was commenced at 25 mg/d, increasing by 25 mg fortnightly to a maximum dose of 150 mg/d (2 mg/kg). Thiopurine methyl-transferase (TPMT) activity level was 51 nmol 6-MTG/g Hb per hour (normal range 25-55 nmol 6-MTG/g Hb per hour). He had one minor flare of his disease, but subsequently entered clinical remission using mesalazine 800 mg *bid* and azathioprine 150 mg/d. Twelve months later, a routine blood test demonstrated a low total white blood cell count of $2.2 \times 10^9/L$ (normal range $3.8-10.6 \times 10^9/L$), with a neutrophil count of $1.34 \times 10^9/L$ (normal range $1.8-6.5 \times 10^9/L$) and a lymphocyte count of $0.4 \times 10^9/L$ (normal range $1.1-3.5 \times 10^9/L$). These results were confirmed on a repeat sample. He was advised to reduce azathioprine dose to 100 mg/d, and these cell counts recovered.

Our patient then travelled to Australia to participate in a cricket tour and for the next 3 mo he remained well and his blood monitoring was satisfactory. However, in February 2008, whilst on tour, he had an acute flare of his colitis, which required hospital admission. Admission blood tests demonstrated CRP 121 mg/L, hemoglobin $9.0 \times 10^9/L$ and total white blood cell count $4.2 \times 10^9/L$. He underwent flexible sigmoidoscopy, which showed a large anal fissure and severe colitis, with extensive mucosal loss to the splenic flexure. A chest X-ray on admission demonstrated pneumomediastinum, but no cause was identified following a barium swallow and subsequent computed tomography (CT) scan of

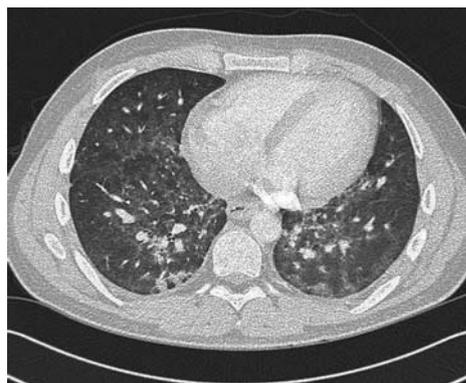


Figure 1 CT scan demonstrating parenchymal abnormalities caused by PCP.

his thorax, abdomen and pelvis. He was treated with intravenous and rectal hydrocortisone and azathioprine was increased to 150 mg/d. His symptoms resolved and he was discharged, returning to the UK on a reducing course of steroids and azathioprine.

On arrival back in the UK, his colitis rapidly relapsed and he was re-admitted to hospital. On admission, he was passing six bloody liquid stools per day. Blood tests demonstrated hemoglobin 9.9 g/dL, total white blood cell count $2.0 \times 10^9/L$, neutrophil count $1.60 \times 10^9/L$, lymphocyte count $0.22 \times 10^9/L$ and CRP 61 mg/L. An abdominal X-ray did not show evidence of colonic dilatation. He was recommenced on intravenous hydrocortisone and azathioprine was stopped in view of the leukopenia. After 48 h, his stool frequency and urgency had much improved, his CRP fell to 11 mg/L and total white cell count increased to $2.8 \times 10^9/L$ (lymphocyte count $0.39 \times 10^9/L$, neutrophil count $2.2 \times 10^9/L$). However, on converting to oral steroids on day 3, CRP rose to 146 mg/L and he became febrile. He was noted to have a dry cough, and reported breathlessness on exertion, which on direct questioning he admitted had been present immediately prior to leaving Australia. No abnormal chest signs were elicited, but his oxygen saturation was noted to be 95% at rest (on room air), falling to 90% on exertion. A chest X-ray was normal, but he was clearly at risk of opportunistic infection because of his recent leukopenia and long history of immunosuppression, and was also at risk of thromboembolic disease in view of his recent long-haul flight and active colitis. A CT pulmonary angiogram with full lung views did not show pulmonary emboli, but demonstrated bilateral, small-volume hilar lymphadenopathy (8 mm) and widespread patchy ground glass changes in both lungs, with more confluent consolidation within the basal segments (Figure 1). Bronchoalveolar lavage washings were positive for *P. jirovecii* (*carinii*) on immunofluorescence. An HIV test was negative. The patient was commenced on a 2-wk course of intravenous co-trimoxazole (2.4 g *qds*) and was maintained on a reducing course of oral steroids (prednisolone 40 mg/d) and high dose oral mesalazine (Asacol™ 2.4 g *bid*). His respiratory and gastrointestinal symptoms improved and his inflammatory markers and white cell count normalized within 5 d. He was discharged

at 14 d with a further week of oral co-trimoxazole. Three days later he was re-admitted with a severe blanching rash on his trunk and limbs and worsening diarrhea. A drug reaction to co-trimoxazole was suspected and he was switched to oral clindamycin (600 mg *tds*) and primaquine (30 mg/d), with slow resolution of the rash and improvement in his stool frequency. Blood tests for cytomegalovirus serology and polymerase chain reaction were negative. He was discharged and remains under close follow up. He had no recurrence of his respiratory symptoms and his colitis remains in clinical remission on high dose oral and rectal mesalazine.

DISCUSSION

We report the case of a 21-year-old Caucasian man, naïve to anti-TNF α therapy, who presented with PCP following treatment with azathioprine and steroids for ulcerative colitis. Azathioprine is a commonly used immunosuppressive agent. It acts by inhibiting purine synthesis by disrupting normal purine incorporation into ribonucleic acids. It is a pro-drug, which is converted in the body to the active metabolites 6-mercaptopurine and 6-thioinosinic acid. Its recognized side effects include hepatitis, pancreatitis and bone marrow suppression. The incidence of these side effects relates in part to the activity of a genetically moderated enzyme involved in the metabolism of thiopurine compounds including 6-mercaptopurine. This enzyme is thiopurine methyltransferase (TPMT). TPMT activity assays are thus used to identify patients with deficient and low activity (approximate 8.5% population), who are at increased risk of bone marrow toxicity from thiopurine drugs, enabling dose adjustments or avoidance. In 2007, in the Royal Devon and Exeter Hospital, 104 patients with inflammatory bowel disease were commenced on azathioprine. In our department, this is the first case of PCP in a patient with inflammatory bowel disease who was naïve of anti-TNF α therapy. In this case, the patient's TPMT was normal (51 nmoL 6-MTG/g Hb per hour). From relatively early in the disease course, he was noted to have steroid-dependent disease, and 9 mo following diagnosis, was commenced on 2 mg/kg azathioprine. This dose was tolerated well initially with no evidence of agranulocytosis, pancreatitis or abnormal liver function on regular blood tests. However, after 1 year of receiving this dose, a routine blood test showed leukopenia, which was confirmed on a repeat sample. The dose was, therefore, reduced to 100 mg/d and the leukopenia resolved. Three months later, he had a further acute flare of his ulcerative colitis whilst in Australia and his azathioprine dose was increased to 150 mg/d (2 mg/kg per day). On returning to the UK, he was subsequently admitted because of symptom recurrence and was noted to be leukopenic, at which point his azathioprine was stopped. In total, this patient received azathioprine for 2 years and 6 mo, with a maximum dose of 2 mg/kg. He became leukopenic on two occasions, the second corresponding with the onset of his respiratory symptoms.

Steroids have been the cornerstone of the treatment

of acute ulcerative colitis since Truelove and Witts first reported their pioneering trial results in 1955^[9]. Steroid use has, however, been associated with PCP in HIV-negative patients^[10]. Since being diagnosed with ulcerative colitis, this patient received two courses of oral steroids, which were prolonged (12 mo and 8 mo) because of symptom recurrence on weaning the dose. However, he had not received any steroids for 9 mo prior to his admission in Australia. During this admission, he received 5 d of intravenous steroids and he received a further 2 d on returning to the UK. These were the first courses of intravenous steroids that he had received since the initial course that was used at diagnosis. Interestingly, adjunctive steroid use in the treatment of PCP has been shown to reduce the incidence of death and respiratory failure associated with severe infection^[11,12]. This patient developed respiratory symptoms immediately prior to his return to the UK and we hypothesize that his initial improvement with high dose intravenous steroids and subsequent deterioration on lower dose oral steroids, was to the result of partial treatment of PCP by steroids. It is also noteworthy that his colitis symptoms and elevated inflammatory markers improved significantly once his PCP was treated with intravenous co-trimoxazole, without any need to increase his steroid dose. We, therefore, conclude that both the symptoms and blood test abnormalities were likely to have been caused by PCP, rather than active colitis *per se*. This case demonstrates the potential of steroid-responsive opportunistic infections to mimic worsening colitis symptoms in patients with ulcerative colitis. It is, therefore, important to consider opportunistic infection in immunosuppressed patients with inflammatory bowel disease, whose inflammatory markers appear disproportionate to the severity of the disease activity.

It is difficult to be sure exactly when this patient contracted PCP. It is noteworthy that pneumomediastinum was recognised on a chest X-ray shortly after he was admitted in Australia, before the introduction of intravenous steroids. Pneumomediastinum is the presence of extra-alveolar air in the mediastinum, which is thought to arise from free air leaking from ruptured alveoli. Historically, the incidence of spontaneous pneumomediastinum has been reported to be 1 in 32896^[13]. However, in HIV infection, spontaneous pneumomediastinum has been well described in association with PCP^[14-17]. Indeed, in one series, the incidence was reported to be as high as 9.5% of cases^[17]. If our patient had contracted PCP prior to his admission in Australia, it is likely to have occurred at a time when he was taking 100 mg/d azathioprine without supplementary steroids.

The advent of anti-TNF α therapy has resulted in improved disease control in many patients with inflammatory bowel disease. Unfortunately, it has also resulted in an increase in the reported number of patients with inflammatory bowel disease who present with opportunistic infections^[4-6]. This has resulted in increased vigilance for such infections in this group of patients. In the case described, the patient is likely to

have developed PCP either as a result of treatment with 100 mg azathioprine (1.25 mg/kg per day) or following a 5-d course of intravenous steroids and 2 mg/kg azathioprine. The presence of pneumomediastinum, a recognised complication of PCP, before he was exposed to intravenous steroids would appear to make the former possibility more likely. He had not received steroids for 9 mo prior to this admission, and at no stage did he receive rescue therapy. During the 6 mo that preceded this admission, he was maintained on a stable dose of azathioprine (100 mg/d) and was not leukopenic. He never received anti-TNF α therapy. With the trend towards increasing use of early immunomodulation, this degree of immunosuppression would not, we believe, be considered excessive nor particularly likely to cause an otherwise fit 21-year-old man to develop PCP. However, this case highlights the importance of considering such diagnoses in any patients with inflammatory bowel disease who are immunosuppressed, irrespective of which agents they may have received.

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A case of primary isolated non-Hodgkin's lymphoma of the esophagus in an immunocompetent patient

Ioannis V Kalogeropoulos, Athanasios N Chalazonitis, Sofia Tsolaki, Fotios Laspas, Nikolaos Ptohis, Ioannis Neofytou, Dimitra Rontogianni

Ioannis V Kalogeropoulos, Sofia Tsolaki, Dimitra Rontogianni, Department of Radiology "Evangelismos" General Hospital, Hypsilantou 45-47, 10676, Athens, Greece

Athanasios N Chalazonitis, Fotios Laspas, Nikolaos Ptohis, Ioannis Neofytou, Department of Radiology "Hippocratio" General Hospital, Thisseos 29, 15234, Halandri, Athens, Greece

Author contributions: Kalogeropoulos IV and Tsolaki S wrote the manuscript; Chalazonitis AN organized and revised the manuscript; Laspas F assisted in writing the manuscript and is the corresponding author; Ptohis N and Neofytou I carried out the literature analysis and assisted in writing the manuscript; Rontogianni D provided data and figures relating to the patient.

Correspondence to: Fotios Laspas, MD, MSc, Department of Radiology, "Hippocratio" General Hospital, Thisseos 29, 15234, Halandri, Athens, Greece. fotisdimi@yahoo.gr

Telephone: +30-21-06011821 Fax: +30-21-06857071

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INTRODUCTION

Although lymphomas are malignant neoplasms which are usually confined to the lymph nodes, one fifth of lymphomas present with extranodal localization^[1]. The esophagus is an uncommon localization accounting for less than 1% of patients with lymphoma and is usually seen secondary to mediastinal nodes or gastric lymphoma^[2,3]. Isolated primary lymphoma of the esophagus is exceptionally rare, and when seen, is usually the non-Hodgkin's type^[4].

The imaging findings of esophageal lymphoma have shown a diverse spectrum of abnormalities, similar to those of lymphoma elsewhere in the gastrointestinal tract. We present a distinctly rare case of primary diffuse non-Hodgkin's lymphoma of the esophagus with a unique, to our knowledge, endoscopic ultrasonographic appearance, of a mainly hyperechoic mass.

CASE REPORT

A 77-year old man was admitted because of paroxysmal atrial fibrillation and epigastric pain. His physical examination was normal without clinical evidence of lymph node enlargement or hepatomegaly. Complete blood cell count and routine serum chemistry levels were also normal. A double-contrast barium swallow showed enlargement of the mucosal folds and mild dilatation of the esophageal lumen below the level of the aortic arch (Figure 1); the stomach and duodenum were normal. Chest CT showed extensive thickening of the esophagus wall extending from the middle to the lower portion, without enlargement of mediastinal lymph nodes (Figure 2). CT of the abdomen and pelvis were negative.

Endoscopic ultrasonography (EUS) was performed in order to evaluate the esophageal wall thickening. EUS showed a transmural wall thickening along the middle and lower portion of the esophagus with a heterogeneous, mainly hyperechoic mass confined to the sub-mucosal layer, without erosions or ulcers. There was

Abstract

Primary non-Hodgkin's lymphoma of the esophagus is a rare disease. A case of primary isolated non-Hodgkin's lymphoma of the esophagus in a 77-year-old man without acquired immunodeficiency syndrome is presented. We describe the clinical features and the imaging findings (barium swallow, endoscopic ultrasonography and CT) of a biopsy proven B-cell lymphoma with diffuse transmural involvement of the esophagus wall, which was discovered incidentally. We also briefly review the literature.

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Key words: Endoscopic ultrasonography; Esophagus; Lymphoma; Non-Hodgkin's lymphoma

Peer reviewers: George Triadafilopoulos, Professor, Clinical Professor of Medicine, Division of Gastroenterology and Hepatology, Alway Building, Room M 211, 300 Pasteur Drive, MC: 5187, Stanford, CA 94305-5187, United States; Jia-Yu Xu, Professor, Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, 197 Rui Jin Er Road, Shanghai 200025, China

Kalogeropoulos IV, Chalazonitis AN, Tsolaki S, Laspas F, Ptohis N, Neofytou I, Rontogianni D. A case of primary isolated non-Hodgkin's lymphoma of the esophagus in an



Figure 1 Double-contrast barium swallow, showing enlargement of the mucosal folds and mild dilation of the esophageal lumen below the level of the aortic arch.



Figure 2 CT of the chest shows marked thickening of the wall of the esophagus from the aortic arch to the gastrointestinal junction. There was no enlargement of the mediastinal or hilar lymph nodes.

no enlargement of adjacent lymph nodes (Figure 3).

Endoscopy showed excessive rigid folds extending 20-30 cm from the incisors, along the length of the middle and lower esophagus. The stomach and duodenum were normal. Biopsy of the lesion was performed. Histologic examination revealed the presence of a B-cell non-Hodgkin's lymphoma (Figure 4A-C).

Bone marrow biopsy specimens showed no evidence of lymphoma; hence, the patient was diagnosed as isolated B-cell lymphoma with diffuse primary involvement of the esophagus.

DISCUSSION

The gastrointestinal tract is the most common extranodal site of non-Hodgkin's lymphoma accounting for 5%-20% of all cases^[1,5,6]. Non-Hodgkin's lymphoma of the esophagus is an extremely uncommon localization of non-Hodgkin's disease, accounting for less than 1% of patients with lymphoma and occurs more often in the distal esophagus^[1,4,7]. In a review of the literature, Okerbloom *et al*^[8] found only 4 primary lymphomas of the esophagus in a large series of 1235 cases of non-Hodgkin's lymphoma, representing an incidence of 0.3%. Freeman *et al*^[11], in another large series found only 3 esophageal lymphomas. Taal *et al*^[9] found 37 cases of esophageal non-Hodgkin's lymphoma and only two were primary lymphomas. Isolated primary lymphoma of the esophagus without an extra-esophageal location is very rare and only about 20 cases have been described in the

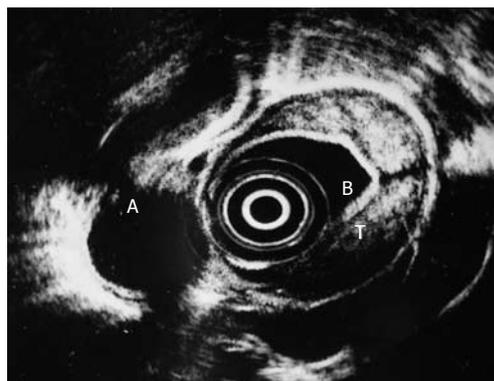


Figure 3 Endoscopic ultrasonography shows a transmural thickening of the esophageal wall and a heterogeneous, mainly hyperechoic, submucosal lesion. A: Thoracic aorta, B: Water-filled balloon, T: Tumor.

literature^[10,11].

Although lymphoma of the esophagus is often asymptomatic, most patients present with dysphagia. Other less common clinical manifestations of esophageal lymphoma are odynophagia, fever and weight loss^[12-15]. Although this disease is extremely rare, it should be considered in the differential diagnosis of patients with acquired immunodeficiency syndrome presenting with dysphagia and weight loss. In our case, the patient complained of epigastric pain. This symptom is nonspecific and should be differentiated from other esophageal or gastric diseases. Our patient had no fever, dysphagia or odynophagia and he was not immunocompromised. Furthermore, our patient fulfilled all Dawson's criteria to identify primary gastrointestinal lymphoma, such as, no palpable superficial lymphadenopathy, no enlargement of mediastinal or hilar lymph nodes and no splenic involvement^[16].

Previously described cases have reported a variety of radiographic appearances for primary esophageal lymphoma. The most common include polypoid masses with or without erosions, stricture with ulceration mimicking esophageal cancer, thickening of the mucosal folds producing a varicoid appearance, narrowed distal segments (achalasia-like appearance), or submucosal nodules^[15,12,17,18]. Given such a diverse spectrum of radiographic appearance, EUS and CT must be performed and biopsy of the esophageal wall are needed in order to confirm the diagnosis.

In our case double-contrast esophagography showed diffuse mucosal fold thickening, without luminal narrowing, suggesting an intramural, benign lesion. CT revealed the precise extent of the lesion, involving the middle and lower third of the esophagus and the absence of mediastinal lymph nodes. With the clinical application of EUS, great progress has been made in the diagnostic accuracy of structural abnormalities and depth of invasion in various gastrointestinal diseases, including lymphoma^[19]. EUS provides more accurate information on the involvement of the wall layers by such an intrinsic process. In our case, all layers were thickened, but not disrupted. Furthermore, a submucosal-located heterogeneous, mainly hyperechoic mass was imaged,

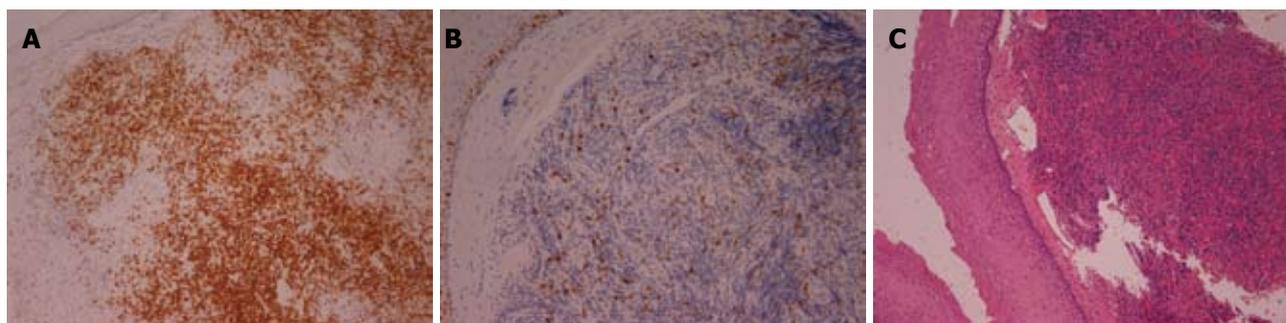


Figure 4 Histologic examination shows the presence of a B-cell non-Hodgkin's lymphoma (A) infiltration of the esophageal mucosa by lymphoplasmotoid cells; (B) expression of CD20 antigen by lymphoid cells; (C) Ki-67 antigen (index of proliferation) in lymphoma cells.

which had gradually elevated margins.

In a previous study esophageal lymphoma was described to involve the whole length of the esophagus^[20]. However, in this study, EUS showed lymphomatous involvement of the gastrointestinal wall to produce a typical hypo-echogenic transmural thickening. To our knowledge, our case of primary diffuse esophageal lymphoma is the first described in the literature to have a mainly hyperechoic appearance on EUS.

Given the non-specific clinical and radiological appearances, endoscopic biopsy of the lesion was necessary. Endoscopy showed extensive rigid folds without erosions and biopsy was performed. Pathologic evaluation of the endoscopic biopsy showed a B-cell non-Hodgkin's lymphoma. The treatment of esophageal lymphoma depends on the histological tumor type and its initial location. Because of the extent of the lesion, our patient was treated with chemotherapy and showed clinical improvement. The chemotherapy regimen was a combination of chemotherapeutic agents. The cycle was repeated every 28 d. The patient received six cycles and achieved complete remission (CT scan of thorax and barium swallow did not reveal any residual lesion).

In summary, esophageal lymphomatous involvement should be considered in the differential diagnosis of immunocompetent patients presenting with epigastric pain. A transmural, mainly hyperechoic, diffuse thickening of the esophageal wall in the EUS is another possible appearance of gastrointestinal lymphoma.

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CASE REPORT

Variceal bleeding from ileum identified and treated by single balloon enteroscopy

Mario Traina, Iliaria Tarantino, Luca Barresi, Filippo Mocchiari

Mario Traina, Iliaria Tarantino, Luca Barresi, Gastroenterology Department, ISMETT/UPMC, Palermo 90100, Italy
Filippo Mocchiari, Department of Internal Medicine, O.V.Cervello, Palermo 90100, Italy

Author contributions: Mocchiari F and Tarantino I wrote the paper; Traina M and Barresi L approved the final document.

Correspondence to: Iliaria Tarantino, Professor, Istituto Mediterraneo Trapianti e Terapie ad Alta Specializzazione (ISMETT), via tricoli 1, 90129, Italy. itarantino@ismett.edu

Telephone: +39-91-2192111 Fax: +39-91-2192192-400

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of varices are the distal esophagus, stomach, and rectum, although varices may develop at any level of the gastrointestinal tract below the esophagus. In this infrequent site of varices, development of acute bleeding may be profuse, but very hard to detect and treat. Endoscopic diagnosis is impossible to achieve with a standard endoscope, and mesenteric angiography often gives a negative result. Recently, a novel endoscopic technique involving a single balloon assisted enteroscopy has emerged. In this report, we showed that the injection of a sclerosant solution in bleeding varices of the deep small intestine can be accomplished using a freehand technique *via* the single-balloon enteroscopy.

Abstract

We report a case of acute uncontrolled gastrointestinal bleeding in a patient with liver cirrhosis. The upper and lower endoscopy were negative for bleeding lesions. We decided to perform the examination of the small bowel using single-balloon enteroscopy. The lower enteroscopy revealed signs of bleeding from varices of the ileum. In this report, we showed that the injection of a sclerosant solution can be accomplished using a freehand technique *via* the single balloon enteroscopy.

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Key words: Ectopic varices; Ileal bleeding; Enteroscopy; Portal hypertension; Glue injection

Peer reviewer: Juan G Abraldes, MD, Hepatic Hemodynamic Laboratory, Liver Unit, Hospital Clinic, University of Barcelona, Villarroel 170, Barcelona 08036, Spain

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INTRODUCTION

Variceal bleeding occurs in 25 to 40 percent of patients with cirrhosis and each episode of active variceal bleeding is associated with a high percentage of mortality (up to 30%). The most common sites for development

CASE REPORT

A 58-year-old woman with HBV-related cirrhosis was transferred to our unit for acute uncontrolled gastrointestinal bleeding. The woman was on beta-blocker therapy for primary prophylaxis against variceal bleeding because of F3-grade oesophageal varices. The upper and lower endoscopy performed upon admission were negative for bleeding lesions. We, therefore, decided to complete the examination of the small bowel using single-balloon enteroscopy. Upper enteroscopy was negative until approximately two meters below the Treitz ligament. The lower enteroscopy, about 1.5 meters from the ileo-cecal junction, revealed signs of recent bleeding from varices of the ileum (Figure 1). On this evidence, sclerotherapy with cyanoacrylate was performed (Figure 2). Bleeding stopped and the patient underwent placement of a trans-jugular intrahepatic portosystemic stent shunt (TIPS).

DISCUSSION

A major cause of cirrhosis-related morbidity and mortality is the development of variceal bleeding due to portal hypertension. Variceal bleeding occurs in 25 to 40 percent of patients with cirrhosis^[1] and each episode of active variceal bleeding is associated with a high percentage of mortality (up to 30%)^[2,3]. The most common sites for development of varices are the distal oesophagus, stomach, and rectum, although varices may develop at any level of the gastrointestinal tract below the oesophagus. In this infrequent site of varices, development of acute bleeding may be profuse, but



Figure 1 Varices in the ileum with signs of recent bleeding.



Figure 2 Varices after cyanoacrylate injection.

very hard to detect and treat. Endoscopic diagnosis is impossible to achieve with a standard endoscope, and mesenteric angiography often gives a negative results. Consequently TIPS placement^[4,5] or major surgery^[6] are the treatment of choice to resolve the bleeding. Reports showed that ectopic varices can re-bleed despite a reduction of the porto-systemic pressure gradient to ≤ 12 mmHg or by 25%-50% of the baseline^[7]; otherwise careful patient selection is vital to a successful outcome, as patients with severe liver dysfunction tend to die post-TIPS despite a functioning shunt. All patients who require TIPS for treatment of complications of cirrhosis should be referred for consideration of liver transplant.

Recently, a novel endoscopic technique involving a single balloon assisted enteroscopy has emerged^[8]. This enteroscopy system consists of a high-resolution endoscope with a latex-free balloon attached at the tip of the silicon over the tube. The balloon is inflated and deflated with air from a pressure controlled pump system. The scope is threaded into the small bowel with

push-pull movements under fluoroscopy control. This is not easy to perform, but can safely examine the deep small intestine with the possibility of both diagnostic and therapeutic approaches during bleeding from varices developed in the small bowel^[9]. Endoscopic therapy is currently the treatment of choice for active variceal bleeding (sclerotherapy and variceal band ligation), but with a standard endoscope only upper and lower variceal bleeding can be treated^[10]. In this report, we showed that the injection of a sclerosant solution in bleeding varices of the deep small intestine can be accomplished using a freehand technique *via* the single-balloon enteroscopy. This novel technique may be used alone or with other treatments of active variceal bleeding, such as TIPS placement. Unfortunately, the difficulty and length of the procedure restricts the use of enteroscopy to referral centers with expert endoscopists.

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CASE REPORT

Giant hepatobiliary cystadenoma in a male with obvious convex papillate

Zhao-Wei Qu, Qiang He, Ren Lang, Fei Pan, Zhong-Kui Jin, Qin-Song Sheng, Dong Zhang, Xiao-Sheng Zhang, Da-Zhi Chen

Zhao-Wei Qu, Qiang He, Ren Lang, Fei Pan, Zhong-Kui Jin, Qin-Song Sheng, Dong Zhang, Xiao-Sheng Zhang, Da-Zhi Chen, Department of Hepatobiliary and Pancreatosplenic Surgery, Beijing Chaoyang Hospital Affiliated to Capital Medical University, Beijing 100020, China

Author contributions: Chen DZ and Qu ZW contributed equally to this work; Chen DZ, He Q, Lang R, and Pan F performed the operation; Jin ZK, Sheng QS, Zhang D and Zhang XS assisted in the reference research; Qu ZW wrote the paper.

Correspondence to: Da-Zhi Chen, Department of Hepatobiliary and Pancreatosplenic Surgery, Beijing Chaoyang Hospital Affiliated to Capital Medical University, Beijing 100020, China. chen-dazhi@hotmail.com

Telephone: +86-10-85231503 Fax: +86-10-85231503

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INTRODUCTION

Hepatobiliary cystadenoma is an uncommon lesion, which is mainly seen in females. No more than 10 male cases were reported in the world literature prior to 2005^[1]. It is difficult to make an accurate diagnosis of hepatobiliary cystadenoma before surgery. We recently treated a male with a preoperative diagnosis of hepatobiliary cystadenoma. We, in this paper, report this case to elucidate its clinical presentation, preoperative evaluation and treatment. To the best of our knowledge, such a case with obvious convex papillate has very rarely been reported in the literature.

CASE REPORT

A 35-year-old man was admitted because of a multilocular cyst in his liver detected by computerized tomography (CT) during the course of health examination 2 wk ago. His past medical history was unremarkable. Physical examination was negative at admission. Laboratory tests were within normal limits, serology for hepatitis B infection was negative, and serum carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9 and alpha-fetoprotein (AFP) levels were normal. CT showed an area measuring 11 cm × 9 cm in which multiple density areas were grouped together. The internal septations and convex papillate were visible, and enhanced after intravenous administration of contrast medium (Figure 1).

Abdominal magnetic resonance imaging (MRI) revealed a large cystic tumor measuring approximately 10 cm in diameter originating from the left liver lobe. On T1-weighted images (T1WI), low signal intensity was apparent within the cystic spaces. On corresponding T2-weighted images (T2WI), the tumor was characterized by a medium-high intensity signal clearly delineated from the surrounding liver tissue with internal septal

Abstract

Hepatobiliary cystadenoma is an uncommon lesion that is most often found in middle-aged women and difficult to diagnose preoperatively. Here, we report a case of giant hepatobiliary cystadenoma in a male patient with obvious convex papillate. On the basis of imaging examinations, the patient was diagnosed as hepatobiliary cystadenoma prior to operation. Left hepatectomy was performed and the patient was symptom-free during a 6-mo follow-up period, suggesting that imaging examination is the major diagnostic method of hepatobiliary cystadenoma, and operation is its best treatment modality.

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Key words: Hepatobiliary cystadenoma; Liver neoplasm; Biliary ductal tumour; Diagnosis; Treatment

Peer reviewers: Hiroaki Nagano, MD, PhD, Associate Professor, Division of Hepato-biliary-pancreatic Surgery and Transplantation, Department of Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita 565-0871 Osaka, Japan; Taku Aoki, MD, Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

Qu ZW, He Q, Lang R, Pan F, Jin ZK, Sheng QS, Zhang D,

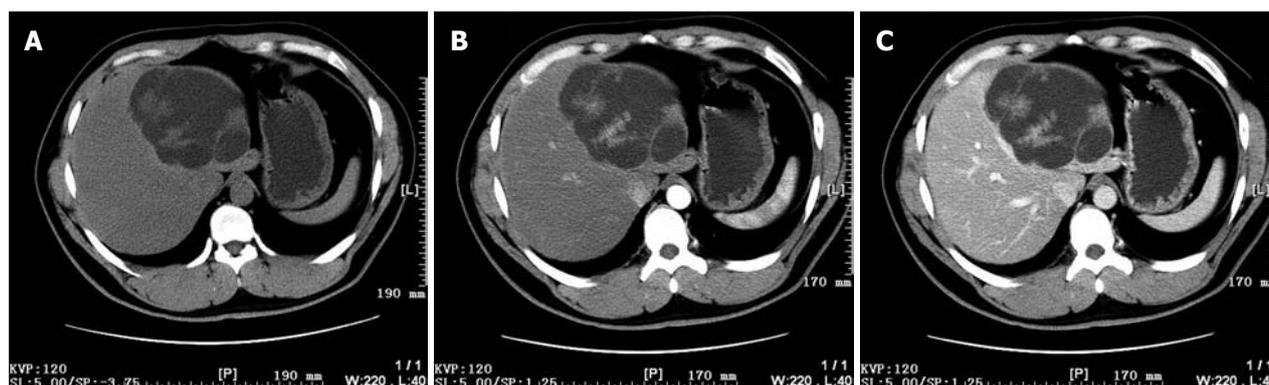


Figure 1 Transverse CT scan (A) showing a combined multiple low-density area and contrast CT showing enhanced septum (B) and convex papillate (C) of the tumor.

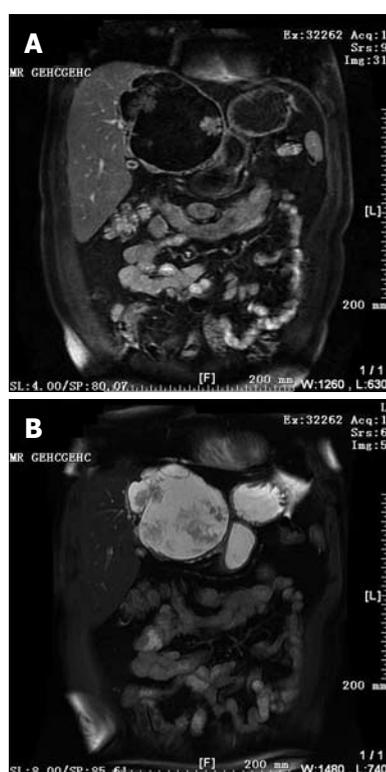


Figure 2 Magnetic resonance imaging showing a low intensity area on T1WI (A), and a medium-high intensity signal area on T2WI (B) as a multilocular cystic lesion with convex papillate.

structures separating the fluid-filled spaces (Figure 2). On the basis of these findings, the patient was diagnosed with a hepatobiliary cystadenoma.

Left hepatectomy was performed (Figure 3A). On gross examination, the resected specimen showed a multilocular cystic lesion with a solid part measuring 12 cm × 9 cm × 9 cm (Figure 3B). The cyst contained mucinous fluid with no connection to the bile duct. CT and MRI showed papillary projections inside the cyst.

Histological examination revealed the cystic wall, most of which was lined with a single layer of columnar and cuboidal cells. The cells were not pleomorphic (Figure 4A). The epithelium showed strong and diffuse cytoplasmic staining with antibodies to CK7, CK8, CK18, and CK19 (Figure 4B), but was negative for AFP,

CEA and CK20. Ki-67 labeling index was lower than 5%. A final diagnosis of hepatobiliary cystadenoma was established.

The patient was discharged on day 9 after surgery. No evidence of recurrence was found during the 6-mo follow-up period.

DISCUSSION

Hepatobiliary cystadenoma is rare neoplasm that arises in the liver, or less frequently in the extrahepatic biliary system, accounting for less than 5% of all cystic neoplasms found in the liver^[2]. However, it is believed to be premalignant. Because its rarity and cystic aspect are similar to other cystic liver lesions^[3], diagnosis is often delayed and may result in inaccurate treatment modalities, such as aspiration, thus further causing unnecessary morbidity and mortality^[4]. Sometimes, a two-stage operation is needed, due to misdiagnosis of the disease^[5].

The lesion is mainly seen in females and more than 80% of the reported patients are over 30 years of age^[2]. Our patient was a male. No more than 10 male cases were reported in the world literature prior to 2005^[1]. This lesion is usually located in the right lobe of liver^[6]. However, all the cases reported by Lewin were found in the left lobe of liver^[7]. In our patient, the lesion was also located in the left lobe of liver.

Clinical manifestations of the tumor are non-specific. The chief complaints are usually an abdominal mass, abdominal pain and distension. When the tumor compresses the porta hepatis or the extrahepatic bile duct, obstructive jaundice may occur^[8]. Nevertheless, some patients are asymptomatic and discovered incidentally at imaging examination, like ours. Thus, clinical manifestations are considered less reliable in the diagnosis of hepatobiliary cystadenoma.

Laboratory results are normal in most patients with hepatobiliary cystadenoma, though serum liver enzyme levels may be mildly elevated occasionally. Serum alpha-fetoprotein and CEA levels are usually within the normal range. It was recently reported that CA 19-9 may be elevated in the cystic fluid and contributes to the diagnosis of hepatobiliary cystadenoma before operation; but, carcinomatosis may occur following cyst

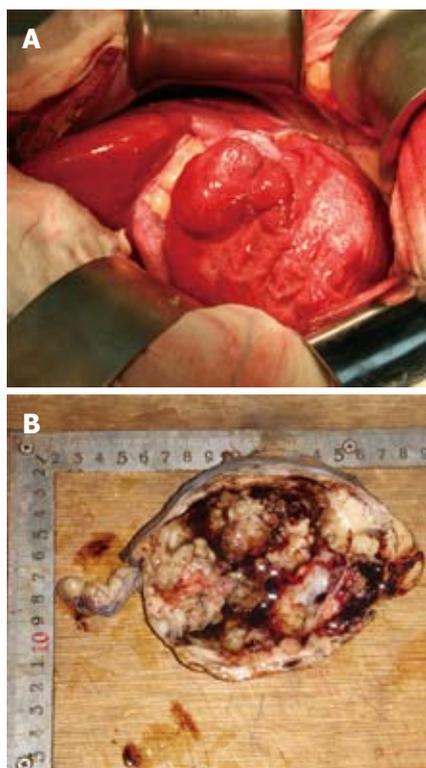


Figure 3 A large cystic hepatobiliary cystadenoma in the left hepatic lobe (A) and a multilocular hepatobiliary cystadenoma with papillary projections (B) observed during and after operation.

aspiration or biopsy^[9]. It has been shown that serum CA 19-9 level can be used as a parameter of tumor activity during follow-up after resection^[10]. In another high CA 19-9 level may be related to focal dysplasia of epithelial cells^[11]. For this reason, the presence of elevated CA 19-9 level cannot be considered a significant parameter for the discrimination between malignant and benign hepatic tumors.

Imaging examination is the key to the diagnosis of hepatobiliary papillary cystadenoma. Preoperative imaging examination, particularly CT and MRI, plays an important role in recognizing and characterizing the disease. Patients are often incidentally diagnosed by imaging examination. The typical CT features of cystadenoma are usually a well-defined mass with low-density and internal septa. Its fibrous capsule and internal septations are often visible and help distinguish the lesion from a simple cyst. The convex papillate can be seen on the septation, although it is more common in cystadenocarcinoma. Bile duct dilatation commonly results from extrinsic compression. Calcifications along the wall and internal septum are uncommon. Unilocular lesions have been reported^[12,13], and are often incorrectly diagnosed as a simple cyst, thus resulting in inadequate therapy^[2]. Contrast CT demonstrates enhanced internal septations and mural nodules. MRI may improve tissue characterization because of its high contrast resolution. Signal intensity may vary depending on the property of cyst fluid^[13,14]. On T1WI, the signal intensity may increase with protein concentration. The signal intensity of serous fluid and bile is low. In rare cases, the intensity of serous cystic

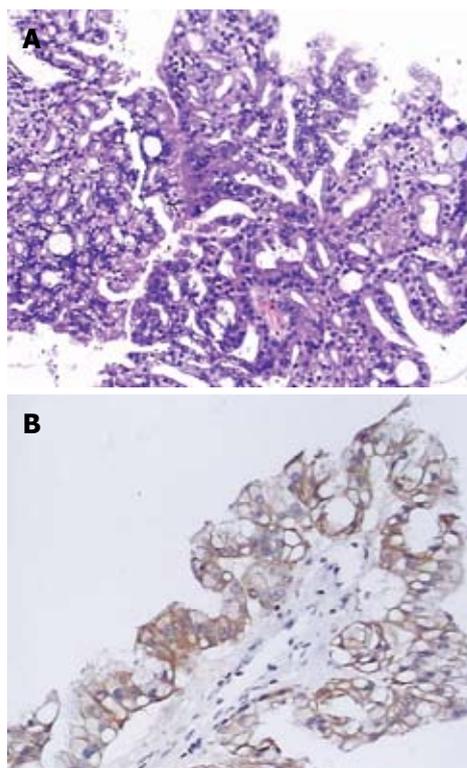


Figure 4 Locules were lined by simple columnar-to-cuboidal epithelium with no nuclear atypia (HE, $\times 100$) (A), and CK7 strongly and diffusely stained cystadenomas (immunostaining, $\times 400$) (B). Similar results were also observed with CK8, CK18 and CK19 staining.

content can be raised by intracystic hemorrhage and fluid-fluid level can be present^[3]. On T2WI, septations with low signal intensity are better visualized in contrast to the high signal intensity of cystic fluid. Kubo *et al*^[15] reported that monitoring changes in radiological appearance of the tumor may be useful for the differential diagnosis of cystadenoma and cystadenocarcinoma. Endoscopic retrograde cholangiopancreatography (ERCP), even if rarely employed, may show a cystic cavity communicating with the biliary tree^[4].

According to their histology, cystadenoma is classified into two subgroups: cystadenoma with and without mesenchymal stroma. Hepatobiliary cystadenoma without mesenchymal stroma can cause malignant alterations, to which our case belongs. The prognosis of patients, especially male patients with hepatobiliary cystadenoma is poor^[16]. Because of its malignant potential, natural history of progressive enlargement and recurrence after partial excision^[17], total excision of the cyst with a wide margin (> 2 cm) of normal liver tissue is widely supported.

In summary, our experience demonstrates that imaging examination contributes to the diagnosis of hepatobiliary cystadenoma before operation. Complete excision is its best treatment modality.

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CASE REPORT

Imatinib mesylate neoadjuvant treatment for rectal malignant gastrointestinal stromal tumor

Ying-Yong Hou, Yang Zhou, Shao-Hua Lu, Wei-Dong Qi, Cheng Xu, Jun Hou, Yun-Shan Tan

Ying-Yong Hou, Yang Zhou, Shao-Hua Lu, Wei-Dong Qi, Cheng Xu, Jun Hou, Yun-Shan Tan, Department of Pathology, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, China

Author contributions: Hou YY and Zhou Y contributed equally to this work; Hou YY, Zhou Y, Lu SH, Hou J, Tan YS designed the research; Qi WD and Xu C performed the research; Hou YY and Zhou Y analyzed the data; and Hou YY and Zhou Y wrote the paper.

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Correspondence to: Yun-Shan Tan, Department of Pathology, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, China. tan10794ys@yahoo.com.cn
Telephone: +86-21-64041990-2732 Fax: +86-21-64038472

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INTRODUCTION

Imatinib mesylate (IM) is a small molecule inhibitor of tyrosine kinase that has been evaluated for efficacy in the treatment of gastrointestinal stromal tumors (GISTs)^[1]. The role of IM neoadjuvant therapy has been discussed^[2] and explored in many clinical trials, since it may potentially offer benefits in the treatment of rectal malignant GIST. The most obvious benefit is to increase the likelihood of complete gross resection of the tumor, and, as a result, the likelihood to minimize the sacrifice of normal tissue avoiding a radical excision. In this report, we describe the case of a patient with rectal malignant GIST who was successfully treated with IM neoadjuvant and followed up 57 mo with a disease-free survival.

CASE REPORT

A 72-year-old man presented at the hospital with a 6-mo history of intermittent melena, mild lower abdominal discomfort and intussusception. At the time of admission, his complete blood count (CBC) revealed anemia with a low hemoglobin concentration of 75 g/L. An abdominal ultrasound and a computerized tomography (CT) scan of the abdomen and pelvis revealed a solitary irregular and low-density mass of 7 cm × 6 cm in the rectum, extending from the anterior rectal wall into the prostate. The urinary bladder was compressed anteriorly and tightly attached. X-ray showed no intra-abdominal lymphadenopathy or liver metastases (Figure 1) or any lung abnormality. Then, colonoscopy and ultrasound endoscopy revealed a submucosal mass in the rectum without any visible mucosal abnormality or intraluminal blood. The mass was localized at about 6.5 cm from the anal verge. However, the histological examination of

Abstract

Surgical treatments including radical resection and local excision remain the main treatment for primary rectal gastrointestinal stromal tumors (GISTs). However, since patients with high-grade rectal GISTs have a higher risk of tumor recurrence and a shorter life expectancy, neoadjuvant treatment is necessary. In this case report, the efficacy of imatinib mesylate (IM) as a neoadjuvant therapy was assessed in an old man with malignant rectal GIST. The patient received IM preoperative treatment for a short period of one and a half months; at the end of the IM treatment, computed tomography scanning showed a markedly reduced tumor size and cystic changes of the tissue. At that time, a function sphincter-sparing surgery was performed. The histological examination of the resected specimen detected no tumor cells, but residual blood vessels and scattered inflammatory lymphocytes. After surgery, the patient has been followed up without additional IM treatment and remained disease-free for 57 mo. This case indicates that IM neoadjuvant therapy can dramatically improve the prognosis of rectal malignant GIST.

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Key words: Gastrointestinal stromal tumor; Imatinib mesylate; Neoadjuvant



Figure 1 CT scan image at pelvic showing a large low-density lesion arising from the rectum.

the rectal biopsy, performed three times at other two hospitals, revealed a normal tissue with a mild infiltrate of inflammatory cells. A provisional diagnosis of rectal GIST was suspected by the CT scan.

The patient was admitted at the hospital and transfused with 3 units of packed red blood cells. Considering the patient's age, the tumor localization and size and its relationship with the neighbouring organs, a definitive histopathological diagnosis was mandatory in order to adopt appropriate treatment. To do so, a rectal subserosa fine-needle deep biopsy was successfully obtained under the guidance of ultrasound endoscopy. Histologically, spindle cells were detected in one of five sections of the tissue biopsy. The cells exhibited moderate to severe atypia and active mitoses, with a total of six mitotic figures in two high-power fields (Figure 2A). Immunohistochemistry showed strong and diffuse staining for CD117 (Figure 2B) and CD34, but proved negative for all other differentiation markers such as α -SMA, MSA, desmin, S-100 protein, and HMB45. Based on the histopathological exam, the final diagnosis of a high grade malignant GIST was made. There was insufficient tumor tissue to examine the genetic mutations for the *c-kit* and *PDGFR α* genes.

Because of the size and the localization of the lesion, both surgical intervention and the possibility of IM neoadjuvant were recommended to the patient and his family members. All declined radical excision and consented to IM neoadjuvant. The patient received IM therapy with a single dose of 400 mg administered daily for one and a half months, and was followed up by CT scans of the chest, abdomen and pelvis to monitor the outcome. The tumor dramatically shrunk to 5 cm after one month, and then developed complete cystic changes and sharp demarcation with respect to neighbouring organs after another half a month, when an exploratory laparotomy was carried out, followed by a segmental colectomy resection. The surgery was performed more easily than we would have anticipated for a similar case without IM therapy. Due to a delayed postoperative recovery, the patient was discharged one week later. Visual observation of the resected tissue found a grayish tumor of 4 cm \times 3.5 cm \times 3 cm in the muscularis propria (Figure 2C). Histopathology examination

revealed that the tumor had completely responded to IM neoadjuvant since there was no viable tumour cells found in the extirpated specimen (Figure 2D). Satisfied with the histopathologic results, the patient declined continuous IM adjuvant therapy. He has been followed up and his disease free survival is currently at 57 mo after the combined therapy: no signs of recurrence or relapse of the tumor have occurred so far.

DISCUSSION

GISTs are the most common mesenchymal tumors of the gastrointestinal tract, most frequently found in the stomach (60%) and the small intestine (30%). The incidence of the rectal GIST is low (4%)^[3,4]. Although abdominoperineal resections of large sized GISTs are not sufficient to prevent the highly frequent recurrence of the tumor^[5], surgical treatments, including radical resection and local excision, remain the main treatment for primary rectal GISTs. However, for rectal GISTs, its rarity makes it difficult to assess the role of the extension of the surgical resection of the tumor.

IM selectively inhibits the enzymatic activity of tyrosine kinases, such as ABL, PDGFR, and KIT. Its development was a landmark event in the history of cancer therapy. With IM adjuvant treatment, approximately 60%-80% of patients with recurrent or metastatic GISTs achieve a partial response or stable disease; but, complete response induced by IM is rare. The lack of complete response in many patients with partial response may be due to the drug resistance developed with a prolonged treatment. In fact, studies showed that half of the patients no longer respond to IM after approximately two years of continuous treatment^[6-8], and the likelihood of developing IM resistance is proportional to the size of residual viable GIST. Therefore, alternative and more efficient therapies for primary GISTs are required in the IM era. Currently, a potential paradigm shift in the management of GISTs has been proposed and explored, and whether IM neoadjuvant can improve outcome has been broadly evaluated.

Until now, the evaluation criteria for IM neoadjuvant therapy have not been well defined. GISTs comprise a wide range of biological behaviors, including benign, borderline and malignant variants^[9]. Surgery remains the treatment of choice for benign and borderline GISTs. Preoperative diagnosis is the most critical criterion for selecting the appropriate therapy. Most alimentary tract diseases can be initially diagnosed by endoscopy, imaging and other methods; pathologic examination of tissue specimens is the most valuable final confirmation of the diagnosis. GISTs are submucosal tumors with the surface covered with normal mucosa. A common problem for the diagnosis of GISTs is that conventional endoscopic biopsies using forceps are often unsuccessful in sampling submucosal mass, because they are too superficial and only obtain biopsy samples of the mucosal tissue. EUS-guided aspiration or core biopsy for submucosal tumors had a success rate of 60%-70% in the earlier studies,

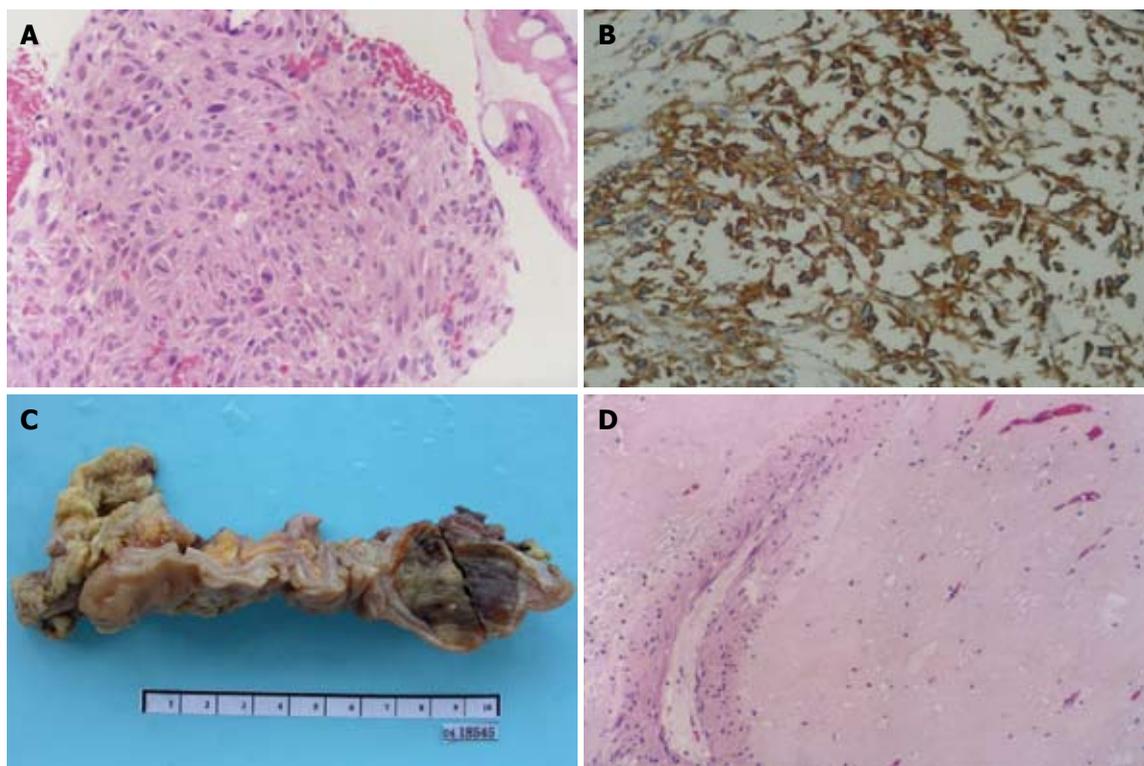


Figure 2 Different images of the rectal tumor. A: Histopathological microphotograph of biopsy tissue showing spindle-shaped cells with obvious atypia and active mitoses (HE, $\times 200$); B: Envision immunohistochemical stained tumor cells showing strong and diffuse positive staining for CD117 (HE, $\times 200$); C: Macroscopic image of the resected tissue showing a submucosal tumor grayish and uniformly soft tissue texture, measuring 4 cm \times 3.5 cm \times 3 cm in size; D: Histological examination of the resected tissue showing no residual tumor cells, except for blood vessels and scattered lymphocytes (HE, $\times 200$).

but this rate is now gradually reaching 80%-90%, which is significantly higher than that of a simple endoscopic biopsy^[10-12]. The usefulness of biopsy in diagnosis can be enhanced by immunohistochemical staining on aspirates or tissues for tumor-specific markers such as CD117 and CD34, which are crucial for a diagnosis of GISTs^[13]. Severe nuclear atypia and active mitotic figures indicating malignancy can help the decision in favor of a neoadjuvant therapy. For this reason, four biopsies were obtained from our patient to confirm the diagnosis before starting the therapy.

IM neoadjuvant therapy for primary GISTs has been reported^[14-18]. Among the reports, there are five gastric and one intestinal GISTs, where the tumor mass size made it unresectable, two primary rectal GISTs^[19,20], and one recurrent rectal GIST where the abdominoperineal resection of the rectum could not be done^[21]. Sparing surgery with preservation of the anal sphincter was possible in all of the three rectal GIST cases reported previously as well as in our case. In the case reported by Lo *et al*^[19], residual tumor cells were found 2.5 mo after IM therapy. In another case, reported by Shah *et al*^[20], no data are available concerning any residual tumor 6.5 mo after IM neoadjuvant therapy. One recurrent rectal case, reported by Salazar *et al*^[21], showed complete histopathological response 12 mo after IM therapy. In our case, the patient achieved complete response after only one and half months of IM neoadjuvant therapy. To our knowledge, this is the shortest time to achieve complete response by IM therapy. Since the patient declined

postoperatively IM adjuvant treatment, he was then closely followed up. The patient has been asymptomatic and disease-free since then, i.e. 57 mo after the combined treatment. It is reasonable to speculate that the 57 mo disease-free survival from the combined treatment is largely due to the preoperative IM therapy.

Recently, Andtbacka *et al*^[22] compared the outcomes of preoperative IM therapy combined with surgery with locally advanced and metastatic GISTs among 46 patients, including 11 with locally advanced primary tumors and 35 with metastatic tumors. Complete surgical resection after IM neoadjuvant was accomplished in all of the advanced primary tumors, but only 31.4% (11/35) of the metastatic tumors. Based on these results, IM neoadjuvant combined with surgery can be considered as more effective for patients with primary GISTs.

Based on our prior experience and the findings from this case, we believe that the approach of IM neoadjuvant treatment combined with surgery should offer the patient a longer event-free and overall survival.

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LETTERS TO THE EDITOR

Furazolidone therapy for *Helicobacter pylori*: Is it effective and safe?

Vincenzo De Francesco, Enzo Ierardi, Cesare Hassan, Angelo Zullo

Vincenzo De Francesco, Enzo Ierardi, Gastroenterology Unit, "Riuniti" Hospital, Viale L. Pinto, 71100 Foggia, Italy
Cesare Hassan, Angelo Zullo, Gastroenterology and Digestive Endoscopy, Nuovo Regina Margherita Hospital, Via E. Morosini 30, 00153 Rome, Italy

Author contributions: Authors equally contributed to the letter.
Correspondence to: Vincenzo De Francesco, Gastroenterology Unit, "Riuniti" Hospital, Viale L. Pinto, 71100 Foggia, Italy. vdefrancesco@alice.it

Telephone: +39-881-702023 Fax: +39-881-702021

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Abstract

Some aspects related with the use of furazolidone as a rescue therapy for *Helicobacter pylori* (*H pylori*) infection should be remarked, especially regarding its potential oncologic risk. The inclusion of furazolidone in a treatment regimen for *H pylori* infection is, at least, controversial, and it does not appear to be safe.

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Key words: *Helicobacter pylori*; Therapy; Furazolidone; Rescue therapy

Peer reviewer: Julio H Carri, Professor, Internal Medicine-Gastroenterology, Universidad Nacional de Córdoba, Av.Estrada 160-P 5-Department D, Córdoba 5000, Argentina

Francesco VD, Ierardi E, Hassan C, Zullo A. Furazolidone therapy for *Helicobacter pylori*: Is it effective and safe? *World J Gastroenterol* 2009; 15(15): 1914-1915 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1914.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1914>

TO THE EDITOR

We have read with great interest the study performed in Brazil by Felga *et al*^[1] on the efficacy and safety of a 'rescue' therapy to cure *Helicobacter pylori* (*H pylori*) infection. Briefly, following a quadruple therapy including PPI, bismuth salts, amoxicillin and furazolidone, a 68.8% eradication rate was achieved, a side-effect incidence of 31.4% was observed, and treatment interruption occurred in 3 (6.7%) out of

45 controlled patients. The authors concluded that 'it is an effective, cheap and safe option for salvage therapy'. We have some concerns about these conclusions. First, in the manuscript, the eradication rate was calculated only at 'per protocol' analysis (68.8%; 31 of 45). However, by calculating the success rate at 'intention to treat' analysis (all the 51 treated patients), the eradication rate was as low as 60.8%. Therefore, this quadruple therapy would not appear so 'effective' as declared. Indeed, it has been found that a simpler, levofloxacin-amoxicillin triple therapy achieved a higher eradication rate as a second-line therapy or even 'rescue' therapy^[2,3]. The reported side-effect incidence (31.4%) was much higher than that observed following furazolidone-free therapies, which was lower than 10% with an interruption rate as low as 0.003%-0.007% in thousands of patients^[4]. This observation suggests that treatment with furazolidone is not so 'safe' as declared. Last but not the least, some crucial ethical concerns arise with the use of furazolidone. This is an antibiotic used in the 1980s for parasitic infections and some studies described its use in human subjects to treat *H pylori* infection. However, different studies were published that raised several concerns about this agent and its potential for causing tumors^[5,6]. Moreover, the company that made the agent in the United States (Roberts Pharmaceuticals) was sold to Shire Pharmaceuticals and the FDA withdrew its approval for furazolidone in March 2005. The drug was ordered to be removed even from animals as an antibiotic by the FDA in 2002. The FDA has subsequently sued companies that illegally imported the drug from Mexico for use in animals. Simultaneously, the European Medicinal Agency (EMA; the equivalent of the FDA in the European Union) banned the drug in Europe. Although the drug continues to be available in some developing countries such as Iran, Pakistan, India, Mexico and Brazil, a number of public and press campaigns from concerned individuals have urged governments to ban the drug in those countries. Therefore, can we consider a therapy including furazolidone to be 'safe' as the authors declared? Although it has been stated that study was approved by the Ethical Committee, were patients informed of possible genotoxic and carcinogenic effects for which furazolidone is not currently approved by both FDA and EMA?

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Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

Kyoichi Adachi, MD

Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan

Frank J Burczynski, Professor

Faculty of Pharmacy, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba, R3T 2N2, Canada

Arno J Dormann, PD, MED

Habil, Medizinische Klinik, Krankenhaus Holweide, Kliniken der Stadt Köln gGmbH, Neufelder St. 32, 51067 Köln, Germany

Volker F Eckardt, MD, Professor, Chief

Department of Gastroenterology, Deutsche Klinik für Diagnostik, Aukammallee 33, 65191 Wiesbaden, Germany

Alfred Gangl, Professor

Department of Medicine 4, Medical University of Vienna, Allgemeines Krankenhaus, Währinger Guertel 18-20, Vienna A-1090, Austria

Alan W Hemming, MD, MSc, FRCSC, FACS, Professor of Surgery, Director of Hepatobiliary Surgery

Department of Surgery, Division of Transplantation, PO Box 100286, University of Florida, Gainesville, FL, 32610, United States

Tom H Karlsen, MD

Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

Jin-Hong Kim, Professor

Department of Gastroenterology, Ajou University Hospital, San 5, Wonchon-dong, Yeongtong-gu, Suwon 442-721, South Korea

Peter L Lakatos, MD, PhD, Assistant Professor

1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Chris JJ Mulder, Professor

Department of Gastroenterology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands

George Papatheodoridis, MD, Assistant Professor in Medicine & Gastroenterology

2nd Department of Internal Medicine, Athens University Medical School, Hippokraton General Hospital of Athens, 114 Vas. Sophias Ave., 115 27 Athens, Greece

Alberto Piperno, Professor

Department of Clinical Medicine and Prevention, Clinical Medicine, San Gerardo Hospital, Via Pergolesi 33, 20052, Monza, Italy

Dr. Massimo Raimondo

Division of Gastroenterology and Hepatology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States

Nageshwar D Reddy, Professor

Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad - 500 082, India

Dr. Markus Reiser, Professor

Gastroenterology-Hepatology, Ruhr-Universität Bochum, Bürkle-de-la-Camp-Platz 1, Bochum 44789, Germany

Gerardo Rosati, MD

Medical Oncology Unit, "S. Carlo" Hospita, Via Potito Petrone, 1, Potenza 85100, Italy

Masayuki Sho, MD, PhD, Professor

Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, 634-8522, Japan

Shingo Tsuji, MD, PhD, AGAF, Professor

Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine(A8), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Christer S von Holstein, Associate professor

Department of Surgery, Lund University Hospital, SE-221 85 Lund, Sweden

Alastair John Watson, Professor

The Henry Wellcome Laboratory, Nuffield Building, University of Liverpool, Crown St., Liverpool, L69 3GE, United Kingdom

Özlem Yilmaz, PhD, Associate Professor of Microbiology

Dokuz Eylul University, School of Medicine, Department of Microbiology and Clinical Microbiology, Inciralti 35340, Izmir, Turkey

Kentaro Yoshika, Associate Professor

Division of Gastroenterology, Department of I, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukade, Toyoake 470-1190, Japan

Yuan Yuan, Professor

Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

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.apdwc.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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- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

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Statistical data

Write as mean \pm SD or mean \pm 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 ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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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 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; 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 23243641.

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Italics

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

^[3]Passed away on June 14, 2008



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EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No.62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
 Telephone: +86-10-59080039
 Fax: +86-10-85381893
 E-mail: wjg@wjgnet.com
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Surgical treatment of anal stenosis

Giuseppe Brisinda, Serafino Vanella, Federica Cadeddu, Gaia Marniga, Pasquale Mazzeo, Francesco Brandara, Giorgio Maria

Giuseppe Brisinda, Serafino Vanella, Gaia Marniga, Pasquale Mazzeo, Giorgio Maria, Department of Surgery, Catholic University Hospital "Agostino Gemelli", Rome 00168, Italy

Federica Cadeddu, Department of Surgery, University Hospital "Tor Vergata", Rome 00133, Italy

Francesco Brandara, Department of Surgery, Hospital "Maria Paternò Arezzo", Ragusa 97100, Italy

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Correspondence to: Giuseppe Brisinda, MD, Department of Surgery, Catholic University Hospital "Agostino Gemelli", Largo Agostino Gemelli 8, Rome 00168, Italy. gbrisin@tin.it
Telephone: +39-6-30155398 Fax: +39-6-30156520

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Abstract

Anal stenosis is a rare but serious complication of anorectal surgery, most commonly seen after hemorrhoidectomy. Anal stenosis represents a technical challenge in terms of surgical management. A Medline search of studies relevant to the management of anal stenosis was undertaken. The etiology, pathophysiology and classification of anal stenosis were reviewed. An overview of surgical and non-surgical therapeutic options was developed. Ninety percent of anal stenosis is caused by overzealous hemorrhoidectomy. Treatment, both medical and surgical, should be modulated based on stenosis severity. Mild stenosis can be managed conservatively with stool softeners or fiber supplements. Sphincterotomy may be quite adequate for a patient with a mild degree of narrowing. For more severe stenosis, a formal anoplasty should be performed to treat the loss of anal canal tissue. Anal stenosis may be anatomic or functional. Anal stricture is most often a preventable complication. Many techniques have been used for the treatment of anal stenosis with variable healing rates. It is extremely difficult to interpret the results of the various anaplastic procedures described in the literature as prospective trials have not been performed. However, almost any approach will at least improve patient symptoms.

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Key words: Anal canal surgery; Anal stenosis; Ano-

plasty; Hemorrhoidectomy; Complications; Lateral internal sphincterotomy; Surgical flap

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INTRODUCTION

Anal stenosis is an uncommon disabling condition^[1-5]. It is a narrowing of the anal canal. This narrowing may result from a true anatomic stricture or a muscular and functional stenosis. In anatomic anal stenosis, the normal pliable anoderm, to a varying extent, is replaced with restrictive cicatrized tissue. Stenosis produces a morphologic alteration of the anal canal and a consequent reduction of the region's functionality, leading to difficult or painful bowel movements^[6-8].

Anal stenosis is a serious complication of anorectal surgery. Stenosis can complicate a radical amputative hemorrhoidectomy in 5%-10% of cases^[9-14], particularly those in which large areas of anoderm and hemorrhoidal rectal mucosa from the lining of the anal canal is denuded, but can also occur after other anorectal surgical procedures.

Treatment, both medical and surgical, should be modulated based on stenosis severity^[4,15]. Mild stenosis can be managed conservatively with stool softeners or fiber supplements. Daily digital or mechanical anal dilatations may be used. Sphincterotomy may be quite adequate for a patient with a mild degree of narrowing. For more severe anal stenosis, a formal anoplasty should be performed to treat the loss of anal canal tissue. Several techniques have been described for the treatment of moderate to severe stenosis refractory to non-operative management. In the literature, several studies have been conducted on anal stenosis treatment, but there is not yet universal consent on the anaplastic procedure to use. This review examines some of the evidence concerning the surgical treatment of anal stenosis.

ETIOLOGY AND PATHOPHYSIOLOGY

Stenosis may be caused by an intrinsic or extrinsic pathologic process of the anorectum. Anal stenosis may follow almost any condition that causes scarring of the anoderm. The causes of anal stenosis include surgery of the anal canal, trauma, inflammatory bowel disease, radiation therapy, venereal disease, tuberculosis, and chronic laxative abuse. We focus on the treatment of postsurgical anal stenosis.

Ninety percent of anal stenosis is caused by overzealous hemorrhoidectomy^[16]. Removal of large areas of anoderm and hemorrhoidal rectal mucosa, without sparing of adequate muco-cutaneous bridges, leads to scarring and a progressive chronic stricture. The surgical procedure influences the incidence of anal stenosis, particularly after "Whitehead hemorrhoidectomy" because, later, surgeons misinterpreted Whitehead's description and anchored the mucosa to the anal verge (Whitehead deformity)^[14,17-19]. After Milligan-Morgan and stapled rectal mucosectomy (SRM), stenosis is less frequent. In a study of 1107 patients treated with stapled hemorrhoidectomy, 164 of 1107 patients registered a complication: anal stenosis was observed in only 0.8% of cases^[19]. Stenoses caused by SRM are presumably rectal stenoses, since the causing event was a resection of rectal mucosa. The stenosis rate following stapled mucosectomy generally ranges from 0.8%-5.0%. The calculated actuarial one-year stenosis rate is 6%, which is higher than the above-mentioned published stenosis rates.

In addition, anal fissure surgery can lead to anal stenosis, if an internal sphincterotomy is not performed. Stenosis may follow anterior resection of the rectum, if complicated by anastomotic dehiscence. Inflammatory bowel diseases may cause anal stenosis, particularly Crohn's disease. These stenoses are characterized by a transmural scarred inflammatory process. Patients with anal fissure or who abuse paraffin laxatives may develop a disuse stenosis. Radiotherapy treatment for pelvic tumors (i.e. uterine carcinoma, prostatic carcinoma, *etc.*) promotes anal stenosis formation. Also sepsis, ischemia from occlusion of lower mesenteric artery or upper rectal artery, AIDS, venereal lymphogranuloma, gonorrhoea, amoebiasis and anorectal congenital disease may lead to anal stenosis. Finally, chronic abuse of ergotamine tartrate for the treatment of migraine headache attack may lead to anorectal stricture^[20].

In the natural anatomic configuration, the anal canal is an upside down funnel, where its diameter is lower than the diameter of the anal verge. Physiologically, during evacuation, the internal sphincter relaxes and dilates to the cutaneous side, where the diameter is greater, to allow the regular passage of stool. On this subject, it is important to distinguish acute from chronic anal stenosis. Acute anal stenosis is determined by a severe and sudden spasm of persistent pain (i.e. in the anal fissure). These spasms are dynamic and reversible. In this case, the ano-rectal passage is cylindrical. Chronic anal stenosis, occurs secondary to surgical procedures, infections and fibrosis, and spasms are adynamic and irreversible^[3,4].

Thus, the anal canal progressively reduces its diameter. In patients who use laxatives improperly, physiologic regular dilatation is abolished. Gradual and irreversible fibrosis occurs in the sub-cutaneous space of the anal canal with a pathologic funnel-shaped configuration in which the diameter of the anal canal is greater than the diameter of the anal verge.

DIAGNOSIS

Diagnosis of this condition is straightforward. The patient usually reports difficult or painful bowel movements. The patient may also have rectal bleeding and narrow stools. The fear of fecal impaction or pain usually causes the patient to rely on daily laxatives or enemas. Suspicion of anal stenosis is heightened by a history of hemorrhoidectomy, Crohn's disease, or excessive laxative use.

Physical examination confirms the diagnosis. Visual examination of the anal canal and perianal skin, along with a digital rectal examination, is usually suffice to establish the presence of anal stenosis. Occasionally the patient is too anxious or the anal canal too painful to allow an adequate examination. In this situation, anesthesia is needed to perform a proper examination of the anal canal. The anesthetic abolishes the spasm associated with an acute fissure but will not produce an increased luminal diameter in a patient with a true stenosis. Anorectal manometry is an objective method for assessing anal musculature tone, rectal compliance, anorectal sensation, and verifying the integrity of the rectoanal inhibitory reflex. Several methods are available for obtaining this information. No single method is universally accepted and manometric data from different institutions are difficult to compare. Manometry has been widely used to document sphincter function prior to procedures, such as lateral internal sphincterotomy, which may affect continence.

It is important to ascertain the cause of the stricture in order to determine proper therapy; a malignant disease must be treated by excision or resection, and anal Crohn's disease is an absolute contraindication to anoplasty^[4].

CLASSIFICATION AND TOPOGRAPHY

When planning treatment of anal stenosis, it is useful to categorize the severity of the stenosis. Anatomic anal stenosis may be classified on the grounds of stricture severity, its structure and the level of involvement in the anal canal. On the basis of severity, Milsom and Mazier^[6] distinguished mild (tight anal canal can be examined by a well-lubricated index finger or a medium Hill-Ferguson retractor), moderate (forceful dilatation is required to insert either the index finger or a medium Hill-Ferguson retractor), and severe anal stenosis (neither the little finger nor a small Hill-Ferguson retractor can be inserted unless a forceful dilatation is employed). Furthermore, stenosis may be diaphragmatic (after inflammatory bowel disease, characterized by a thin strip of constrict-

tor tissue), ring-like or anular (after surgical or traumatic lesions, of length less than 2 cm), and tubular (length more than 2 cm). On the basis of the anal canal levels, stenosis may also be distinguished as low stenosis (distal anal canal at least 0.5 cm below the dentate line, 65% of patients), middle (0.5 cm proximal to 0.5 cm distal to the dentate line, 18.5%), high (proximal to 0.5 cm above the dentate line, 8.5%), and diffuse (all anal canal, 6.5% of cases)^[6].

TREATMENT

The best treatment of postsurgical anal stenosis is prevention. Adequate anorectal surgery reduces the incidence of anal stenosis^[3,16]. It is essential to treat tissues delicately and not to draw them. Also it is important to use absorbable sutures and minimal resection of tissues. Khubchandani^[3] condemned the use of manual dilatation under anesthesia for the non-operative treatment of mild to moderate stenosis because the resultant hematoma in the sphincter apparatus may cause fibrosis and progressive stenosis. In Milligan-Morgan hemorrhoidectomy, internal sphincterotomy, if necessary, associated with preservation of adequate muco-cutaneous bridges, prevents anal stenosis. However, if anal stenosis is present, treatment should be modulated based on severity, cause and localization^[6].

Non-operative treatment is recommended for mild stenosis and for initial care of moderate stenosis. Also, with severe stenosis, conservative treatment can lead to good results, however, surgery is always necessary. The use of stool softeners and fiber supplements with adequate gain of fluids is the basis of non-operative treatment. This gradual and natural dilation is very effective in most patients. Anal dilatation is another important part of this treatment. Anal dilation can be performed daily both digitally or with any of a number of graduated mechanical dilators. Patients are instructed to sit down on the toilet, bear down, and gradually insert the smallest dilator with ample lubrication. If the patient can persist with the dilations on a regular basis, the result is usually excellent. Many patients do not tolerate this procedure. On the other hand, a dilator may tear the canal. In fact, a complication from the use of dilators may itself precipitate the need for surgical intervention. However, it would be a rare circumstance when mild stenosis would require surgery^[4].

Moreover, if the patient remained symptomatic with the usual measures, it is important to be certain that anal stenosis is indeed the cause of the patient's complaints; particularly in the postoperative patient, anal fissure must be ruled out as a possible source of the problem. If stenosis is refractory to non-operative management, surgery represents the last solution. However, a long course of conservative, medical management is indicated in the treatment of mild anal stenosis before resorting to a surgical approach.

Many different surgical techniques have been described for the management of moderate to severe anal stenosis. Moderate stenosis is generally treated initially in

the same fashion as mild stenosis. Fiber supplementation is initiated and dilations are carried out if necessary. Furthermore, partial lateral internal sphincterotomy may be quite adequate for a patient with a mild degree of narrowing. This technique is simple and safe and use is limited to functional stenosis. It is important that the sphincterotomy is done in the open fashion so that the associated scarred anoderm is divided at the same time to allow full release of the scar. The resulting wound is then left open and allowed to heal by secondary intent. This provides relief of the partial obstruction and pain caused by the stenosis, but the relief will be short-lived without appropriate medical management. The importance of a high-fiber diet and fiber supplements must be emphasized to the patient and instituted immediately after surgery. Although the results have been reported as excellent^[21,22], it is difficult to interpret whether the patients had significant narrowing or spasm associated with the anal fissure.

For more severe anal stenosis, a formal anoplasty should be performed to treat the loss of anal canal tissue. Various types of flaps have been described for anal stenosis which allow delivery of the more pliable anoderm into the anal canal to replace the scarred lining at that level. A lateral internal sphincterotomy is also usually necessary at the time of anoplasty.

Lateral mucosal advancement flap

This is a modification of Martin's anoplasty (Figure 1A)^[1,23,24]. A midlevel stenosis is corrected by excision of the scar tissue. An undermining of the proximal rectal and anal mucosa through a transverse incision at the dentate line is performed. An internal sphincterotomy is performed if a functional component is present. The resulting flap is advanced to the distal edge of the internal sphincter near the anal verge. The vascular supply is maintained through the submucosal plexuses. The external part of the wound is left open to minimize ectropion formation.

Y-V advancement flap

This procedure is performed in the gynaecological prone position. It is important to administer adequate antibiotic therapy (cephazoline and metronidazole) at the time of surgery. A mechanical bowel preparation is usually done the day before surgery. After anal dilatation with a medium Hill-Ferguson retractor, the initial incision overlying the area of stricture is the vertical limb of the Y (Figure 1B). This incision is extended on the perianal skin in two directions for creating a V flap. Incisions are carried proximally for 5 to 8 cm. The V flap is incised with fatty subdermal tissue, providing an adequate blood supply. The resulting V advancement flap is sutured into the vertical limb of the Y incision in the anal canal with the internal apex of the triangular flap sutured to the internal sphincter and to rectal mucosa (dentate line) with interrupted long-term absorbable sutures^[5,20,23,25-30]. This flap can be done in the posterior midline or in either lateral position. It can also be done bilaterally if needed to relieve the stenosis. Postsurgical management consists of fiber supplements and pain control. Sitz baths can also

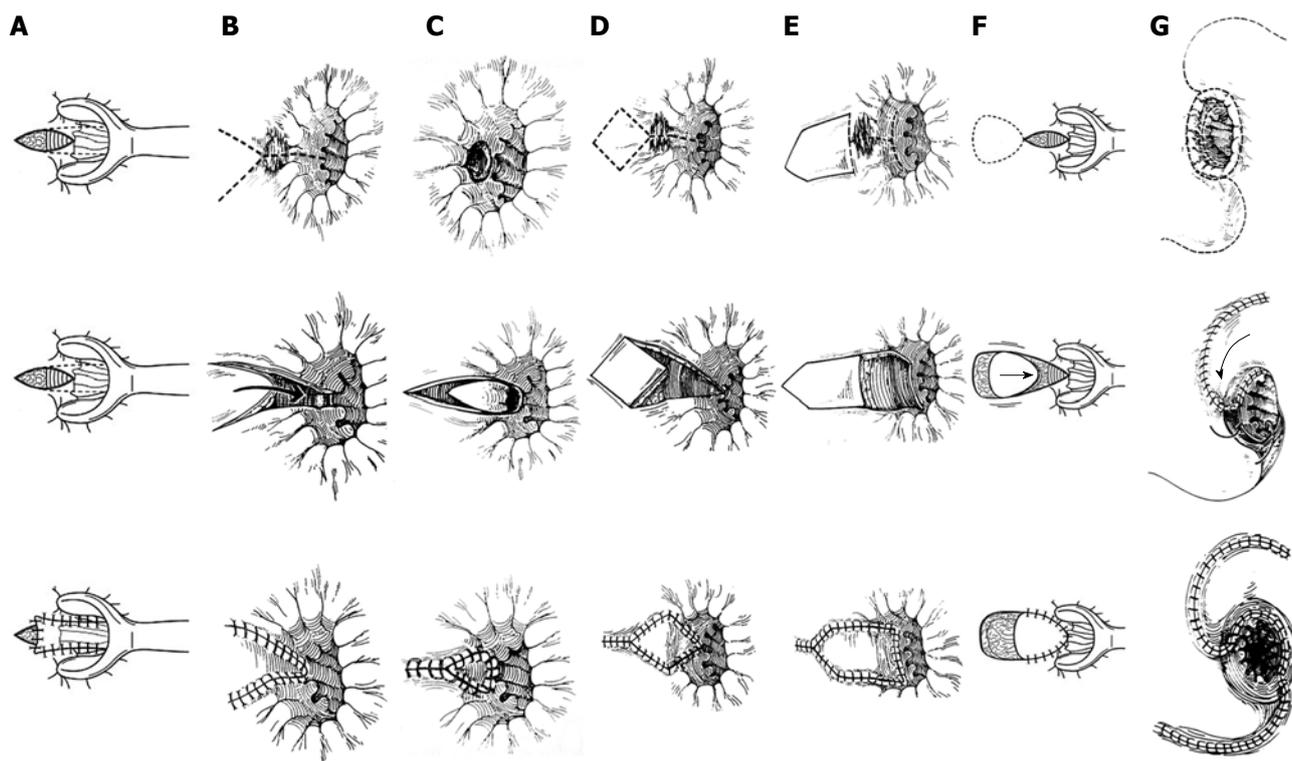


Figure 1 Operative procedure for the surgical treatment of anal stenosis. A: Martin's anoplasty; B: Y-V advancement flap; C: V-Y advancement flap; D: Diamond-shaped flap; E: House-shaped flap; F: U-shaped flap; G: Rotational S-flap.

be instituted to assist with local hygiene. In the post-operative period, a constipating regimen is recommended for 2 d. Antibiotic therapy is usually continued for 7 d. This technique is simple and quite useful for stenosis associated with an anal fissure. However, if more than 25% of the circumference of the anal canal needs to be covered, another anaplastic procedure is indicated^[4].

V-Y advancement flap

This procedure is an alternative to Y-V anoplasty. The base of the triangular V flap is sutured to the dentate line (Figure 1C). In addition, the underlying vascular pedicle is contained in the subcutaneous fat. Thus, it is necessary to preserve fatty subcutaneous tissue with wide mobilization to maintain flap viability. The skin is then closed behind the V at the external portion of the perineum to push the V into the anal canal and widen the stenotic area^[5].

Diamond-shaped flap

After adequate mechanical bowel preparation and antibiotic therapy in the pre-operative period, this procedure is performed in the gynaecological prone position. To avoid bleeding, epinephrine can be used. On the basis of stenosis severity, one or two flaps can be created. The scar tissue is incised leaving a diamond-shaped defect (Figure 1D). A diamond-shaped flap is designed so that it will cover the intra-anal portion of the defect^[5,23,29]. The preparation of the flap is a crucial step in the procedure: the flap should be well mobilized to reduce tension and to provide enough blood supply to preserve the underlying vascular pedicle.

House flap

After the use of stool softeners in the pre-operative period, enema is performed on the day of surgery. This technique is performed in the gynaecological prone position. If stenosis is extended from the dentate line to perianal skin, a house flap is recommended (Figure 1E). With the use of a Hill-Ferguson retractor, a longitudinal incision is made toward the perianal skin, from the dentate line to the end of the stenosis. The length of incision corresponds to the length of the flap wall. Proximal and distal transverse incisions are centered on the longitudinal incision. The flap is then designed in the shape of a house with the base oriented proximally. The width of the base of the house is designed to match the transverse incisions and hence the width of the mucosal defect to be replaced. It is necessary to preserve the subcutaneous vascular pedicle^[2,7,26,31,32]. The flap is then easily advanced into the anal canal and sutured. This procedure offers two advantages: (1) the creation of a wide flap increases the anal canal diameter along its length, (2) the technique allows primary closure of the donor site.

U flap

This procedure is used for the treatment of anal stenosis associated with mucosal ectropion. A U-shaped incision is made in the adjacent perianal skin (Figure 1F). Mobilization and suture of the flap are the same as for diamond-shaped anoplasty. The donor site is left open, and covered with fatty gauzes.

C flap

This procedure is performed in the lithotomy position.

Table 1 Anoplasty for anal stenosis

Procedures	Indications	Advantages/Disadvantages
Partial lateral internal sphincterotomy	Functional stenosis; mild and low stricture in the anal canal	This technique is simple and safe. Use is limited to functional stenosis
Mucosal advancement flap	Middle or high localized stricture	Ectropion formation if the flap is sutured at the anal verge
Y-V advancement flap	Low and localized stricture below the dentate line	Proximal part of the flap is very narrow and will not allow for a significant widening of the stricture above the dentate line. Also, the tip of the V within the anal canal is subject to ischemic necrosis from lack of mobilization, tension of the flap or loss of vascularization
V-Y advancement flap	Mild to severe stricture at the dentate line. Middle or high localized strictures, associated with mucosal ectropion	The tip of the V is subject to ischemic necrosis
Diamond flap	Moderate to severe long stricture, localized or circumferential stricture above the dentate line, associated with mucosal ectropion	A diamond-shaped flap is designed so that it will cover the intra-anal portion of the defect. The flap is mobilized with minimal undermining to preserve the integrity of the subcutaneous vascular pedicle
House flap	Moderate to severe long stricture, localized or circumferential or diffuse, and stricture above the dentate line, associated with mucosal ectropion	It allows primary closure of the donor site and increases anal canal diameter along its length. Because of the wide base, it avoids the pitfall of having a narrow apex present inside the anal canal that may become ischemic
U flap	Moderate to severe stricture, localized or circumferential, associated with mucosal ectropion	This technique is particularly useful when there is need to excise a significant area of ectropion. The donor site is left open
C flap	Moderate to severe stricture, localized or circumferential, associated with mucosal ectropion	The donor site is left open
Rotational S flap	High severe stricture, circumferential or diffuse, associated with mucosal ectropion	It provides for adequate blood supply, avoids tension, and can be performed bilaterally if necessary for coverage of large areas of skin. Complex technique: high morbidity and longer hospital stay

With the use of a small Hill-Ferguson retractor, a radial lateral incision is made from the dentate line to the anal verge. Then a C-shaped incision is made in the perianal skin starting from the distal point of radial incision. Preparation of a C flap should guarantee an adequate blood supply.

Rotational S-flap

The S-plasty is best used for the treatment of Bowen's disease or Paget's disease, where a large amount of skin has to be excised and new skin rotated into the area^[29,33]. The S-plasty does not open a stricture as well as the advancement flap. In the gynaecological prone position, after scar tissue has been excised, a full-thickness S-shaped flap is made in the perianal skin, with the size of the base as great as its length, starting from the dentate line for approximately 8 cm to 10 cm. The flap is then rotated and sutured to the normal mucosa (Figure 1G).

Internal pudendal flap anoplasty

A solitary case report has been reported where extensive coverage was required concomitant with excision of Paget's disease of the anal canal^[34].

Foreskin anoplasty

This interesting operation has been described for the treatment of mucosal ectropion. The procedure uses the foreskin (suitable prepuce) to provide a full-thickness skin graft to the anal canal. Since the initial report by Freeman with six children in 1984^[35], no further publications have been noted.

Choice of procedure

The choice of an adequate procedure is related to the

extent and severity of the stenosis (Table 1) as it may involve the skin, transitional zone to the dentate line, anal canal, or all of these. Y-V anoplasty is not used in the treatment of stricture above the dentate line. V-Y anoplasty has been used in the treatment of severe low anal stenosis with good results.

Various types of anoplasties with adjacent tissue transfer flaps have been devised to relieve anal stenosis. All of these flaps share the concept of an island of anoderm that is incised completely around its circumference. A significant advantage of these flaps over the Y-V anoplasty is that there is significantly greater mobility of the flap, so it can be advanced well into the anal canal. The diamond flap, House flap, and island flap have all been reported to yield excellent results^[3-8,10,15,19,20,22-32,34-44]. The type of flap to be used is based on the surgeon's familiarity and choice as well as the patient's anatomy and the availability of adequate perianal skin for use in the various flaps. For any of these flaps, the preoperative preparation is the same as for the Y-V flap. A partial lateral internal sphincterotomy is often required as well. Once the flap is fully mobilized, it can be advanced into the anal canal and sutured in place with interrupted long-term absorbable sutures. Similar to the Y-V flap, these flaps can be done in any location and can be done bilaterally if needed.

The House flap is recommended if stenosis extends from the dentate line to perianal skin, allowing primary closure of the donor site and an increase in anal canal diameter along its length. U flap anoplasty is used for the treatment of anal stenosis associated with mucosal ectropion. If less than 50% of the anal circumference is involved, an advancement flap should suffice; however, if 50% or more of the anal canal needs to be recon-

structed, a rotational flap of skin should be considered, as it is necessary to cover a large area providing adequate blood supply and avoiding tension.

Postoperative care

Single and limited flaps may be performed in the out-patient setting. For the simplest procedures, patients are started on a high-fiber diet, bulk laxatives and mineral oil for a short time in the postoperative period. Sitz baths and showers are recommended for comfort and hygiene. Flaps with multiple and extensive dissection or reconstruction will require hospital admission. These extensive procedures may require bowel confinement for 3 d to 5 d after which a high-fiber diet is initiated.

Complications

In the literature, various complications have been reported after anoplasty. These include flap necrosis from loss of vascular supply, infection or local sepsis, suture dehiscence from excessive suture line tension, failure to correct the stenosis, donor site problems, sloughing of the flap, ischemic contracture of the edge of the flap, pruritus, urinary tract infection subsequent to *Clostridium difficile* enterocolitis only in a few cases, fecal incontinence, constipation without stenosis, urinary retention, restenosis and ectropion if the flap is advanced too far and sutured at the anal verge^[4-7,10,15,19,20,22,23,25,26,31,32,34,36-39,41,42,44].

COMMENTS

Anal stenosis, although rare, is one of the most feared and disabling complications of anorectal surgery. It has been documented that hemorrhoidectomy is the most frequent cause, but stenosis may be a consequence of other causes. Several operative techniques to treat hemorrhoids have been described. Milligan-Morgan's open hemorrhoidectomy is most commonly used; other procedures, such as Ferguson's closed hemorrhoidectomy and Parks' submucosal hemorrhoidectomy, are technically more complex. We feel that the surgeon's choice of technique is primarily based on personal experience and technical training, and only a competently performed technique produces satisfying results: hemorrhoidectomy needs skilled operators. If technical guidelines are rigorously followed, the feared complications associated with surgical procedures, such as anal stricture and sphincteric injuries, are largely reduced.

Furthermore, anal stenosis became a focus of interest after the introduction of SRM. Anorectal stenosis is not a specific problem of SRM but is a considerable problem after all anal interventions. In a direct comparison in prospective randomized trials there was no significant difference in stenosis rate between conventional hemorrhoidectomy and SRM. Nevertheless, a substantial rate of stenoses was observed following conventional hemorrhoidectomy, and probably the highest stenosis rate was described after Whitehead hemorrhoidectomy. One potential mechanism that might cause stenosis following SRM is ring dehiscence followed by submucous inflammation. Another theoretical cause is that the

stapled ring is placed too deep in the anal canal and that the squamous skin cells react by scarring and shrinking. One major aspect of the potential risk of developing a stenosis is the distance to the anal verge. A full thickness excision of the rectal wall is another potential cause for stenosis after SRM.

A number of corrective surgical procedures have been designed aiming to bring a healthy lining to the narrowed portion of the anal canal. Since more complex techniques, such as S-plasty, have now been abandoned due to high morbidity and longer hospital stay, easier techniques are still being performed with good results (Table 2). The ideal procedure should be simple, should lead to no or minimal early and late morbidity, and should restore anal function with a good long-term outcome.

Each of the surgical techniques described can be performed safely and have been used with variable healing rates. It is extremely difficult to interpret the results of the various anaplastic procedures in the literature for the obvious reason that prospective trials have not been performed. There are no controlled studies on the advantages and disadvantages of the various anaplastic maneuvers; however, almost any approach will at least improve symptoms in the patient. Oh and Zinberg^[41] used C anoplasty in 12 patients with anal stenosis (10 by previous hemorrhoidectomy, 1 by fistulectomy and 1 by fissurectomy), and 11 patients obtained satisfactory results with a total healing rate of 91%. Khubchandani^[3] published a study in which 53 patients underwent mucosal advancement flap anoplasty with a healing rate of 94%. Similar results have been reported in a total of 33 patients treated with Y-V anoplasty in two studies^[23,28]. A total healing rate of 100% was obtained using diamond flap anoplasty in a total of 23 patients affected by anal stricture and mucosal ectropion. The healing rate was 91.5% in 53 patients who suffered from anal stenosis and ectropion treated with island flap anoplasty^[29,39].

Aitola and coworkers^[25] conducted a retrospective study in 10 patients who had undergone Y-V anoplasty combined with internal sphincterotomy between 1991 and 1995. After a median follow up period of 12 mo, all but one patient improved. Six patients had good results, three had fair results and in one the result was poor. This patient later developed a restenosis. Total healing rate was 60% with improvement rate of 30%. In a recent study^[5], a Y-V anoplasty was performed in 29 cases and a diamond flap anoplasty in the remaining 13 cases. At 2 years follow-up, all patients who underwent diamond flap anoplasty had complete resolution of the stenosis (healing rate 100%). Among 29 patients who underwent Y-V anoplasty, 26 (90%) judged their clinical results as excellent while 3 patients (10%) required periodical use of anal dilators. Those three patients had post-operative complications (two suture dehiscence and one ischemic contracture of the edge of the flap).

Rakhmanine and colleagues^[24] published a study in which 95 patients underwent lateral mucosal advancement anoplasty. Mean follow up was 50 mo. Only 63% of patients had undergone previous surgery: 35 patients

Table 2 Experiences in literature

Authors	No. of cases	Procedure	Results			Healing rate (%)
			Good	Fair	Poor	
Sarner <i>et al</i> ^[45]	21	Sarner's flap	-	-	-	100
Nickell <i>et al</i> ^[46]	4	Advancement flap anoplasty	4	-	-	100
Oh <i>et al</i> ^[41]	12	C anoplasty	11	-	1	90
Khubchandani ^[40]	53	Advancement flap anoplasty	Nr	Nr	Nr	94
Milsom <i>et al</i> ^[6]	24	V-Y anosplasty and Sarner's anoplasty	-	-	-	90
¹ Gingold <i>et al</i> ^[28]	14	Y-V anoplasty	9	5	-	64
Caplin <i>et al</i> ^[11]	23	Diamond flap anoplasty	23	-	-	100
Ramanujam <i>et al</i> ^[30]	21	Y-V anoplasty	18	2	1	95
Pearl <i>et al</i> ^[29]	25	Island flap anoplasty	16	7	2	92
² Angelchik <i>et al</i> ^[23]	19	Y-V anoplasty (12 cases)	8	4	0	100
		Diamond flap anoplasty (7 cases)	7	-	-	100
Pidala <i>et al</i> ^[42]	28	Island flap anoplasty	25	-	3	91
Eu <i>et al</i> ^[38]	9	Lateral internal sphincterotomy (5 patients) and anoplasty (4 cases)	9	-	-	100
Gonzalez <i>et al</i> ^[39]	17	S anoplasty (6 cases) and advancement flap anoplasty (11 cases)	16	-	1	92
Sentovich <i>et al</i> ^[32]	29	House advancement flap	26	-	3	90
Saldana <i>et al</i> ^[34]	1	Internal pudendal flap anoplasty	1	-	-	100
Aitola <i>et al</i> ^[25]	10	Y-V anoplasty with internal sphincterotomy	6	3	1	60
de Medeiros ^[47]	30	Sarner's flap or Musiani's flap	-	-	-	100
Maria <i>et al</i> ^[5]	42	Y-V anoplasty (29 cases)	26	-	3	90
		Diamond flap anoplasty (13 cases)	13	-	-	100
Stratmann <i>et al</i> ^[44]	3	-	-	-	-	100
Ettorre <i>et al</i> ^[26]	1	House advancement flap	1	-	-	100
Saylan ^[20]	3	Y-V anoplasty	-	-	-	100
³ Rakhmanine <i>et al</i> ^[24]	95	Lateral mucosal advancement anoplasty	74	-	8	90
Carditello <i>et al</i> ^[36]	149	Internal sphincterotomy and mucosal flap anoplasty	Nr	Nr	Nr	97
Habr-Gama <i>et al</i> ^[15]	77	Sarner's flap (58 patients) and Musiani's flap (19 patients)	-	-	-	87
Filingeri <i>et al</i> ^[27]	7	Y-V anoplasty	-	-	-	100
Casadesus <i>et al</i> ^[11]	19	Y-V anoplasty (7 patients) and lateral mucosal advancement flap anoplasty (12 patients)	-	-	-	100
Alver <i>et al</i> ^[31]	8	House advancement flap (14 flaps: 1 flap for 2 patients, and 2 flaps for 6 patients)	6	-	-	100

Nr: Not reported. ¹No sphincterotomies were done; ²Not all patients underwent sphincterotomy, and some of the procedures were for anal ectropion; ³Depending on the degree of stenosis, patients initially underwent either unilateral (62%) or bilateral (38%) anoplasty. Thirteen patients with a follow up of less than 6 mo were excluded from the analysis for restenosis.

had had hemorrhoidectomy, 10 operations for anal fissure, 4 for fistula, 1 transversal excision of a neoplasm and 10 other operations. The overall complication rate was 3% (one abscess and two seepage of liquid stool).

CONCLUSION

Anal stenosis is most often a preventable complication. A well-performed hemorrhoidectomy is the best preventative measure. Anoplasty should be part of the armamentarium of colorectal surgeons for treating severe anal stenosis. The anatomic configuration of the anorectum and perianal region is very complex and knowledge of this area is essential before performing any surgical procedure. Most post-anoplasty complications can be avoided by respecting the rectal wall anatomy in the execution of surgical procedures. The preparation of flaps is important for treatment success. In all cases, in fact, it is necessary to preserve as much sub-cutaneous fat as possible with wide mobilization, and to maintain viability and to avoid excessive suture line tension. In addition, it is important to treat tissues delicately and not to draw them, to use absorbable sutures and perform minimal tissue resection.

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Measurement of serum paraoxonase-1 activity in the evaluation of liver function

Jordi Camps, Judit Marsillach, Jorge Joven

Jordi Camps, Judit Marsillach, Jorge Joven, Centre of Biomedical Research, University Hospital Sant Joan, Institute of Health Research Pere Virgili, University Rovira i Virgili, 43201 Reus, Catalonia, Spain

Author contributions: Camps J drafted the manuscript; All the authors collected the scientific information, edited the manuscript and approved the final version.

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Correspondence to: Dr. Jordi Camps, Centre of Biomedical Research, University Hospital Sant Joan, C. Sant Joan s/n, 43201 Reus, Catalonia, Spain. jcamp@grupsagessa.cat

Telephone: +34-977-310300 **Fax:** +34-977-312569

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Department of Surgery, Division of Trauma and Critical Care, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati OH 45267-0558, United States

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Abstract

Paraoxonase-1 (PON1) is an esterase and lactonase synthesized by the liver and found in the circulation associated with high-density lipoproteins. The physiological function of PON1 seems to be to degrade specific oxidized cholesteryl esters and oxidized phospholipids in lipoproteins and cell membranes. PON1 is, therefore, an antioxidant enzyme. Alterations in circulating PON1 levels have been reported in a variety of diseases involving oxidative stress including chronic liver diseases. Measurement of serum PON1 activity has been proposed as a potential test for the evaluation of liver function. However, this measurement is still restricted to research and has not been extensively applied in routine clinical chemistry laboratories. The reason for this restriction is due to the problem that the substrate commonly used for PON1 measurement, paraoxon, is toxic and unstable. The recent development of new assays with non-toxic substrates makes this proposal closer to a practical development. The present editorial summarizes PON1 biochemistry and function, its involvement with chronic liver impairment, and some aspects related to the measurement of PON1 activity in circulation.

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Key words: Lipoproteins; Liver cirrhosis; Liver function tests; Oxidative stress; Paraoxonase-1

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INTRODUCTION

The paraoxonase (PON) enzyme family comprises 3 members, PON1, PON2 and PON3, whose genes are located adjacent to each other on chromosome 7q21-22^[1]. In humans, PON1 and PON3 are mainly found in the circulation bound to high-density lipoproteins (HDL)^[2]. Conversely, PON2 is an intracellular enzyme^[3]. Their physiological roles have not been completely ascertained. PON1 has esterase and lactonase activities^[4] and is involved in protection against xenobiotic toxicity^[5]. PON2 and PON3 have only lactonase activity^[6]. All the PONs are able to reduce low density lipoprotein (LDL) oxidation^[7], while PON2 reduces cellular oxidative stress and prevents apoptosis in vascular endothelial cells^[8]. PON1 is the best known among these enzymes. Alterations in circulating PON1 levels have been reported in a variety of diseases involving oxidative stress. These include cardiovascular disease, Alzheimer's disease, chronic renal failure, HIV-infection, metabolic syndrome, and chronic liver impairment^[9]. As such, increased knowledge of the physiological significance of PON1 and its involvement in human pathology would be of critical importance in the years to come. In the present article we review fundamental concepts regarding PON1 biochemistry and function, and the relationships with chronic liver diseases. We also discuss the possible application of its measurement in serum for an improved evaluation of hepatic function.

PON1 IS AN ANTIOXIDANT ENZYME

The first approximation to the physiological role of PON1 was suggested by Mackness *et al*^[10]. The authors investigated the protection against copper-induced LDL oxidation *in vitro* provided by purified PON1. They observed that this enzyme prevents the generation of

lipoperoxides during the process of LDL oxidation. Further studies from this and other groups reached the conclusion that PON1 protects LDL and HDL from lipid peroxidation by degrading specific oxidized cholesteryl esters and specific oxidized phospholipids contained in oxidized lipoproteins^[11-13]. PON1 is, in turn, inactivated by oxidized lipids. This was shown by Aviram *et al*^[14], who demonstrated that the incubation of PON1 *in vitro* with oxidized palmitoyl arachidonoyl phosphatidylcholine, lysophosphatidylcholine, and oxidized cholesteryl arachidonate, inactivated PON1 activity, as well as did oxidized LDL. Cysteine-284 was required for this effect of oxidized lipids on PON1 because, in recombinant PON1 in which this amino acid had an induced mutation, no inactivation was observed. A further article from the same group showed that, under oxidative stress, PON1 may be inactivated by *S*-glutathionylation, a redox regulatory mechanism characterized by the formation of a mixed disulfide between a protein thiol (i.e. cysteine-284) and oxidized glutathione^[15].

Identifying the native function of PON1 has, for a long time, been hampered by confusion with respect to the structure and mechanism-of-action of this enzyme. Several studies established the primordial function of PON1 as that of a lipolactonase^[16-18] which subsequently evolved new substrate specificities. These studies also established that the preferred substrates of PON1 are 5- and 6-membered ring lactones, typically with aliphatic side-chains^[19]. A model has been proposed to link lactonase activity and the degradation of lipid peroxides^[20] by which oxidized lipids containing hydroxyl groups at the 5'-position could be lactonized by PON1 to yield lysophosphatidylcholine and δ -valerolactone products. As such, according to this hypothesis, the PON1 ability to degrade lipid peroxides is secondary to its lipolactonase activity.

PON1 IS ESSENTIALLY SYNTHESIZED BY THE LIVER

PON1 is found mainly in serum and in the liver. Northern blot analysis performed in human and rabbit tissues detected *PON1* mRNA only in the liver, although reverse transcriptase PCR (RT-PCR) studies in mice identified *PON1* mRNA in liver, kidney, heart, brain, intestine, and lung^[1]. It seems highly likely that the liver is the main source of serum PON1 since it is the organ with the highest *PON1* gene expression, and where an important percentage of HDL is synthesized and secreted into the circulation. Over the last 15 years, there have been several attempts to purify hepatic PON1 to homogeneity with the aim of comparing its properties with those of the serum enzyme. This task is complicated by the hepatic PON1 being an enzyme associated with membrane vesicles derived from the endoplasmic reticulum^[21].

In 1993, the first method for the partial purification of rat liver PON1 was published^[22]. Essentially, the process consisted of the preparation of microsomes, solubilization with Triton X-100, adsorption on to hydroxyapatite, and chromatography with DEAE-52

cellulose to yield a 77-fold purified product. Later, Huang *et al*^[23] isolated PON1 from mouse hepatic microsomes, and Rodrigo *et al*^[24] in 1997, purified rat liver PON1 to homogeneity. They achieved a 415-fold purified product by using hydroxyapatite adsorption followed by three chromatography steps including DEAE-Sephacrose, affinity chromatography, and Mono Q HR fast-performance liquid chromatography. The N-terminal sequence and two internal sequences of the purified protein were similar to those of rabbit and human PON1 of serum and mouse liver PON1. Subsequent studies with rat and human liver PON1 demonstrated many biochemical characteristics in common with those of serum PON1. These included optimum pH, substrate affinity (K_M), kinetic constants, heat inactivation, and calcium requirement^[25]; all of which strongly suggested a high degree of identity between both enzymes.

What is the true role of hepatic PON1? If HDL-bound serum PON1 is an antioxidant enzyme, it may not seem illogical to infer that a similar function could apply to intracellular PON1. Indeed, liver microsomes are the major sites for the catabolism of xenobiotic compounds, in the course of which process an increased production of free radical species is observed. Rodrigo *et al*^[26] observed PON1 protein expression mainly in the hepatocytes from the centrolobular region, thus supporting the hypothesis of intrahepatic PON1 participation in oxidative by-product inactivation.

OXIDATIVE STRESS AND CHRONIC LIVER IMPAIRMENT

Increased oxidative stress and inflammation play a fundamental role in the onset and development of liver diseases. The most important causes of chronic liver disease are alcohol abuse, obesity, and hepatitis C virus infection.

Alcoholic liver disease (ALD) comprises a broad spectrum of hepatic alterations ranging from steatosis and minimal injury to advanced fibrosis and cirrhosis^[27]. The involvement of oxidative injury in ethanol toxicity has emerged from reports showing that alcohol-fed animals and patients with ALD present with a high content of lipid peroxidation products in their livers and in the circulation^[28]. Oxidative stress associated with ethanol intake comes mainly from reactive oxygen species (ROS) generated by the mitochondrial respiratory chain and cytochrome P4502E1 from hepatocytes, and the NADPH oxidase from Kupffer cells and recruited macrophages^[29]. The impairment of mitochondrial lipid oxidation is one of the mechanisms responsible for hepatic fat accumulation^[30]. Pan *et al*^[31] reported that lipid peroxidation reduces hepatic lipoprotein secretion by enhancing the degradation of newly synthesized apolipoproteins and this effect, together with alterations in lipoprotein glycosylation in the Golgi apparatus^[28], might contribute to microvesicular steatosis. Further evidence suggests that alcohol-induced oxidative stress interferes with the regulation of lipid synthesis by the peroxisome proliferator-activated receptor- α and the sterol

regulatory element binding protein 1^[32]. The possible role of oxidative stress in promoting an inflammatory reaction in ALD has emerged from the observation that ethanol-induced lipid peroxidation increases the hepatic production of cytokines, growth factors, and collagen^[33-35].

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are hepatic lesions which appear frequently in obese and diabetic individuals despite the fact that they may not have a history of alcohol abuse. These lesions resemble those of ALD, and are characterized by steatosis, hepatocyte hydropic degeneration, and inflammatory infiltrates. In addition, alterations in mitochondrial shape and function, and varying degrees of fibrosis are usually found^[36]. NAFLD is an emerging lesion in modern societies, and will become more prevalent in the future, as it is associated with insulin resistance, metabolic syndrome, diabetes, and obesity. Oxidative stress plays a pivotal role in the evolution of "benign" steatosis to the more severe NASH. Several studies have shown that mitochondria in patients with NASH are abnormal from both the morphological and the functional points of view and, as in ALD, alterations in the fatty acid β -oxidation promote increased free radical production and lipid peroxidation^[36]. The consequences of oxidative stress in NASH would be similar to those of ALD, with altered lipoprotein synthesis and secretion, an inflammatory reaction, and fibrosis.

Hepatitis C virus (HCV) is a major cause of viral hepatitis. In the USA, about 4 million people are infected, and 35 000 new HCV cases are estimated to occur every year^[37]. The infection by this virus frequently does not resolve, and about 80% of the infected individuals become chronic carriers who may then progress to the most severe forms of liver impairment, as cirrhosis or hepatocellular carcinoma. Lipid peroxidation products, aldehydes as 4-hydroxynonenal, and 8-hydroxyguanosine (a marker of oxidative DNA damage) are elevated in HCV infection^[38]. The increased oxidative stress may be explained by chronic inflammation and the generation of free radicals by Kupffer cells and recruited macrophages. The NS3 protein of HCV has been found to activate Nox 2 protein from macrophages, leading to increased generation of ROS that can exert oxidative stress to the nearby cells^[39]. Furthermore, studies have indicated that HCV can directly induce oxidative stress in hepatocytes. HCV core gene expression has been associated with increased ROS, decreased reduced glutathione content, and increased thioredoxin in parenchymal cells. Recent studies showed that HCV core proteins bind to the outer mitochondrial membrane resulting in mitochondrial dysfunction by Ca^{2+} accumulation. These alterations would inhibit electron transport and promote ROS production^[37]. Another HCV protein, NS5A, has also been reported to increase free radical production by Huh7 cells^[40]. As in ALD and NASH, increased oxidative stress would produce a multifactorial reaction involving the synthesis of pro-inflammatory and pro-fibrogenetic cytokines and chemokines.

Therefore, it seems evident that chronic liver diseases share common biochemical alterations irrespective

of their etiology. They are all accompanied by an increased oxidative stress secondary to mitochondrial abnormalities, promoting changes in lipid and lipoprotein metabolism, fat accumulation, an exacerbation of the inflammatory reaction due to increased cytokine synthesis, and extracellular matrix deposition.

THE MEASUREMENT OF SERUM PON1 ACTIVITY

There are no standardized methods for measuring PON1 esterase activity. The most widely used method is the hydrolysis of paraoxon. However, this method is not free of drawbacks, because paraoxon is very unstable and extremely toxic. The solution to the former problem is to prepare the reagent immediately before use. The solution to the latter problem requires that the stock solutions be handled in an air-extraction cupboard and the operator to take appropriate safety precautions such as wearing masks and gloves to protect against accidental contact or inhalation of the toxic fumes. Recent significant advances in the search for reliable PON1 lactonase activity assays may facilitate the measurement in a routine clinical chemistry laboratory setting. A new serum test based on this capacity of PON1, and employing 5-thiobutyl butyrolactone (TBBL) as a substrate, was recently proposed^[41,42]. TBBL is a synthetic chromogenic lactone that resembles the natural lipolactone substrate of PON1. The method enables PON1 activity to be measured using a more 'physiological-like' substrate.

SERUM PON1 ACTIVITY IN CHRONIC LIVER IMPAIRMENT

In chronic liver diseases, oxidative stress influences the pathophysiological changes leading to liver cirrhosis and to hepatocellular carcinoma. Since PON1 exerts a protective effect against oxidative stress, it is logical to find an association between this enzyme and liver impairment. Ferre *et al.*^[43] observed, in rats with carbon tetrachloride-induced fibrosis, that an inhibition of hepatic PON1 activity was an early biochemical change related to increased lipid peroxidation and liver damage. They investigated the relationships between hepatic microsomal PON1 activity, lipid peroxidation and the progress of the disease in this experimental model. They found that PON1 activity decreased while lipid peroxidation increased in carbon tetrachloride-administered rats while the addition of zinc, which possesses antioxidant and anti-fibrogenetic properties, was associated with enhanced PON1 activity and a normalization of lipid peroxidation. This study suggested that PON1 activity may be involved in the defence against free radical production in liver organelles.

Pioneer studies in the 1970's observed for the first time a significant decrease in serum PON1 activity in small groups of patients with liver cirrhosis^[44,45]. This results were confirmed by Ferre *et al.*^[46,47] in a wider series of patients with various degrees of chronic liver damage. These latter studies noted a significant decrease of serum

PON1 activity in patients with chronic hepatitis, and an even greater decrease in cirrhotic patients, compared to a control group. In alcoholic patients, the effects of alcohol intake on serum PON1 levels depend on the degree of liver dysfunction. In a study conducted in chronic alcohol abusers, subjects were classified into several sub-groups according to their degree of liver disease. The results demonstrated that serum PON1 activity was decreased in alcoholic patients, and that the magnitude of the alteration was related to the degree of liver damage^[48]. These findings differ from those described in normal volunteers reporting moderate alcohol consumption, and in whom serum PON1 activity and HDL cholesterol were found to be slightly increased^[49]. Changes in serum PON1 activity has also been studied in relation to outcomes of liver transplantation in patients with severe liver disease^[50]. The serum PON1 activity was low, but tended to increase, in liver transplanted patients when the hepatic arteries had become blocked. Since PON1 activity is closely related to the recovery of liver function, its measurement could provide more accurate information on the success, or otherwise, of the liver transplant.

Serum PON1 measurement has been proposed as an useful test for the evaluation of the degree of liver impairment. Clinical diagnosis of chronic liver impairment and/or liver fibrosis is currently conducted *via* the invasive procedure of needle biopsy followed by histological evaluation. This procedure has important drawbacks, including a significant mortality rate (1/10000-1/1000), sampling error, and subjectivity. Therefore, the development of non-invasive tests for the diagnosis of liver disease and the extent of the disease is an important goal of current research. Unfortunately, most of the individual laboratory tests to assess liver impairment have low specificity and sensitivity and, hence, the standard approach is to perform a battery of several tests followed by an algorithmic evaluation of the results. It is for this reason that several years ago, Ferre *et al*^[46] proposed the addition of serum PON1 paraoxonase activity measurement as a biomarker of liver impairment. Serum PON1 measurement has an important feature in that the measured value is inversely related to the degree of liver derangement i.e. it decreases while most of the standard laboratory test values increase with the extent of the disease. Thus, PON1 measurement makes an additional contribution in improving current algorithms, and the ratios between tests. These authors estimated, by multiple logistic regression analysis, that the addition of paraoxonase measurement to a battery of standard liver function tests increased the overall sensitivity up to $\geq 90\%$, while keeping the specificity close to 100%. However, the measurement of this enzyme is, to-date, restricted to research laboratories and has not been extensively applied as yet in routine clinical chemistry laboratories, due to the problems associated with the use of paraoxon as a substrate. These drawbacks preclude full automation of PON1 measurement and, as such, can rarely be justified for inclusion in panels of standard biochemical tests. The recent development of new assays, such as the TBBL lactonase assay, makes this proposal closer to practical development. The TBBL assay has been

shown to be equivalent to the paraoxon assay in terms of diagnostic accuracy^[42], but with better safety of the TBBL substrate for the laboratory worker, and makes the lactonase measurement a strong candidate for inclusion into routine clinical laboratory testing of liver impairment, or for the study of other diseases involving oxidative stress.

CONCLUSION

Research into paraoxonases has flourished over the last 10 years. It seems now evident that PON1 is a lactonase with the ability to degrade lipid peroxides in lipoproteins and in cells, and that plays a protective role against oxidative stress and inflammation, which are key processes involved in the pathophysiology of chronic liver diseases. In the years to come, more reliable, practical, and accurate methods to measure PON activity and concentration will become available and these will facilitate more research in this field, and also enable the addition of PON measurement to the battery of routine analyses in clinical chemistry laboratories.

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TOPIC HIGHLIGHT

Yusuf Bayraktar, Professor, Series Editor

Five years' experience with capsule endoscopy in a single center

Taylan Kav, Yusuf Bayraktar

Taylan Kav, Yusuf Bayraktar, Department of Medicine, Division of Gastroenterology, Hacettepe University School of Medicine, Sıhhiye, Ankara 06100, Turkey

Author contributions: Kav T and Bayraktar Y contributed equally to this work.

Correspondence to: Taylan Kav, MD, Department of Medicine, Division of Gastroenterology, Hacettepe University School of Medicine, Sıhhiye, Ankara 06100, Turkey. tkav@hacettepe.edu.tr
Telephone: +90-312-3051712 Fax: +90-312-4429429

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Georgia, BBR 2544, Medical College of Georgia, 15th Street, Augusta, United States

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Abstract

Capsule endoscopy (CE) is a novel technology that facilitates highly effective and noninvasive imaging of the small bowel. Although its efficacy in the evaluation of obscure gastrointestinal bleeding (OGIB) has been proven in several trials, data on uses of CE in different small bowel diseases are rapidly accumulating in the literature, and it has been found to be superior to alternative diagnostic tools in a range of such diseases. Based on literature evidence, CE is recommended as a first-line investigation for OGIB after negative bi-directional endoscopy. CE has gained an important role in the diagnosis and follow-up of Crohn's disease and celiac disease and in the surveillance of small bowel tumors and polyps in selected patients. Capsule retention is the major complication, with a frequency of 1%-2%. The purpose of this review was to discuss the procedure, indications, contraindications and adverse effects associated with CE. We also review and share our five-year experience with CE in various small bowel diseases. The recently developed balloon-assisted enteroscopies have both diagnostic and therapeutic capability. At the present time, CE and balloon-assisted enteroscopies are complementary techniques in the diagnosis and management of small bowel diseases.

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Key words: Capsule endoscopy; Small bowel diseases; Obscure gastrointestinal bleeding; Crohn's disease; Celiac disease; Indications; Contraindications

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INTRODUCTION

Examination of the small bowel (SB) has been considered a challenge for several anatomical (i.e. distance from external orifices, length) and physiological (i.e. active peristalsis) reasons. Conventional techniques of endoscopy are limited by length while radiologic examinations, such as barium studies, are insensitive for the evaluation of pathology in the SB. An ingestible miniature camera device capable of obtaining images of the whole small intestine was developed due to a need for the exploration of this "final frontier". Video capsule endoscopy (CE) is a breakthrough in medical history for noninvasive imaging of the entire small intestine^[1-3]. It was first introduced in 2000, and since then more than 700 studies have been published, which is indicative of its ease and the widespread acceptance of this new diagnostic tool^[4]. According to reports by Given Imaging, more than 650 000 CEs have been performed, representing an increase in the utilization of this technology of approximately 15% over the previous year^[5]. Problems with reimbursement, physician training, time requirements for interpretation and lack of therapeutic capability limit the further widespread use of this technology.

A wide range of uses for CE has been reported in the literature, but the majority of the studies have aimed to evaluate the cause of obscure gastrointestinal bleeding (OGIB). Recent studies showed the superiority of CE over conventional methods, but passive features such as inability to insufflate the bowel and to biopsy and lack of therapeutic capability have generated a debate on its advantages^[6-14]. Newly developed balloon assisted enteroscopes are also available and have the potential to outscore CE in terms of diagnostic indications and therapeutic applications.

The purpose of this article was to review and share our institution's results using small bowel CE, with special reference to the existing literature.

PROCEDURE

Technical features of the capsule

The Given M2A (Given Imaging; Yoqneam, Israel) video CE is a pill-shaped wireless device with a slippery coating for easy ingestion and measures 11 mm × 26 mm. It is composed of a white light-emitting diode as light source, lens, imaging chip, batteries and a radio transmitter with internal antenna. The image field is 140 degrees and magnification is × 8^[4]. Once swallowed, the capsule moves thorough the intestine *via* peristalsis and is excreted in the stool. The camera takes two images per second as it sweeps the intestine and transmits these to eight lead sensor arrays, arranged in a specific manner and taped to the anterior abdominal wall, connected to a recording device in the belt for the duration of the battery life, which is 6-8 h. Once the study is completed, the recording device and sensor arrays are removed and the images (50 000-60 000 images total) are downloaded to a computer with reporting and processing of images and data (Rapid, Given Imaging) software that displays the video images on a computer monitor. This software includes a localizing system, blood detector and some features to assist the interpreter. The suspected blood indicator is quite good at detecting active bleeding, but is not so useful at detecting other lesions and does not replace careful examination of the CE. It is recommended that patients avoid magnetic fields such as magnetic resonance imaging (MRI), and metal detectors until the capsule is excreted in the stool, which usually occurs in 24-48 h.

Bowel preparation

Pre-procedure bowel preparation is a controversial issue. Some favor the bowel preps and prokinetics. Incomplete SB transit during the examination occurs in about 20% of patients^[6]; however, according to data from the international conference on capsule endoscopy, it was suggested that there was no need for routine use of bowel preparations^[11]. We performed CE in an ambulatory outpatient setting, but there were some inpatients. All of the patients undergoing CE examination had bowel preparations before the procedure. Each patient was administered 3 L of polyethylene-glycol solution for bowel cleansing. Patients fasted overnight for at least 12 h before taking the capsule. After ingestion of the capsule, patients were allowed to drink clear liquids after 2 h and eat a light meal after 4 h and were observed for 8 h at the study site.

INDICATIONS

Capsule endoscopy is mainly indicated for the evaluation of SB diseases, particularly for the diagnosis of OGIB. CE can be used in a variety of conditions including Crohn's disease (CD), malabsorption, chronic diarrhea, evaluation

Table 1 Indications and contraindications of capsule endoscopy

Indications	Contraindications
Small bowel	Absolute
Obscure gastrointestinal bleeding	Bowel obstruction
Overt GI bleeding	Extensive and active Crohn's
Occult (positive FOBT)	Disease ± strictures
Evaluation of iron deficiency anemia	Intestinal pseudo-obstruction
Crohn's disease	Young children (< 10 years)
Suspected Crohn's disease	Relative
Indeterminate colitis	Cardiac pacemakers
Assessment of mucosal healing	Implanted electromedical
Determine post-operative recurrence	Devices
Abdominal pain	Dysphagia
Graft-versus-host disease	Previous abdominal surgery
Surveillance of polyposis syndromes	Pregnancy
Celiac disease	Diverticulosis
Suspected small bowel tumors	
Follow-up of small intestine transplantation	
Evaluation of abnormal small bowel imaging	
Evaluation of drug induced injury	
Esophagus	
Barrett esophagus	
Esophagitis	
Variceal evaluation	

of refractory iron deficiency anemia, abdominal pain, polyposis syndromes, celiac disease, and detection of SB tumors. Graft versus host disease (GVHD) and follow-up of small intestine transplantation are rare indications, but our experience thus far did not include such patients. CE with high frame rate (PillCam Eso, Given Imaging) can be used for esophageal disorders, such as noninvasive evaluation of esophageal varices, esophagitis and Barrett's esophagus^[11]. Table 1 shows the indications and contraindications for Capsule Endoscopy.

We reviewed our database in a retrospective evaluation of the characteristics and findings of patients who underwent CE examination between 2003 and 2008. All patients had upper and lower GI endoscopies before the CE study. There was no clinical sign of intestinal obstruction, but patients with suspected CD had radiologic examinations to exclude obstruction. A total of 120 CE examinations were performed from 2003 to 2008 for various indications. The average patient age was 47.7 ± 18.2 (min: 13 - max: 97), 45 were female (37.5%) and 75 male (62.5%). The CE completely evaluated the entire SB in 89 patients (74.2%). Indications for CE were OGIB (57.5% of cases), diarrhea (15%), abdominal pain (5.8%), other indications such as known CD, and surveillance for polyposis syndromes. CE study was normal without any finding in 22.5% of patients. We did not use CE for esophageal disorders and there were no findings suggestive of esophageal diseases.

OGIB

Gastrointestinal bleeding is a common problem encountered by gastroenterologists during clinical practice. Proximal and distal bleeding sites are mostly identified by means of endoscopy and colonoscopy. The bleeding

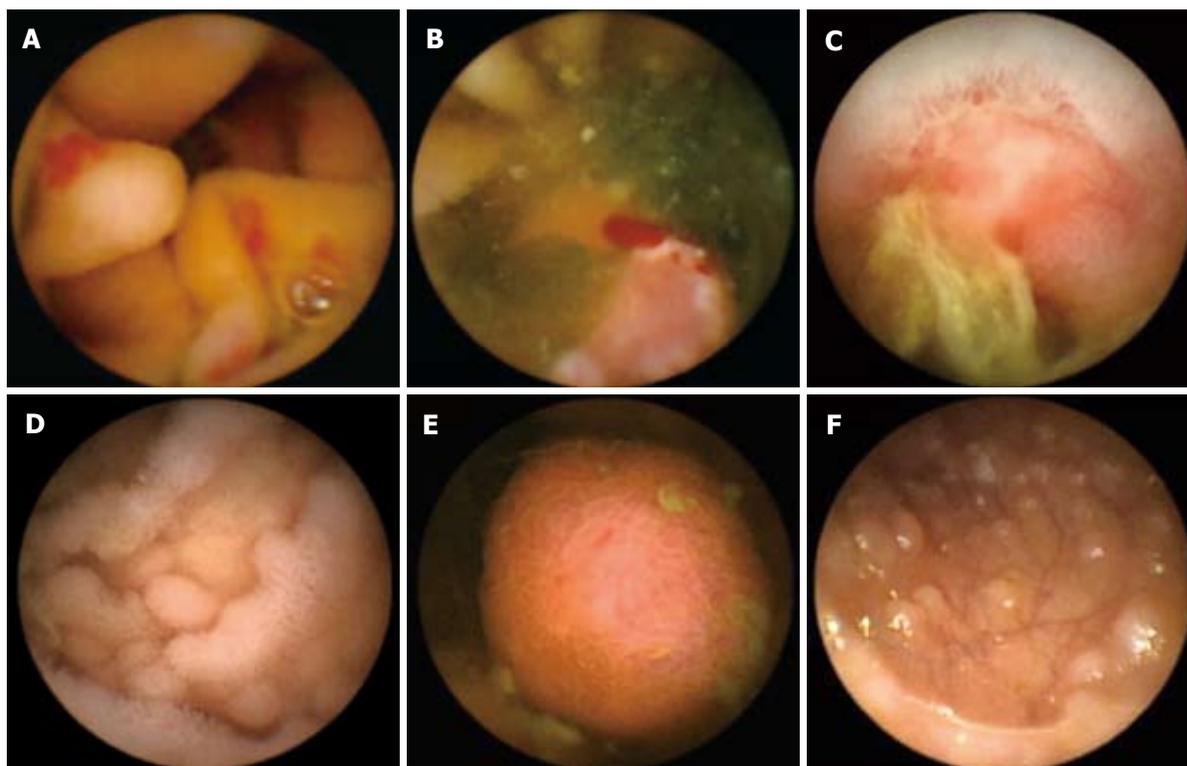


Figure 1 VCE images of lesions found in patients with obscure-overt GI bleeding. A: Multiple angiodyplasias in the jejunum; B: A jejunal mass with active bleeding; C: An ileal ulcer in a patient with newly diagnosed Crohn's disease. VCE images of small bowel polyps; D: Benign lymphoid hyperplasia located diffusely through the GI tract in a patient with CVID; E: A jejunal polyp in a patient with peutz-jeghers disease; F: Multiple small polyps in the ileum in the same patient depicted in Figure 1 E.

source is not identified in 3%-5% of cases despite the utilization of multiple studies^[15,16]. OGIB is defined as bleeding from an unidentified source that persists and recurs after a negative endoscopy examination^[16,17].

Obscure GI bleeding is the most common indication for CE examination. CE has a high diagnostic yield in OGIB, which may lead to early diagnosis and revision of the management strategy. CE facilitates effective decision-making regarding subsequent investigations and treatments^[4].

Diagnostic yield of CE for OGIB varied between 31% and 91%^[9,17-31]. Lema and Ruano-Ravina^[32] reviewed the published studies of CE for OGIB and reported that sensitivity ranged from 79% to 95% and specificity from 75% to 100%. The positive predictive value (PPV) varied from 94% to 100% and the negative predictive value (NPV) from 80% to 100%. CE led to a change in therapeutic management in 9%-77% of patients. A recent study by Albert *et al*^[33] reported that CE detected the bleeding source in 76.8% of patients.

The diagnostic yield of CE in OGIB depends on the type of bleeding. Pennazio *et al*^[17] found that the highest yield of CE was in patients with active bleeding (92.3%) compared to those with obscure occult bleeding (44.2%). Researchers observed a reverse relationship between findings and time after last bleeding episode. The longer the time from last bleed, the lower the diagnostic yield. Do the lesions discovered by CE have any bleeding potential or clinical importance in terms

of management change? Saurin *et al*^[18] showed that CE detects more lesions, but only half of them have true bleeding potential.

Several studies examined the diagnostic role of CE in OGIB and mostly compared the diagnostic yield of CE to other diagnostic modalities. CE is superior to other techniques in diagnosing the source of bleeding. The yield for CE is 63% and 67% compared with 28% for push enteroscopy (PE) and 8% for barium study^[34].

Obscure GI bleeding was the most common (57.5% of cases) indication for CE study in our cohort. SB ulcerations were found in 25.8% of patients. Angiodysplasias were present in 12.5% of cases (Figure 1A). Active bleeding was observed in 8.3% of patients. Figure 1B shows a jejunal mass, which was found to be adenocarcinoma, with active bleeding. Diagnostic yield of CE for OGIB was 72.5% in our series. We have been performing single balloon enteroscopy (SBE) (Olympus; Tokyo, Japan) and a few patients underwent both CE and SBE. CE revealed angiodysplasias in two patients with OGIB who were treated with argon plasma coagulation during SBE examination. Balloon assisted enteroscopy and CE should be used as complementary studies. It is advisable to use CE to detect lesions and direct enteroscopy for the therapeutic interventions.

Crohn's disease

Crohn's disease is a chronic inflammatory disease that can involve any part of the GI system, and disease is

confined to the SB in about one-third of the patients. There is no single test to diagnose CD completely, so CD diagnosis can be established with a combination of clinical, endoscopic and histological findings. Most imaging studies lack sensitivity to identify early changes, and endoscopy does not allow total examination of the bowel. CE is able to identify mucosal changes before other technologies. It has a valuable role in the evaluation of the SB in patients with suspected or known CD. The use of CE in the diagnosis of small bowel CD has been examined in several studies. Triester *et al.*^[35] compared the yield of CE with other modalities in patients with suspected small bowel CD. Diagnostic yield of CE was 63% compared with 23% for barium radiography. When compared with ileocolonoscopy, CE had a higher yield (61% *vs* 46%). Compared with PE, CE had a 38% higher yield, and when compared with CT enterography, the yield of CE was 69% to 30%. Due to its high diagnostic yield, CE will have a very important place in the diagnostic workup of patients with CD, but more studies are needed to make such suggestions. Triester *et al.*^[35] reported in their meta analysis that there was no statistical significance in the incremental yield between CE and other diagnostic modalities in patients suspected of having CD. However, there was a significant difference in yield of CE over alternative methods in patients with known CD who were being evaluated for SB recurrence^[35]. Yield of CE is low when performed in patients with abdominal pain alone; when other criteria are added, this yield is increased^[34].

Capsule endoscopy can be used for the assessment of mucosal healing after treatment. The only limitation of CE is its inability to offer biopsy for histological examination. A scoring system has been proposed to evaluate CD on the basis of CE findings of villous structure, ulceration and stenosis. Each variable is assessed by size and extent of the change^[36]; however, further studies are needed to clarify the helpfulness of this system. The score provides a common language to quantify mucosal changes associated with any inflammatory process. The index does not diagnose or measure a disease, it measures mucosal change. In addition, this scoring index does not have the discriminatory ability to differentiate between illnesses. This index could be helpful in determining mucosal healing after therapy in CD^[34]. Mucosal breaks and aphthous ulcers or erosions are also seen in asymptomatic healthy volunteers. Since non-steroidal antiinflammatory drugs (NSAIDs) may cause ulcerations resembling those of CD, patients should be advised to stop such drugs at least one month before the CE examination^[10]. It is difficult to differentiate these findings with the presence of CD.

Mucosal ulcerations were the most common finding in our patient series, determined in almost one out of four patients. CD was the third most common indication for CE study (6.7% of patients). Patients with CD had severe ulcerations and two patients had strictures

that resulted in regional transit abnormality. However, no capsule retention occurred in this group. Moreover, CE changed the management strategy in 10% of patients with a new diagnosis of CD. Another interesting finding was that 37.5% of the patients diagnosed as suspected CD did not have complete examination. Nonspecific jejunoileitis and NSAID-induced erosions were observed in 6.7% of patients. Figure 1C shows a mucosal ulceration.

Celiac disease

Celiac disease is an immune-mediated disease characterized by chronic SB inflammation that may result in mucosal atrophy, malabsorption and related clinical manifestations. Diagnosis is based on the combination of serologic, endoscopic and typical histological changes of the SB biopsy in clinically suspected patients. Its prevalence is around 1% in the United States. There are four endoscopic changes suggestive of villous atrophy: loss of mucosal folds, mosaic mucosal pattern, scalloping of the duodenal folds and nodularity of the mucosa^[37]. It is no surprise that CE provides high resolution images that contain such changes. Rondonotti^[38] evaluated 43 patients with signs or symptoms suggestive of celiac disease and positive serological markers. Patients underwent both CE and upper GI endoscopy. Characteristic histological changes were observed in 32 patients. Using this as a gold standard, 87.5% of patients were diagnosed by CE. Mucosal changes beyond the duodenum were detected in 18 (66.6%) patients and in 3 (11.1%) patients the whole SB was affected.

Another newly published study, searching for celiac disease in older adults, also showed that duodenal mucosa was normal in appearance on CE in 71% of patients, but classic abnormalities of celiac disease were present distally^[39].

Overall, CE can detect endoscopic markers of celiac disease. In addition, CE seems to be able to recognize the extent of disease and may be a tool for follow-up. CE has a high sensitivity (range, 70%-95.2%), specificity (range, 63.6%-100%) and high PPV and NPV (96.5%-100% and 71.4%-88.9%, respectively)^[38,40-43]. When an atrophic pattern is detected by CE, the patient has a high probability of having celiac disease^[37]. CE has also been reported to be able to demonstrate diseases such as adenocarcinoma, lymphoma or ulcerative jejunoileitis, which may complicate the course of celiac disease. A limitation is that CE is able to detect Marsh III lesions, which are associated with clear mucosal abnormalities, but may not distinguish between Marsh I and II lesions^[37]. At present, CE is an alternative to endoscopy with biopsy in patients with suspected celiac disease who do not consent to the conventional methods.

Chronic diarrhea was the second most common indication for CE study in our series. Half of these patients did not have any condition that may cause diarrhea. Lymphoid hyperplasia and nodularity were observed in 6.7% of patients. Lymphoid hyperplasia due to common variable immune deficiency was detected in three pa-

tients. Celiac disease was investigated in only one patient but CE examination was completely normal. One patient with iron deficiency anemia had mucosal atrophy on CE examination and was diagnosed as having celiac disease. Figure 1D shows benign lymphoid nodular hyperplasia in a CVID patient.

Small bowel tumors and polyps

Capsule endoscopy is a major advance in the diagnosis of SB tumors. Before the introduction of CE, malignant neoplasms of the SB were often diagnosed at a later stage of the disease, mostly during the work-up of obstructive symptoms. Diagnosis is delayed because conventional imaging techniques fail to detect small neoplasms in almost half of the patients. SB tumors are a rare disease, accounting for 1%-3% of all primary GI tumors. SB mass lesions are responsible for OGIB in up to 10% of patients^[44-48]. Early clinical studies of CE have reported a frequency of SB tumors ranging between 6% and 9%^[49-54]. This has led to an idea that CE doubled the rate of diagnosing SB tumors. However, a recent multicenter European study showed that the frequency of SB tumors was 2.4% and the most common indication for CE was OGIB^[55,56]. SB tumors appear as masses or polyps in most patients and ulcer or stenoses in a minority of patients. It is not possible to distinguish the type of tumor based only on CE pictures. Most of the tumors reside in the mid SB^[56].

Capsule endoscopy is also useful for the surveillance of polyps in patients with inherited GI polyposis syndromes (familial adenomatous polyposis and Peutz-Jeghers syndrome), who are at increased risk of developing polyps in the SB. Several studies comparing the yield of CE to other imaging modalities in patients with polyposis syndromes have shown that CE is accurate in the detection of polyps. The same studies also emphasized that CE is not reliable for sizing and determining localization of polyps^[57-60]. The duodenum is a potential blind point of CE because the capsule passes quickly with tumble and results in inadequate examination. Wong *et al*^[61] reported that CE underestimated the total number of polyps and did not reliably detect larger polyps in that portion.

In our series, SB masses were diagnosed in 4.2% of patients who had tumor resection, and two patients had benign tumors. CE examination was done in only one patient with Peutz-Jeghers disease. CE revealed a few proximal jejunal polyps measuring < 2 cm (Figure 1E and F). Subsequent enteroscopy showed multiple jejunal polyps with diameters up to 8 cm. CE definitely has a potential for use in patients with polyposis syndromes, but more studies are needed.

Other indications

Abdominal pain is one of the most common symptoms of patients referred to the gastroenterologist. Use of CE for the evaluation of abdominal pain is debated. Although some serious causes are identified in such patients, CE is mostly unyielding. If patients with other signs and symptoms of inflammation were selected, than

the diagnostic yield was considerably higher^[62].

Capsule endoscopy may be helpful in the diagnosis of the following diseases: surveillance for NSAID side effects, Henoch-Schönlein purpura, indeterminate colitis, protein losing enteropathy, intestinal lymphangiectasia, Meckel's diverticulum, follow-up of SB transplantation, GVHD, and bowel changes in refractory pouchitis^[1-10,62].

COMPLICATIONS, LIMITATIONS AND SAFETY ISSUES OF CAPSULE ENDOSCOPY

Capsule endoscopy is a safe and well-tolerated procedure for patients, with very low complication rates. Contraindications to CE include the presence of intestinal obstruction, fistulas and strictures. Swallowing abnormalities and esophageal stricture are other contraindications for the procedure. Capsule retention is the major complication of CE. Retention is defined as the indefinite presence of a capsule in the SB. This is different from slow transit, incomplete transit or regional transit abnormalities. In these cases, the capsule stays in the ileum but ultimately passes *via* peristalsis. Retention can cause symptoms of SB obstruction that in turn lead to need for endoscopic or surgical removal of the capsule^[63,64].

Retention risk is high in patients with known CD, NSAID stricture, radiation enteritis and SB tumors. The capsule retention rate ranges from 0% to 13%. The rate of retention in patients with OGIB is 5% and in suspected CD 1.4%, and it can be as high as 8% in patients with known CD. Interestingly, no capsule retention was reported in healthy volunteers. The overall frequency of capsule retention is usually 1%-2%^[10,63,64]. A negative SB series does not prevent capsule impaction^[17]. It is advisable to perform abdominal radiographs within two weeks to identify capsule retention if the capsule did not enter the colon. Therapeutic intervention can be instituted anytime unless the patient becomes symptomatic^[4].

The patency capsule (Agile Patency System, Given Imaging Ltd; Yoqneam, Israel) has been developed for the detection of high-risk patients before the procedure. This capsule is identical to the video capsule, with the same dimensions, and is made of lactulose and 5% barium, which make the capsule radiopaque and it dissolves spontaneously after 40 h. The capsule has a radiofrequency identification tag that enables easy detection by a special handheld device. In a recent study that included patients with known strictures, no CE retention occurred if the patency capsule passed safely^[65]. Although there are promising data on patency capsule use before CE, it is still not definitive to predict capsule retention based on results of barium studies or patency capsule.

Another theoretic risk is electromagnetic interference with implantable medical devices, pacemakers, *etc.* In a small series of patients, no adverse cardiac effect or

image distortion due to interference was noted. Large sample sized studies are needed to confirm the safety of capsule in this context^[66].

Reading the procedure is a time-consuming process and reading time is another limitation of this procedure. The optimal review rate is 15 images/s and it takes over 1 h to read a full 8-h procedure^[62]. The reliable interpretation of the CE procedure requires experienced readers (experience of reading at least 20 studies).

Another clinical problem is sizing and locating SB lesions, since location and size are important findings for subsequent management. CE underestimates the number of SB polyps and does not reliably detect large polyps^[61]. Technical problems related to the battery and failure of image downloading are also reported. The overall rate of technical failure is around 9%^[10]. Incomplete study occurs due to delayed gastric emptying, previous SB surgery, hospitalization and poor bowel cleansing. A gastric transit time longer than 45 min was identified as a risk factor^[67]. Reported incompleteness rates vary between 0% and 50%, approximately 20% to 30% in most studies^[67]. Effect of prokinetic drugs on completion rates is uncertain. Real time viewers of CE may help to identify prolonged gastric stay and in such case, endoscopy can be done to push the CE into the SB.

The overall miss rate of CE is about 11%, ranging between 0.5% for ulcerative disease and 18.9% for neoplastic disease. Of course, this rate is much lower than conventional examinations^[47]. Inability to take biopsy or perform any therapeutic procedure is also a limitation of the CE, which makes balloon assisted enteroscopies a good choice for a number of indications.

In our patient cohort, the most common cause for an incomplete examination was premature battery failure in 20 patients (16.7%), followed by technical problems, of the capsule itself, in seven patients (5.8%). No complication related to the CE procedure was observed. There was no capsule retention event. Two patients' studies showed regional transit abnormality. One was due to severe CD with stricture, and the other patient had an ileal adenocarcinoma that was diagnosed after operation for ileal perforation. Although there was a temporal relation of perforation to CE study (2 d after the study), no capsule was detected in the preoperative radiograms and CE was not the likely cause of perforation. There was no patient with an implantable cardio defibrillator or pacemaker among our cohort, but it seems safe to use the capsule in these patients. Based on our data, we can say CE is a safe procedure. Placing the capsule directly in the duodenum by means of dedicated devices or endoscopy may lower the incomplete examination rate. However, by doing so, we can miss esophageal and gastric disorders in which CE is also informative. Therefore, if selective placement of the capsule is preferred, the proximal GI tract should be carefully re-examined. Higher capture rate and longer battery life could resolve these obstacles.

OTHER TYPES OF CAPSULE ENDOSCOPE

The Olympus Endo Capsule (Olympus; Tokyo, Japan) has been in the Turkish market for a while, but there is not yet sufficient experience with its use. It differs from the PillCam by having a high resolution image chip and an external real time viewer. There are additional SB capsule systems that are not currently available in Turkey. One is from China, the OMOM pill (Jinshan Science and Technology; Chongqing, China) and there is also a Korean model (MicroCam, Intromedic; Seoul, Korea)^[68,69]. Both the capsule endoscopes are similar to the PillCam in terms of battery life, dimensions, field of view and picture intervals. The first trials of the MiRo capsule and OMOM capsule were published in 2008 but they were without FDA approval. The MiRo capsule uses a novel telemetry technology known as "electric-field propagation", which uses the human body as a conductive medium for data transmission. A pair of gold plates coated on the surface of the capsule acts as a transmitter. This is claimed to be superior in terms of battery life since the CE has few power-consuming components. Bang *et al.*^[68] used this new capsule in 45 healthy adults and it produced good image quality and capture rates. This capsule may also be used for the colon due to the long battery life. The first trial of the OMOM CE revealed comparable results to the PillCam. The authors express the cost advantage over other CEs, which could affect the choice of CE systems because of reimbursement problems^[69]. PillCam SB2 and EndoCapsule have real time viewer capability that may shorten the examination once the cecum is seen. PillCam ESO was specially designed for investigation of esophageal disorders. It may be an accurate noninvasive method for detection of esophageal varices and portal hypertensive gastropathy, but it may not be suitable as a screening tool for Barrett's esophagus^[12]. PillCam COLON is bigger than the standard PillCam SB capsule (11 mm × 31 mm). It was developed for detection of colonic neoplasia. It is a promising tool but further studies and improvements are needed before its regular use^[70].

In summary, capsule endoscopy is a new diagnostic modality for the diagnosis and management of GI disorders. It is a simple and well-tolerated procedure. Capsule retention is the major complication. Care must be taken in patients with symptoms suggesting partial obstruction and CD. SB series and computerized tomography enteroclysis before CE may reveal stenosis. The newly developed patency capsule may be an alternative for detection of stenoses.

The value of CE in patients with OGIB appears to be high and is supported by high yields in the literature. CD and celiac disease appear to be areas where use of CE would be helpful. There may also be an indication for CE in CD surveillance and follow-up. The diagnostic role of CE extends beyond the SB. PillCam ESO and COLON showed promising outcomes in diagnosing esophageal and colonic diseases. More

research is needed to explore the feasibility of CE in these contexts.

Blind spots of CE such as the duodenum should be examined by a second look endoscopy before the CE procedure, especially in patients with OGIB. After negative endoscopic examinations, CE should be recommended as a first-line investigation over balloon assisted enteroscopies in view of its noninvasiveness, higher probability of visualizing the entire small intestine and the similar diagnostic yield of both investigations. Such an approach may decrease the time between diagnosis and intervention. A second look CE may reveal more findings in up to 35% of patients who had prior nondiagnostic CE.

CONCLUSION

The newly announced CEs would fire up the competition for new innovations and possible cost reductions, making possible the widespread use of this technology. Improvement in capsule design for better luminal visualization by coupling with a second backward camera, higher frame rates for viewing and longer battery life will definitely overcome the blind spots resulting in complete and detailed examination of the whole GI tract from the mouth to anus with just one capsule, as the capsule named M2A has denoted.

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Effects and mechanisms of silibinin on human hepatocellular carcinoma xenografts in nude mice

Wei Cui, Fan Gu, Ke-Qin Hu

Wei Cui, Ke-Qin Hu, Division of Gastroenterology and Chao Family Comprehensive Cancer Center, University of California, Orange School of Medicine, Orange, CA 92868, United States
Fan Gu, Department of Surgery, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Author contributions: Cui W and Gu F contributed to the study design, analyzed and interpreted the data, and prepared the manuscript; Cui W performed the experiments; Hu KQ designed the study, interpreted the data and wrote the manuscript.

Correspondence to: Ke-Qin Hu, Division of Gastroenterology, University of California, Irvine Medical Center, 101 The City Drive, Building 53, Suite 113, Orange, CA 92868, United States. kqhu@uci.edu

Telephone: +1-714-4566745 Fax: +1-714-4563283

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CONCLUSION: Silibinin reduces HCC xenograft growth through the inhibition of cell proliferation, cell cycle progression and PTEN/P-Akt and ERK signaling, inducing cell apoptosis, and increasing histone acetylation and SOD-1 expression.

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Key words: Apoptosis; Cell cycle; Chemoprevention; Hepatocellular carcinoma; Histone acetylation; Silibinin

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Abstract

AIM: To investigate the *in vivo* effects and mechanisms of silibinin on the growth of hepatocellular carcinoma (HCC) xenografts in nude mice.

METHODS: Nude mice bearing HuH7 xenografts were used to assess the anti-HCC effects and mechanisms of silibinin.

RESULTS: Silibinin resulted in a potent dose-dependent reduction of HuH7 xenografts in association with a significant decrease in Ki-67 and α -fetoprotein production, nuclear NF- κ B content, polo-like kinase 1, Rb phosphorylation, and E2F1/DP1 complex, but increased p27/CDK4 complex and checkpoint kinase 1 expression, suggesting that the *in vivo* effects of silibinin are mediated by inhibiting G1-S transition of the cell cycle. Silibinin-induced apoptosis of HuH7 xenografts was associated with inhibited survivin phosphorylation. Silibinin-reduced growth of HuH7 xenografts was associated with decreased p-ERK, increased PTEN expression and the activity of silibinin was correlated with decreased p-Akt production, indicating involvement of PTEN/PI3K/Akt and ERK pathways in its *in vivo* anti-HCC effects. Silibinin-reduced growth of HuH7 xenografts was also associated with a significant increase in AC-H3 and AC-H4 expression and the production of superoxide dismutase (SOD)-1.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents approximately 6% of all human cancers^[1,2]. The global incidence of HCC has risen significantly in the past 2 decades^[2], and prognosis of HCC is usually poor^[3]. Limited treatment options and the poor prognosis of HCC emphasize the importance of developing an effective chemoprevention for this disease.

Milk thistle (*Silybum marianum*) is a popular dietary supplement that has been reported to be safe, well-tolerated, and protects the liver from drug or alcohol-related injury^[4]. Silibinin, the major biologically active compound of milk thistle is a polyphenolic flavonoid, and a strong antioxidant and radical scavenger^[5-7]. Studies have demonstrated the inhibitory effects of silibinin on multiple cancer cell lines, including prostate, lung, colon, skin, and bladder cancers^[8-16]. Recently, we and Varghese *et al*^[17] reported the *in vitro* anti-HCC effects of silibinin^[18], however, additional studies are needed to further determine its *in vivo* inhibitory effects and mechanisms on the growth of human HCC. Clearly, nude mice bearing human hepatoma xenografts represent a suitable model for such a study^[19,20].

Plasma α -fetoprotein (AFP) has been used as a

clinical marker in the diagnosis and monitoring of HCC^[21-23]. We demonstrated that silibinin reduces AFP production and secretion from human hepatoma cells, but the AFP value in monitoring the *in vivo* anti-HCC effects of silibinin has not yet been tested.

Hepatocarcinogenesis is a complicated process that alters cell cycle progression and apoptosis. This may be mediated by altering signal transduction through cell cycle modulators, phosphatase and tensin homolog deleted on chromosome ten (PTEN), phosphatidylinositol 3'-kinase (PI3K) and Akt (PTEN/PI3K/Akt) pathways^[24-30], and histone acetylation^[31-33]. p-Rb, p21, and p27 are molecules that are involved in cell cycle regulation^[17]. Nuclear factor (NF)- κ B activation stimulates G1 to S phase progression and transcription of a wide variety of genes that are involved in cell proliferation^[34,35]. Checkpoint kinase 1 (Chk1) and polo-like kinase 1 (Plk1) are the up-stream molecules. Chk1 controls cell cycle progression and inhibits mitosis^[36]. Plk1 has long been recognized as a potential target for cancer therapy. Inhibition of Plk1 function may increase anti-tumor activity *in vivo*^[37]. It is unclear whether these pathways are involved in silibinin-mediated anti-HCC effects.

Studies have also indicated that signals related to reactive oxygen species (ROS) may play important roles in the development of HCC^[38]. The cellular levels of ROS are regulated by the antioxidant defense systems, that is, the enzymatic activities of superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase^[39]. Altered expression of SOD has been associated with the development and differentiation of HCC^[40,41]. Silymarin significantly increased suppressed SOD activity in patients with chronic alcoholic liver disease^[42]. However, it is unclear whether silibinin-reduced growth of HCC cells is mediated by enhanced expression of SOD.

In the present study, we demonstrated that silibinin can effectively inhibit growth of HuH7 xenografts, a human HCC cell line, in nude mice and examined the related mechanisms.

MATERIALS AND METHODS

Reagents

The cell culture media were the same, as previously reported^[19,20]. Anti-activated caspase-3 antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibodies against human Ki-67, AFP, p-Rb, E2F1, DP1, CD1, CDK4, p21 and p27, active caspase-9, phosphorylated-AktThr308, PTEN, AC-histone3 and AC-histone4, survivin phosphorylation (p-survivin), Plk1, Chk1, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The NF- κ B assay kit was from Panomics, Inc. (Redwood City, CA, USA).

Cell culture

HuH7 cells, a human HCC cell line, were cultured in DMEM with 10% FBS, as previously reported^[17,19,20], and used to establish HCC xenografts in nude mice as described below.

Development and treatment of nude mice bearing HuH7 xenografts

After subcutaneous inoculation of $5 \times 10^6/0.25$ mL of HuH7 cells^[19,20], the mice were randomized to 3 groups, 10 mice in each group, respectively. The control group received daily gavage of a vehicle solution. The other 2 groups received silibinin at a dose of 80 mg/kg per day and 160 mg/kg per day, respectively, started 24 h after inoculation. The silibinin dose was adjusted weekly based on changes in body weight. Tumor volumes were recorded weekly and the experiment lasted for 5 wk. At the end of the experiments, xenograft tumors were measured, isolated, and weighted after euthanasia. Blood specimens were collected from the tail vein and plasma was used to quantify AFP. Three HCC xenograft specimens which were closest to the mean volume were taken from each group. Three hundred milligram of tumor tissue from each xenograft was homogenized with lysing buffer. After centrifuging, the clarified supernatants were stored in -80°C and used for the experiments described below.

Quantification of plasma and tissue AFP levels

The plasma AFP level was quantified using an enzyme immunoassay (EIA) kit as previously reported^[20]. A standard curve was obtained using the manufacturer's internal control and was used to calculate plasma AFP levels.

Analysis of apoptosis

Apoptosis was quantified using an EIA kit, as previously reported^[19,20]. The degree of apoptosis was expressed based on the ratios of absorbance of the treated *vs* control xenograft tissue specimens.

Immunoprecipitation and Western blotting analysis

The supernatants of xenograft lysates were used to detect Ki-67, p21, p27, E2F1, CDK4, p-Rb, activated caspase-3 and caspase-9, PTEN, AC-H3, AC-H4, p-Akt, p-survivin and p-ERK, Plk1, Chk1, and SOD1. To determine whether silibinin could affect binding of p21 and p27 with CDK4, and binding of DP1 with E2F1, an immunoprecipitation technique was used. β -actin was used as an internal control. The relative amount of each protein was quantified by digitally scanning its hybridizing bands, and the optical density of the scanned Western blotting results, as previously reported^[19,20].

PTEN activity assay

PTEN protein was immunoprecipitated with 10 μL of rabbit anti-human antibodies at 4°C overnight, followed by the addition of 25 μL of anti-rabbit IgG-conjugated agarose beads at 4°C for 2 h. The phosphatase reaction was performed using the PTEN activity assay kit in accordance with the manufacturer's instructions^[19,20].

NF- κ B assay

NF- κ B was quantified using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Briefly, after incubation

Table 1 Silibinin reduced the frequency and volume of HuH7 xenografts in nude mice

Groups	Treatment	Tumor frequency (%)	Tumor volume (cm ³)
Group 1	Placebo	100	4.0 ± 0.9
Group 2	80 mg/kg per day	50 ^a	2.1 ± 0.3 ^a
Group 3	160 mg/kg per day	30 ^a	0.6 ± 0.2 ^a

^a*P* < 0.05 vs Group 1, *n* = 10/group.

for 1 h with 10 μL of the sample solution at room temperature the sample was washed 3 times, NF-κB p50 antibody (1:1000) was added and incubated for another hour at room temperature, followed by anti-rabbit HRP antibody (1:1000) and substrate reaction. The *A* absorbance at 450 nm was recorded.

Statistical analysis

The descriptive statistics are provided with mean ± SD. *t*-test was used to assess the effect (i.e. mean differences) of silibinin treatment on AFP production, apoptosis, as well as the scanning data of Western blots. *P* < 0.05 was considered statistically significant.

RESULTS

Silibinin reduced the frequency and volume of HuH7 xenograft growth

As shown in Table 1, silibinin treatment significantly reduced the frequency and volume of HuH7 xenografts in a dose-dependent fashion. The frequency of HuH7 xenografts was reduced by 50% in the group treated with silibinin 80 mg/kg per day and by 70% in the group treated with 160 mg/kg per day. The mean reduction in HuH7 xenograft volume was 48% in the group treated with silibinin 80 mg/kg per day and was 85% in the group treated with 160 mg/kg per day. The silibinin-reduced frequency and volume of HuH7 xenografts was associated with a significant decrease in Ki-67 expression (Figure 1A). These findings demonstrated that silibinin produced a significant *in vivo* inhibition of HCC growth through a reduction in HCC cell proliferation.

Silibinin-reduced growth of HCC xenografts was associated with decreased AFP production and secretion

Consistent with our previous *in vitro* report^[17], we found that silibinin treatment significantly reduced AFP levels in both xenograft tissue and plasma obtained from the mice (Figure 2A and B). This indicated that silibinin-reduced HuH7 xenograft growth was associated with decreased production of AFP in xenograft tissue and secretion of AFP into blood circulation.

Effects of silibinin on cell cycle progression

Uncontrolled progression of the cell cycle promotes multiplication of cancer cells. We have reported the inhibitory effects of silibinin on p-Rb formation *in vitro*^[17]. In the present study, we demonstrated that silibinin

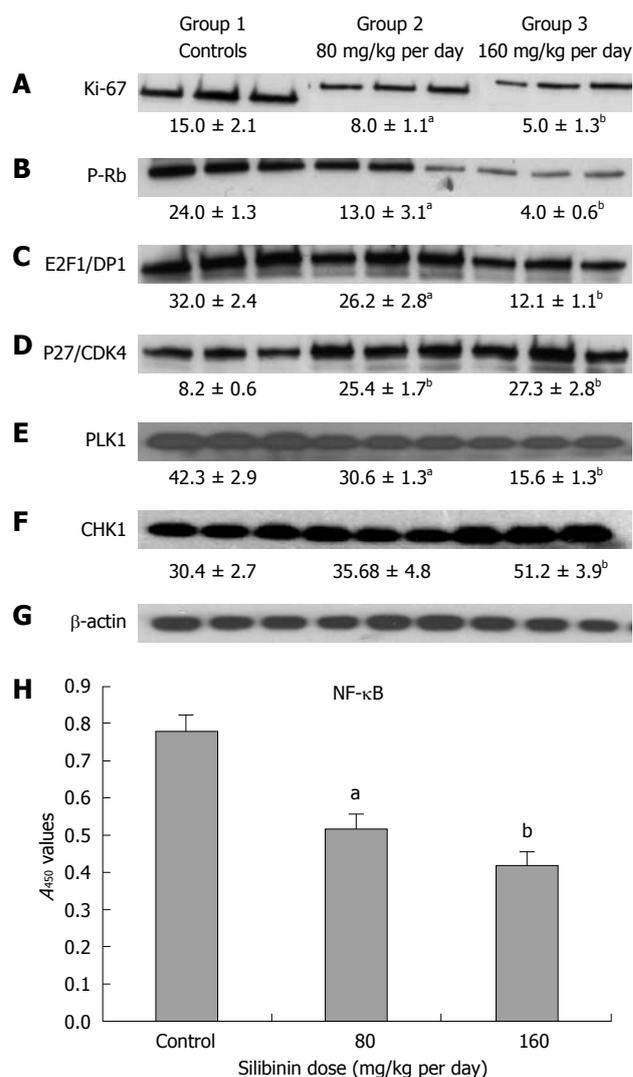


Figure 1 Effects of silibinin on proliferation and cell cycle progression in HuH7 xenograft tissue specimens. A: Silibinin inhibited Ki-67 expression; B: Silibinin inhibited Rb phosphorylation; C: Silibinin inhibited E2F1/DP1 complex formation; D: Silibinin increased p27/CDK4 complex formation; E: Silibinin inhibited Plk1 expression; F: Silibinin increased Chk1 expression; G: β-actin as internal control; H: Silibinin inhibited nuclear NF-κB content. ^a*P* < 0.05; ^b*P* < 0.01 vs control.

resulted in a significant and dose-dependent inhibition of p-Rb production (Figure 1B), which was associated with decreased E2F1/DP1 complex formation in HuH7 xenograft tissue (Figure 1C).

By binding to the cyclin/CDK complexes, cyclin dependent kinase inhibitors, such as p21 and p27, halt uncontrolled cell proliferation. P21/CDK4 and p27/CDK4 complexes are involved in the transition from G1 into S phase. Consistent with our *in vitro* report^[17], silibinin treatment significantly and dose-dependently increased p27/CDK4 complex (Figure 1D), but did not affect p21/CDK4 complex formation (data not shown) in HuH7 xenograft tissue. To further determine whether silibinin could also alter the levels of up-stream molecules that control cell cycle progression, the changes in Plk1, Chk1 and nuclear NF-κB were determined. As shown in Figure 1E-H, silibinin increased Chk1 expression, but inhibited Plk1 expression and nuclear NF-κB level.

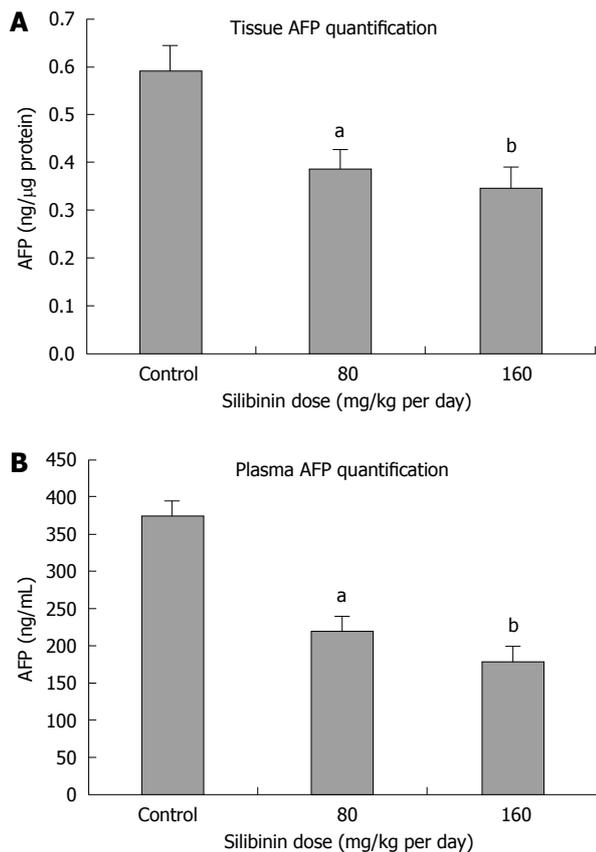


Figure 2 Effects of silibinin on AFP production and secretion. A: Silibinin reduced AFP production; B: Silibinin reduced AFP secretion. ^a*P* < 0.05; ^b*P* < 0.01 vs control.

Silibinin-reduced HuH7 xenograft growth was associated with increased apoptosis and reduced survivin phosphorylation

Apoptosis is another important mechanism that controls cancer cell growth. We previously reported that silibinin promotes HuH7 cell apoptosis *in vitro*^[17]. In the present study, we examined apoptosis in HuH7 xenograft tissue specimens. As showed in Figure 3A, we observed that silibinin significantly increased apoptosis in HuH7 xenograft tissue. To further define the mechanisms involved in the apoptosis pathway, activated caspase-3 and 9, Bcl-2, and p-survivin expression were assessed. We demonstrated that silibinin treatment significantly inhibited p-survivin (Figure 3B), as previously reported in the *in vitro* system^[17]. However, inconsistent with our previous *in vitro* findings, silibinin did not affect production of activated caspase 3 and 9, or Bcl-2 (data not shown).

In vivo effects of silibinin on p-Akt and P-ERK pathways

P-Akt and p-ERK pathways are involved in modulating cancer development and growth^[24-30]. Our previous study indicated that silibinin increased PTEN activity and reduced p-Akt expression *in vitro*^[17]. In the present study, we found that significantly reduced p-Akt production was only seen in HuH7 xenograft tissue treated with silibinin at a dose of 160 mg/kg per day, but not 80 mg/kg per day (Figure 4A). However, silibinin-

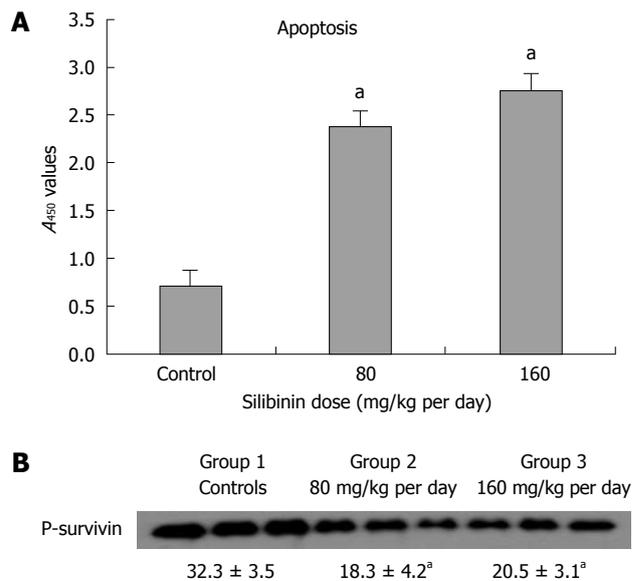


Figure 3 Effects of silibinin on apoptosis in HuH7 xenograft tissue. A: Silibinin induced apoptosis; B: Silibinin inhibited survivin phosphorylation. ^a*P* < 0.05 vs control.

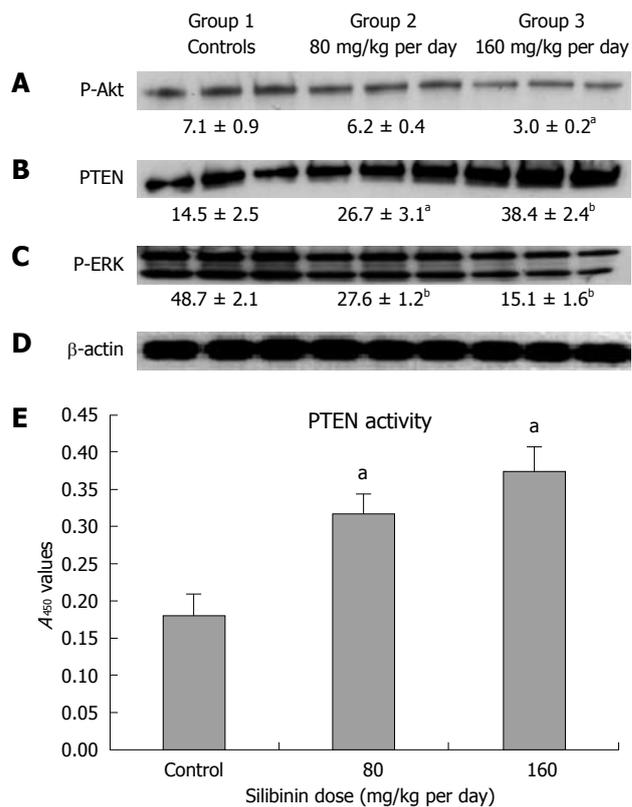


Figure 4 Effects of silibinin on P-Akt and P-ERK pathways in HuH7 xenograft tissue. A: Silibinin inhibited p-Akt expression; B: Silibinin increased PTEN expression; C: Silibinin inhibited P-ERK expression; D: β-actin as internal control; E: Silibinin increased PTEN activity. ^a*P* < 0.05; ^b*P* < 0.01 vs control.

reduced HuH7 xenograft growth was associated with a silibinin dose-dependent increase in PTEN production (Figure 4B) and its activity (Figure 4E). In addition, silibinin-reduced HuH7 xenograft growth was also dose-dependently associated with a decrease in p-ERK (Figure 4C).

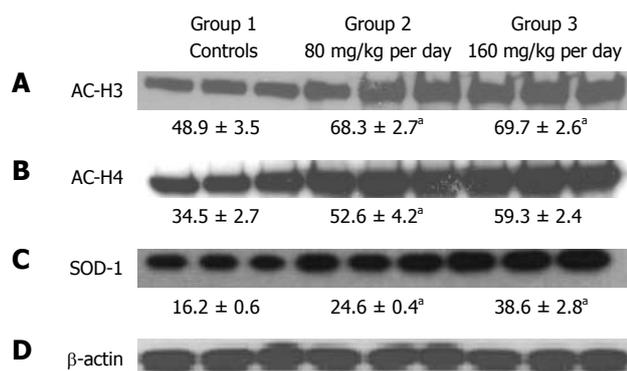


Figure 5 Effects of silibinin on AC-H3, AC-H4, and SOD1 in HuH7 xenograft tissue. A: Silibinin increased AC-H3 expression; B: Silibinin increased AC-H4 expression; C: Silibinin increased SOD1 expression in HuH7 xenograft tissue; D: β-actin as internal control. ^a*P* < 0.05 vs control.

In vivo effects of silibinin on histone acetylation

Histone acetylation plays an important role in controlling cell proliferation and cell cycle progression^[31-33]. Our previous *in vitro* results indicated silibinin increases AC-H3 and AC-H4^[17]. In the present study, we found that silibinin-reduced HuH7 xenograft growth was associated with significantly increased AC-H3 and AC-H4 production (Figure 5A and B). These results further confirmed the *in vivo* effects of silibinin on AC-H3 and AC-H4 production, indicating their potential role in HCC growth.

In vivo effects of silibinin on SOD1 expression

SOD1 is one of the most important enzymes in reducing ROS levels. It was reported that SOD1 may play a role in the effects of silymarin on alcoholic-induced liver injury^[42]. We demonstrated that silibinin-reduced growth of HuH7 xenografts was associated with a significant and dose-dependent increase in SOD1 production in the xenograft tissue (Figure 5C). Our results indicate a possible mechanistic role of SOD1 in silibinin-reduced growth of HuH7 xenografts.

DISCUSSION

HCC is one of the most common malignancies globally. A rise in incidence, limited treatment options, and poor prognosis of this disease emphasize the importance of developing effective chemoprevention for this disease. Silibinin is the major biologically active compound of milk thistle which has been reported to be safe and well-tolerated, and protects the liver from drug or alcohol-related injury^[5-7]. Recently, the potent *in vitro* anti-HCC effects of silibinin have been demonstrated^[15,17], which have provided us with a rationale to further define the *in vivo* effects and mechanisms of silibinin on HCC growth.

In the present study, we examined the *in vivo* effects and mechanisms of silibinin on HCC growth using the nude mouse model bearing human HCC xenografts following inoculation of HuH7 cells^[19,20]. We demonstrated that silibinin treatment resulted in a

significant dose dependent decrease in both frequency and mean volume of HuH7 xenograft growth. Our previous data recently reported the *in vivo* anti-HCC effects of silymarin^[43]. The silibinin dose used in our study was lower than that of silymarin^[43]. The fact that silibinin is a purified bioactive component from silymarin may explain why silibinin at the lower dose can achieve a potent anti-HCC effect. The anti-HCC effects of silibinin were associated with a significant reduction in Ki-67 expression, a biomarker of cell proliferation. These findings were consistent with our previous *in vitro* results and those of Varghese *et al.*^[17], and were further supported by the recent reported effects of silibinin on colorectal cancer^[14,17]. Thus, our data suggest that silibinin-reduced *in vivo* growth of human HCC xenografts is associated with down regulation of cell proliferation.

Plasma AFP has been widely used as a noninvasive biomarker for HCC^[21-23]. As we previously reported in the cell culture system, it was demonstrated that silibinin treatment resulted in a significant decrease in xenograft production and plasma levels of AFP which was correlated with growth inhibition of HCC xenografts. Since AFP overexpression has been associated with uncontrolled growth of HCC, our data provided additional *in vivo* evidence that silibinin-reduced growth of human HCC is associated with down regulation of cell proliferation. These findings also indicate the potential value of using plasma AFP as a non-invasive biomarker to determine the *in vivo* anti-HCC effects of silibinin.

Uncontrolled G1-S progression results in continued proliferation with potential malignant transformation and carcinogenesis. Increased E2F1/DP1 complex promotes cell cycle progression. Our results indicated that silibinin could significantly inhibit E2F1/DP1 complex formation in association with inhibition of HCC xenograft growth. Consistent with these findings, we also demonstrated that silibinin significantly decreased p-Rb expression, an important modulator that induces E2F1/DP1 formation.

P21 and p27 inhibit cell cycle progression by forming p21/CDK4 or p27/CDK4 complexes. Consistent with our previous *in vitro* report^[17], we demonstrated that silibinin significantly increased p27/CDK4 complexes in HuH7 xenograft tissue. Similar effects of silibinin were previously reported in a skin carcinogenesis model. In contrast to the *in vitro* data^[17], silibinin did not enhance p21/CDK4 complex formation in HuH7 xenograft tissue.

Chk1 is a critical enzyme in DNA damage-induced G2/M arrest, and blocks mitosis by phosphorylating Cdc25C and has been proposed as a novel tumor suppressor^[36]. Both Plk1 and NF-κB promote cell cycle progression. NF-κB mediates activation of cyclin D1 gene transcription, induces cell cycle progression and inhibits cell apoptosis^[34]. Inhibition of NF-κB activation induced an early G1 cell cycle arrest in primary rat hepatocytes^[35]. In human cells, Plk1 has

been implicated in the regulation of different processes, including mitotic entry, spindle formation, and plays a role at multiple points during the restart of the cell cycle following DNA damage^[37]. Our results demonstrated that silibinin at 80 mg/kg and 160 mg/kg significantly reduced Plk1 expression and the level of nuclear NF- κ B. The higher dose of silibinin (160 mg/kg) also increased Chk1 expression. Taken together, our data indicate that silibinin reduced *in vivo* HCC xenograft growth by decreasing HCC cell proliferation and cell cycle progression which was mediated by inhibiting translocation of NF- κ B to the nucleus, Plk1, p-Rb expression, E2F1/DP1, and increasing Chk1 expression and formation of the p27/CDK4 complex.

Increasing cell apoptosis is another important step that inhibits tumor growth^[44]. We demonstrated that silibinin promotes *in vivo* apoptosis in HuH7 xenografts, which reconfirmed the previous *in vitro* findings^[17,18]. Survivin is an apoptosis inhibitor that is overexpressed in most cancers in a cell cycle-dependent manner. P-survivin is necessary for cancer cell viability^[45]. Our results demonstrated that silibinin inhibited p-survivin in association with increased apoptosis in HuH7 xenograft tissue. These results reconfirmed our previous *in vitro* findings^[17] and indicated the important role of the survivin-mediated decrease in apoptosis in HCC growth.

We reported that silibinin-enhanced apoptosis of cultured HuH7 cells was associated with increased production of activated caspase 3 and 9, however, these changes were not reproducible in HuH7 xenograft tissue. Additionally, silibinin seemed not to alter Bcl-2 expression, another modulator of apoptosis, in HuH7 xenograft tissue. These data indicated that a discrepancy of silibinin-mediated apoptosis signaling may occur in these two systems.

Studies have indicated the important roles of PTEN/PI3K/Akt and ERK signaling in carcinogenesis and cancer progression^[24-30,46]. Phosphorylation of Akt results in its activation, which promotes cell cycle progression by phosphorylating several other key proteins^[47-51]. PTEN is an up-stream molecule that inhibits p-Akt. We found that silibinin significantly increased PTEN expression and activity that was further associated with reduced p-Akt production in HCC xenograft tissue. These results indicate a possible pathogenic role of the PTEN/PI3K/p-Akt pathway in HCC growth that may also serve as an important silibinin target. Increased p-ERK activates transcription of the mitogenic and cell regulatory genes and promotes oncogenesis^[46]. P-ERK was reportedly increased in HCC^[52], suggesting its involvement in HCC development. A previous *in vitro* study reported that silibinin can inhibit ERK phosphorylation in human osteosarcoma^[53]. In the present study, we found silibinin-reduced HuH7 xenograft growth was also associated with a significant inhibition of p-ERK production. These results are also in agreement with an effect on colorectal cancer reported by Singh *et al*^[14]. The results revealed that the p-ERK pathway is likely involved in silibinin-reduced HCC growth, another possible novel

target of HCC chemoprevention and therapy in future research.

Histone acetylation has been reported to be involved in cell proliferation, differentiation, and cell cycle regulation. A decrease in acetylation status in the cells is associated with carcinogenesis^[31-33]. Our results demonstrated that silibinin significantly increased AC-H3 and AC-H4 expression, suggesting that increased histone acetylation may mediate silibinin-reduced HCC growth.

ROS stress has been associated with the development of HCC. SOD is one of the important enzymes in reducing ROS levels. Altered expression of SOD has been associated with the development and differentiation of HCC. Although the effects of silibinin on SOD were reported in patients with alcoholic liver disease^[42], it is unknown whether the same mechanism has any role in the anti-HCC effects of silibinin. We demonstrated that silibinin-reduced growth of HuH7 xenografts was associated with a significant increase in the production of SOD1 in the xenograft tissue of nude mice. This was particularly evident when a higher dose of silibinin was used. Thus, our results indicate a possible mechanistic role of SOD1 in silibinin-reduced growth of HuH7 xenografts.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies. Silibinin has been reported to be safe, well-tolerated, and protects the liver from drug or alcohol-related injury. A recent demonstration of the potent *in vitro* anti-HCC effects of silibinin has provided us with a rationale to further assess the *in vivo* effects of silibinin on HCC growth. The present study examined the *in vivo* effects and mechanisms of silibinin on HCC growth using a nude mouse model bearing HuH7 (a human HCC cell line) xenografts.

Research frontiers

The search for safe and well-tolerated chemopreventive agents is one of the significant research frontiers in HCC chemoprevention. Many studies have demonstrated that silibinin can effectively inhibit the growth of various types of tumor cells, however, little is known about the *in vivo* effects and mechanisms of silibinin on HCC growth.

Innovations and breakthroughs

Previous study demonstrated that silibinin can inhibit HCC cell growth *in vitro*. In the present study, we confirmed that silibinin can effectively inhibit growth of human HCC xenografts in mice by affecting cell cycle progression, apoptosis, and several other pathways.

Applications

These results provide a rationale to further pre-clinical investigations which may result in clinical trials assessing the application of silibinin in HCC chemoprevention.

Terminology

Xenografts: Tissue or organs from an individual of one species inoculated, transplanted into or grafted onto an organism of another species, genus, or family. Chemoprevention: The use of chemical compounds to intervene in the early stage of carcinogenesis and thereby reverse tumor formation.

Peer review

This is a well-designed and very interesting study, methods are appropriated and results are consistent with the conclusions.

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Histological and biochemical alterations in early-stage lobar ischemia-reperfusion in rat liver

Hossein Ali Arab, Farhang Sasani, Mohammad Hossein Rafiee, Ahmad Fatemi, Abbas Javaheri

Hossein Ali Arab, Mohammad Hossein Rafiee, Ahmad Fatemi, Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran, Tehran 14155, Iran

Farhang Sasani, Department of Pathology, Faculty of Veterinary Medicine, University of Tehran, Tehran 14155, Iran

Abbas Javaheri, Faculty of Veterinary Medicine, University of Semnan, Semnan 35195, Iran

Author contributions: Arab HA designed research; Arab HA, Rafiee MH and Fatemi A performed the research; Sasani F and Javaheri A contributed to the histological and immunohistochemistry examinations; Arab HA and Sasani F analyzed data; Arab HA and Sasani F wrote the manuscript.

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Correspondence to: Hossein Ali Arab, PhD, Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran, Tehran 14155, Iran. harab@ut.ac.ir

Telephone: +98-21-6117086 Fax: +98-21-6693322

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the numbers of apoptotic bodies was significantly enhanced. The amounts of lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, creatinine and urea were significantly increased in serum obtained from animals exposed to hepatic IR.

CONCLUSION: Inflammation and subsequent apoptotic cell death were the most important changes in early-stage hepatic reperfusion injury, and the number of apoptotic bodies increased with time of reperfusion.

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Key words: Lobar ischemia; Liver; Reperfusion injury; Apoptosis; Immunohistochemistry

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Abstract

AIM: To investigate the structural and biochemical changes in the early stage of reperfusion in the rat livers exposed to lobar ischemia-reperfusion (IR).

METHODS: The median and left lobes of the liver were subjected to 60 min ischemia followed by 5, 10, 30, 45, 60 and 120 min reperfusion. Blood samples were taken at different time intervals to test enzyme activities and biochemical alterations induced by reperfusion. At the end of each reperfusion period, the animals were killed by euthanasia and tissue samples were taken for histological examination and immunohistochemistry.

RESULTS: Cell vacuolation, bleb formation and focal hepatitis were the most important changes occur during ischemia. While some changes including bleb formation were removed during reperfusion, other alterations including portal hepatitis, inflammation and the induction of apoptosis were seen during this stage. The occurrence of apoptosis, as demonstrated by apoptotic cells and bodies, was the most important histological change during reperfusion. The severity of apoptosis was dependent on the time of reperfusion, and by increasing the time of reperfusion,

INTRODUCTION

Reperfusion of a previously ischemic tissue is associated with additional injury that leads to structural and functional alterations in many organs including the liver. The hepatic injury that occurs during reperfusion has been shown to be the major problem associated with stroke, shock, cirrhosis, liver surgery and transplantation^[1-4]. The mechanisms of reperfusion-induced pathological and functional alterations are under intensive investigation, but the results of different studies are controversial. Some studies suggest that the reintroduction of oxygen to the ischemic (hypoxic) tissues stimulates the production of reactive oxygen species (ROS), which contribute to cell damage. Others have argued that the liver tissues are basically resistant to the oxidative stress followed by ischemia-reperfusion (IR)^[5-7]. However, many studies have shown that the ischemic livers undergo moderate^[8,9] to severe structural and functional alterations by IR^[10,11].

It has been shown that the injury induced during

reperfusion has a biphasic pattern that consists of an early stage that starts upon reoxygenation and a delayed phase. The early stage is associated with hepatocellular damage during 2-6 h after reperfusion (reoxygenation), and the delayed phase occurs 18-24 h after reperfusion and is accompanied with a massive neutrophil infiltration^[12-15]. The injury in early stage (acute phase) is mediated by ROS, but the damage in the delayed stage (subacute phase) is associated with the inflammatory responses mediated by neutrophil activity. It is thought that ROS formation during reperfusion induces a cascade of cellular events that eventually leads to hepatocellular injury, including inflammation, necrosis, and/or apoptosis^[16-19]. However, the detailed mechanisms of cell death and the structural alterations induced during different stages of reperfusion injury are not yet completely determined. Some studies have reported that the morphological changes induced by reperfusion are predominantly limited to non-parenchymal cells^[20-23], whereas others have shown that some changes are seen in parenchymal cells^[8,24].

While necrosis has been shown to be the cause of hepatic IR injury, many studies have shown that programmed cell death or apoptosis is the cause of cell death during liver reperfusion after long-term ischemia^[20,25,26]. However, the role of apoptosis as the main cause of the injury and the level of morphological changes induced by this type of cell death have not been determined in detail.

The present study was designed to characterize the features of the injury induced in the early stage of reperfusion in rat liver. The ischemia was established by a lobar model and the ischemic liver was exposed to different reperfusion times. The hepatic alterations were assessed by both histological and biochemical observations.

MATERIALS AND METHODS

Animals and experimental groups

Female Sprague-Dawley rats weighing 230-280 g were used in all experiments. The animals were group-housed with a 12-h light-dark cycle and fed a standard laboratory diet. All experiments were performed according to the standard procedures outlined by our institutional guidelines. Rats were fasted overnight for at least 16 h prior to the experiments, but access to water was uninterrupted.

A group of four animals were subjected to 60 min lobar ischemia only. They were sacrificed at the end of this period and then liver tissue samples were taken for histology and immunohistochemistry (IHC). Seven groups of animals underwent 60 min ischemia followed by 5, 10, 15, 30, 54, 60 or 120 min reperfusion. A sham-operated group was selected for each test groups as a control.

In vivo (lobar) models of IR

This term refers to the model in which the ischemia is induced in the anesthetized animals through application of a vascular clamp simultaneously to branches of the hepatic portal vein, hepatic artery and bile duct. The

reperfusion is commenced by removal of the clamp, thus restoring normal blood flow. Anesthesia was induced by a single intraperitoneal (i.p.) injection of ketamine (80 mg/kg) plus xylazine (10 mg/kg). After injection of 300 U of heparin *via* the femoral vein, the right jugular veins were catheterized by polyethylene tubing for blood sampling and infusion of normal saline solution to replace the removed blood. After laparotomy, the median and left lobes of the liver were removed from the abdominal cavity. Then, *in vivo* lobar ischemia was induced by clamping the left branches of the hepatic portal vein, hepatic artery and bile duct with a microvascular occlusion clip for a period of 60 min. This, caused occlusion of all blood vessels supplying the median and left lobes of the liver, which is reported to produce approximately 70% (partial) liver ischemia^[27]. Upon release of the clamp, reperfusion was commenced and the blood flow was continued for different times as described above. Control (sham-operated) groups underwent the same surgical procedure, except that the blood supply to the liver lobes was not interrupted.

Biochemical assays

Blood samples were taken at different times before ischemia, during ischemia and after reperfusion. The plasma was separated by centrifuging the blood, which was kept in the freezer until analysis. The release of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes, as well as the level of glucose, urea and creatinine were measured by a Hitachi 747 analyzer (Boehringer Mannheim).

Histological examination

Small pieces of liver were taken from both left and median hepatic lobes. Parts of samples were fixed in 10% formalin for light microscopy. Paraffin embedded sections of 5- μ m thickness were stained with hematoxylin and eosin and/or periodic acid-Schiff. The remaining samples were fixed with 3% glutaraldehyde/4% paraformaldehyde in 0.1 mmol/L sodium cacodylate buffer for electron microscopy. They were transferred into sodium cacodylate buffer on the following day and then stored at 4°C until processing.

IHC

IHC was carried out to detect the presence of apoptosis protease-activating factor 1 (APAF-1) as a marker of apoptosis induction in tissue samples. Serial embedded sections were prepared from formalin-fixed samples, which were cut at a thickness of 3 μ m and dried at 37°C overnight. IHC was performed by the avidin-biotin complex (ABC) procedure, including heat-induced epitope-retrieval and enzymatic antigen-retrieval procedures. Incubation with the primary antibody (NCL-APAF-1; Novocastra; 1:100 dilutions) was carried out in a moist chamber at 37°C for 1 h. Negative controls were treated identically, with the primary antibody omitted; positive controls consisted of normal hepatic tissue.

Table 1 Data analysis of different biochemical tests obtained from livers subjected to 60 min ischemia followed by 30-120 min reperfusion

Time of sampling	Glucose (µg/dL)	Urea (µg/dL)	Creatinine (µg/dL)	AST (IU/L)	ALT (IU/L)	LDH (IU/L)
Before ischemia	261.33 ± 36.16 <i>n</i> = 5	17.66 ± 2.88 <i>n</i> = 5	0.55 ± 0.058 <i>n</i> = 5	54.82 ± 19.82 <i>n</i> = 5	78.2 ± 24.3 <i>n</i> = 5	153.75 ± 0.11 <i>n</i> = 16
During ischemia	189.66 ± 18.8 <i>n</i> = 5	18.33 ± 1.2 <i>n</i> = 5	0.57 ± 0.075 <i>n</i> = 7	12.74 ± 9.51 <i>n</i> = 5	18.19 ± 4.39 <i>n</i> = 5	39.69 ± 8.58 <i>n</i> = 16
After reperfusion	160.83 ± 10.63 <i>n</i> = 14	26.50 ± 1.66 <i>n</i> = 14	2.62 ± 1.29 <i>n</i> = 13	61.58 ± 9.12 <i>n</i> = 10	142.28 ± 30.94 <i>n</i> = 7	200.52 ± 60.52 <i>n</i> = 23

The values are expressed as the mean ± SE taken from at least 12 samples in test.

Table 2 Comparison of data obtained from biochemical analysis of blood samples taken from the animals exposed to 60 min ischemia followed by different times of reperfusion

Sampling phase	P-value					
	Glucose	Urea	Creatinine	AST	ALT	LDH
Before ischemia/ during ischemia	0.38	0.72	0.79	0.043	0.049	0.034
Before ischemia/ after reperfusion	0.31	0.05 ^a	0.006 ^a	0.08	0.043 ^a	0.008 ^a
During ischemia/ after reperfusion	0.17	0.05 ^a	0.05 ^a	0.08	0.005 ^a	0.014 ^a

^a*P* < 0.05.

Statistical analysis

Data from biochemical assays are expressed as the mean ± SD obtained from at least four experiments in each group. They were analyzed by ANOVA using the SPSS program and the significance of the differences between groups were tested by Tukey’s post-hoc test, with *P* < 0.05 considered statistically significant. Pathological changes in both untreated and treated groups were scored semi-quantitatively from + (mild) to +++++ (severe).

RESULTS

Biochemical changes induced by reperfusion injury

There were significant changes in enzyme release and blood urea and creatinine level in the animals exposed to hepatic IR, compared to the controls. The data obtained from analysis of biochemical assays are summarized in Table 1, and comparison of the results at different times of reperfusion is shown in Table 2. As seen in the tables, while enzyme release was significantly reduced during ischemia (*P* < 0.05), the level of glucose, creatinine and urea did not change in the blood of animals exposed to lobar hepatic ischemia. However, plasma level of LDH, AST, creatinine and urea was significantly increased during reperfusion (Table 2).

Morphological findings

The histological changes induced by IR were examined, based on portal inflammation, focal inflammation, sinusoidal congestion, cytoplasmic vacuolation, bleb formation, apoptotic cells and apoptotic bodies production. The liver samples from the sham-operated group did not show significant histological alterations,

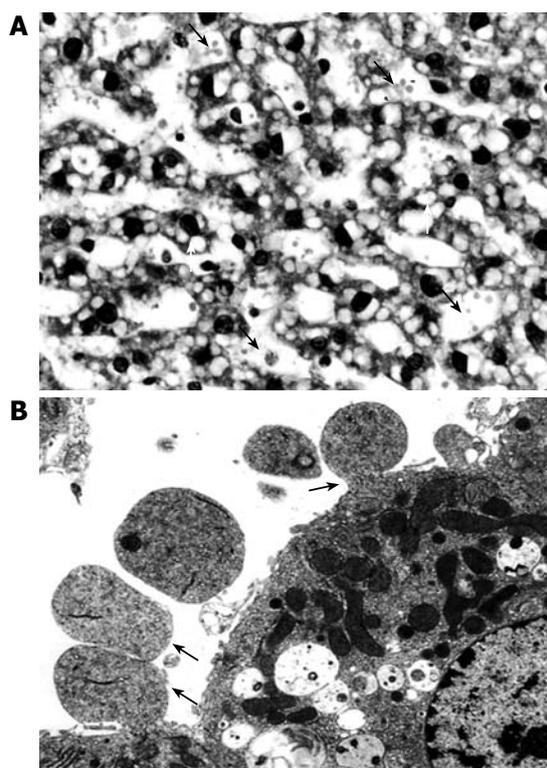


Figure 1 Zone 3 cytoplasmic vacuolation and hepatocyte bleb formation in the liver subjected to 60 min ischemia only. A: The white arrow shows cytoplasmic vacuolation and the black arrows show the cytoplasmic blebs formation with light microscopy; B: The arrows shows the cytoplasmic blebs with electron microscopy.

similar to those of the non-operated control group. Changes observed in the liver exposed to lobar ischemia alone were limited to mild to moderate focal hepatitis, sinusoidal congestion, vacuolation and bleb formation (Figure 1).

During reperfusion, whilst some changes including blebbing of hepatocytes were improved, induction of portal hepatitis and mild apoptosis were added at 5 min of reperfusion. By increasing the time of reperfusion, the induction of portal and local hepatitis were reduced, but the amount of apoptosis was moderately increased, so that at 30 min reperfusion, the presence of apoptotic cells but not apoptotic bodies was the most important change in the majority of tissue samples (Figure 2A). Sixty minutes ischemia followed by 45 or 60 min reperfusion caused an increased amount of apoptosis

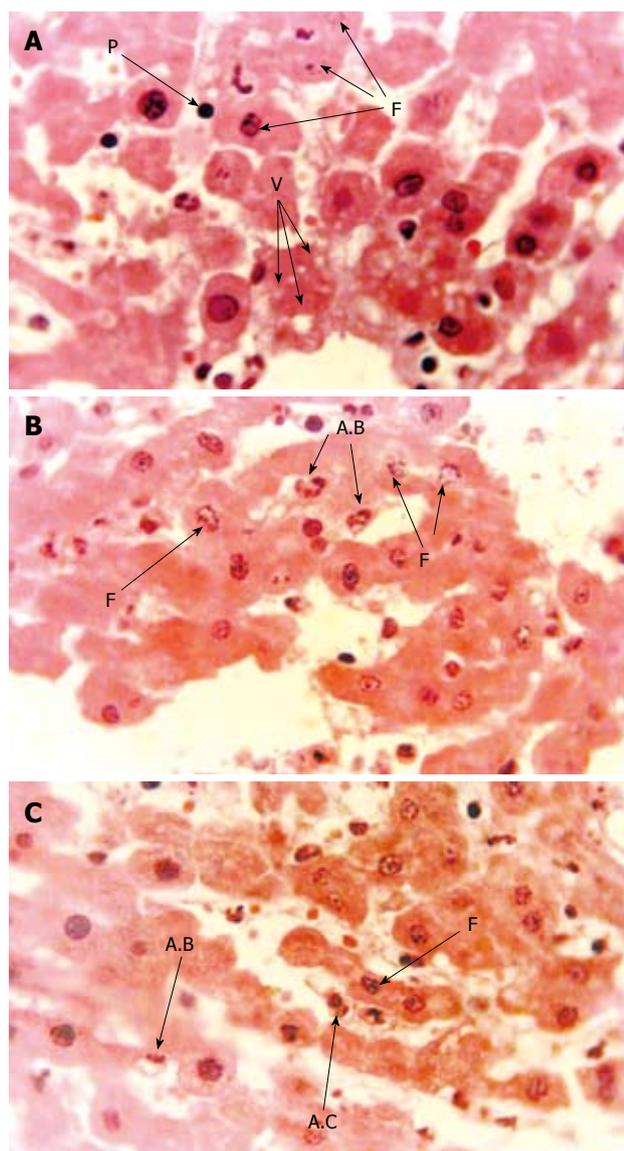


Figure 2 Histological changes in the livers exposed to 60 min lobar ischemia followed by different times of reperfusion. A: Nuclear pyknosis (P), nuclear fragmentation (F) and cytoplasmic vacuolation in the liver exposed to 60 min ischemia followed by 30 min reperfusion; B: Apoptotic bodies (A.B) and nuclear fragmentation (F) in the liver exposed to 60 min ischemia followed by 60 min reperfusion; C: Nuclear fragmentation (F), apoptotic cell (A.C) and apoptotic bodies (A.B) in the liver exposed to 60 min ischemia followed by 120 min reperfusion.

with phagocytic apoptotic bodies and sinusoidal congestion (Figure 2B). However, in the livers that underwent ischemia and 60 or 120 min reperfusion, phagocytic apoptotic bodies were seen in most tissue samples (Figures 2C and 3). To organize different groups exposed to different reperfusion times, they were classified into three durations: short time, 5 and 10 min; middle time, 30, 45 and 60 min; and long time (120 min). Statistical analysis of the histological alterations and the severity of hepatic changes in the liver subjected to 60 min ischemia followed by short, middle and long times of reperfusion in comparison with the changes induced by 60 min ischemia only are summarized in Figure 4.

The occurrence of apoptosis was confirmed by



Figure 3 Apoptotic bodies of an endothelial origin apoptotic cell phagocytosed by a hepatocyte in the liver exposed to 60 min ischemia followed by 60 min reperfusion.

IHC, in which the expression of APAF-1 was positive in the stained sections of livers exposed to IR, and the presence of apoptosis and/or apoptotic bodies was seen by light and electron microscopy (Figure 5A). Representative staining patterns for APAF-1 expression showed that the occurrence of apoptosis was limited to the pericentral area (Figure 5B). The presence of cells labeled with brown color indicated that the expression of APAF-1 was increased during 5-15 min of reperfusion. This showed that the occurrence of apoptosis occurred upon the initiation of reperfusion. However, during 30-60 min of reperfusion, the presence of cells with a brown-colored cytoplasm was accompanied with apoptotic bodies in which, when reperfusion time was increased (120 min), the number of apoptotic bodies also increased (Figure 5B).

DISCUSSION

In the present study, an *in vivo* lobar (partial) model of rat liver IR injury was established by clamping the vessels to the left lateral and median hepatic lobes, which account for 70% of the rat liver mass. This hepatic insult is similar to the clinical situation when the liver is rendered ischemic during total vascular exclusion for liver resection^[28]. We tried to illustrate the pathological profile of the liver exposed to 60 min ischemia followed by different periods of 5, 10, 30, 45, 60 and 120 min reperfusion. It was found that cell vacuolation, bleb formation and focal hepatitis were the most important changes induced by *in vivo* lobar ischemia in rat liver. However, during reperfusion, not only some changes including bleb formation was reduced, but some other alterations including portal hepatitis, inflammation and the induction of apoptosis, occurred. It appears that the occurrence of apoptosis, as demonstrated by the formation of apoptotic cells and bodies, is the most important histological change during the early stage of reperfusion. The severity of apoptosis was dependent on the time of reperfusion, so that by increasing the time of reperfusion, the number of apoptotic bodies was significantly enhanced. To accompany these changes,

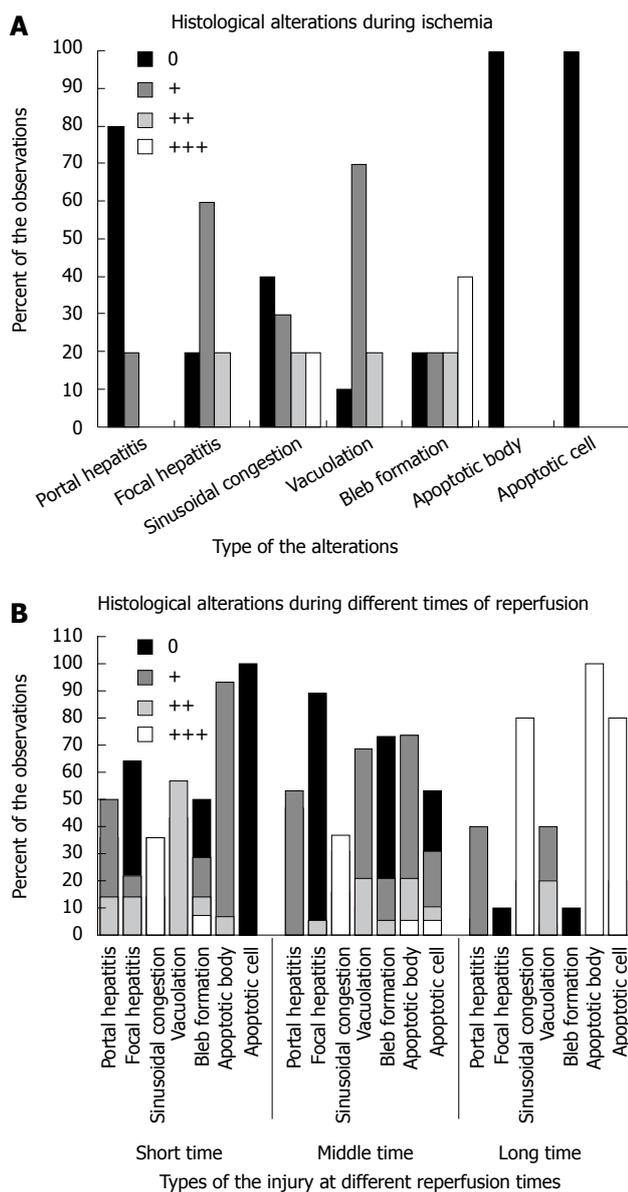


Figure 4 A summary of statistical analysis of histological alterations in the livers exposed to ischemia or ischemia-reperfusion. A: Hepatic changes in the liver exposed to 60 min lobar ischemia only; B: Changes in the liver subjected to 60 min ischemia followed by short, middle and long times of reperfusion in which: 0= Normal or no changes, +: Mild injury, ++: Moderate injury, +++: Severe injury; $0 < A.C/A.B \leq 2 = +$, $2 < A.C/A.B \leq 5 = ++$, $5 < A.C, A.B = +++$.

the serum level of LDH, ALT, creatinine and urea was significantly increased in rats exposed to hepatic IR, which indicates the induction of cell injury in the liver and other organs, including the kidneys.

Hepatic IR injury is a common pathological phenomenon that may be induced after severe liver trauma, extensive hepatic lobe excision, liver transplantation, and shock. By initiation of reperfusion injury, a series of functional, humoral and structural alterations occur in the liver tissues that directly influence the prognosis of patients^[3-5,9]. The mechanisms by which the reperfusion injury induces pathological and functional alterations and the methods of intervention have been under intensive investigation. However, the detailed

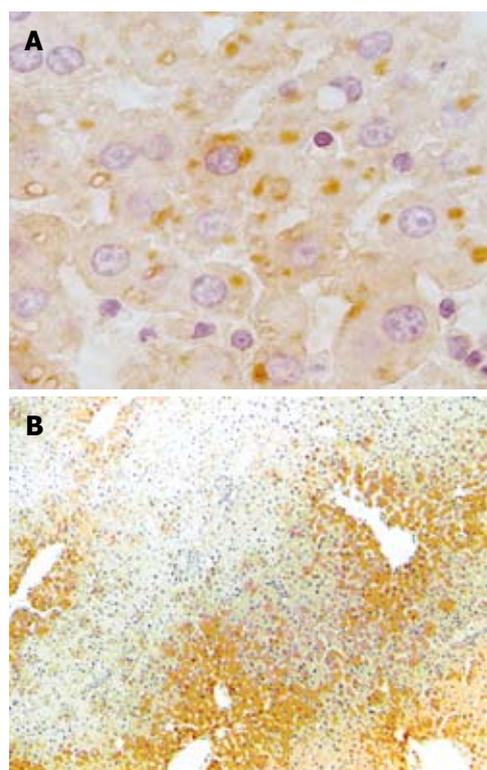


Figure 5 Confirmation of apoptosis by IHC assay in the staining sections of livers exposed to ischemia-reperfusion and the presence of apoptosis cells and/or apoptotic bodies. A: Representative staining patterns for APAF-1 positive, illustrating the occurrence of apoptosis in the sections of the liver exposed to 60 min ischemia followed by long time of reperfusion; B: APAF-1 positive staining that shows the high level of apoptosis incidence in the pericentral area of the liver exposed to 60 min ischemia followed by long time of reperfusion.

pathological mechanisms of liver injury induced by this phenomenon are complex and not yet fully understood. Different *in vivo* and *in vitro* models have been used to establish the pathological process of hepatic IR injury, such as the liver transplantation model, the partial warm or cold IR model, and the total hepatic IR model^[6,28-30].

There is evidence that the pathogenesis of reperfusion injury involves a series of events, including Kupffer cell activation, cytokine release, neutrophil activation, increased expression of adhesion molecules, sinusoidal endothelial cell death, and hepatocyte injury^[15,19,31-33]. Among these, the inflammatory process and activation of Kupffer cells, which result in the release of excessive quantities of cytokines and ROS formation, play a major role^[4,17]. The inflammatory aspect of the injury includes cellular and humoral components. A growing body of evidence, primarily from animal models of IR and preliminary human studies, has revealed that the inflammatory mechanisms may play a major role in the pathogenesis of the injury induced by reperfusion. It has been shown that hepatocyte injury followed by reperfusion is partly dependent on Kupffer cell activation and production of inflammatory mediators. This occurs in a biphasic pattern that consists of acute-phase (ROS-mediated) and subacute-phase (neutrophil-mediated) damage^[8,19,20,24].

Our findings strongly suggest the occurrence of inflammation and the subsequent cell death by apoptosis as an important morphological change observed in the early stage (acute phase) of reperfusion. It is proposed that in the early stage of hepatic reperfusion injury, these inflammatory reactions and the different stress processes that follow may result in the activation of the apoptotic pathway mediated by mitochondria. This may lead to an increased number of apoptotic cells and apoptotic body formation, which is associated with a reduction in the total number of parenchymal cells, thus damaging the hepatic tissues and resulting in liver dysfunction^[20,27].

The role of apoptosis as a cell death mechanism in reperfusion injury has been shown by some studies previously^[20,34,35]. Although other alterations including hepatocyte blebbing, sinusoidal congestion, and portal and focal hepatitis were shown have been seen in liver histology, the integrity and organizational arrangement of the hepatic acinus remains intact. This suggests that the liver parenchyma is able to resist against these types of insults^[9]. This has been confirmed with further studies that have demonstrated that parenchymal cells are not susceptible to damage under some conditions of ischemia-reperfusion IR^[21-23].

In the present study, the occurrence of apoptosis was confirmed by IHC of the liver for APAF-1 expression. Positive APAF-1 staining in most sections of liver exposed to IR confirmed the role of apoptosis as the main cause of cell death in early-stage hepatic reperfusion injury. APAF-1 has been identified as a key protein that plays an essential role in the induction of apoptosis in different mammalian cells. In response to apoptotic stimuli, such as ROS, Ca²⁺ and cytokines released by reperfusion, APAF-1 binds to cytochrome c and procaspase 9, to yield a complex entitled the apoptosome. Activation of procaspase 9 through an autocatalytic process initiates a cascade of downstream effector caspases, which finally leads to mitochondrial apoptosis. The mitochondrial/cytochrome c apoptotic pathway and the expression of APAF-1 have attracted close attention of researchers to determine the induction of apoptosis^[36-39]. Using IHC, we found that the occurrence of apoptosis was started in the initial phase of reperfusion, and it was completed as the reperfusion time increased. This was shown by the abundance of apoptotic bodies phagocytosed by macrophages or neighboring hepatocytes during long periods of reperfusion. Increased expression of APAF-1 in zone 3 of the liver indicated the greater susceptibility of this area of the liver to reperfusion injury.

In conclusion, we showed that inflammation and apoptosis were the major histological alterations induced by early-stage reperfusion injury in the liver exposed to lobar ischemia. It appears that apoptosis is the most important histological change induced in the early stage of hepatic reperfusion injury, during which the number of apoptotic bodies was increased with the time of reperfusion.

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COMMENTS

Background

Reperfusion of a previously ischemic tissue is associated with additional injury that leads to structural and functional alterations in many organs, including the liver. The mechanisms of reperfusion-induced pathological and functional alterations are under intensive investigation, but the results of different studies are controversial.

Research frontiers

The injury induced during reperfusion evolves in a biphasic pattern that consists of an early stage that starts with reoxygenation and a delayed phase. The early stage is associated with hepatocellular damage at 2-6 h after reperfusion, and the delayed phase occurs at 18-24 h after reperfusion, and is accompanied by massive neutrophil infiltration. The injury in the early stage is mediated by reactive oxygen species (ROS), but the damage in the delayed stage is associated with the inflammatory responses mediated by neutrophil activity. It is thought that ROS formation during reperfusion induces a cascade of cellular events that eventually leads to hepatocellular injury. However, the detailed mechanisms of cell death and the structural alterations induced during different stages of reperfusion injury are not yet completely understood.

Innovations and breakthroughs

In the present study, the authors demonstrated that the occurrence of inflammation and the subsequent cell death by apoptosis were the most important changes in the early stage of hepatic reperfusion injury.

Applications

By characterizing the feature of the injury induced in the early stage of reperfusion, this study may represent a future strategy for therapeutic intervention of reperfusion injury induced under different conditions, such as stroke, shock, cirrhosis, liver surgery and transplantation.

Terminology

Lobar ischemia refers to the model in which the ischemia is induced in the anesthetized animals through application of a vascular clamp simultaneously to branches of the hepatic portal vein, hepatic artery and bile duct.

Peer review

The authors examined the effects of 60 min lobar ischemia followed by different periods of 5, 10, 30, 45, 60 and 120 min reperfusion. It was found that the occurrence of apoptosis, as demonstrated by apoptotic cells and bodies, was the most important histological change during reperfusion. The severity of apoptosis was dependent on the time of reperfusion, such that by increasing the time of reperfusion, the number of apoptotic bodies was significantly enhanced.

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Rocket "*Eruca sativa*": A salad herb with potential gastric anti-ulcer activity

Saleh Alqasoumi, Mohammed Al-Sohaibani, Tawfeq Al-Howiriny, Mohammed Al-Yahya, Syed Rafatullah

Saleh Alqasoumi, Tawfeq Al-Howiriny, Mohammed Al-Yahya, Syed Rafatullah, Department of Pharmacognosy and Medicinal, Aromatic and Poisonous Plants Research Center, College of Pharmacy, PO Box 2457, King Saud University, Riyadh 11451, Saudi Arabia

Mohammed Al-Sohaibani, Department of Pathology (32), PO Box 2925, King Khalid University Hospital, King Saud University, Riyadh 11461, Saudi Arabia

Author contributions: Alqasoumi S designed and performed some experiments, and collected the material; Al-Howiriny T and Al-Yahya M provided vital reagents and are responsible for the preliminary phytochemical screening and extraction of the plant material; Rafatullah S contributed in performing some of the experiments and wrote the manuscript; Al-Sohaibani M carried out the histopathology.

Correspondence to: Dr. Syed Rafatullah, MD (Unani), MF Hom, Medicinal, Aromatic and Poisonous Plants Research Center (MAPPRC), College of Pharmacy, PO Box 2457, King Saud University, Riyadh 11451, Saudi Arabia. srafat@ksu.edu.sa
Telephone: +966-1-4677207 Fax: +966-1-4677245

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Abstract

AIM: To validate gastric anti-ulcer properties of Rocket "*Eruca sativa*" on experimentally-induced gastric secretion and ulceration in albino rats.

METHODS: Gastric acid secretion studies were undertaken using pylorus-ligated rats. Gastric lesions in the rats were induced by noxious chemicals including ethanol, strong alkalis, indomethacin and hypothermic restraint stress. The levels of gastric wall mucus (GWM), nonprotein sulfhydryls (NP-SH) and malondialdehyde (MDA) were also measured in the glandular stomach of rats following ethanol administration. The gastric tissue was also examined histologically. The extract was used in two doses (250 and 500 mg/kg body weight) in all experiments.

RESULTS: In pylorus-ligated Shay rats, the ethanolic extract of Rocket "*Eruca sativa* L." (EER) significantly and dose-dependently reduced the basal gastric acid secretion, titratable acidity and ruminal ulceration. Rocket extract significantly attenuated gastric ulceration induced by necrotizing agents (80% ethanol, 0.2 mol/L NaOH, 25% NaCl), indomethacin and

hypothermic restraint stress. The anti-ulcer effect was further confirmed histologically. On the other hand, the extract significantly replenished GWM and NP-SH levels, as well as the MDA level significantly reduced by extract pretreatment.

CONCLUSION: Rocket extract possesses anti-secretory, cytoprotective, and anti-ulcer activities against experimentally-induced gastric lesions. The anti-ulcer effect is possibly through prostaglandin-mediated activity and/or through its anti-secretory and antioxidant properties.

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Key words: Cytoprotection; *Eruca sativa*; Gastric ulcer and secretion; Malondialdehyde; Rocket; Sulfhydryls

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INTRODUCTION

Gastric ulcer is an illness that affects a considerable number of people worldwide. The etiological factors of this disorder include: stress, smoking, nutritional deficiencies, infections, frequent and indiscriminate use of nonsteroidal anti-inflammatory drugs (NSAIDs)^[1]. The pathogenesis of gastroduodenal ulcers are influenced by various aggressive and defensive factors, such as mucus secretion, mucosal barrier, acid-pepsin secretion, blood flow, cellular regeneration and endogenous protective agents (prostaglandins and epidermal growth factor)^[2]. Although the introduction of proton-pump inhibitors to the classic anti-ulcer therapy had revolutionized treatment of peptic ulcers and other gastrointestinal disorders, but there is still no complete cure for this disease. It has been shown that long term use of these drugs leads to various adverse

and side effects. Relapses of the malady, ineffectiveness of different drug regimens and even resistance to drugs are emerging^[3]. Thus, there is an urgent requirement to identify more effective and safe anti-ulcer agents. During the past few decades, a widespread search has been launched to identify new anti-ulcer therapies from natural sources. Herbs, medicinal plants, spices, vegetables and crude drug substances are considered to be a potential source to combat various diseases including gastric ulcer. In the scientific literature, a large number of medicinal plants with gastric anti-ulcer potential have been reported^[4-8]. In recent years, Rocket "*Eruca sativa* L." (EER), a member of the Brassicaceae family, has gained greater importance as a salad vegetable and spice, especially among Middle Eastern populations and Europeans^[9]. It is believed that plants belonging to the Brassicaceae family possess diversified medicinal and therapeutic properties including inhibition of tumorigenesis^[10], anti-ulcer^[11], and hepatoprotective^[12] activities. Rocket, locally known as Jarjeer, is used in salads, by local herbal practitioners and in Unani medicine, and is used as a diuretic, stimulant, and in the treatment of stomach disorders and scurvy^[13]. The seeds and tender leaves are known in Arabian countries to increase sexual desire and are considered to be an aphrodisiac. It is also used as a carminative and to alleviate abdominal discomfort and improve digestion. It has been reported that the rocket seed ethanolic extract possesses potent antioxidant and renal protective and diuretic activities^[14-16]. Phytochemical studies of rocket leaves and seeds have revealed the presence of glucosinolates^[17,18]. Weckerle *et al.*^[19] isolated and identified three new quercetins from *Eruca sativa* leaves. In view of the acclaimed medicinal value of rocket in Unani, Ayurvedic and Arab traditional medicine as well as its diversified therapeutic uses, we have undertaken the present study to evaluate the anti-ulcerogenic property of EER in different ulcer models in rats.

MATERIALS AND METHODS

Plant material and preparation of extract

Fresh *Eruca sativa* leaves were purchased from a local vegetable market in Riyadh, and the identity of these leaves was confirmed by an expert taxonomist of the Department of Pharmacognosy, where a voucher specimen (No. 8208) of the plant has been kept in the Herbarium of the College of Pharmacy, KSU, Riyadh. Shade-dried, coarsely pulverized rocket leaves were placed in a glass percolator with ethanol and were allowed to stand at room temperature for about 72 h. The percolate was collected and dried under reduced pressure in vacuo. The extract obtained was later used and dissolved in distilled water for evaluation of anti-ulcer activity.

Animals and dosing

Albino Wistar rats of either sex, approximately the same age, weighing 150 to 200 g and fed on a diet of standard chow were used in this study. They were randomly

divided into experimental groups of 6 rats each. Aqueous solutions of ulcerogens and EER were freshly prepared before administration. EER at doses of 250 and 500 mg/kg were given orally in the anti-ulcer studies and intraperitoneally for gastric secretion evaluation. The rats were sacrificed, and the stomachs removed and opened along the greater curvature. After washing with saline, the gastric lesions were quantified by a person unaware of the treatments. The animal study protocol was approved by the Research and Ethics Committee of the Experimental Animal Care Society, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Pylorus-ligated rats

Rats were fasted for 36 h with access to water *ad libitum* before pylorus ligation under ether anesthesia was carried out. Care was taken not to cause bleeding or to occlude blood vessels^[20]. EER was administered intraperitoneally immediately after pylorus ligation (Shay). The rats were sacrificed at 6 h after pylorus ligation. The stomachs were removed, the contents were collected, volumes measured, centrifuged and analyzed for titratable acidity against 0.01 mol/L NaOH at pH 7.

Gastric lesions induced by necrotizing agents (cytoprotection)

Each rat was administered 1 mL of a necrotizing agent (80% ethanol, 0.2 mol/L NaOH or 25% NaCl). Rocket extract was given 30 min before the administration of necrotizing agents. One hour after the administration of ethanol and the alkalis, the rats were sacrificed and examined for stomach lesions. The scoring of stomach lesions was as follows: Patchy lesions of the stomach induced by ethanol were scored according to the method described by Robert *et al.*^[21] using the following scale: 0 = normal mucosa; 1 = hyperemic mucosa or up to 3 small patches; 2 = from 4 to 10 small patches; 3 = more than 10 small or up to 3 medium-sized patches; 4 = from 4 to 6 medium-sized patches; 5 = more than 6 medium-sized or up to 3 large patches; 6 = from 4 to 6 large patches; 7 = from 7 to 10 large patches; 8 = more than 10 large patches or extensive necrotic zones. "small" was defined as up to 2 mm across (max. diameter), "medium-sized" between 2 and 4 mm across and "large" more than 4 mm across.

Gastric lesions induced by indomethacin

Indomethacin was suspended in 1.0% carboxymethylcellulose (CMC) in water (6 mg/mL) and administered orally to the 36 h fasted rats at a dose of 30 mg/kg body weight. Control rats were treated similarly with an equivalent amount of vehicle^[22]. The rocket extract was given 30 min prior to indomethacin administration at a dose of 250 and 500 mg/kg. The animals were sacrificed 6 h after treatment. The stomachs were excised, rinsed with normal saline and examined for ulceration.

Hypothermic restraint stress-induced ulcers

The method described by Senay *et al.*^[23] was adopted

with slight modifications. Animals were fasted for 36 h but had access to water *ad libitum*. Thirty minutes after the oral administration of EER (250 and 500 mg/kg), the rats were immobilized in restraint cages and placed inside a ventilated refrigerator maintained at $3 \pm 1^\circ\text{C}$ for 3 h. The animals were then sacrificed and the stomachs were excised. They were examined for ulceration and the severity of intraluminal bleeding according to the following arbitrary scale described by Chiu *et al*^[24]. 0 = no blood detectable; 1 = thin blood follows the rugae; 2 = thick blood follows the rugae; 3 = thick blood follows the rugae with blood clots in certain areas and 4 = extensive covering of the whole gastric mucosal surface with thick blood.

Determination of gastric wall mucus (GWM)

Gastric wall mucus was determined according to the modified procedure of Crone *et al*^[25]. The glandular segment of the stomach was separated from the rumen of the stomach, weighed, and transferred immediately to 10 mL of 0.1% w/v Alcian blue solution (in 0.16 mmol/L sucrose solution buffered with 0.05 mL sodium acetate at pH 5). Tissue was stained for 2 h in Alcian blue, and excess dye was removed by two successive rinses with 10 mL of 0.25 mmol/L sucrose, firstly after 15 min and then after 45 min. Dye complexed with the gastric wall mucus was extracted with 10 mL of 0.5 mmol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract were then vigorously shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 r/min for 10 min and the absorbance of the aqueous layer was recorded at 580 nm. The quantity of Alcian blue extracted per gram of wet glandular tissue was then calculated.

Estimation of non-protein sulfhydryls (NP-SH)

Gastric mucosal non-protein sulfhydryls were measured according to the method of Sedlak and Lindsay^[26]. The glandular part of the stomach was homogenized in ice-cold 0.02 mmol/L ethylenediaminetetraacetic acid (EDTA). Aliquots of 5 mL of the homogenates were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10 min and centrifuged at 3000 r/min. Two milliliters of supernatant were mixed with 4 mL of 0.4 mol/L Tris buffer at pH 8.9. 0.1 mL of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The absorbance was measured within 5 min of DTNB addition at 412 nm against a reagent blank.

Determination of malondialdehyde (MDA)

The method reported by Utley *et al*^[27] was followed. The animals were killed 1 h after ethanol administration. The stomachs were removed and each was homogenized in 0.15 mol/L KCl (at 4°C) in a Potter-Elvehjem type C homogenizer to give a 10% w/v homogenate. Aliquots

of homogenate 1 mL in volume were incubated at 37°C for 3 h in a metabolic shaker. Then 1 mL of 10% aqueous TCA was added and mixed. The mixture was then centrifuged at 800 g for 10 min. One milliliter of the supernatant was removed and mixed with 1 mL of 0.67% w-thiobarbituric acid in water and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 mL distilled water. The absorbance of the solution was then read at 535 nm. The content of malondialdehyde (nmol/g wet tissue) (index of the magnitude of lipid peroxidation) was then calculated, by reference to a standard curve of malondialdehyde solution.

Histopathological evaluation

Gastric tissue samples were fixed in neutral buffered formalin for 24 h. Sections of gastric tissue were histopathologically examined to study the ulcerogenic and/or anti-ulcerogenic activity of EER. The tissues were fixed in 10% buffered formalin and processed using a VIP tissue processor. The processed tissues were embedded in paraffin blocks and sections about 5 μm thick were cut using an American optical rotary microtome. These sections were stained with haematoxylin and eosin using routine procedures^[28]. The slides were examined microscopically for pathomorphological changes such as congestion, hemorrhage, edema, and erosions using an arbitrary scale for severity assessment of these changes.

Statistical analysis

Values in tables and figures are given as mean \pm SE. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Student's *t*-test.

RESULTS

Effect of EER on gastric secretions in 6 h pylorus-ligated rats

When the rats were subjected to pylorus ligation for 6 h, a considerable amount of basal gastric acid secretion was noted (10.83 ± 1.16 mL) in the control group. In the same control group, the titratable acidity was found to be 196.57 ± 15.50 mEq/L and the ulcer index was recorded as 2.33 ± 1.5 . EER at both doses (250 and 500 mg/kg) significantly reduced gastric acid secretion, titratable acidity and ulcer formation (6.33 ± 1.63 mL, 4.16 ± 1.16 mL $P < 0.05$, $P < 0.01$; 132.77 ± 17.43 , 55.55 ± 10.46 mEq/L $P < 0.05$, $P < 0.001$, 0.66 ± 0.51 , 0.50 ± 0.54 , $P < 0.05$, $P < 0.05$), respectively (Table 1).

Effect of EER on necrotizing agents-induced gastric lesions

The treatment of rats with 80% ethanol, 0.2 mol/L NaOH and 25% NaCl produced extensive gastric lesions in the glandular mucosa of the stomach in all the control rats. The ulcer index was 6.00 ± 0.89 , 6.66 ± 1.36 and 6.66 ± 0.51 , respectively in control rats 1 h after administration of the necrotizing agents. Pretreatment of rats with EER at doses of 250 mg/kg (ulcer index in 80% ethanol, 0.2 mol/L

Table 1 Effects of EER on gastric secretion, acidity and gastric lesion index in pylorus-ligated shay rats (mean \pm SE)

Group serial	Treatment	Dose (mg/kg, i.g.)	Volume of gastric content (mL)	Titratable acidity (mEq/L)	Ulcer index
1	Control (distilled water)	-	10.83 \pm 1.16	196.57 \pm 15.50	2.33 \pm 1.50
2	EER	250	6.33 \pm 1.63 ^a	132.77 \pm 17.43 ^a	0.66 \pm 0.51 ^a
3	EER	500	4.16 \pm 1.16 ^b	55.55 \pm 10.46 ^d	0.50 \pm 0.54 ^a

Six rats were used in each group. ^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ vs control (distilled water) group, Student's *t*-test.

Table 2 Effect of EER on gastric lesions induced by necrotizing agents (mean \pm SE)

Group serial	Treatment	Dose (mg/kg, i.g.)	Ulcer index		
			80% EtOH	0.2 mol/L NaOH	25% NaCl
1	Control (distilled water)	-	6.00 \pm 0.89	6.66 \pm 1.36	6.66 \pm 0.51
2	EER	250	2.00 \pm 0.89 ^b	2.66 \pm 1.21 ^a	2.83 \pm 0.98 ^b
3	EER	500	1.66 \pm 1.03 ^b	1.50 \pm 0.54 ^b	2.16 \pm 0.75 ^d

Six rats were used in each group. ^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ vs control (distilled water) group, Student's *t*-test.

Table 3 Effect of EER on indomethacin-induced gastric mucosal lesions (mean \pm SE)

Group serial	Treatment	Animals (n)	Dose (mg/kg, i.g.)	Ulcer Index
1	Control (indomethacin only)	6	-	44.50 \pm 5.82
2	EER	6	250	25.50 \pm 6.88
3	EER	6	500	13.50 \pm 4.84 ^b

Six rats were used in each group. ^b $P < 0.01$ vs control (indomethacin only) group, Student's *t*-test.

NaOH and 25% NaCl = 2.00 \pm 0.89, $P < 0.01$, 2.66 \pm 1.21, $P < 0.05$ and 2.83 \pm 0.98, $P < 0.001$; 500 mg/kg (ulcer index = 1.66 \pm 1.03, $P < 0.01$, 1.50 \pm 0.54, $P < 0.01$ and 2.16 \pm 0.75, $P < 0.001$), respectively, significantly inhibited the formation of gastric lesions as shown in Table 2.

Effect of EER on gastric lesions induced by indomethacin

The oral administration of indomethacin induced marked damage in the rat glandular stomach. EER at the 500 mg/kg dose significantly prevented the development of gastric lesions in the rat stomach ($P < 0.01$), however, no significant preventive effect of EER at the 250 mg/kg dose, in indomethacin-treated rats was observed (Table 3).

Effect of EER on hypothermic restraint stress-induced gastric mucosal lesions

Table 4 shows that EER at a dose of 500 mg/kg body weight significantly inhibited intraluminal bleeding and ulcer formation induced by hypothermic restraint stress ($P < 0.05$). Although the intraluminal bleeding and ulcer index was reduced at a dose of 250 mg/kg body weight, this reduction was not found to be statistically significant.

Effect of EER on ethanol-induced changes in gastric wall mucus (GWM)

Rats treated with ethanol showed a significant decrease

Table 4 Effect of EER on hypothermic restraint stress-induced intraluminal bleeding and gastric lesions in rats (mean \pm SE)

Group serial	Treatment	Dose (mg/kg, i.g.)	Intraluminal bleeding score	Gastric lesion ulcer index
1	Control (distilled water)	-	2.60 \pm 0.50	24.00 \pm 3.24
2	EER	250	1.20 \pm 0.83	15.20 \pm 3.96
3	EER	500	0.83 \pm 0.40 ^a	7.66 \pm 6.12 ^a

Six rats were used in each group. ^a $P < 0.05$ vs control (distilled water) group, Student's *t*-test.

in the Alcian blue binding capacity of gastric wall mucus (148.41 \pm 18.81 μ g/g of tissue, $P < 0.001$) as compared to control (normal) rats (426.73 \pm 39.15 μ g/g). Pretreatment of rats with EER at doses of 250 mg/kg (263.87 \pm 32.65 μ g/g, $P < 0.05$) and 500 mg/kg (313.90 \pm 24.30 μ g/g, $P < 0.001$) significantly enhanced the Alcian blue binding capacity of gastric mucosa (Figure 1).

Effect of EER on ethanol-induced depletion of gastric mucosal NP-SH

The level of NP-SH in the gastric mucosa of control rats was 5.26 \pm 0.63 mmol/g of tissue, which was significantly decreased to 2.38 \pm 0.33 mmol/g ($P < 0.001$) following the administration of 80% ethanol. Pretreatment of rats with EER at both doses (250 and 500 mg/kg) significantly replenished the ethanol-induced depletion of NP-SH (3.63 \pm 0.22, $P < 0.001$; 4.53 \pm 0.56, $P < 0.01$), respectively (Figure 2).

Effect of EER on ethanol-induced increase in MDA

As depicted in Figure 3, MDA levels in the gastric mucosa used as an index of lipid peroxidation were significantly higher in the ethanol only treated group than in the untreated control group (7.09 \pm 0.60 μ mol/g of tissue; 2.77 \pm 0.19 μ mol/g of tissue), respectively. EER at both doses (250 and 500 mg/kg) significantly

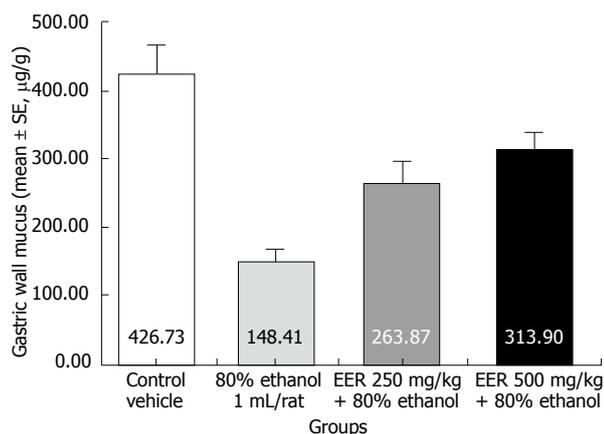


Figure 1 Effect of EER on the changes in gastric wall mucus induced by 80% ethanol.

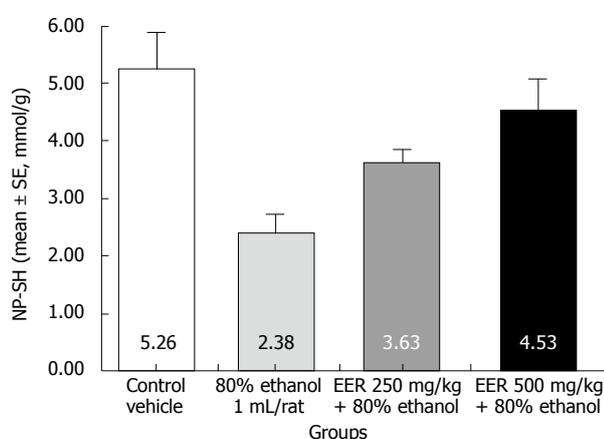


Figure 2 Effect of EER on NP-SH concentration in gastric ulcer induced by 80% ethanol.

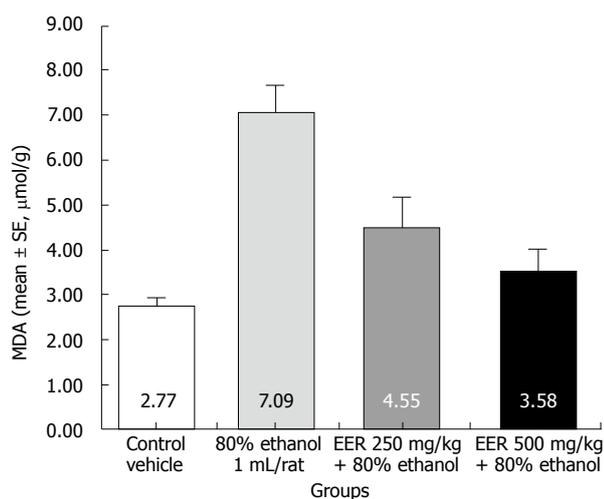


Figure 3 Effect of EER on MDA concentration in gastric ulcer induced by 80% ethanol.

decreased the MDA content ($4.55 \pm 0.66 \mu\text{mol/g}$ and $3.58 \pm 0.49 \mu\text{mol/g}$), respectively.

Effect of EER on histopathological evaluation

Histopathological studies (Figure 4) further confirmed that pretreatment with rocket extract prevented ethanol-

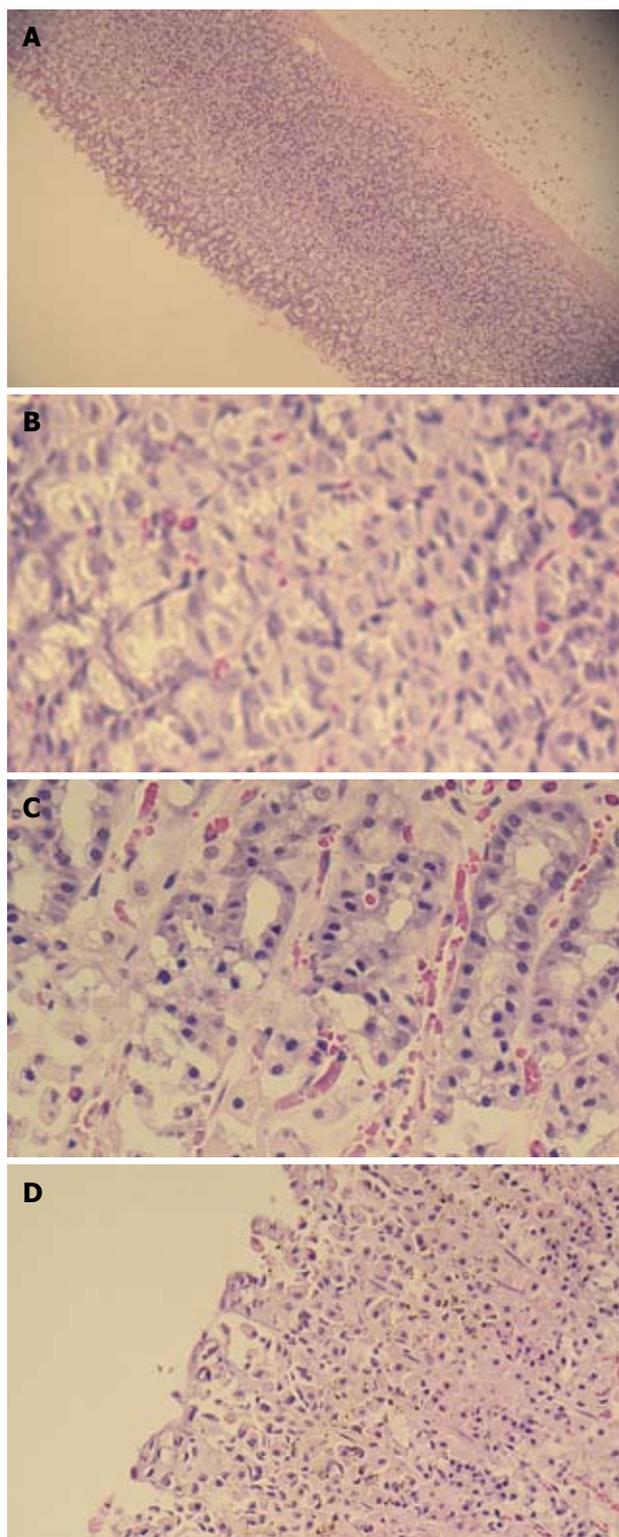


Figure 4 Light micrographs showing the effect of EER on ethanol-induced gastric lesions of rats. A: Normal mucosa; B: Ethanol-induced gastric mucosal congestion and necrosis; C: Pretreatment of rats with EER 250 mg/kg; D: Pretreatment of rats with EER 500 mg/kg.

induced necrosis in the superficial layers of the gastric mucosa with congestion.

DISCUSSION

The results of this study show that the ethanolic extract

of Rocket possesses significant anti-secretory, anti-ulcer and cytoprotective properties in rats. Pretreatment with EER produced a dose-dependent decrease in the volume of basal gastric secretion, titratable acidity and lesions in pylorus-ligated Shay rats. It has been reported that anti-secretory agents such as histamine H₂-receptor antagonists ameliorate the decrease in gastric mucosal blood flow caused by factors that disturb the gastric mucosa such as NSAIDs and ethanol^[29]. Gastric acid is an important factor in the genesis of ulceration in pylorus-ligated rats^[20]. The activation of the vagus-vagal reflux by stimulation of pressure receptors in the antral gastric mucosa in the hypersecretion model of pylorus ligation is believed to increase gastric acid secretion^[30]. Since EER markedly inhibited gastric acid secretion and ruminal ulcers in pylorus-ligated rats, this observed effect could be related, at least in part, to the ability of EER to reduce gastric acid secretion. It is now accepted that gastric acid secretion plays an important role in the progression from an erosive mucus layer to a gastric lesion. On the other hand, substances which have the ability to suppress gastric acid secretion, such as proton pump inhibitors and histamine H₂-receptor antagonists are believed to accelerate the healing process of the gastric lesions or inhibit the formation of mucosal injury^[31]. Rocket extract was found to offer the gastric mucosa, a statistically significant and dose-dependent protection against ulceration caused by various necrotizing agents including ethanol and strong alkalis. Ethanol-induced gastric ulcers have been widely used in the evaluation of gastroprotective activity. Ethanol is metabolized in the body and releases superoxide anion and hydroperoxy free radicals. It has been found that oxygen-derived free radicals are implicated in the mechanism of acute and chronic ulceration in the stomach^[32]. The genesis of ethanol-induced gastric lesions is of multifactorial origin with a decrease in gastric mucus, and is associated with the significant production of free radicals leading to increased lipid peroxidation which in turn causes damage to cells and cell membranes^[33]. The cytoprotective effect of EER may be related to its ability to prevent gastric acid secretion and/or enhance the mucosal defensive factors such as prostaglandins and decrease lipid peroxidation^[34]. Treatment of rats with indomethacin, a non-selective cyclooxygenase inhibitor is known to induce gastric damage through multiple mechanisms which include suppression of prostaglandin generation, overproduction of leukotrienes, acting as a topical irritant and by reducing the local blood-flow^[35]. Rats pretreated with EER produced significant protection in this model. It is possible that an enhanced level of gastric mucus, generating prostaglandins and inhibiting leukotriene may contribute to the gastroprotective effect of rocket extract.

Hypothermic-restraint stress ulcers have been used as an experimental model in the evaluation of anti-ulcer activity in rats due to data reproducibility^[36]. Disturbances of gastric mucosal microcirculation, enhancement of acid secretion and reduction in mucus production are mediated by histamine release and abnormal gastric motility^[37]. It is also reported that

free radicals may play a major role in stress-induced gastric injury^[38]. Stress is reported to inactivate mucosal prostaglandin syntheses by accumulating hydrogen peroxide, a prostaglandin biosynthesis inhibitor, which also causes reactive oxygen species (ROS) generation^[39]. In addition, a positive correlation has been reported between the level of gastric mucosal lipid peroxidation products, a marker of oxidative stress, and stomach damage in cold restraint-stressed rats^[40]. The protective efficacy against cold restraint-stress may be due to the antioxidant activities of EER, as an antioxidant activity was reported earlier in *Eruca sativa*^[14], this together with its antisecretagogue potential, thereby strengthens the animals physiological capabilities to decrease stress ulcers.

Our results revealed that the rocket ethanol extract significantly protected gastric mucosa against the depletion of gastric wall mucus. The mucus gel adhering to the gastric mucosal surface protects the underlying epithelium against acid^[41,42], pepsin^[43] and necrotizing agents such as ethanol and indomethacin^[11]. Gastric wall mucus however, plays a more important role in the defense of the gastric mucosa against chemical or mechanical aggression than the soluble mucus in the lumen of the stomach^[44]. The gastric mucus coat is thought to be important in facilitating the repair of the damaged gastric epithelium^[45]. It seems likely that the cytoprotective activity of EER could result, at least in part, from interaction with the adhering gastric mucus layer.

Sulfhydryl compounds in living organisms plays a central role in the maintenance of gastric integrity, particularly when ROS are involved in the pathogenesis of tissue damage^[46]. A significant decrease in gastric NP-SH following ethanol administration indicated massive generation of oxygen derived free radicals (ODFR). Our findings are in agreement with earlier reports showing depletion of sulfhydryls in ethanol-induced gastric lesions^[3,47]. Treatment of rats with glutathione depletors has been shown to significantly potentiate ulcerogen-induced gastric mucosal injury^[48], whereas an increase in mucosal NP-SH exerts a gastroprotective effect^[49]. Our observations clearly point towards the mediation of sulfhydryls in EER gastric mucosal protection.

Furthermore, the extract also showed significant inhibition of lipid peroxidation. The generation of MDA from lipids that react with thiobarbituric acid was found to be inhibited by the EER. Thus, it appears that the antioxidant property of the rocket extract may possibly counteract oxidative damage caused by alcohol toxicity. The observed anti-ulcerogenic activity may be due to its antioxidant effects and appears to strengthen the mucosal barrier, which is the first line of defense against endogenous and exogenous ulcerogenic agents.

The preliminary phytochemical screening of rocket revealed the presence of flavonoids, sterols and/or triterpenes. Moreover, quercetin and its derivatives were also reported in rocket leaves. Previous studies have shown that flavonoids may be related to the anti-ulcer activity^[50], and play a major role in the mechanism of

gastroprotection^[51,52]. In addition to flavonoids, other constituents in rocket such as sterol and/or triterpenes are known for their antioxidant activities, which may contribute to some of the anti-ulcer mechanisms^[53].

In conclusion, the data obtained confirm the traditional indications for this salad herb and present a new therapeutic option for the treatment of gastric ailments. The exact mechanism(s) underlying this anti-ulcerogenic effect remain unknown, but the extract contains substances, which might increase endogenous prostaglandins and mucus synthesis through its potent antioxidant activity. Furthermore, the anti-secretory mechanism can not, however, be dismissed.

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COMMENTS

Background

Gastric ulcer is an illness that affects a considerable number of people worldwide. The etiological factors of this disorder include: stress, smoking, nutritional deficiencies, infections, frequent and indiscriminate use of nonsteroidal anti-inflammatory drugs (NSAIDs). Herbs, medicinal plants, spices, vegetables and crude drug substances are considered to be a potential source to combat various diseases including gastric ulcer. In the scientific literature, a large number of medicinal plants with gastric anti-ulcer potential have been reported.

Research frontiers

Although the introduction of proton-pump inhibitors to the classic anti-ulcer therapy has revolutionized treatment of peptic ulcers and other gastrointestinal disorders, there is still no complete cure for this disease. It has been shown that long term use of these drugs leads to various adverse and side effects. Relapses of the malady, ineffectiveness of different drug regimens and even resistance to drugs are emerging. Thus, there is an urgent requirement to identify more effective and safe anti-ulcer agents. During the past few decades, a widespread search has been launched to identify new anti-ulcer therapies from natural sources.

Terminology

The anti-secretory, cytoprotective and antioxidant properties of the rocket extract caused an inhibition of chemically and stress-induced gastric ulceration in rats.

Peer review

The authors examined an ethanolic extract of rocket "*Eruca sativa* L." for its claimed beneficial effects on gastrointestinal disorders. The results are interesting and may provide a better understanding and give a clue for further investigations and innovations for an effective and safe phytotherapy for peptic ulcer disease.

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***In vitro* and *in vivo* suppression of hepatocellular carcinoma growth by midkine-antisense oligonucleotide-loaded nanoparticles**

Li-Cheng Dai, Xing Yao, Xiang Wang, Shu-Qiong Niu, Lin-Fu Zhou, Fang-Fang Fu, Shui-Xin Yang, Jin-Liang Ping

Li-Cheng Dai, Xiang Wang, Shu-Qiong Niu, Shui-Xin Yang, Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China
Xing Yao, Department of General Surgery, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China
Lin-Fu Zhou, Fang-Fang Fu, Zhejiang University School of Medicine, Hangzhou 310016, Zhejiang Province, China
Jin-Liang Ping, Department of Pathology, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China

Author contributions: Yao X, Niu SQ, Zhou LF and Wang X performed the majority of the experiments; Ping JL, Fu FF and Yang SX provided vital reagents and analytical tools; Dai LC designed the study and wrote the manuscript.

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Correspondence to: Li-Cheng Dai, Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China. dlc@hzhospital.com

Telephone: +86-572-2033020 Fax: +86-572-2033020

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Abstract

AIM: To synthesize antisense oligonucleotides (ASODNs) of midkine (MK), package the ASODNs with nanoparticles, and to inhibit hepatocellular carcinoma (HCC) growth using these nanoparticles.

METHODS: HepG2 cell proliferation was analyzed *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. The *in vivo* activity of nanoparticles delivering the MK-ASODNs was analyzed by histopathological and immunohistochemical staining and quantitative real time polymerase chain reaction (PCR).

RESULTS: The *in vitro* proliferation of HepG2 cells was significantly inhibited by the nanoparticles packaged with MK-ASODNs (NANO-ASODNs). Furthermore, the NANO-ASODNs significantly inhibited the growth of HCC in the mouse model.

CONCLUSION: NANO-ASODNs can significantly suppress the growth of HCC *in vitro* and *in vivo*.

INTRODUCTION

Hepatocellular carcinoma (HCC), a primary malignancy of the liver, is one of the most common tumors worldwide. The mortality rate from HCC is the third highest worldwide for any cancer-related diseases, and since the 1990s, HCC has been the cause of the second highest mortality rate due to cancer in China^[1]. Globally, the 5-year survival rate of HCC is less than 5% and 598 000 HCC patients die each year^[2], mainly because no satisfactory treatment is available and chemotherapy has been extremely ineffective. Recent insights into the biology of HCC suggest that certain pathways and molecular alterations are likely to play essential roles in HCC development by promoting cell growth and survival. Growth factors and the downstream signaling factors are often overexpressed in tumors and will become the targets of diagnosis or treatment.

Midkine (MK), a heparin-binding growth factor or cytokine, has been reported to be generally overexpressed in malignant tumors^[3-5], whereas in normal adult tissues, MK levels are low or undetectable^[6-10]. MK exhibits several cancer-related activities, including fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic and chemotactic functions. The antisense oligonucleotide (ASODN) that targets MK has been reported to suppress the growth of tumors in nude mice^[11,12]. Additionally,

siRNA or an ASODN that targets MK inhibits neointima formation^[13] and renal injury after ischemia^[14]. MK has been suggested to play an important role in carcinogenesis, thus MK can serve as a novel tumor marker or therapeutic target.

At present, the delivery tools of siRNAs or ASODNs are ineffective and toxic. Although lentiviral technology is a proven tool in the laboratory setting, it has several adverse effects, such as toxic immune responses and genetic alterations. Therefore, in the present study, we have used the systemic delivery of nanoparticles incorporated with MK-ASODNs (NANO-ASODNs). This delivery approach is a much safer and more effective alternative to viral therapy for the treatment of tumors, such as HCC. NANO-ASODNs have been found to play an important role in the suppression of HCC growth *in vitro* and *in vivo*, and provide insights into their future clinical application to tumor therapy.

MATERIALS AND METHODS

ASODN synthesis

Antisense phosphorothioate oligonucleotide MK-ASODN (5'-CCCCGGGCCGCCCTTCTTCA-3') targeting 108-127 base positions of MK mRNA was synthesized by an Applied Biosystems Model 391 DNA synthesizer (Amersham, Piscataway, NJ, USA) and purified by HPLC (Waters Delta Prep 4000, Milford, MA, USA) using a SOURCE 15Q column (Amersham). In our previous study^[15], this antisense sequence has been identified to be the most effective sequence for the down-regulation of MK. Consequently, this sequence was synthesized and applied in this study.

Nanoparticles liposome packaging

Acyl-chloride-cholesterol 2.25 g was dissolved in 5 mL anhydrous chloroform and transferred to a 25-mL three-necked flask. Two milliliters of N', N'-dimethylethanediamine was dissolved into another 5 mL of anhydrous chloroform. The solution was added to the acyl-chloride-cholesterol solution at a constant temperature of 0°C. After dropwise addition, the chloroform was removed by reduction vaporization and the product was purified three times by recrystallization with dehydrated alcohol, and finally eluted with dehydrated alcohol. Following recrystallization, the product was dehydrated by vacuum dehydration for 12 h and the white DC-Chol was obtained. This target product was confirmed by thin-layer chromatography and ¹H-NMR exosynonim analysis. Ten milligrams of DC-Chol and 8 mg of dioleophosphatidyl-ethanolamine were dissolved into chloroform and transferred into a pear shape. The shape was filled with nitrogen gas on a rotary evaporation instrument. Organic solvent was removed by reduction vaporization at a constant temperature of 40°C, until an even lipid film formed on the shape wall. Fifteen milliliter chloroform and 6 mL PBS (pH 7.4) were added into the shape to produce a water-in-oil emulsion adjuvant, using water-bath ultrasound. Chloroform was removed by reduction vaporization on a rotary evaporation instrument to produce a proteoliposome suspension. The suspension

was filled with nitrogen gas, made up to a volume of 10 mL with PBS, shattered using a transducer-ultrasound (1 s ultrasound with 2 s breaks for 150 times, with a work rate of 200 W) and filtered using a 0.1- μ m polycarbonate membrane five times using a minitruder. Nanometer liposomes were finally produced. One milliliter of the stock solution (5% manicol) was stored at -70°C for more than 3 h before vacuum dehydration. The shape and size of the nanometer liposomes were detected by transmission electron microscopy (TEM) and dynamic light scattering.

Cell culture and transduction assay

Human liver HCC cell line HepG2 (HepG2 cells were purchased from the Chinese Academy of Medical Sciences, Beijing, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C and 5% CO₂. Cells (3×10^3) were seeded in each well of a 96-well microtiter plate and allowed to attach overnight. ASODNs and NANO-ASODNs with different concentrations were added to the cells at different time points. Furthermore, the transduction rate of NANO-ASODNs was analyzed using a confocal microscope (Leica, Heidelberg, Germany).

Cell proliferation assay

NANO-ASODNs with concentrations of 0.1, 0.2, 0.4 and 0.6 μ mol/L were added into the HepG2 cell cultures. ASODNs (0.6 μ mol/L) transfected into the cells with Lipofectin (Invitrogen), following the manufacturer's instructions, acted as a positive control. Free nanoparticles were added as a negative control. The effects of ASODNs on cellular viability were measured by an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. After 48 h of incubation following transfection, 20 μ L MTS (Sigma, St Louis, MO, USA) was added to each well and incubated at 37°C for 2 h. The absorbance values were determined at 490 nm using a MR600 microplate reader (Wallac 1420 Multilable counter; Wallac, Turku, Finland).

In vivo tumor studies

Athymic nude mice (BALB/c-nu/nu females, 6-8 wk old) were purchased from the Academy of Military Medical Science (Beijing, China) and housed in a controlled environment at 22°C on a 12 h light/12 h dark cycle. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The *in situ* HCC models were established as described previously^[16]. Three days after the *in situ* HCC models were established, mice were randomly divided into six groups and each experimental group consisted of eight mice. The mice were intravenously injected with PBS (negative control group), 5-fluoro-2, 4 (¹H, ³H) pyrimidinedione (5-FU, positive control group) at 10 mg/kg per day and free nanoparticles as a second positive control group. 25, 50 and 100 mg/kg per day of NANO-ASODNs or

free ASODNs were injected into the mice for 20 d. The distribution of NANO-ASODNs was detected with the *in vivo* imaging systems (ICE-FM-1024B, LumazoneFM). Briefly, mice were anesthetized and FAM-NANO-ASODNs or Free ASODNs were intravenously injected through the tail vein. Images were obtained using the YAP-(S) PET scanner (ISE) at 0, 30, 60 and 90 min after injection. The data were acquired in list mode from 256 views over an angle range of 360 degrees. Images were reconstructed using an iterative reconstruction algorithm that provided transaxial, coronal and sagittal slices. At the end of the second *in vivo* imaging study, animals were sacrificed and tumors were removed for radioactivity counting. The body weights of the animals were recorded weekly. Two days after the intravenous injections were completed, mice were sacrificed and the tumors were removed and weighed. Tumor sizes were monitored with calipers; the tumor volume (V , mm^3) was calculated as: $V = \text{length} \times \text{width} \times \text{depth}/2$. The percentage of tumor growth inhibition was calculated as: Inhibitory rate = $(W_{\text{control}} - W_{\text{treat}})/W_{\text{control}} \times 100\%$. The blood of the mice was taken for routine blood tests and the α fetoprotein (AFP) test. The tissues of livers and tumors were taken for hematoxylin and eosin (HE) staining and histological examination.

Animal HCC model

The virgin female BALB/c mice used in this experiment were obtained from the Academy of Military Medical Science (Beijing, China). All animal experiments were carried out according to the standards of animal care as outlined in the NIH guide for the Care and Use of Laboratory Animals. The human HCC tumor model was described previously^[17]. Briefly, the HCM-Y89 tumor derived from a surgical specimen of HCC was cut into 1 mm \times 1 mm \times 1 mm pieces, and implanted into the liver of mice. Twenty days later, the mice treated with or without drugs were killed. The tumors were removed and fixed in neutral buffered 10% formalin, processed by standard methods, embedded in paraffin and sectioned and stained with HE. The HCC model maintains various important features similar to clinical liver cancer patients, including local growth, regional invasion, lymph nodes and pulmonary metastasis, peritoneal seeding with bloody ascites, and secretion of AFP in the recipient animals^[17].

Histopathological and immunohistochemical analysis

The liver and tumor specimens were fixed and frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC, USA). Five-micrometer sections were cut and stained with HE for histopathological analysis. The immunohistochemical demonstration of anti-MK protein binding was achieved with a rabbit polyclonal anti-MK antibody and an LSAB 2 kit, with visualization of the binding using 3,3'-diaminobenzidine tetrahydrochloride. Staining intensities were classified according to the proportions of positive cells as: negative, none; slightly positive, 50%; positive, 50%-90%; and strongly positive, > 90%. The specificity of the binding was confirmed by negative control staining using a rabbit non-immune serum rather than the primary antibody.

RNA isolation and real-time polymerase chain reaction (PCR)

Total cellular RNA from cell cultures and tissues isolated from the livers and the tumors of mice were extracted using the RNeasy kit according to the manufacturer's protocol. cDNA of the tissue was synthesized from 5 μg of total RNA using a reverse transcription kit. Subsequently, the first strand of cDNA was used as a PCR template. Aliquots of 1 μL of 10-fold diluted cDNA solutions were subjected to PCR in a 20- μL reaction mixture (2 μL PCR buffer; 2 μL dNTP mix; 0.1 μL Taq DNA polymerase; 0.2 μL primers; 14.7 μL autoclaved, distilled water). The primers were as follows: MK, sense primer: 5'-CTCCGCGGTTCGCCAAAAAGAAAGA-3'; anti-sense primer: 5'-CCCCATCACACGCACCCCA GTT-3'. GAPDH, sense primer: 5'-GGAGCCAAAAG GGTCCATCT-3'; anti-sense primer: 5'-AGGGGCC ATCCACAGTCTTCT-3'. PCR was conducted using a SYBR Premix ExTaq™ kit (TakaRa, Dalian, China) with the following conditions: pre-heating at 95°C for 2 min, 40 cycles of 30 s denaturation at 94°C, a 20 s annealing at 56°C, and a 40-s extension at 72°C.

Statistical analysis

All parameters were analyzed by analysis of variance. Analysis of variance and Student's *t* test were used to compare each post operative result of each level among all groups. A minimum level of 0.05 was chosen.

RESULTS

Generation of nanoparticles

The produced nanometer liposomes were used to package the MK-ASODNs using a ratio of 1.8 μL of the nanometer liposomes to 1 μg MK-ASODNs (Figure 1A). The nanoparticles packaged with MK-ASODNs were stained with 1% uranyl acetate and examined with an electron microscope. The size of the micelles was determined with a Zetasizer 5000 (Malvern Instruments, Malvern, Worcestershire, UK). The morphology of the nanoparticles packaged with MK-ASODNs was examined using dynamic light scattering and TEM (Figure 1B).

Inhibition of growth of HCC *in vitro*

In order to determine the transduction of NANO-ASODNs, the ASODNs were conjugated with FAM and the rate of transduction under the confocal microscope at different time points was observed. Figure 2A shows that the NANO-ASODNs were effectively transduced into the HepG2 cells at the indicated times (6, 12 and 18 h). Since the NANO-ASODNs readily enter into the cells, we wanted to know whether they could decrease expression of MK in HepG2 cells. Figure 2B shows that the NANO-ASODNs (0.1, 0.2, 0.4 and 0.8 $\mu\text{g}/\text{mL}$) could significantly down-regulate the MK mRNA levels (Figure 2B). The MTS assay was used to analyze the effect of NANO-ASODNs on HCC cell proliferation. Figure 2C shows that the inhibition rates ranged from 20% to 80%, which correlated with the ASODNs concentrations.

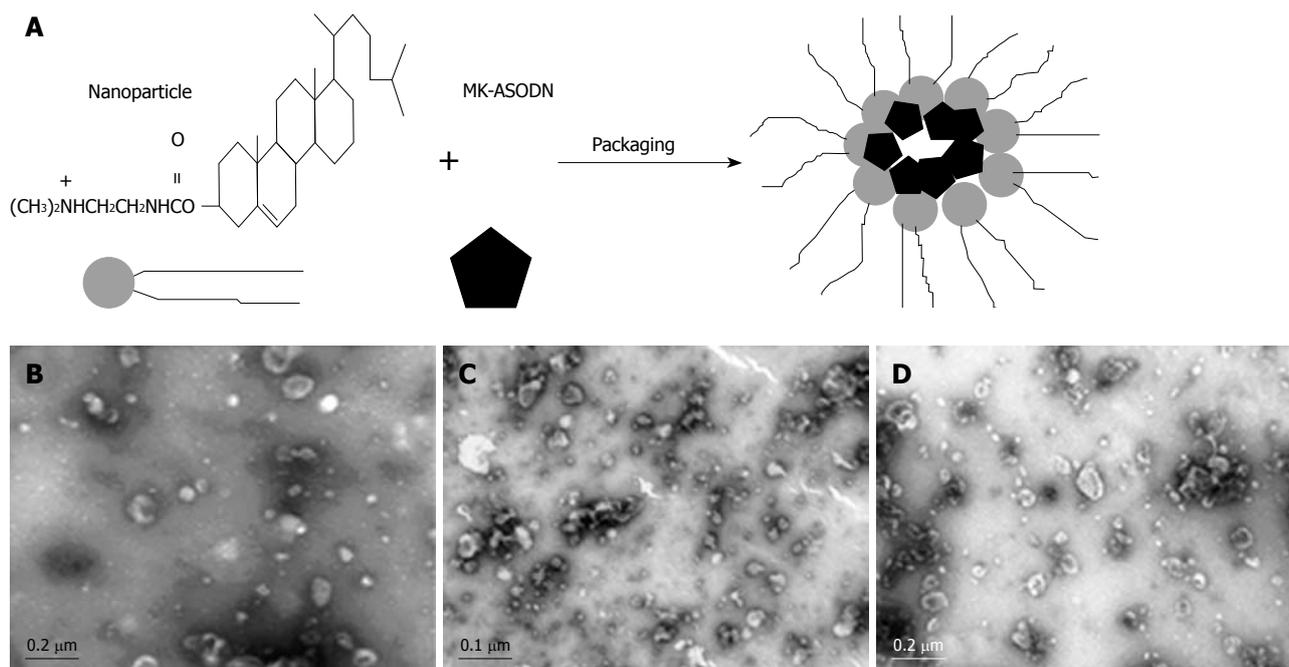


Figure 1 Nano-assembly of MK-ASODNs and nanoliposomes and characterization of NANO-ASODNs. A: Schematic illustration of the self-assembly of MK-ASODN and nanoliposomes; B: TEM image of the empty nanoliposomes stained with 1% uranyl acetate; C, D: TEM image of the NANO-ASODNs.

Table 1 NANO-ASODNs inhibit growth of HCC *in vivo*

Group (mg/kg)	Tumor volume (mm ³)	Tumor weight (g)	Tumor inhibition (%)	AFP (ng/mL)
Control (PBS)	1633.38 ± 525.93	1.92 ± 0.45	-	457.63 ± 141.47
ASODN-100	321.56 ± 85.55 ^b	0.57 ± 0.16 ^b	70.31	97.63 ± 23.79 ^b
ASODN-50	509.29 ± 300.85 ^b	0.73 ± 0.22 ^b	61.98	179.86 ± 210.27 ^b
ASODN-25	835.25 ± 263.33 ^a	1.14 ± 0.12 ^b	40.63	428.63 ± 141.47
5-FU10	717.19 ± 281.25 ^b	0.98 ± 0.16 ^b	48.96	315.25 ± 195.77
Nano-ASODN-100	225.81 ± 128.75 ^b	0.35 ± 0.17 ^b	81.77	58.25 ± 30.83 ^b
Nano-ASODN-50	457.88 ± 249.29 ^b	0.52 ± 0.21 ^b	72.92	89.38 ± 61.75 ^b
Nano-ASODN-25	584.00 ± 261.92 ^b	0.83 ± 0.20 ^b	56.77	205.38 ± 125.16 ^b
Nano control	1319.25 ± 340.70	1.59 ± 0.18	17.18	419.25 ± 148.46

^a $P < 0.05$, ^b $P < 0.01$ vs control (PBS).

Inhibition of growth of HCC *in vivo*

NANO-ASODNs mainly target the liver. In the present study, we used an *in situ* mouse HCC model to evaluate the antitumor activity of NANO-ASODNs. Figure 3 shows that NANO-ASODNs mainly targeted the liver after injection through the tail vein. We also found that the concentration of the NANO-ASODNs reached a peak 90 min after the injection, and then slowly decreased.

Effects of NANO-ASODNs treatment on *in situ* HCC xenograft growth: After establishing the mouse HCC model for 2 d, PBS, free nanoparticles, 10 mg/kg per day 5-FU, various doses of NANO-ASODNs or ASODNs (25, 50 and 100 mg/kg per day) were administered through the tail vein for 20 d. The tumors were removed after sacrificing the mice. The tumors were measured and weighed. Table 1 and Figure 4 show the final tumor volumes and weights after 20 d of treatment. The results showed that the tumor volumes decreased in both free ASODNs and NANO-ASODNs treated groups compared with the PBS control group ($P < 0.01$).

Additionally, the effect of NANO-ASODNs on tumor growth inhibition was superior to the free ASODNs ($P < 0.05$). Moreover, the effect of NANO-ASODNs on inhibiting tumor proliferation was dose-dependent (Table 1). In addition, the NANO-ASODNs treatment also resulted in a significant inhibition of tumor weight compared with the PBS- and free-nanoparticle-treated mice. In contrast to the PBS group, it had the highest inhibitory efficacy for the tumor weight which was 81.77% and 10 mg/kg 5-FU had an inhibitory efficacy of 48.96%; however, for the free nanoparticles treatment, the inhibitory rate was only 17.18% ($P < 0.01$) (Table 1).

Histopathological analysis: The morphology of the tumors treated by NANO-ASODNs, ASODNs, 5-FU and PBS were evaluated by HE staining. The tumors were excised at the endpoint of the treatment of each protocol. Figure 5 shows the representative sections of the tumors from each experimental group. The tumors from the mice treated with NANO-ASODNs, ASODNs and 5-FU showed a marked increase in the necrotic

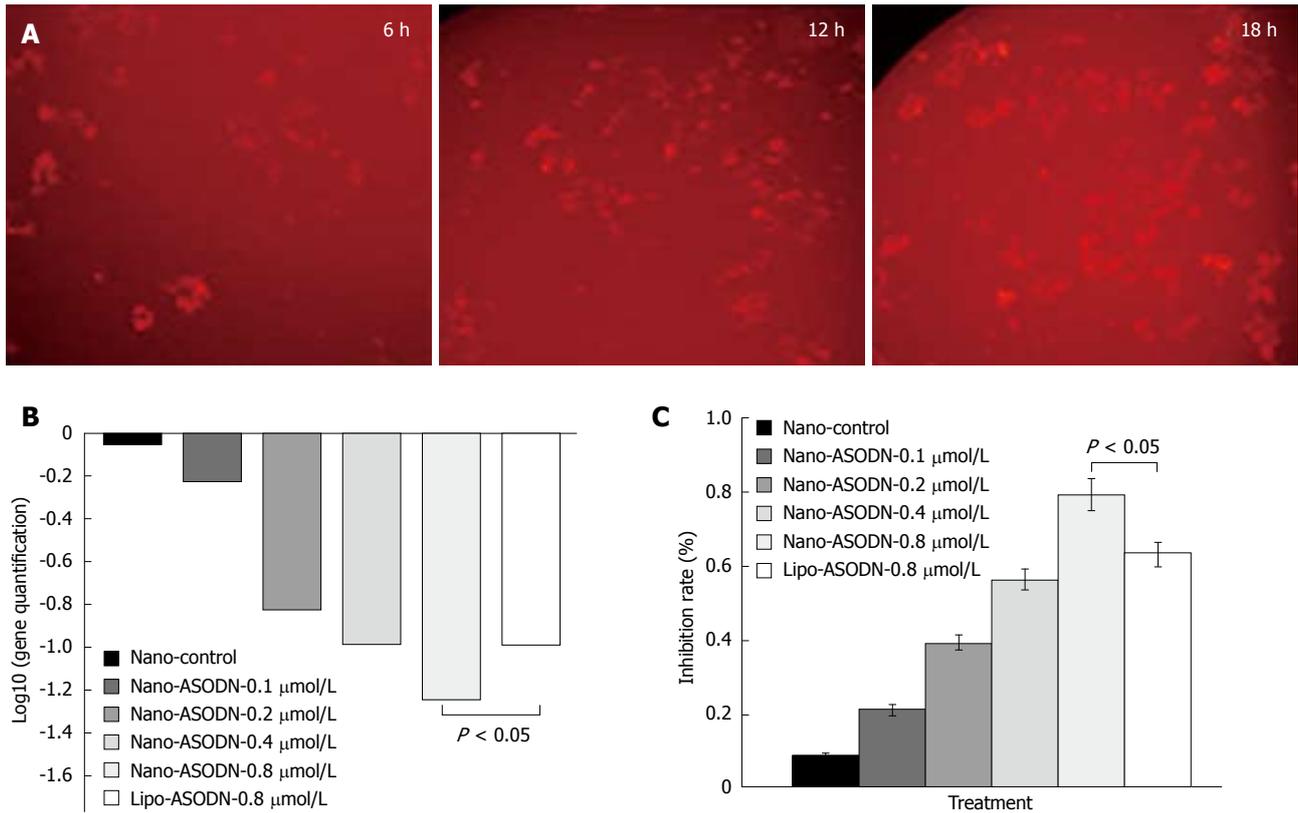


Figure 2 Transduction and function of NANO-ASODNs *in vitro*. A: 0.2 μmol/L FAM-conjugated NANO-ASODNs transduced into HepG2 cells. The results were observed under a confocal microscope at indicated times of 6, 12 and 18 h; B: NANO-ASODNs down-regulated expression of MK mRNA; C: The proliferation of HepG2 cells was significantly inhibited by NANO-ASODNs.

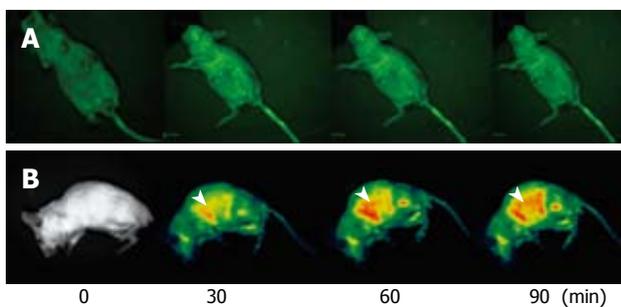


Figure 3 NANO-ASODNs target the liver. The kinetic results of the NANO-ASODNs were observed through *in vivo* imaging systems at indicated times after NANO-ASODNs were injected through the tail vein. A: Free ASODNs did not concentrate within the liver and these ASODNs disappeared quickly; B: NANO-ASODNs were found to mainly target the liver (the arrow represents the NANO-ASODNs).

area compared with the PBS-treated animals. This result suggests that the NANO-ASODNs, ASODNs and 5-FU treatment induced HCC necrosis *in vivo*.

Inhibition of plasma AFP secretion: AFP is often expressed in high levels in fetal liver, the gastrointestinal tract and the yolk sack, but AFP is transcriptionally down-regulated after birth and frequently re-expressed in HCC. Therefore, it is often used as an indicator of HCC^[18]. In this experiment, we used radioimmunoassay to detect serum AFP concentrations at the endpoint of the treatment. Table 1 shows that NANO-ASODNs at the dose of 100, 50 and 25 mg/kg per day significantly

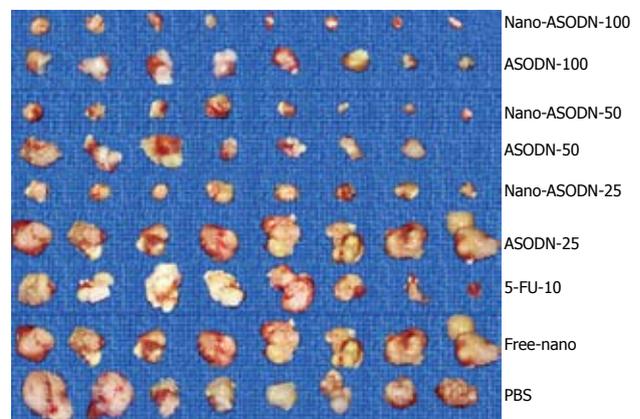


Figure 4 Morphological changes of HCC following treatment with NANO-ASODNs. The volume of HCC decreased significantly following treatment with 100, 50 and 25 mg/kg per day of NANO-ASODNs for 20 d. MK-ASODNs were the positive control. The PBS or free nanoparticles represent the negative controls.

decreased AFP secretion compared with the ASODNs or control groups. This result suggests that there were fewer liver tumor cells in the NANO-ASODNs-treated mice and this treatment reduced circulating AFP.

Systemic toxicity of NANO-ASODNs: Drug treatment of cancer is usually associated with terrible side effects, which result in severe reduction of white blood cell counts or weight loss. In order to evaluate the toxicity of the NANO-ASODNs, we compared the systemic toxicity

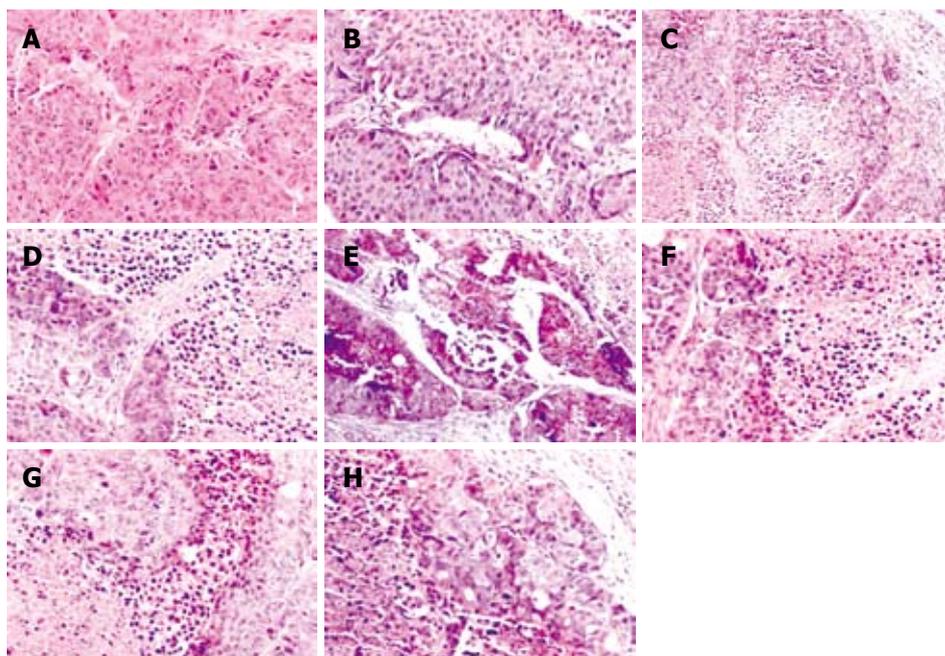


Figure 5 Histopathological analysis.

A: Tissue sections of the tumors from *in situ* xenograft HCC; B: Tissue sections of the tumors from nanoparticles; C: Tissue sections of the tumors from 5-FU (10 mg/kg per day); D: Tissue sections of the tumors treated with ASODNs 50 mg/kg per day; E-G: Tissue sections of tumors treated with NANO-ASODNs 100, 50 and 25 mg/kg per day of NANO-ASODN treated tumors, respectively; H: Tissue sections of the tumors treated with 5-FU (10 mg/kg per day) and 50 mg/kg per day of NANO-ASODNs. Regions showing an increase of necrosis and fibrosis were observed in the 5-FU or ASODN treatment groups (C-H, $\times 200$) compared with the free nanoparticles and untreated groups (A and B, $\times 200$).

of free ASODNs, 5-FU and NANO-ASODNs. Mice without tumors were administered a dose of 10 mg/kg per day ASODNs, 5-FU and NANO-ASODNs, and PBS as the control. The body weight was subsequently monitored. The results showed that the mean body weight decrease of the NANO-ASODNs group was not significantly different compared with the ASODNs or PBS groups ($P > 0.05$). However, the decrease in the weight of ASODN- and 5-FU-treated mice was more significant (data not shown). In addition, no inflammatory infiltrate was observed surrounding the solid tumor after treatment with different concentrations of the NANO-ASODNs (data not shown).

All the above studies suggest that nanoparticles packaged with ASODNs were safer than free ASODNs or chemical drugs.

DISCUSSION

MK is a heparin-binding growth factor identified as a product of a retinoic acid response gene^[19,20]. MK is overexpressed in a wide range of human carcinomas and believed to contribute to tumorigenesis and tumor progression. HCC is the most common primary liver malignancy, with a rising incidence worldwide. At present, although surgery and chemotherapy are effective in patients with localized tumors, the prognosis of patients with advanced or metastatic tumors is not ideal. Therefore, novel treatment approaches for the cancer are urgently needed. Recently, HCC tumor cells were found to overexpress MK. In our previous studies, ASODNs that target MK were demonstrated to play an important role in anti-tumor functions^[15,16]. However, the anti-tumor effect was not satisfactory and found to be toxic because of the absence of smart and safer delivery tools. At present, the strategies that have been adopted to improve the uptake of various nucleic-acid-based therapeutic agents are microinjection, passive diffusion, endocytosis (i.e. receptor

mediated endocytosis, fluid phase pinocytosis, adsorptive endocytosis) and artificially enhanced uptake (i.e. using delivery vectors like liposomes, micro- or nanoparticles or dendrimers)^[21,22].

In the present study, we used more effective and less toxic nanoparticle liposomes that have previously been used effectively to deliver siRNA for the treatment of lymphoma and ovarian cancer, as well as colorectal carcinoma. Liposomes, the first nanotechnology to benefit cancer patients, are continuing to evolve as tools for delivering potentially useful therapies for the treatment against tumors. In this study, MK-ASODNs incorporated into nanoliposomes have been used effectively *in vivo*. Evidence from these experiments showed that nanoliposomes packaged with MK-ASODNs could suppress HCC growth both *in vitro* and *in vivo*. In addition, the data also indicated that nanoliposomes could effectively deliver MK-ASODNs and showed less systematic toxicity. Consequently, nanoliposomes incorporated with MK-ASODNs should represent an effective and less toxic approach for treatment of HCC, and potentially, other tumor types.

In summary, our results suggest that nanoliposomes packaged with MK-ASODNs can increase the therapeutic effect of MK-ASODNs, both *in vivo* and *in vitro*, for the treatment of HCC. The combination of nanoliposomes and MK-ASODNs showed a more effective and less toxic tool for therapy against HCC and should provide a novel strategy for cancer treatment.

COMMENTS

Background

Midkine (MK) is a 13-kDa protein with a heparin-binding growth factor function. MK has been found to play important roles in carcinogenesis, including mitogenic, anti-apoptotic, transforming, fibrinolytic, chemotactic and angiogenic cancer-related activities.

Research frontiers

MK plays an important role in tumor development and progression.

Nanoliposomes packaged with MK-antisense oligonucleotides (ASODNs) can inhibit growth of hepatocellular carcinoma (HCC), both *in vitro* and *in vivo*, and potentially represents a significant clinical benefit.

Innovations and breakthroughs

In this study, nanoliposomes effectively delivered MK-ASODNs *in vitro* and *in vivo* with low toxicity. Additionally nanoliposomes packaged with MK-ASODNs inhibited proliferation of HepG2 cells and the growth of HCC xenografts.

Applications

In this study, the authors addressed the potential therapeutic effect of nanoliposomes packaged with MK-ASODNs on the suppression of HCC growth. Significant inhibition of HCC growth was achieved using the nanoliposomes packaged with MK-ASODN. This observation indicates that the nanoliposomes packaged with MK-ASODN are an effective anti-tumor agent.

Terminology

MK is a growth protein, which is overexpressed in HCC and promotes the growth of tumors. Nanoparticles represent powerful delivery tools, which can effectively deliver drugs or molecules into cells.

Peer review

The authors studied the growth inhibition of HCC *in vitro* and *in vivo* with nanoparticles delivering MK-ASODNs. MK-ASODNs have been shown to inhibit the growth of HCC. They found that the proliferation of HepG2 cells was significantly inhibited in the presence of different concentrations of nanoparticles packaged with MK-ASODNs (NANO-ASODNs). Furthermore, in the HCC mouse model, the NANO-ASODNs mainly accumulated in the liver and significantly inhibited the growth of the HCC tumors.

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Colonoscopic polypectomy in anticoagulated patients

Shai Friedland, Daniel Sedehi, Roy Soetikno

Shai Friedland, Daniel Sedehi, Roy Soetikno, Division of Gastroenterology, GI-111, VA Palo Alto and Stanford University, 3801 Miranda Avenue, Palo Alto, CA 94304, United States

Author contributions: Friedland S and Sedehi D performed data collection, analysis and writing of the manuscript; Soetikno R performed data analysis and writing of the manuscript.

Correspondence to: Shai Friedland, MD, Division of Gastroenterology, GI-111, VA Palo Alto and Stanford University, 3801 Miranda Avenue, Palo Alto, CA 94304, United States. shai_friedland@yahoo.com

Telephone: +1-650-4935000 Fax: +1-650-8490505

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Assistant Professor, Department of Surgery, University of Chicago, 5841 S. Maryland Ave, MC 5031, Chicago, IL 60637, United States

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Abstract

AIM: To review our experience performing polypectomy in anticoagulated patients without interruption of anticoagulation.

METHODS: Retrospective chart review at the Veterans Affairs Palo Alto Health Care System. Two hundred and twenty five polypectomies were performed in 123 patients. Patients followed a standardized protocol that included stopping warfarin for 36 h to avoid suprathreshold anticoagulation from the bowel preparation. Patients with lesions larger than 1 cm were generally rescheduled for polypectomy off warfarin. Endoscopic clips were routinely applied prophylactically.

RESULTS: One patient (0.8%, 95% CI: 0.1%-4.5%) developed major post-polypectomy bleeding that required transfusion. Two others (1.6%, 95% CI: 0.5%-5.7%) had self-limited hematochezia at home and did not seek medical attention. The average polyp size was 5.1 ± 2.2 mm.

CONCLUSION: Polypectomy can be performed in therapeutically anticoagulated patients with lesions up to 1 cm in size with an acceptable bleeding rate.

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Key words: Colon cancer; Colonic polyps; Colonoscopy; Early detection of cancer; Endoscopy; Hemorrhage; Thrombosis; Warfarin

Peer reviewer: Alessandro Fichera, MD, FACS, FASCRS,

INTRODUCTION

Current guidelines for the management of anticoagulants during colonoscopic polypectomy recommend that clotting parameters should be normalized at the time of the procedure^[1,2]. These guidelines are based largely on expert opinion: polypectomy is considered to be a high risk procedure, and the risk of temporary discontinuation of anticoagulants was previously considered low^[3-5]. However, recent data suggest that the risk of thromboembolic events is significant when anticoagulants are discontinued for endoscopic and other procedures: the risk of stroke was 1% in a study of 987 patients with atrial fibrillation undergoing 1137 endoscopic procedures, and the risk of thromboembolic events was 0.7% in a study of 1293 warfarin interruptions in 1024 patients^[6,7].

One strategy that may decrease this risk by shortening the period of subtherapeutic anticoagulation, is to use intravenous heparin or subcutaneous low-molecular-weight heparin in the perioperative period, rather than simply holding warfarin for several days before and resuming warfarin after the procedure^[8,9]. An alternative strategy to decrease the thromboembolic risk is to perform polypectomy on lesions up to 1 cm in size while patients remain anticoagulated. In 2007, we reported a series of 41 polypectomies performed in 21 patients with an average international normalized ratio (INR) of 2.3 (range 1.4-4.9)^[10]. The patients were maintained on warfarin until 36 h before the procedure; the medication was held for 36 h in order to avoid suprathreshold anticoagulation as a result of dietary restriction during colonoscopy preparation. Endoscopic clipping was performed prophylactically immediately after polypectomy. There were no episodes of post-polypectomy bleeding in that small case series. In this study we report our experience of performing polypectomy in a significantly larger number of patients who were anticoagulated.

MATERIALS AND METHODS

We reviewed available data from all anticoagulated patients from July 2004 to May 2008 who underwent colonoscopy at the Veterans Affairs Palo Alto Health Care System. Informed consent for the procedure was obtained from all patients, including discussion of the potentially high risk of bleeding due to anticoagulation. Institutional review board approval was obtained for retrospective data analysis. Our clinical protocol, which was followed by all patients, was to continue warfarin until 36 h before the procedure, when a clear liquid diet was initiated in preparation for the procedure. Warfarin was not taken while patients were on a clear liquid diet in order to avoid supratherapeutic anticoagulation during this period of potentially low vitamin K intake. In the first 21 patients, as reported in our original case series, INR was measured on the day of the procedure^[10]. Subsequently, INR was no longer routinely measured. Colonoscopic polypectomy was generally only performed on polyps up to 1 cm in size (the size was estimated by comparison to a fully opened 1 cm snare), and patients with larger lesions were rescheduled at a later date with cessation of anticoagulation. On five occasions, lesions larger than 10 mm were removed and these patients were also included in the series. Immediately after polypectomy, one or more endoclips were placed prophylactically to close the polypectomy defect. Following the procedure, warfarin anticoagulation was continued on the patient's standard schedule. Follow-up was available on all patients *via* telephone and/or clinic visits.

RESULTS

Two hundred and twenty five polypectomies were performed in 123 patients (Table 1). The most common indications for colonoscopy were screening, history of polyps, iron-deficiency anemia, hematochezia and occult bleeding. The most common indications for warfarin therapy were atrial fibrillation, history of thromboembolism and mechanical heart valves. Characteristics of resected polyps are described in Table 2. The average diameter of resected polyps was 5.1 ± 2.2 mm, with a range of 2-15 mm. Most of the polyps were removed by cold snare (snare removal without cautery or submucosal saline injection) or by snare with cautery following submucosal saline injection. Seventy percent of the resected polyps were neoplastic, consisting mainly of tubular adenomas. Twenty percent of the resected polyps were non-neoplastic. Ten percent of the specimens were lost.

One patient (0.8%, 95% CI: 0.1%-4.5%) developed major post-polypectomy bleeding. He was a 79-year-old man with atrial fibrillation, dilated cardiomyopathy, emphysema and a history of alcohol abuse who had 6 mm, 8 mm and 12 mm tubular adenomas removed. The smallest polyp was removed by cold snare and the larger two were removed by snare with cautery after saline injection. A subsequent upper endoscopy on the same day as the colonoscopy demonstrated

Table 1 Patient characteristics

Patient characteristics	Number or percentage
Number of patients	123
Average age (range)	68.4 ± 9 (49-90) yr
Male, female	122, 1
Indication for procedure	Screening (48%) History of polyps (24%) Iron def anemia (9%) Hematochezia (7%) Occult-blood positive stool (7%) Other (5%)
Indication for warfarin	Atrial fibrillation (65%) Thromboembolism (16%) Mechanical valve (9%) Other indications (13%)

Table 2 Polypectomy characteristics

Polyp characteristics	Number or percentage
Number of polyps	225
Polyps per patient	1.8
Average polyp size (mm)	5.1 ± 2.2
Range of polyp size (mm)	2-15
Polypectomy method	Cold snare (48%) Snare/cautery after saline injection (30%) Snare/cautery, no saline injection (16%) Cold biopsy (4%) Cold snare after saline injection (1%)
Polyp histology	Neoplastic (70%) Non-neoplastic (20%) Lost specimen (10%)

portal hypertensive gastropathy. On post-procedure day 4, he developed hematochezia. He was admitted to a local hospital and received 2 units of packed red blood cells. Repeat colonoscopy was not performed, and the bleeding resolved without further treatment. Two patients (1.6%, 95% CI: 0.5%-5.7%) had self-limited hematochezia at home and did not seek medical attention; these were classified as minor bleeding complications. No thromboembolic events were observed.

DISCUSSION

Current practice guidelines for the management of anticoagulation during colonoscopy are largely based on expert opinion. According to current guidelines, colonoscopy with or without biopsy can be performed in anticoagulated patients, but polypectomy is considered a high risk procedure for which anticoagulation must be temporarily discontinued in order to achieve normalization of coagulation function at the time of the polypectomy. The risk of withholding warfarin for several days has generally been estimated based on extrapolation from the annual incidence of thromboembolic events in patients with various clinical conditions who do not receive anticoagulants. However, more recent data suggest that actual observed thromboembolic complication rates in patients who have interruption of anticoagulation for endoscopic

procedures are higher than the theoretical predictions; this may be due to a rebound increase in clotting factors in this setting^[6-7,11].

Post-polypectomy bleeding is a relatively common complication of colonoscopic polypectomy, with a reported incidence of approximately 0.3%-2% that depends on multiple factors including lesion size^[12,13]. A multivariate regression analysis suggested that anticoagulation increases this risk^[14]. Post-polypectomy bleeding is generally divided into two types: immediate bleeding following the polypectomy, and delayed bleeding that can occur up to 2-3 wk following the procedure. Immediate bleeding is familiar to therapeutic endoscopists, as it is particularly common following endoscopic mucosal resection of large lesions^[15]. In this situation, it is generally treated very effectively by methods such as clip application^[15-17]. In contrast, delayed bleeding typically occurs when the patient is already at home and is therefore a significant concern when polypectomy is undertaken in patients who require anticoagulation. Even when current guidelines are followed and warfarin is held in anticipation of polypectomy, it is quite possible that the patient will be therapeutically anticoagulated at the time that delayed bleeding occurs several days after the procedure. The major issues with performing polypectomy while patients are anticoagulated are therefore as follows: will there be difficulty in controlling any immediate bleeding, will there be a significantly increased risk of delayed bleeding, and are the bleeding risks outweighed by the risk of thromboembolic events that could occur if anticoagulation was interrupted? This study systematically evaluated the risks associated with performing polypectomy while patients were anticoagulated.

Our study demonstrated that polypectomy of lesions up to approximately 1 cm in size can be performed with relative safety in anticoagulated patients. Because we anticipated some degree of immediate bleeding, our uniform practice was to immediately apply endoscopic clips to the polypectomy sites rather than observing the site and waiting to see if bleeding developed. With this strategy, we did not observe any extraordinary episodes of immediate bleeding that could not be controlled with clip application. There was only 1 episode of major delayed bleeding out of 123 patients and 225 polypectomies, a major bleeding rate of 0.8% (CI: 0.1%-4.5%). There were no thromboembolic events, although follow-up was often performed by telephone and typically did not include a neurological exam. The complication profile observed in this study suggests that the strategy of removing small lesions up to 1 cm in size may compare favorably with the standard practice of discontinuation of warfarin for elective procedures, where two major recent studies found thromboembolic rates of 0.7% and 1%^[6,7].

It must be emphasized that in our patients warfarin was withheld for approximately 36 h in order to avoid supratherapeutic anticoagulation due to dietary restriction and possibly other factors relating to bowel

preparation. We previously published our experience with the first 21 patients in our series, in whom INR was routinely measured before the procedure^[10]. In those patients the average INR at the time of colonoscopy was 2.3. Following this experience, we stopped routine measurement of INR before colonoscopy as this involved significant logistical difficulty. The polypectomy techniques utilized in our series varied, with cold snare, standard snare with cautery and inject & cut mucosectomy comprising the majority of polypectomies. Although our data suggest that all three techniques may be safe, our current preference is to perform cold snare in lesions smaller than 5 mm and to perform submucosal injection for larger lesions in which cautery is used in order to minimize potential injury to the bowel wall. In addition, prophylactic clipping was performed immediately at all polypectomy sites. While there is no data to demonstrate the efficacy of clipping in this circumstance, and a randomized study of clipping in average-risk patients demonstrated no benefit, we felt compelled to perform clipping because of the absence of data in anticoagulated patients^[18]. The drawbacks of clipping include additional endoscopy time, clip cost, and in our case also the practice of immediate clipping before specimen retrieval may have contributed to a relatively large fraction, 10%, of lost specimens.

Limitations of our study include the retrospective, single center design. It is possible that the bleeding rate could be higher in different patient populations, with alternative polypectomy techniques, and/or with less experience in clip placement. Anecdotally, we have observed that endoscopists in our unit apply significantly less cautery during polypectomy than many of our colleagues and it is possible that this will influence delayed bleeding rates due to cautery ulcers. An additional limitation is the absence of INR measurements on patients after the first 21 in the series. However, there were no changes in our colonoscopy preparation during the study period so we expect that the INR levels in subsequent patients would have been similar.

Our study suggests that a reasonable strategy for screening and surveillance colonoscopy in anticoagulated patients may be to perform the procedure while patients are anticoagulated. Small polyps can be removed with a relatively low risk of bleeding. The proportion of patients with lesions larger than 1 cm is relatively low in most clinical settings, so only a relatively small number of patients would undergo a repeat colonoscopy with normalization of coagulation parameters to resect these larger lesions^[19,20]. A more refined strategy, which would be ideal, would be to develop a highly predictive algorithm to determine which patients are likely to harbor a lesion larger than 1 cm and proceed directly to colonoscopy with interruption of anticoagulation only in these select patients. This strategy could potentially resolve a dilemma facing endoscopists and patients who follow current American Society for Gastrointestinal Endoscopy guidelines: whether to perform screening/surveillance colonoscopy while patients are anticoagulated and repeat

the procedure off anticoagulation in the large number of patients who have small polyps, or to risk thromboembolic complications by normalizing coagulation parameters even in screening/surveillance colonoscopies where potentially no polyps may be found.

A recent survey of endoscopists in the United Kingdom demonstrated a wide variation in the management of anticoagulants for colonoscopy, with 49% of responding physicians routinely stopping anticoagulants and 37% routinely continuing anticoagulants^[21]. This wide variation in practice suggests that the management of anticoagulants is a significant clinical dilemma. A prospective randomized trial would be the ideal method to resolve this dilemma, and by demonstrating that polypectomy of small lesions can be performed with relative safety, our series demonstrated that it would be reasonable to prospectively compare a strategy of anticoagulant interruption to one of colonoscopy in anticoagulated patients where small polyps can be removed at the discretion of the endoscopist.

COMMENTS

Background

Current guidelines recommend discontinuation of anticoagulation prior to colonoscopic polypectomy. However, small colon polyps are exceedingly common and the risk of significant complications from anticoagulant interruption may outweigh the benefit in these patients.

Research frontiers

In this study, the authors report their experience with resection of small colon polyps up to 1 cm in diameter without interruption of anticoagulation.

Innovations and breakthroughs

The authors report that the bleeding risk is very low (0.8%) when small polyps are removed in this setting. This suggests that it is reasonable to perform polypectomy without interruption of anticoagulation.

Applications

Patients who are on chronic anticoagulation and are undergoing screening colonoscopy can be considered for colonoscopy without interruption of anticoagulation, and if small polyps are found then polypectomy can be considered.

Terminology

Polyps are growths in the colon that are often precancerous. During colonoscopy, the colon is examined for polyps using an endoscope. Polyps are usually removed using a variety of instruments during colonoscopy. Bleeding is a relatively common complication of polyp removal, so there is widespread concern about performing polyp removal in patients who are taking anticoagulants.

Peer review

The authors performed a retrospective review that demonstrated that the risk of post-polypectomy bleeding is low in patients with small polyps who are anticoagulated. A comparison to published risk estimates for anticoagulant interruption suggests that it may be favorable to perform polypectomy in this setting rather than interrupting anticoagulation.

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Effect of dephytinization on bioavailability of iron, calcium and zinc from infant cereals assessed in the Caco-2 cell model

Carmen Frontela, Maria Laura Scarino, Simonetta Ferruzza, Gaspar Ros, Carmen Martínez

Carmen Frontela, Gaspar Ros, Carmen Martínez, Department of Food Science and Nutrition, Faculty of Veterinary Science and Food Science and Technology, Murcia University, Murcia 30071, Spain

Maria Laura Scarino, Simonetta Ferruzza, INRAN, National Research Institute for Food and Nutrition, Via Ardeatina 546, Rome 00178, Italy

Author contributions: Frontela C and Ferruzza S performed the research; Scarino ML, Martínez C and Ros G designed the research in addition to providing financial support for this work; Frontela C recorded the data and wrote the paper.

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Correspondence to: Carmen Frontela, Department of Food Science and Nutrition, Faculty of Veterinary Science and Food Science and Technology, Murcia University, Murcia 30071, Spain. carmenfr@um.es

Telephone: +34-968-364798 Fax: +34-968-398767

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Abstract

AIM: To test the effect of the dephytinization of three different commercial infant cereals on iron, calcium, and zinc bioavailability by estimating the uptake, retention, and transport by Caco-2 cells.

METHODS: Both dephytinized (by adding an exogenous phytase) and non-dephytinized infant cereals were digested using an *in vitro* digestion protocol adapted to the gastrointestinal conditions of infants younger than 6 mo. Mineral cell retention, transport, and uptake from infant cereals were measured using the soluble fraction of the simulated digestion and the Caco-2 cells.

RESULTS: Dephytinization of infant cereals significantly increased ($P < 0.05$) the cell uptake efficiency (from 0.66%-6.05% to 3.93%-13%), retention (from 6.04%-16.68% to 14.75%-20.14%) and transport efficiency (from 0.14%-2.21% to 1.47%-6.02%), of iron, and the uptake efficiency (from 5.0%-35.4% to 7.3%-41.6%) and retention (from 4.05%-20.53% to 14.45%-61.3%) of zinc, whereas calcium only cell

uptake showed a significant increase ($P < 0.05$) after removing phytate from most of the samples analyzed. A positive relationship ($P < 0.05$) between mineral solubility and the cell uptake and transport efficiencies was observed.

CONCLUSION: Removing phytate from infant cereals had a beneficial effect on iron and zinc bioavailability when infant cereals were reconstituted with water. Since in developing countries cereal-based complementary foods for infants are usually consumed mixed with water, exogenous phytase additions could improve the nutritional value of this weaning food.

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Key words: Infant cereals; Phytate; Iron; Calcium; Zinc; Caco-2 cells; Bioavailability

Peer reviewer: Silvana Zanlungo, Professor, Departamento de Gastroenterología, Pontificia Universidad Católica de Chile, Marcoleta 367, Casilla 114-D, Santiago, Chile

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INTRODUCTION

Insufficient mineral intake during infancy is responsible for many diseases which can not only influence immediate health, but may also have an adverse impact on adult health. Anaemia, rickets, osteoporosis, and immune diseases are caused by a deficiency of iron, calcium and/or zinc^[1]. An adequate intake of these minerals is important for meeting infant nutritional needs^[2]. Cereals are considered a rich plant source of carbohydrate, proteins, vitamins, and minerals, and are therefore usually introduced to an infant's diet between the ages of 4 and 6 mo. However, cereals are also rich in phytate, which can decrease the bioavailability of critical nutrients such as iron, calcium, and zinc because of its high ability to chelate and precipitate minerals^[3,4]. Dephytinization by adding an

exogenous phytase or by activating the naturally occurring plant phytases has been proposed as a sustainable strategy for reducing mineral deficiency by increasing mineral bioavailability in infant complementary cereal-based foods^[5]. An estimate of mineral bioavailability from infant cereals is important because not only must the absolute amounts of minerals be increased in the edible portions of foods, but they must also be in forms bioavailable to infants. Bioavailability should preferably be determined by *in vivo* testing, however, these studies could also be based on preliminary *in vitro* methods^[6]. Caco-2 cells are human intestinal adenocarcinoma cells exhibiting biochemical and morphological characteristics of small intestinal absorptive enterocytes, and together with a simulation of gastrointestinal digestion, have been used widely for mineral bioavailability studies^[7-9]. Caco-2 cells grown on microporous supports, allow the measurement of mineral uptake and transport across cell monolayers, improving the estimation of bioavailability by *in vitro* methods used until now (solubility and dialysis)^[10,11]. In western countries, infants are usually fed with infant cereals reconstituted with follow-on formula; nevertheless in developing countries, where mineral deficiencies are particularly frequent, cereal-based complementary foods destined to infants are usually consumed mixed with water^[12]. Given the importance of an adequate intake of minerals during infancy, the purpose of the current investigation was to study the effect of dephytinization on iron, calcium and zinc solubility, retention, transport, and uptake by Caco-2 cells from infant cereals when reconstituted with water, with the aim of obtaining data on mineral bioavailability from infant cereals with this kind of reconstitution.

MATERIALS AND METHODS

Chemicals

Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO): pepsin (porcine, catalogue no. P-7000), pancreatin (porcine, catalogue no. P-1750), and bile extract (porcine, catalogue no. B-8756). Pepsin solution was prepared by dissolving 1.6 g of pepsin in 10 mL of 0.1 mol/L HCl. Pancreatin-bile extract solution was prepared by dissolving 0.2 g of pancreatin and 1.25 g of bile extract in 50 mL of 0.1 mol/L NaHCO₃. Millipore Milli-Q distilled-deionized water (Millipore Ibérica S.A., Barcelona, Spain) was used throughout the experiments. Cell culture media, antibiotics (penicillin and streptomycin), glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(N-morpholino) ethanesulfonic acid (MES), and Hank's Balanced Salt Solution (HBSS) were obtained from Gibco BRL Life Technologies (Paisley, Scotland).

Inositol phosphate content

Inositol phosphates were determined by HPLC using a Merck Hitachi chromatograph [pump L-7100, refraction index (RI)-detector L-7490, and L-7350 column oven] according to the method of Lehrfeld^[13]. Inositol phosphates were extracted from the different samples with 0.5 mol/L HCl at room temperature for 2 h. Because

of the high binding capacity of inositol pentaphosphate (IP₅) and inositol hexaphosphate (IP₆) to minerals, we consider the sum of IP₅ and IP₆ to determine phytate content^[4,13]. The molar ratios of phytate to iron, calcium, and zinc were calculated as the millimoles of phytate present in the sample divided by the millimoles of iron, calcium, and zinc present in the sample, respectively. To find the phytate × (Ca/Zn) molar ratio, the total amount of Ca (mmol) in 100 g of infant cereal was multiplied by the phytate/Zn molar ratio.

Samples

Both commercial and dephytinized infant cereals were dried in an oven at 120°C overnight to obtain the dry weight, and were then milled. Infant cereals were reconstituted according to the recommendations of the manufacturer: 200 mL of water was mixed with 35 g of infant cereal. The infant cereals were dephytinized using an exogenous phytase from *Aspergillus oryzae* (EC 3.1.3.26 from Stern-Enzym GmbH & Co. KG, Ahrensburg, Germany, 2500 PU/g). The phytase was added to the aqueous slurry at a concentration of 3.2 U/g of sample and incubated at pH 5.5 with stirring at 55°C for 20 min. The dephytinized samples were dried in an oven at 120°C overnight to obtain the dry weight, and then ground in an electrical mill to a fine powder similar to that of commercial infant cereals. Dephytinization was checked by HPLC^[13].

Caco-2 cells

Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK) and used in assays at passages 28-55. For iron, calcium, and zinc uptake assays, cells were seeded onto polycarbonate membrane chamber inserts (24 mm diameter, 0.4 μm pore size; Transwell, Costar Corp.) at a density of 50000 cells/cm² and allowed to differentiate on filters for 21 d. During this period, cells were maintained in minimum essential medium (MEM) with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% v/v nonessential amino acids, 1% v/v L-glutamine and 1% antibiotic solution (penicillin-streptomycin) at 37°C in an incubator with 5% CO₂, 95% air atmosphere and 95% relative humidity. The medium was changed every 2 d. During the cell differentiation period, monolayer formation and tight junction maturation and sealing were assessed by measuring the passage of phenol red across the monolayer according to Ferruzza *et al*^[14]. Briefly, following three washes of cell monolayers with phosphate-buffered saline (PBS), 0.5 mL of 1 mmol/L phenol red was added in the apical compartment, whereas 1 mL of PBS was added in the basolateral compartment. After 1 h of incubation at 37°C, 0.9 mL of basolateral medium was collected, treated with 0.1 mL of 0.1 mol/L NaOH, and read at 560 nm using a molecular absorption spectrophotometer (UV-Vis, U-200, Hitachi Ltd. Tokyo, Japan) to determine the phenol red concentration. The passage of phenol red was expressed as apparent permeability (P_{app}) and obtained from the following formula: $P_{app} = C_t \times V_{BL} / \Delta t \times C_0 \times A$, where V_{BL} is the volume of the basolateral compartment

(cm^3), A is the filter area (cm^2), Δt is the time interval (s), C_t is the phenol red concentration in the basolateral compartment at the end of time interval, and C_0 is the phenol red concentration in the apical compartment at time zero. Apparent permeability of monolayers at the end of differentiation period was $1.71 \times 10^{-5} \text{ cm/s}$, indicating that tight junctions were functionally mature^[14]. The experiments were conducted on day 21 from seeding. Microscopic examination of the cultures revealed that confluence was reached after 3–4 d of growth. Cell viability 3 h after the addition of the soluble fraction was assessed by trypan blue exclusion and was typically 85%–95%.

***In vitro* gastrointestinal digestion**

Gastrointestinal digestion was applied to infant cereals, whether or not dephytinized, and reconstituted with deionized distilled water using the *in vitro* method described by Miller *et al.*^[15] with modifications aimed at reducing the amounts of the enzymes used because the gastrointestinal tract in the early stages of life is not yet fully developed^[16,17]. The method consisted of two phases: gastric and intestinal. Prior to the gastric stage, the pH of 17.5 g of each infant cereal homogenized with 100 mL of deionized-distilled water was lowered to pH 4 with 6 mol/L HCl. Then, 3 g of pepsin solution was added, and the sample was incubated in a shaking water bath at 37°C and 120 strokes/min for 2 h to allow pepsin digestion. The digest was then maintained in ice for 10 min to stop pepsin digestion. For intestinal digestion, the pH of the gastric digests was raised to 5.0 by dropwise addition of NaHCO_3 1 mol/L. Then a freshly prepared pancreatin-bile solution sufficient to provide 0.005 g of pancreatin and 0.03 g of bile salts/g of sample was added, and incubation was continued for 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. Then the pH was adjusted to 7.2 by dropwise addition of 0.5 mol/L NaOH. The intestinal digest was heated for 4 min at 100°C to inhibit the sample proteases and then cooled by an ice bath. The gastrointestinal digest were centrifuged at $9187 \times g$ for 30 min at 4°C. The supernatant fraction was filtered through a centrifugal filter devices with a 30000 MW cut-off (Millipore Corporation Bedford, MA 01730, USA) and then centrifuged at $4000 \times g$ for 90 min at 4°C using a Sorvall centrifuge (Model RC5C, with SS-34 rotor; Sorvall instruments, DuPont, Mississauga, ON, Canada). Prior to addition of the soluble fraction to the cells, glucose (5 mmol/L final concentration), HEPES (50 mmol/L final concentration), and MES (30 mmol/L final concentration) (pH 6.5–6.9) were added to make the soluble fraction similar to the culture media; and finally, water was added to adjust the osmolarity to $310 \pm 10 \text{ mOsm/kg}$ (Freezing point osmometer 030, Berlin, Germany) according to Ekmekcioglu^[18]. Then the supernatants (soluble fraction) were analyzed for mineral content and used in cell uptake assays.

Uptake, retention and transport experiments

The soluble fraction obtained from gastrointestinal digestion was used to carry out uptake, retention, and

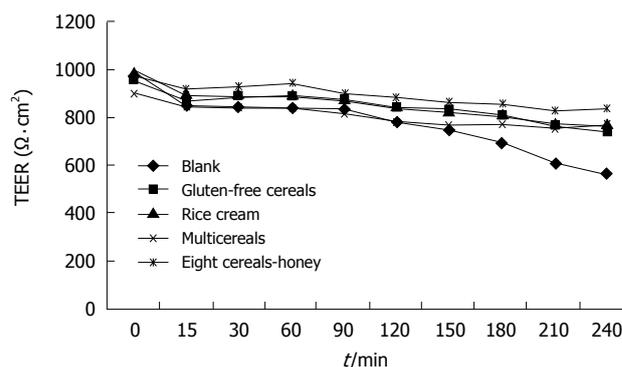


Figure 1 TEER values of Caco-2 monolayers incubated in the presence of digested infant cereals.

transport experiments with Caco-2 cells because it is more similar to the *in vivo* digests^[19]. Before each experiment growth medium was removed and apical and basolateral cell surfaces of the monolayers were washed three times with phosphate-buffered saline (PBS) at 37°C. One millilitre of soluble fractions was added to the apical chamber and 1.5 mL of HBSS (pH 7.4) was added to the basal chamber of each cell monolayer. After incubation, the apical samples were collected, and the monolayers were carefully washed three times with 1 mL ice-cold HBSS to remove any nonspecific-bound mineral and residual soluble fractions. Cells on filters were lysed by the addition of 0.5 mL of deionized water to each well, and then harvested; HBSS in basal chamber also was removed. Total mineral content was measured in the apical solutions, cell monolayer, and basal solutions.

Assessment of cell monolayer integrity during experiments

To investigate possible adverse effects of infant cereal components or digestive enzymes from the supernatants on Caco-2 cells, the integrity of the monolayer was assessed by measuring the transepithelial electrical resistance (TEER) according to the method of Okada *et al.*^[20]. At the end of differentiation period control, monolayers used for experiments had a resistance higher than $900 \Omega \cdot \text{cm}^2$ (Figure 1). During the uptake, retention, and transport experiments measurements of TEER were taken every 30 min. Background resistance was determined by measuring across a filter without cells in Hank's Balanced Salt Solution (HBSS). Monolayers with resistances $< 500 \Omega \cdot \text{cm}^2$ were discarded.

Iron, calcium, and zinc determination

To estimate mineral bioavailability, iron, calcium, and zinc contents in the sample (before digestion), soluble fraction (apical solution), blank (HBSS), basal solution and in cell homogenates were determined by flame atomic absorption spectrophotometry (AAS; Perkin-Elmer, mod. 3100 Norwalk, U.S.A) according Perales *et al.*^[10] with slight modifications; dry organic matter destruction (450°C) was applied prior to analysis. An amount of lanthanum chloride sufficient to obtain a final content of 0.1% was added to eliminate phosphate

Table 1 Mineral content (per 100 g), phytate (IP₅ + IP₆) content (per 100 g) and molar ratios of phytate to iron, calcium and zinc, and phytate × calcium/zinc of commercial infant cereals

Infant cereal	Fe (mg)	Ca (mg)	Zn (mg)	Phytate (mg)	Phytate/Fe	Phytate/Ca	Phytate/Zn	Phytate × Ca/Zn
Eight cereals-honey	8.3 ± 0.4	137.3 ± 5.6	0.6 ± 0.3	319.6 ± 3.1	3.8	0.16	53.1	182.3
Rice cream	8.8 ± 0.1	283.1 ± 27.7	1.2 ± 0.2	167.1 ± 27.5	1.6	0.11	14.4	101.9
Multicereals	8.7 ± 0.2	174.4 ± 21.0	1.5 ± 0.4	143.5 ± 10.6	1.4	0.07	9.8	42.7
Gluten-free cereals	7.5 ± 1.0	154.4 ± 38.9	1.0 ± 0.3	299.8 ± 16.9	3.5	0.18	31.8	122.7

interferences in the calcium determination. To dissolve the ashes, 2 mL of concentrated HCl (sp gr = 1.19) was added, and the vessel was covered with a watch glass and gently warmed (70-75°C) for 4 h, leaving about 1 mL of liquid at the end of heating. The solution was then transferred to a 10 mL volumetric flask, and the volume was completed with water. Solubility percentages were determined as follows: solubility % = 100 × S/C, where S = soluble mineral content (µg of mineral/g of sample), and C = total mineral content of the sample (µg of mineral/g of sample). Differences between the mineral content of the monolayer incubated with soluble mineral fraction and the content of monolayer not exposed (retention blank) yielded an estimation of the cellular retention (micrograms) of minerals. Transport (T) was evaluated by the difference between the mineral amount in basal chamber solutions of treated samples and HBSS. The following calculation was used for retention percentages: retention % = 100 × R/C, where R = mineral retention (µg of mineral/well), and C = mineral soluble added (µg). Transport percentages were calculated as follows: transport % = 100 × T/C, where T = cellular transport (µg of mineral/well), and C = mineral soluble added (µg)/well. The differences between the mineral content of cells cultures incubated with samples or HBSS (blank) gave an estimation of the cellular uptake (cell retention plus transport) of these mineral elements. Uptake percentage values were calculated as the percentage of the mineral applied to the Caco-2 cell monolayer which was taken up by the cells and results were used as a measure of mineral availability. Due to the differences among samples in terms of the solubility of minerals after *in vitro* digestion, mineral transport and uptake were normalized for solubility as follows: % transport efficiency = (% solubility × % transport)/100, % uptake efficiency = (% solubility × % uptake)/100.

Quality control of the iron, calcium, and zinc analyses

The absence of matrix interferences in AAS determination of Fe and Ca in the samples was checked by the addition's method. Community Bureau of reference material CRM-189 (wholemeal flour) (Brussels, Belgium) was used as a control to test the method for accuracy. For Fe, Ca and Zn, the measured mean values were 66.9 µg/g, 519 µg/g and 54.9 µg/g, respectively, which were in accordance with the certified range of 68.3 ± 1.9 µg/g for Fe, 520 µg/g (standard deviation non-certified) for Ca and 56.5 ± 1.7 µg/g for Zn. The detection limit was determined to be 0.6 mg/L. The method was shown to

be linear ($r \geq 0.998$) over the range 1-5 mg/L for both Fe ($y = 2.86 \times 10^{-3} + 4.76 \times 10^{-2}X$) and Ca ($y = 2.67 \times 10^{-3} + 5.24 \times 10^{-2}X$).

Statistical analysis

Results are reported as mean ± SD of five experiments. After testing for normality and equal variances, the mean solubility, retention, transport efficiency, and uptake efficiency percentages of Fe, Ca, and Zn from infant cereals, whether or not dephytinized, were compared by one-way analysis of variance (ANOVA) including the Tukey post-test in the data treatment to determine significant differences among means ($P < 0.05$). A Pearson correlation analysis was performed to investigate the possible correlation between phytate content; Fe, Ca, and Zn contents; mineral solubility (%); retention (%); uptake (%); and transport (%) by Caco-2 cells. Values of $P < 0.05$ were considered significant. All statistical analyses were performed with the Statistical Package for the Social Sciences (version 14.0; SPSS).

RESULTS

Inositol phosphate content

Molar ratios of phytate (IP₅ + IP₆) to iron, phytate to calcium and phytate to zinc, as well as the phytate × (Ca/Zn) molar ratio, are shown in Table 1. For iron, values ranged from 1.4 to 3.8; for calcium, from 0.07 to 0.18; and for zinc, 42.7 to 182.3.

Uptake, retention and transport experiments

The results obtained in the iron retention, transport, and uptake assays by Caco-2 from infant cereals are summarized in Table 2. The iron retention percentage and transport and uptake efficiencies of eight cereals-honey, Multicereals and Gluten-free cereals after dephytinization were significantly higher ($P < 0.05$) than that of commercial infant cereals. The iron solubility percentage was higher from eight cereals-honey and Multicereals after dephytinization; conversely the solubility percentage of commercial rice cream was higher than that of the same sample after phytase treatment. Total iron content of each infant cereal before and after phytase treatment was equal.

Table 3 shows the results obtained for calcium cell retention, transport, and uptake assays by Caco-2 cells. Calcium uptake efficiency percentage was higher from infant cereals analyzed after phytase treatment with the exception of eight cereals honey; however, results were significant ($P < 0.05$) only for Gluten-free cereals.

Table 2 Iron retention, transport and uptake from infant cereals by Caco-2 cells

	Infant cereal	Iron added (μg)	Solubility (%)	Retention (μg)	Retention (%)	Transport (μg)	Transport efficiency (%)	Uptake (μg)	Uptake efficiency (%)
- phytase	A	12.51	17.42 \pm 6.1 ^a	1 \pm 0.1	7.99 \pm 2.2 ^b	1.59 \pm 0.6	2.21 \pm 1 ^a	2.59 \pm 0.8	3.6 \pm 0.9 ^b
	B	13.08	8.72 \pm 2.7 ^b	0.79 \pm 0.4	6.04 \pm 0.6 ^b	0.21 \pm 0	0.14 \pm 0.03 ^c	1 \pm 0.2	0.66 \pm 0.2 ^d
	C	13.13	24.21 \pm 1.9 ^a	2.19 \pm 0.1	16.68 \pm 3.8 ^a	1.09 \pm 0.7	2.01 \pm 0.8 ^a	3.28 \pm 1.1	6.05 \pm 2.2 ^a
	D	11.18	20.93 \pm 4.8 ^a	0.78 \pm 0.2	6.98 \pm 0.8 ^b	0.18 \pm 0.04	0.33 \pm 0.1 ^b	0.96 \pm 0.2	1.8 \pm 0.3 ^c
+ phytase	A	12.51	34.53 \pm 8.6 ¹	2.52 \pm 0.3	20.14 \pm 3.9 ¹	2.18 \pm 0.9	6.02 \pm 1.3 ¹	4.7 \pm 0.9	13 \pm 2.5 ¹
	B	13.08	21.1 \pm 1.8 ¹	2.1 \pm 0.2	16.28 \pm 2.1 ¹	2.49 \pm 0.9	4.01 \pm 0.8 ¹	4.62 \pm 1.6	7.45 \pm 3.9 ¹
	C	13.13	19.19 \pm 3 ¹	2.41 \pm 0.3	18.35 \pm 3.4	1.66 \pm 0.6	2.43 \pm 0.6	4.0 \pm 1.2	5.95 \pm 1.1
	D	11.18	16.64 \pm 6.2	1.65 \pm 0.2	14.75 \pm 1.3 ¹	0.99 \pm 0.2	1.47 \pm 0.3 ¹	2.64 \pm 0.8	3.93 \pm 0.7 ¹

A: Eight cereals honey; B: Multicereals; C: Rice cream; D: Gluten-free cereals. mean \pm SD, $n = 5$. Different letter (a-d) denotes significant differences ($P < 0.05$) between commercial infant cereals (without phytase treatment) to assess the effect of different phytate content. ¹ P vs the same infant cereal dephytinized or not.

Table 3 Calcium retention, transport and uptake from infant cereals by Caco-2 cells

	Infant cereal	Calcium added (μg)	Solubility (%)	Retention (μg)	Retention (%)	Transport (μg)	Transport efficiency (%)	Uptake (μg)	Uptake efficiency (%)
- phytase	A	206	38.9 \pm 11.1 ^{a1}	5.72 \pm 0.3	2.78 \pm 1 ^{a1}	25.99 \pm 8	4.9 \pm 1.2 ^a	31.71 \pm 4.9	5.99 \pm 2 ^a
	B	261.6	15.2 \pm 1.8b ¹	7.33 \pm 0.2	2.8 \pm 0.6 ^a	16.1 \pm 3.9	0.94 \pm 0.1 ^{b1}	23.43 \pm 3.6	0.66 \pm 0.2 ^b
	C	424.7	2.8 \pm 1 ^c	2.18 \pm 0.3	0.51 \pm 0.2 ^b	33.1 \pm 6.1	0.22 \pm 0.02 ^d	35.28 \pm 7.2	0.23 \pm 0.2 ^c
	D	231.6	4.53 \pm 1.3 ^c	4.45 \pm 0.2	1.92 \pm 0.3 ^a	15.64 \pm 4.4	0.31 \pm 0.08 ^c	20.09 \pm 1.8	0.39 \pm 0.1 ^c
+ phytase	A	206	22.5 \pm 2.3	0.7 \pm 0.2	0.34 \pm 0.09	29.59 \pm 7.2	3.24 \pm 1	30.29 \pm 6.8	3.31 \pm 0.9
	B	261.6	8.03 \pm 2.7	7.33 \pm 1.4	2.8 \pm 0.6	23.08 \pm 2.1	0.71 \pm 0.04	30.41 \pm 2.2	0.93 \pm 0.2
	C	424.7	3.72 \pm 1.9	5.5 \pm 0.1	1.3 \pm 0.7	28.1 \pm 4.4	0.25 \pm 0.07	33.6 \pm 7.1	0.43 \pm 0.09
	D	231.6	8.31 \pm 1.2 ¹	2.98 \pm 0.7	1.29 \pm 0.9	66.16 \pm 10.8	2.38 \pm 0.1 ¹	69.14 \pm 8.2	2.48 \pm 0.3 ¹

Table 4 Zinc retention, transport and uptake from infant cereals by Caco-2 cells

	Infant cereal	Zinc added (μg)	Solubility (%)	Retention (μg)	Retention (%)	Transport (μg)	Transport efficiency (%)	Uptake (μg)	Uptake efficiency (%)
- phytase	A	2.12	36.4 \pm 7.1 ^a	0.18 \pm 0.08	8.5 \pm 2 ^b	1.88 \pm 0.6	32.3 \pm 3 ^{a1}	2.06 \pm 0.8	35.4 \pm 4.1 ^a
	B	2.22	18.9 \pm 3.8 ^b	0.09 \pm 0.01	4.05 \pm 2 ^c	0.5 \pm 0.08	4.25 \pm 1.1 ^c	0.59 \pm 0.6	5 \pm 0.9 ^d
	C	2.63	17 \pm 1.9 ^b	0.54 \pm 0.1	20.53 \pm 3.8 ^a	0.62 \pm 0.1	4 \pm 0.8 ^c	1.16 \pm 0.4	7.5 \pm 0.2 ^c
	D	1.63	37.8 \pm 6.2 ^{a1}	0.09 \pm 0.01	5.5 \pm 1.6 ^c	0.71 \pm 0.2	16.5 \pm 0.3 ^b	0.8 \pm 0.4	18.6 \pm 1.2 ^b
+ phytase	A	2.12	46.9 \pm 6.6	0.84 \pm 0.2	22.2 \pm 2.7 ¹	1.04 \pm 0.5	23 \pm 3.6	1.88 \pm 0.9	41.6 \pm 6.5
	B	2.22	18.92 \pm 2.7	0.42 \pm 0.1	18.92 \pm 2.8 ¹	0.44 \pm 0.2	3.74 \pm 0.3	0.86 \pm 0.2	7.3 \pm 0.2 ¹
	C	2.63	27.3 \pm 6 ¹	0.38 \pm 0.1	14.45 \pm 3.4	1.59 \pm 0.6	16.5 \pm 2.6 ¹	1.97 \pm 0.8	20.4 \pm 2.1 ¹
	D	1.63	21 \pm 4.3	0.18 \pm 0.03	61.3 \pm 9.9 ¹	1.43 \pm 0.4	18.4 \pm 4.1	1.61 \pm 0.2	20.7 \pm 0.6 ¹

After phytase treatment, this infant cereal showed that solubility and transport efficiency percentages of calcium increased significantly as well. From samples not dephytinized, significant highest solubility and retention percentages of calcium were observed for eight cereals-honey, and highest solubility and transport efficiency percentages for multicereals. Total calcium content of each infant cereal before and after phytase treatment was equal.

As shown in Table 4, the effect of dephytinization caused an increase ($P < 0.05$) in retention and uptake efficiency percentages of zinc in most of samples analyzed. Rice cream showed an increase in solubility, transport, and uptake efficiencies percentages after phytase treatment. Significant differences on the solubility percentage of zinc were observed for Gluten-free cereals and rice cream between samples, whether or not dephytinized. Total zinc content of each infant

cereal before and after phytase treatment was equal.

With significant values at $P < 0.05$, a negative correlation between phytate content and retention percentages of iron and zinc ($r = -0.730$ and $r = -0.538$) and iron transport ($r = -0.507$), and uptake efficiency ($r = -0.519$) percentages were found. Calcium content showed a negative correlation with transport efficiency percentage of iron ($r = -0.426$) and with uptake efficiency percentage of zinc ($r = -0.855$). Mineral solubility percentage showed for each mineral analyzed (Fe, Ca, and Zn) a positive correlation with transport efficiency percentage ($r = 0.735$, $r = 0.912$, $r = 0.732$, respectively) and with uptake efficiency percentage ($r = 0.794$, $r = 0.923$, $r = 0.838$, respectively).

DISCUSSION

Fe, Ca, and Zn contents of infant cereals analyzed were

in accordance with recommendations of the European Economic Community (Directive 2006/125)^[21]. In the present study, the values found for the phytate (IP₅ + IP₆) to iron molar ratios were higher than 1.4. Although the critical ratio (capable of compromising bioavailability) has not yet been well established, according to Hurrell^[5,12], values obtained in infant cereals of this study have the potential to compromise iron bioavailability. It is interesting to note the very low phytate to calcium molar ratio for all infant cereals analyzed; apparently phytate could not compromise Ca availability since the critical value for which the absorption of calcium is compromised has been reported to be > 0.24 ^[4]. Three of the four infant cereals analyzed showed a phytate to zinc molar ratio above 12, a value implicated in interference with zinc bioavailability in humans^[22,23]. The infant cereal named eight cereals-honey showed phytate \times calcium/zinc molar ratios above the critical value within the range of 150-200, which have been associated with a decrease in zinc bioavailability.

It has been reported that removing phytate increases iron bioavailability in the Caco-2 cell *in vitro* model^[24,25]. Since iron solubility, iron retention, transport efficiency, and uptake efficiency percentages can be used as bioavailability predictors^[8,26,27], our results showed that three of the four infant cereals analyzed after phytase treatment increased iron bioavailability. The source of iron used for the enrichment of infant cereals (elemental iron) plays an important role in iron solubility^[28]; in this regard, values found in our study were lower than those reported by other authors^[29,30] for the same element, probably due to the phytate content or fiber components from cereals, the different pH conditions applied for the assays^[29], or the previously reported the inhibitory effect of calcium on iron availability^[10] since infant cereals used in this study were calcium-enriched. Differences in iron bioavailability parameters between dephytinized infant cereals could indicate that other components of infant cereals can also decrease iron bioavailability; in this regard, it has been reported that some dietary fiber components can bind mineral ions decreasing their bioavailability^[30,31]. Commercial infant cereal (Multicereals) showed the highest values of iron bioavailability parameter measures which can be justified because of its lower molar ratio of phytate to iron, compared to eight cereals-honey, rice cream and Gluten-free cereals.

It should be noted that the positive correlation observed between iron solubility with cell transport and uptake efficiencies percentages found in our study is in agreement with Bergqvist *et al*^[26], studying iron absorption from carrot juice, and with Proulx and Reddy^[32] studying iron bioavailability of maize, both using Caco-2 cells. Meanwhile, it has been reported by other authors^[27,33] that solubility and bioavailability by Caco-2 cells did not show parallel trends.

In our study, a lack of significant effect of dephytinization on calcium bioavailability parameters by Caco-2 cells was found in most of the infant cereals

analyzed, although a great variability was observed. The inhibitory effect of phytate on calcium bioavailability has been reported^[34-36] but only for high ratios. Our observation could be explained by the binding of calcium phytate to the membrane of Caco-2 cells. In this regard, Phyllippy^[36] reported that Caco-2 cells may not be useful for studying the effects of inositol phosphates on the calcium uptake by cells. Calcium solubility from eight cereals-honey and Multicereals decreased after phytase treatment whereas Gluten-free cereals showed a higher calcium solubility after phytase treatment. Since it has been reported that calcium solubility depends on the phytate to calcium molar ratio^[34] the low ratio Ca: phytate ratio (≤ 0.18) of all infant cereals analyzed could explain the lack of effect of phytase on calcium solubility. In fact, only Gluten-free cereals (the sample with the highest phytate/Ca molar ratio) showed a significant increase in transport and uptake efficiencies after dephytinization.

Percentages of soluble zinc did not show significant differences before and after dephytinization for most of the samples analyzed. Zn solubility percentages obtained ($< 37.8\%$ for commercial infant cereals; $< 46.9\%$ for dephytinized infant cereals) were lower than values obtained by Cámara *et al*^[27] in school meals, and by Lyon^[37] in cereal products. The same trend was observed in a previous analysis in our laboratory studying infant cereals^[38], since a lack of phytase effect on zinc solubility was found for most of the samples studied; moreover, Kayode *et al*^[39] observed similar results studying opaque sorghum beer. Probably, as reported Perales *et al*^[10], the calcium added as enrichment to infant cereals that are not Zn-fortified exerted a negative effect on Zn solubility.

However, when bioavailability was evaluated in Caco-2 cells, all infant cereals analyzed showed an increase in the Zn uptake efficiency percentage after phytase treatment. Values obtained for Multicereals, rice cream and Gluten-free cereals were significant ($P < 0.05$). Eight cereals-honey, Multicereals and Gluten-free cereals presented a higher percentage of zinc retention after phytase treatment with respect to the same infant cereal not dephytinized. The retention and uptake efficiencies of zinc data obtained in our study clearly demonstrate that phytate impaired bioavailability, since significant differences were found between samples dephytinized and not dephytinized. The inhibitory effect of phytate on Zn bioavailability by Caco-2 cells has been previously reported^[40,41].

In conclusion, dephytinization of infant cereals by an exogenous phytase resulted in increasing bioavailability parameters of iron and zinc measured in a Caco-2 cell line. However, for calcium, a lack of effect of dephytinization of infant cereals on bioavailability by Caco-2 cells was found in our study.

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COMMENTS

Background

An adequate intake of minerals is particularly important for infants in the first year of life. Although breast-feeding is considered the natural and preferred method for infant feeding, after the 6th mo of age cereals are introduced to supplement breast milk. Given the importance of an adequate nutrition during infancy, a better knowledge of the gastrointestinal conditions of infants and its effects on food components affecting intestinal absorption of minerals are essential.

Research frontiers

The research hotspot is the study and improvement of mineral bioavailability from cereal foods with a different matrix under gastrointestinal conditions of infants and to obtain major knowledge about intestinal absorption of iron, calcium and zinc using the Caco-2 cell line.

Innovations and Breakthroughs

Low absorption of minerals from infant foods is considered to be a factor in the aetiology of mineral deficiencies in infants. Gastrointestinal conditions (pH and digestive enzymes) are keys in mineral absorption. Data indicate that the Caco-2 cell line is a useful tool to study iron and zinc absorption and simultaneously to characterize the effect of some food components on mineral intestinal absorption.

Applications

Better knowledge of intestinal mineral absorption process and interactions with dietary factors at a gastrointestinal level would be helpful to develop infant foods with improved mineral availability.

Terminology

Phytic acid: (my-inositol hexaphosphoric acid), a dietary factor found principally in cereals and legumes which is a potent inhibitor of mineral absorption owing to its strong ability to bind multivalent metal ions. Mineral bioavailability: the proportion of minerals that can be absorbed and used for physiological purposes.

Peer review

This is a descriptive study that shows the effect of dephytinization on the bioavailability of iron, calcium and zinc in different commercial infant cereals using the *in vitro* Caco-2 cell model. The manuscript is easy to understand and in general terms well written. The work described is well done.

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Intussusception in adults: Clinical characteristics, diagnosis and operative strategies

Savas Yakan, Cemil Caliskan, Ozer Makay, Ali Galip Denecli, Mustafa Ali Korkut

Savas Yakan, Ali Galip Denecli, Department of Surgery, Izmir Education and Research Hospital, Izmir 35270, Turkey
Cemil Caliskan, Ozer Makay, Mustafa Ali Korkut, Department of Surgery, Ege University Faculty of Medicine, Izmir 35100, Turkey

Author contributions: Yakan S, Caliskan C and Makay O designed the research; Yakan S and Caliskan C performed the research; Korkut MA, Denecli AG analyzed the data; Yakan S wrote the paper; all of the authors read and approved final version.

Correspondence to: Savas Yakan, Department of Surgery, Izmir Education and Research Hospital, Izmir 35270, Turkey. savasyakan@gmail.com

Telephone: +90-533-4717940 Fax: +90-232-2614444

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is viable or malignancy is not suspected; however, a more careful approach is recommended in colonic intussusception because of a significantly higher coexistence of malignancy.

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Abstract

AIM: To evaluate 20 adults with intussusception and to clarify the cause, clinical features, diagnosis, and management of this uncommon entity.

METHODS: A retrospective review of patients aged > 18 years with a diagnosis of intestinal intussusception between 2000 and 2008. Patients with rectal prolapse, prolapse of or around an ostomy and gastroenterostomy intussusception were excluded.

RESULTS: There were 20 cases of adult intussusception. Mean age was 47.7 years. Abdominal pain, nausea, and vomiting were the most common symptoms. The majority of intussusceptions were in the small intestine (85%). There were three (15%) cases of colonic intussusception. Enteric intussusception consisted of five jejunojejunal cases, nine ileoileal, and four cases of ileocecal invagination. Among enteric intussusceptions, 14 were secondary to a benign process, and in one of these, the malignant cause was secondary to metastatic lung adenocarcinoma. All colonic lesions were malignant. All cases were treated surgically.

CONCLUSION: Adult intussusception is an unusual and challenging condition and is a preoperative diagnostic problem. Treatment usually requires resection of the involved bowel segment. Reduction can be attempted in small-bowel intussusception if the segment involved

INTRODUCTION

Intestinal invagination or intussusception is the leading cause of intestinal obstruction in children, but in adults it accounts for only 5% of all intussusceptions, and 0.003%-0.02% of all adult hospital admissions. In contrast to childhood intussusception, which is idiopathic in 90% of cases, adult intussusception has a demonstrable lead point, which is a well-definable pathological abnormality in 70%-90% of cases^[1-3].

The presentation of pediatric intussusception often is acute with sudden onset of intermittent colicky pain, vomiting, and bloody mucoid stools, and the presence of a palpable mass. In contrast, the adult entity may present with acute, subacute, or chronic non-specific symptoms^[4]. Therefore, the initial diagnosis often is missed or delayed and may only be established when the patient is on the operating table. Most surgeons accept that adult intussusception requires surgical resection because the majority of patients have intraluminal lesions. However, the extent of resection and whether the intussusception should be reduced remains controversial^[5].

Therefore in this paper, we report our experience in an attempt to clarify the cause, clinical features, diagnosis, and management of this uncommon entity.

MATERIALS AND METHODS

The clinical, operative, and pathological records of 20 adult patients (> 18 years of age) with a diagnosis of intussusception, surgically treated between 2000 and 2008 were reviewed retrospectively. Patients with rectal prolapse, prolapse of or around an ostomy and gastroenterostomy intussusception were excluded.

Intussusception was classified as enteric or colonic. When the pathologic lead point was located in the small bowel, including jejunojejunal, ileoileal and ileocolic intussusceptions, it was classified as enteric. Colonic intussusception included ileocecal-colic, colocolonic, sigmoidorectal, and appendicocecal intussusception. Ileocolic and ileocecal-colic intussusception was distinguished by the site of the pathological lead point. When the lead point was at the ileum, intussusception was classified as ileocolic, whereas when the lead point was at the ileocecal valve, it was classified as ileocecal-colic.

RESULTS

Demographics

A total of 20 patients were identified who had a diagnosis of intussusception and were older than 18 years of age. The average age of the patients was 47.7 years, with a range of 21 to 75 years. Nine (45%) were male and 11 (55%) were female.

Clinical manifestations

Pain was the most common presenting complaint and was present in 17 patients (85%). Nausea, vomiting, constipation, rectal bleeding, and diarrhea were other symptoms. Table 1 shows the symptoms and signs. A palpable mass was found in only one patient (5%). The mean duration of symptoms was 7.9 d (range, 1 d to 3 mo). Six patients (30%) had acute symptoms (< 4 d), five (25%) had subacute symptoms (4-14 d), and nine (45%) had chronic symptoms (> 14 d).

Preoperative diagnostic studies

Plain abdominal X-rays were first obtained in patients with acute symptoms, which revealed air-fluid levels that suggested intestinal obstruction in five patients (25%). It was normal in the other 15 patients (75%).

Intussusception was a preoperative diagnosis in 14 patients (70%). Six patients (30%) who were diagnosed with intussusception in the operating room showed serious signs of bowel strangulation and were not diagnosed preoperatively because they were transferred to the operating room without further radiological evaluation. Abdominal computed tomography (CT) scan was performed in 12 patients, of whom 10 (83.3%) were diagnosed with intussusception. The finding on CT was an in-homogeneous soft-tissue mass that was target- or sausage-shaped (Figure 1). Three patients underwent colonoscopy, and intussusception was confirmed in two. A small-bowel series were performed in three patients. Two patients in diagnostic studies had findings

Table 1 Symptoms and signs of intussusception

Symptoms and signs	n (%)
Pain	17 (85)
Nausea	15 (75)
Vomiting	14 (70)
Constipation	3 (15)
Rectal bleeding	1 (5)
Diarrhea	1 (5)
Abdominal mass	1 (5)
Fever	1 (5)



Figure 1 Abdominal CT scan showing an in-homogeneous soft tissue mass that is target or sausage-shaped in a jejunojejunal intussusception (white arrow).

suspicious of intussusception caused by obstruction with polyps or tumors.

Location

The majority of intussusceptions were enteric (17/20 or 85%) (Table 2). There were three (15%) cases of colonic intussusception. Five cases of enteric intussusception were jejunojejunal (Figure 2A), nine were ileoileal, and four had ileocecal invagination detected.

Pathology

The pathological cause of intussusception was identified in 18 (90%) cases (Table 2). Benign pathology was seen in 14 cases (77.8%) and malignancy in four (22.2%). Among enteric intussusception, 14 cases were secondary to a benign process, including submucosal lipoma, Peutz Jeghers polyps, inflammatory fibroid polyp, intussuscepting Meckel diverticulum, fibroid polyp (Figure 2B), and congenital band adhesions. One malignant case was secondary to metastatic lung adenocarcinoma. All colonic intussusceptions resulted from a malignant lesion. The causes of colonic intussusception were secondary to primary adenocarcinoma in two cases and primary colonic lymphoma in one. No colorectal or rectorectal intussusception was identified in this study.

Treatment and consequences

All patients underwent operative treatment (Table 2). No hydrostatic reduction was attempted in any case. The choice of procedure was determined by the location, size, and cause of the intussusception and the viability

Table 2 Location, treatment and pathology

No. of patients	Age	Gender	Location of the lesion	Preoperative diagnosis	Surgical treatment	Pathology
1	33	M	Enteric (jejunojejunal)	+ (small bowel series)	Reduction + enterotomy + polypectomy	Peutz-Jeghers (hamartomatous polyp)
2	49	F	Enteric (ileocecal)	+ (CT)	Right hemicolectomy	Ileal lipoma
3	60	F	Enteric (ileoileal)	-(Urgent)	Reduction + segmental ileal resection	Inflammatory fibroid polyp
4	30	F	Enteric (ileocecal)	+ (CT)	Reduction + segmental ileal resection	Fibrous polyp
5	63	M	Colonic (colocolic)	+ (colonoscopy)	Near totalcolectomy + ileorectal anastomosis	Lenfoma
6	56	F	Enteric (ileocecal)	+ (CT)	Right hemicolectomy	Inflammatory fibroid polyp
7	21	F	Enteric (jejunojejunal)	+ (CT)	Reduction + segmental jejunal resection + enterotomy + polypectomy	Peutz-Jeghers (hamartomatous polyp)
8	36	M	Enteric (ileoileal)	-(Urgent)	Reduction	Idiopathic
9	75	M	Colonic (colocolic)	+ (colonoscopy)	Left hemicolectomy	Adeno CA
10	25	M	Enteric (ileoileal)	-(Urgent)	Reduction	Congenital band
11	60	M	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Ileal lipoma
12	28	F	Enteric (jejunojejunal)	-(Urgent)	Reduction + segmental jejunal resection	Inflammatory fibroid polyp
13	48	M	Enteric (jejunojejunal)	+ (CT)	Reduction + segmental jejunal resection	Idiopathic
14	55	F	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Ileal lipoma
15	62	F	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Inflammatory fibroid polyp
16	26	M	Enteric (jejunojejunal)	+ (small bowel series)	Reduction + enterotomy + polypectomy	Peutz-Jeghers (hamartomatous polyp)
17	48	F	Enteric (ileoileal)	-(Urgent)	Reduction + diverticulectomy	Meckel's diverticulum
18	72	M	Colonic (colocolic)	+ (CT)	Left hemicolectomy	Adeno CA
19	56	F	Enteric (ileoileal)	+ (CT)	Reduction + segmental ileal resection	Metastatic Adeno CA
20	51	F	Enteric (ileoileal)	-(Urgent)	Reduction + diverticulectomy	Meckel's diverticulum

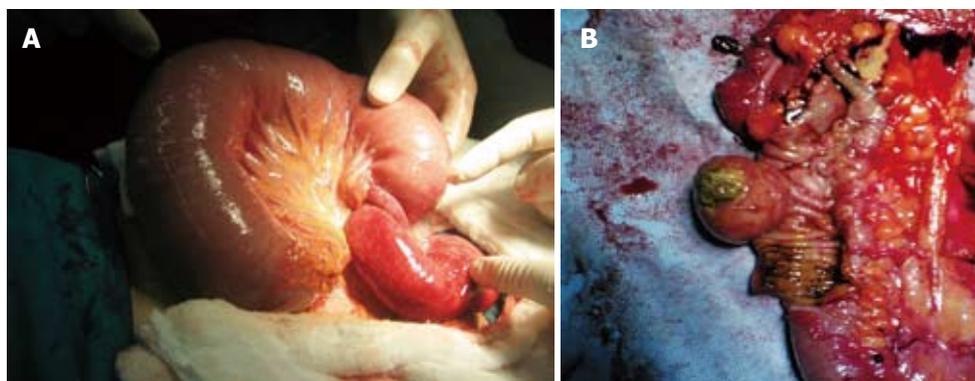


Figure 2 Operative picture. A: Jejunojunal intussusception; B: The fibroid lesion that acted as a lead point for ileocecal intussusception.

of the bowel. All 20 patients underwent laparotomy. En bloc resection (without reduction) was performed in five patients (25%), four of whom underwent right or left hemicolectomy, and one, subtotal colectomy and ileorectal anastomosis because of suspicion of malignancy. Reduction of the intussuscepted bowel was performed on the remaining 15 patients (75%). Among these, segmental resection was performed in nine, two of whom underwent diverticulectomy, two, enterotomy and polypectomy, and one, congenital band excision.

Postoperative complications occurred in four of 20 patients (20%): superficial wound infection in two (10%), pneumonia in one (5%), and severe sepsis in one (5%). There were no anastomotic leaks or intra-abdominal abscesses. There was one perioperative death (5%), which was secondary to severe sepsis complicated by multiple organ failure 6 d after the operation.

DISCUSSION

Adult intussusception is an uncommon clinical entity encountered by surgeons. The exact mechanism is unknown, and it is believed that any lesion in the bowel wall or irritant within the lumen that alters normal peristaltic activity is able to initiate invagination^[2,6]. Ingested food and the subsequent peristaltic activity of the bowel produce an area of constriction above the stimulus and relaxation below, thus telescoping the lead point (intussusceptum) through the distal bowel lumen (intussusciens)^[1,2]. The most common locations are at the junctions between freely moving segments and retroperitoneally or adhesively fixed segments^[6,7].

About 90% of occurrences in adults have a lead point, a well-definable pathological abnormality. In general, the majority of lead points in the small intestine

consist of benign lesions, such as benign neoplasms, inflammatory lesions, Meckel's diverticuli, appendix, adhesions, and intestinal tubes. Malignant lesions (either primary or metastatic) account for up to 30% of cases of intussusception in the small intestine^[2,5]. On the other hand, intussusception occurring in the large bowel is more likely to have a malignant etiology and represents up to 66% of the cases^[2,5,6,8]. In our study malignant etiology was detected in all cases of colonic intussusception.

The clinical presentation in adult intussusception is often chronic, and most patients present with non-specific symptoms that are suggestive of intestinal obstruction. Abdominal pain is the most common symptom followed by vomiting and nausea^[1,2]. Abdominal masses are palpable in 24%-42% of patients, and identification of a shifting mass or one that is palpable only when symptoms are present is suggestive of intussusception or volvulus^[1,2,5]. In our series, an abdominal mass was only palpable in one patient (5%).

The symptoms in cases of adult intussusception are so non-specific that a clinical diagnosis beyond bowel obstruction is rarely made before surgery.

Several imaging techniques may help to precisely identify the causative lesion preoperatively. Plain abdominal X-rays are typically the first diagnostic tool and show signs of intestinal obstruction, and may provide information regarding the site of obstruction^[8,9]. Contrast studies can help to identify the site and cause of the intussusception, particularly in more chronic cases. Upper gastrointestinal series may show a "stacked coins" or "coiled spring" appearance^[8]. Barium enema examination may be useful in patients with colonic or ileocolic intussusception in which a "cup-shaped" filling defect is a characteristic finding^[8]. Barium studies are obviously contraindicated if there is the possibility of bowel perforation or ischemia.

Colonoscopy is also a useful tool for evaluating intussusception, especially when the presenting symptoms indicate a large bowel obstruction^[2,10,11]. It may not be advisable to perform endoscopic biopsy or polypectomy in those individuals with long-term symptoms because of the high risk of perforation, which is more likely to happen in the phase of chronic tissue ischemia, and perhaps necrosis because of vascular compromise in intussusception^[12].

In our series, three patients underwent colonoscopy, and intussusception was confirmed in two (66.6%). A small-bowel series were performed in three patients. Two (66.6%) patients in diagnostic studies had findings suspicious of intussusception because of obstruction with polyps or tumors.

Ultrasonography has been used to evaluate suspected intussusception. The classic features include the "target and doughnut sign" on transverse view and the "pseudokidney sign" in longitudinal view. The major disadvantage of ultrasound is masking by gas-filled loops of bowel, and operator dependency^[10,11,13,14].

In recent years, CT has become the first imaging method performed, after plain abdominal X-rays, in the evaluation of patients with non-specific abdominal

complaints. The characteristics of intussusception on CT are an early "target mass" with enveloped, excentrically located areas of low density. Later, a layering effect occurs as a result of longitudinal compression and venous congestion in the intussusceptum^[15]. Abdominal CT has been reported to be the most useful tool for diagnosis of intestinal intussusception and is superior to other contrast studies, ultrasonography, or endoscopy^[15-18]. The reported diagnostic accuracy of CT scans was 58%-100%, especially in recent series^[1,4,6,16,19]. The diagnosis of intussusception was based on CT findings in the majority of our cases (10/12); two were based on colonoscopy and two on a small-bowel series. The accuracy was 83.3% for CT, 66.6% for colonoscopy, and 66.6% for small-bowel series. The preoperative diagnostic accuracy was 70% in our series.

Although few reports have described magnetic resonance imaging (MRI) of adult intussusception, the general imaging characteristics of intussusception on MRI are similar to those on CT^[18,20], but fast MR examination, unlike CT, is not technically limited by the presence of previously administered barium for small bowel series^[21].

The optimal management of adult intussusception remains controversial. Most of the debate focuses on the issue of primary en bloc resection versus initial reduction, followed by a more limited resection^[1,2,19,22]. Proponents of primary resection cite the high incidence of underlying malignancy, especially in colonic lesions, which mandates en bloc resection. Furthermore, the inability to differentiate malignant from benign etiology preoperatively or intraoperatively also dictates that small bowel intussusception be resected without reduction. The reduction of an intussusception secondary to a malignant lead point is potentially detrimental, as there is the theoretic risk of intraluminal seeding and venous embolization in regions of ulcerated mucosa. Other drawbacks include the increased risk of anastomotic complications (the bowel wall may be weakened during manipulation) and the potential for bowel perforation^[1,5,6,23,24].

On the other hand, some authors have recommended a selective approach to resection, taking into consideration the site of intussusception, which influences the type of pathology^[2,25]. They advocate resection of all colonic lesions but a more selective approach for small bowel pathology, as the lower malignancy rate for small bowel intussusception makes the argument for initial resection less convincing.

Recently, minimally invasive techniques have been applied to the treatment of small or large bowel obstructions, specifically to the diagnosis and treatment of adult intussusception. There are several case reports about laparoscopic small bowel resection because of intussusception^[26,27]. The choice of using a laparoscopic or open approach depends on the clinical condition of the patient, the location and extent of intussusception, the possibility of underlying disease, and the availability of surgeons with sufficient laparoscopic expertise^[28,29]. In the present study, we did not use laparoscopy for diagnosis or treatment.

In conclusion, intussusception in adults is a rare entity and diagnosis may be challenging because of non-specific symptoms. Surgeons should be familiar with the various treatment options, because the real cause of the intussusception often is accurately diagnosed by laparotomy. CT is the most useful imaging modality in the diagnosis of intussusception. Treatment usually requires resection of the involved bowel segment. Reduction can be attempted in small-bowel intussusception if the segment involved is viable or malignancy is not suspected; however, a more careful approach is recommended in colonic intussusception because of a significantly higher chance of malignancy.

COMMENTS

Background

Intestinal intussusception in adults is a rare entity and there is an ongoing controversy regarding the optimal management of this problem. Most surgeons accept that adult intussusception requires surgical resection because the majority of patients have intraluminal lesions. However, the extent of resection and whether the intussusception should be reduced remains controversial.

Research frontiers

Authors aimed to evaluate their experience with 20 adult intussusception cases and to clarify the cause, clinical features, diagnosis, and management of this uncommon entity.

Applications

The present study was retrospective; however, it highlights the clinical features in adult intussusception and may guide surgeons who encounter this problem.

Terminology

Intussusception occurs when a segment of bowel and its mesentery (intussusceptum) invaginates the downstream lumen of the same loop of bowel (intussusciptens). Sliding within the bowel is propelled by intestinal peristalsis and may lead to intestinal obstruction and ischemia.

Peer review

This is an interesting retrospective study with excellent figures. Although the authors do not show any case with laparoscopic approach, the general statement should be more positive and oriented towards the practice in 2009.

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BRIEF ARTICLES

Computer simulation of flow and mixing at the duodenal stump after gastric resection

Nenad Filipovic, Aleksandar Cvetkovic, Velibor Isailovic, Zoran Matovic, Mirko Rosic, Milos Kojic

Nenad Filipovic, Aleksandar Cvetkovic, Velibor Isailovic, Zoran Matovic, Mirko Rosic, Faculty of Mechanical Engineering, University of Kragujevac, Kragujevac 34000, Serbia
Nenad Filipovic, Velibor Isailovic, Milos Kojic, Research and Development Center for Bioengineering, Kragujevac 34000, Serbia

Nenad Filipovic, Milos Kojic, Harvard School of Public Health, Boston, MA 02115, United States

Aleksandar Cvetkovic, Zoran Matovic, Clinical Center Kragujevac, Kragujevac 34000, Serbia

Mirko Rosic, Medical Faculty Kragujevac, Kragujevac 34000, Serbia

Milos Kojic, The University of Texas Health Science Center, Houston TX77030, United States

Author contributions: Filipovic N, Kojic M and Rosic M designed research; Cvetkovic A, Matovic Z and Rosic M designed and performed experimental research; Filipovic N and Isailovic V created and ran computer simulations; Filipovic N, Kojic M and Cvetkovic A wrote the paper.

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Correspondence to: Nenad Filipovic, Faculty of Mechanical Engineering, University of Kragujevac, S Janjica 6, Kragujevac 34000, Serbia. fica@kg.ac.rs

Telephone: +381-34-334379 Fax: +381-34-333192

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Abstract

AIM: To investigate the flow and mixing at the duodenal stump after gastric resection, a computer simulation was implemented.

METHODS: Using the finite element method, two different Billroth II procedure cases (A and B) were modeled. Case A was defined with a shorter and almost straight duodenal section, while case B has a much longer and curved duodenal section. Velocity, pressure and food concentration distribution were determined and the numerical results were compared with experimental observations.

RESULTS: The pressure distribution obtained by numerical simulation was in the range of the recorded experimental results. Case A had a more favorable pressure distribution in comparison with case B. However, case B had better performance in terms of food transport because of more continual food

distribution, as well as better emptying of the duodenal section.

CONCLUSION: This study offers insight into the transport process within the duodenal stump section after surgical intervention, which can be useful for future patient-specific predictions of a surgical outcome.

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Key words: Computer simulation; Gastric resection; Duodenal stump; Billroth II; Pressure distribution

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INTRODUCTION

The duodenum is a short, complex and functionally highly specialized part of the small intestine. It has many motor, sensitive and secretory functions. Transit of chyme through the duodenum is a very complicated process (which includes intestinal peristalsis, gastric emptying, and pyloric sphincter tone) and is regulated by many neurological and hormone-dependent feedback mechanisms^[1-6].

Two layers of smooth muscle cells (inner-longitudinal and internal-circumferential), as well as two neurological intramural networks (*Auerbach's* and *Meisner's* complexes), are responsible for gastroduodenal peristalsis. Electrical activity of the smooth muscle cell syncytium of the duodenum and in other parts of the gastrointestinal tract is represented by two basic types of electrical waves: slow waves and the spikes. Slow waves represent basic electrical activity and they are caused by activity of the Na-K pump. When the resting membrane potential become less negative than -40 mV, the spikes appear on the top of slow waves with a frequency of 1-10 Hz,

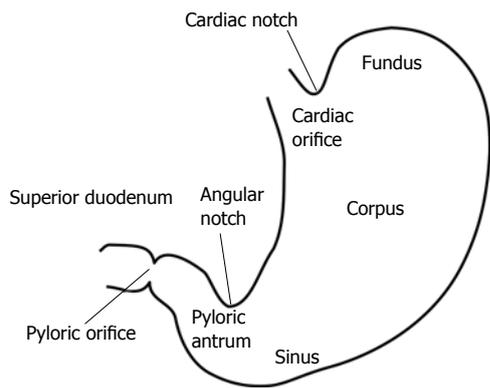


Figure 1 Normal physiological gastric outlet, pylorus, and superior duodenum.

and cause smooth muscle contraction. The number of duodenal contractions is about 12/min^[7]. Several new investigations suggest that slow waves and spikes are regulated and spread through different cell networks^[8].

The pylorus shown in Figure 1 comprises a collection of tissue structures that connect the antrum to the duodenum. Its luminal diameter is controlled by a sphincter muscle complex that sets resistance to the bulk gastric effluent, through regulation of the pyloric orifice tone. During gastric digestion, the proximal and distal pyloric muscle loops occlude the pyloric lumen, preventing premature discharge of unprocessed material into the duodenum. Once the stomach has completed its task of breaking down large solid agglomerates into smaller particles, the pylorus relaxes and peristaltic contractions in the antrum begin to force chyme distally. At this point, antral contraction waves approach the pyloric orifice and, along with the sphincter complex and mucosal folds, cause steady constriction of the pyloric lumen. Chyme continues to be forcibly transported through this lumen until it is fully occluded, a process thought to induce an effluent jet into the superior duodenum^[9]. Dysfunction of the duodenum can occur as a result of many disorders of gastric emptying and dyspeptic complaints, which demonstrates the vital role of the duodenum^[10]. This role becomes especially apparent in surgical interventions on the gastroduodenum^[11].

A large number of studies have investigated the intact gastrointestinal tract anatomically^[12-14], but there have been relatively few studies on the consequences of cutting muscles, nerves and other important anatomical structures of the gastroduodenum. These are unavoidable during surgical interventions, with disturbance of many fine, highly sophisticated feedback systems. These undesirable conditions lead to negative feedback mechanisms^[15,16] that cause changes in physiological processes with respect to the preoperative state^[17,18].

To date, it seems that insufficient attention has been paid to how the geometry and flow conditions affect the gastroduodenal system after distal gastric resection. There are various types of reconstruction

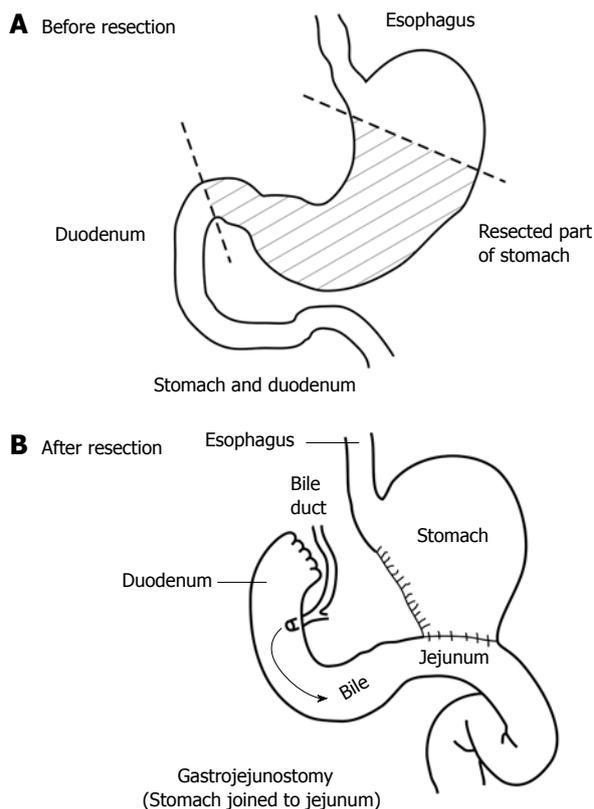


Figure 2 Schematic representation of reconstruction of gastrointestinal continuity after gastric resection. A: Normal anatomy of gastroduodenal region with resection lines; B: Billroth II antiperistaltic anastomosis.

of gastrointestinal continuity after gastric resection. In studying the treatment of gastric cancer, Devin reported in 1968 about 300 types of reconstruction after surgery of the gastroduodenal region^[19]. During recent years, a few types have become more frequent. The first and most physiological variant is a state in which continuity of the gastrointestinal tract is reconstructed with anastomosis between the gastric stump and the duodenum; this procedure is called Billroth I gastric resection (gastroduodenal anastomosis). The other type of reconstruction is the Billroth II operation, which is shown in Figure 2, in which the anastomosis is located between the gastric stump and a loop of jejunum (gastrojejunal anastomosis, gut to side), and this type of intervention is the subject of our study. Anastomosis may include the entire circumference of the gastric stump (today it is a rarely used technique because of many unwanted consequences) or just a part of the circumference when a smaller diameter anastomosis is created. This is a better method of adaptation of the gastric stump and jejunal loop-the Hoffmeister-Finsterer modification. The length of the proximal jejunal loop is variable and depends on anatomical variations (e.g. length of the mesentery of the small intestine, and adhesions), as well as surgeons' ability. The jejunal loop conducts duodenal juice toward the gastric stump and the rest of the intestine. There is a hypothesis that increased intraluminal pressure in the afferent loop is the dominant cause of duodenal suture

dehiscence (caused by the length of afferent jejunal loop, narrow gastrojejunoanastomosis, *etc*^[20-22]). The distal or efferent loop is the part of the duodenum that is downstream of the anastomosis, and it conducts duodenal and gastric stump content distally to the small intestine. The anastomosis is antecolic when the jejunal loop is positioned in front of the transverse colon, or the jejunal loop may be brought up posteriorly through an opening made in the transverse mesocolon (retrocolic anastomosis). Adequate position, shape and diameter of the anastomosis facilitate gastric emptying. The orientation of the jejunal loop may be two-fold, isoperistaltic, when the stomach and jejunum have the same peristalsis direction, and antiperistaltic, when the stomach and afferent loop of the jejunum have the opposite direction of peristalsis.

There are other methods of gastrointestinal tract reconstruction after gastric resection, and one of these is called Roux en Y gastrojejunoanastomosis, in which the small bowel is cut distal to the ligament of Treitz, and the anastomosis is created between the distal limb of the jejunum and remaining gastric tissue (or esophagus in cases of total gastrectomy). The proximal limb of the jejunum is positioned downstream to the jejunum at a distance of about 45 cm, where a termino-lateral end-to-side anastomosis is created^[23,24].

In gastrointestinal tract reconstruction, the small bowel is not prepared to receive acidic content from the stomach, especially when duodenal juice is not present to neutralize it^[25]. Suture dehiscence with postoperative peritonitis can occur as a complication after surgical intervention. Duodenal stump blowout is considered to be a serious postoperative complication, because of its high mortality rate^[26,27]. Suture dehiscence after surgical intervention in the gastroduodenal region has been the subject of many investigations, but still many pathological mechanisms involved in this surgical problem remain unclear^[28].

This study offers a new approach to this problem. Using finite element analysis with computer modeling and clinical experiments, we attempted to determine physiological constants relevant to the above-mentioned surgical complication. In order to achieve this goal, we computed flow and mixing in the duodenal junction after distal gastric surgical resection and a Billroth II procedure.

MATERIALS AND METHODS

A prospective, randomized, placebo-controlled, multicenter study was performed on patients treated with Billroth II Hofmeister-Finsterer subtotal gastrectomy. Our task was to investigate and quantify the effects of increased intraluminal pressure in the duodenal stump on disruption of the duodenal suture.

Subjects

Measurements were performed on five patients after Billroth II Hofmeister-Finsterer surgical intervention. The indication for surgery was a malignant gastric



Figure 3 Schematic representation of the manometry equipment positioned in a patient during pressure measurement.

process in three cases and ulcer disease in two. Patients with no significant co-morbidity were chosen for the study. Each patient gave written consent to our investigation. No complications were observed, and no complaints were made by these patients in relation to our actions for the purpose of this study.

Manometry equipment

We created hardware and software equipment for measurement of pressure in the duodenal stump after distal gastric resection. The manometry assembly consisted of a Miller-Abbott tube, connecting catheters with control valves, transducers, analog-to-digital (AD) converter, and a computer with appropriate software. A Miller-Abbott tube 3.7 m in length, with an outer diameter of 5 mm, was placed in the duodenum after the operation (Figure 3). The volume of the balloon on top of the Miller-Abbott tube was 30 mL and the balloon expanded under pressure. The length of the balloon was 7 cm. The proximal end of the two-lumen tube was divided in two channels: one that connected the balloon with the measurement assembly of catheters, transducers, AD converter and personal computer; and the other used for aspiration of the duodenal stump. Distilled water was injected manually through the first channel into the balloon positioned in the duodenal stump. Data were recorded by an in-house developed software and stored to disk for later analysis. The measuring system was mobile and could be used outside the laboratory.

Protocol

The balloon of the Miller-Abbott tube was positioned at the duodenal bulb with minimum air insufflation, followed by direct visual control. The duodenum and anterior wall of the gastrojejunal anastomosis were sutured after appropriate placing of the balloon. The entire procedure lasted 3-5 min and did not have any influence on the operative procedure itself. The final location of the catheter was documented by checking X-rays before starting measurements and again before removal. Catheter migration from the duodenum was not observed. However, duodenal motility activity was noticed in two cases.

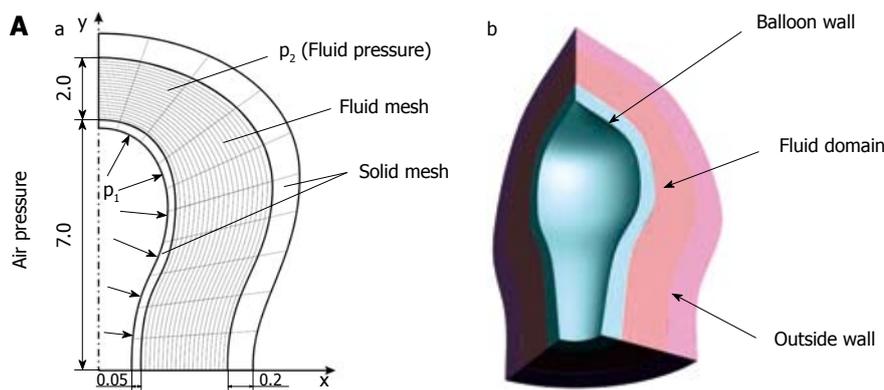


Figure 4 Computer simulation of pressure measurement inside duodenum bulb. A: Schematic representation of the computational model of balloon pressure measurement inside the duodenum. Geometry of the model (a); 3D model view (b); B: Distribution of solid deformation (wall displacement) at the maximum pressure measurement (a); Fluid pressure distribution inside the duodenum (b).

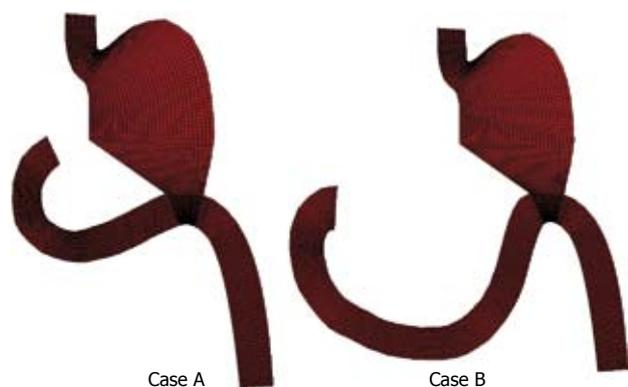
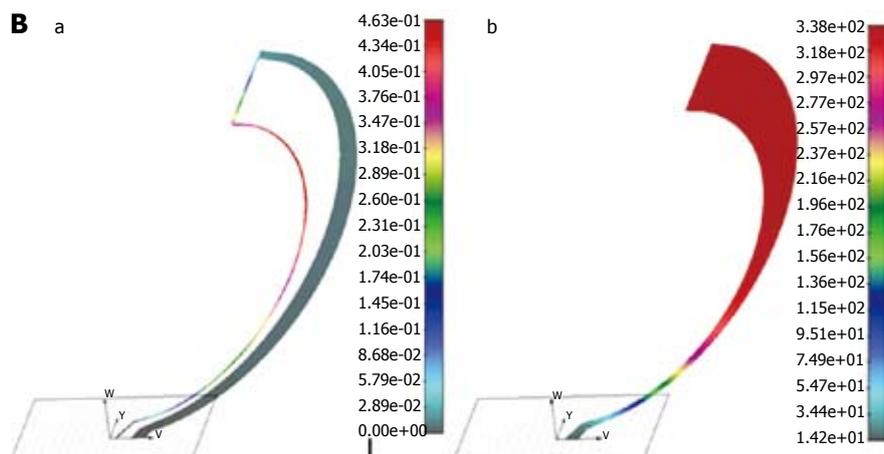


Figure 5 Geometry and finite element mesh of two selected cases. Case A showed a straight duodenal part with shorter length. Case B had a much longer and curved duodenal section.

After the catheter was positioned and each subject was at rest for at least 24 h, all measurements were performed on patients in the supine position. Data files were recorded for each patient during seven postoperative days, starting 24 h after intervention. Data files consisted of pressure measurements over time. Prostigmine was used to stimulate bowel peristaltic movements. Every measurement lasted about 30 min. All external impacts that could cause some changes in duodenal intraluminal pressure were recorded in diagram form, as contractions of the anterior abdominal wall, cough, patient movements and administration of prostigmine.

Patients were discharged from the clinic after 10 d.

They continue to be under observation and come to follow-up clinical examinations.

Details about the calculation of pressure inside the duodenal bulb using finite element fluid-solid analysis are as follows.

Computer simulation of pressure measurement inside duodenum bulb:

We modeled balloon measurement of duodenal intraluminal pressure by using a finite element method^[29,30]. The balloon and the duodenal walls were discretized by 2D solid finite elements, while 2D fluid elements were used for the fluid domain (Figure 4A). A simplified axi-symmetric model was adopted. The thickness of the balloon wall was 0.05 cm and the outer wall was 0.2 cm. The length of the balloon was 7.0 cm, with a distance from the outer wall of 2.0 cm. Fluid density was taken as 1 g/cm³, dynamic viscosity μ was 1000 cP, Young's elasticity modulus E was 1 MPa. Fluid and solid domains had common boundaries at the interfaces on the balloon wall and duodenal wall. Air pressure was applied to the balloon wall, which caused flow of the fluid surrounding the balloon. The air pressure p_1 inside the balloon was measured, and the fluid pressure p_2 between the balloon and duodenal wall was calculated. The balloon deformed as a result of the difference between the air and fluid pressures. The deformed walls and fluid pressure distribution inside the duodenum are shown in Figure 4B. They corresponded to a maximum inlet balloon pressure of 2000 Pa (15 mmHg). The maximum balloon displacement was 0.4 cm, and the maximum

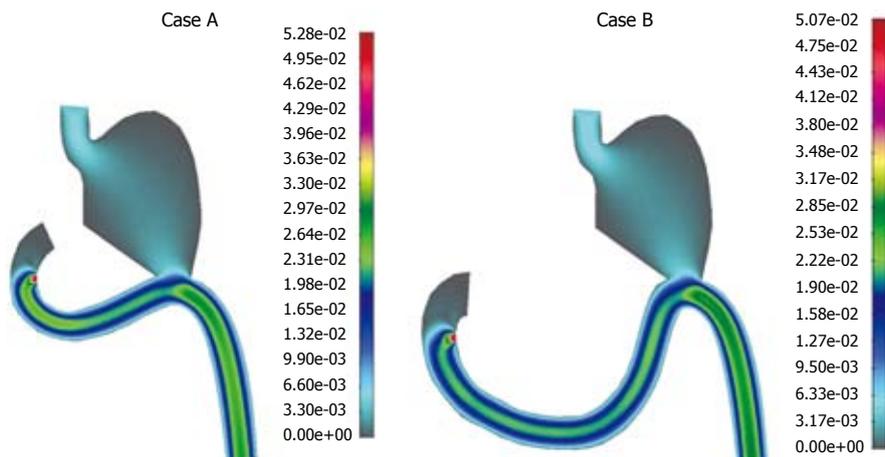


Figure 7 Velocity distribution for the steady state flow conditions [in (cm/s)].

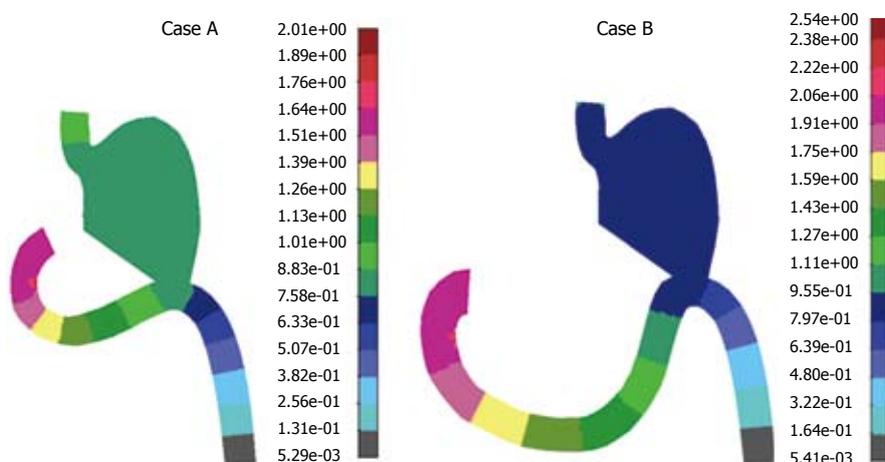


Figure 8 Pressure distribution for the steady state flow conditions [in (mmHg)].

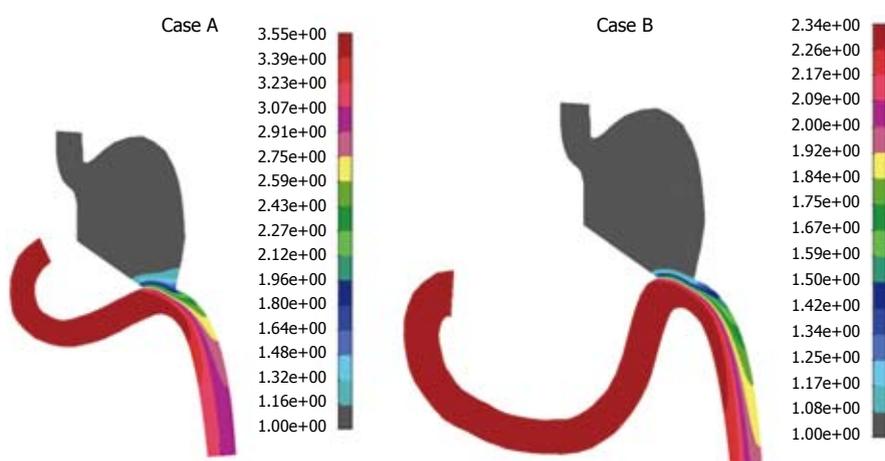


Figure 9 Mass concentration [in (g/cm³)] distribution for the diffusion coefficient $D = 2.0 \times 10^{-3} \text{ cm}^2/\text{s}$.

different (Figure 10). Obviously, for the higher diffusivity coefficient (according to our analysis, $D = 8. \times 10^{-3} \text{ cm}^2/\text{s}$) mass transport of food will go smoothly to the rest of gastrointestinal system, which is a desirable outcome. This effect of diffusivity is expected since diffusion enhances mass transport. There is also a difference in these two cases A and B, when the diffusivity is higher. It can be seen from Figure 9 that in case B, the duodenum section looked empty, while in case A there was approximately uniform mass distribution. This was probably the effect of the duodenal section. Therefore, the conditions in case B were better for the patient. A

lower diffusivity coefficient D caused a more dominant convective term in equation (3), which resulted in food accumulation in the duodenal section. This is clearly shown in Figure 9, in which cases A and B had maximum food concentration in the duodenal zones. This means that a patient after this surgical procedure may have food accumulation in the duodenal zone, which is not desirable.

We tested cases A and B for $D > 8. \times 10^{-3} \text{ cm}^2/\text{s}$ (data not shown), for which computational analysis indicated a slightly better performance in the gastroduodenal connection for case A, for which more food passed

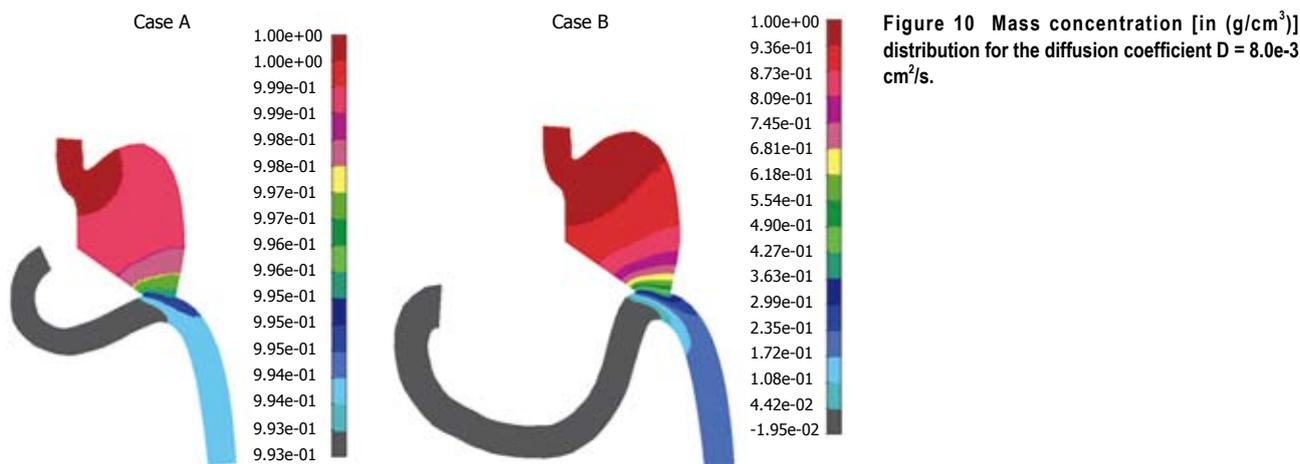


Figure 10 Mass concentration [in (g/cm³)] distribution for the diffusion coefficient $D = 8.0e-3 \text{ cm}^2/\text{s}$.

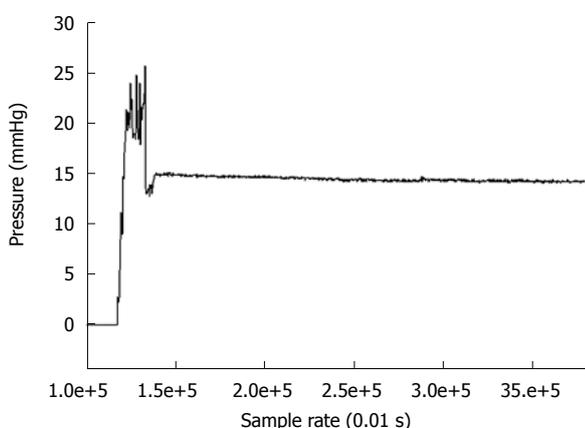


Figure 11 Pressure measurement of Miller-Abbott (MA) tube and connecting catheter in the duodenum at 3 d after gastric resection.

through the duodenal zone.

A measurement technique described in the Methods section and Appendix was implemented in order to measure pressure inside the duodenum a few days after surgical intervention. Patients agreed that this measurement technique could be used at 3-4 d after gastric resection. Among five patients, there was one with duodenal stump leakage, and one died from a non-surgical complication (pulmonary embolism). The contractile events associated with pressure changes were detected and recorded with our in-house software. We recorded pressure changes every 0.1 s. The pressure range shown in Figure 11 for the air inside the balloon was around 15 mmHg, while the fluid pressure inside the duodenum was between 3.5 and 4.5 mmHg (which was in the physiological range^[32]). Also, numerical simulations (Figure 8) for cases A and B showed similar pressure distributions (around 2.5 mmHg).

DISCUSSION

The rate of postoperative gastric resection complication seems to be primarily influenced by increased duodenal pressure. Localization and extent of the malignant process, suture material, surgical technique, presence of potentially pathogenic microorganisms colonizing the digestive tract, and tissue quality, also may play a role in

the pathogenesis of this complication^[33,34]. In this study, we focused on numerical simulation of two different Billroth II antiperistaltic anastomosis cases, with a slightly different geometrical connection of the duodenal and gastric parts during gastric resection. Numerical solutions of velocity and pressure distribution for both cases gave similar results. As expected, the maximum pressure was localized in the duodenal suture area and it was in the range of experimental pressure measurements obtained by using a Miller-Abbott tube and connecting catheter. Case B showed higher pressures, which was the result of the longer duodenal section.

Additional transport analysis showed that the food distribution in the gastric and duodenal connection zone varied depending on the diffusivity coefficient. The results of this analysis implied that case B was more favorable with respect to food transport because of continual food distribution and an empty duodenal section. Therefore, the longer duodenal section in case B provides conditions for food not to stay within the duodenal zone.

Experimental results have shown that intraluminal duodenal stump pressure after gastric resection was higher by an order of magnitude with respect to the pre-operative state. Comparison of models with different lengths of afferent loops showed that the afferent loop syndrome was an important factor in postoperative complications.

By changing input parameters of our interactive computational model (fluid parameters, model geometry), we can improve prediction of the potential *in vivo* events. Also, it is possible to obtain insight into possible outcomes of different types of surgical reconstruction of the digestive tract after operations on the gastroduodenal region. This may include variations of the same surgical intervention, related to surgeons' ability or the specific anatomy of a single patient.

The practical aim of this study was to provide an insight into the physical conditions within the duodenal stump after gastric resection, in relation to the pathogenesis of duodenal stump blowout, and the implications for the surgical technique itself.

New, more comprehensive computational models may first include duodenal pressure quantification,

which causes disruption of the duodenal stump closure. Such models will require determination of shear stress and pressure distribution at the inner surface of the duodenal stump, as well as the stress-strain state within the duodenal wall, especially in the suture area. The stress-strain state and the critical state at the point of disruption depend on tissue quality and tissue material characteristics. Furthermore, these sophisticated models need to establish a correlation between duodenal flow and the wall dynamics of contractions, in order to quantify critical levels of increased intraluminal duodenal pressure.

COMMENTS

Background

A malignant process and ulcer disease in the gastric distal section are the main causes for surgical intervention, which has become a standard procedure over recent years. Duodenal stump dehiscence after gastric resection occurs relatively rarely, but is of particular importance because it is associated with a high rate of mortality. A computer simulation can provide better understanding of this process.

Innovations and breakthroughs

Computer simulation offers better insight into fluid flow, mass transport and pressure distribution in the duodenal section before and after surgical intervention.

Applications

This computer simulation study can be applied to new follow-up studies that will help clinicians with diagnosis and treatment.

Terminology

Computer simulation and a finite element method solve a large number of equations in which physical laws such as fluid flow and mass transport are solved with differential partial equations. This methodology has been well known in industrial applications over several decades, and recently, it has been used for biological systems. Many questions regarding parameters in this computational study are still unknown and future computer simulation will try to answer these.

Peer review

The title describes well the manuscript. The introduction is clear. The description of the materials and methods is clear. The results are reported well. The discussion is well organized. The references are well reported. The tables and figures are clear.

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Transjugular intrahepatic portosystemic shunt in liver transplant recipients

Armin Finkenstedt, Ivo W Graziadei, Karin Nachbaur, Werner Jaschke, Walter Mark, Raimund Margreiter, Wolfgang Vogel

Armin Finkenstedt, Ivo W Graziadei, Karin Nachbaur, Wolfgang Vogel, Department of Internal Medicine II (Gastroenterology and Hepatology), Medical University of Innsbruck, Anichstraße 35, A-6020 Innsbruck, Austria
Werner Jaschke, Department of Radiology, Medical University of Innsbruck, Anichstraße 35, A-6020 Innsbruck, Austria
Walter Mark, Raimund Margreiter, Department of Transplant Surgery, Medical University of Innsbruck, Anichstraße 35, A-6020 Innsbruck, Austria

Author contributions: Finkenstedt A and Graziadei IW analyzed the data and wrote the paper; Graziadei IW and Vogel W designed the study; Nachbaur K, Jaschke W, Mark W and Margreiter R collected the data.

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Correspondence to: Ivo W Graziadei, MD, Department of Internal Medicine II (Gastroenterology and Hepatology), Medical University of Innsbruck, Anichstraße 35, A-6020 Innsbruck, Austria. ivo.graziadei@i-med.ac.at

Telephone: +43-512-50423401 Fax: +43-512-50424052

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Abstract

AIM: To evaluate the efficacy of transjugular intrahepatic portosystemic shunts (TIPSS) after liver transplantation (LT).

METHODS: Between November 1996 and December 2005, 10 patients with severe recurrent hepatitis C virus infection ($n = 4$), ductopenic rejection ($n = 5$) or portal vein thrombosis ($n = 1$) were included in this analysis. Eleven TIPSS (one patient underwent two TIPSS procedures) were placed for management of therapy-refractory ascites ($n = 7$), hydrothorax ($n = 2$) or bleeding from colonic varices ($n = 1$). The median time interval between LT and TIPSS placement was 15 (4-158) mo.

RESULTS: TIPSS placement was successful in all patients. The mean portosystemic pressure gradient was reduced from 12.5 to 8.7 mmHg. Complete and partial remission could be achieved in 43% and 29% of patients with ascites. Both patients with hydrothorax did not respond to TIPSS. No recurrent bleeding was

seen in the patient with colonic varices. Nine of 10 patients died during the study period. Only one of two patients, who underwent retransplantation after the TIPSS procedure, survived. The median survival period after TIPSS placement was 3.3 (range 0.4-20) mo. The majority of patients died from sepsis with multiorgan failure.

CONCLUSION: Indications for TIPSS and technical performance in LT patients correspond to those in non-transplanted patients. At least partial control of therapy-refractory ascites and variceal bleeding could be achieved in most patients. Nevertheless, survival rates were disappointing, most probably because of the advanced stages of liver disease at the time of TIPSS placement and the high risk of sepsis as a consequence of immunosuppression.

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Key words: Portal hypertension; Ascites; Variceal bleeding; Immunosuppression; Liver transplantation

Peer reviewers: Dr. Bijan Eghtesad, Associate Professor, Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland OH 44195, United States; Salvatore Gruttadauria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

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INTRODUCTION

Since the first attempts were made over 30 years ago, placement of transjugular intrahepatic portosystemic shunts (TIPSS) has become an established procedure in patients with complications of portal hypertension^[1]. The two main indications for TIPSS are therapy-refractory ascites and variceal bleeding unresponsive to endoscopic treatment^[2-7]. In many patients with these complications,

TIPS is used as a bridge to liver transplantation (LT). In contrast, there is rather limited experience with the use of TIPS following LT. One reason is the rare occurrence of portal hypertension after LT. The causes of portal hypertension in such patients can be impaired venous outflow, recurrence of the underlying liver disease, size mismatch between donor and recipient organs and/or vessels^[8,9], or increased vascular resistance as a consequence of repeated rejection episodes^[10]. This might result in the development of ascites, hepatic hydrothorax or variceal bleeding comparable to the non-grafted population. Therapeutic options for these conditions are basically the same, including TIPS. The placement of a TIPS can be rendered more difficult by the altered anatomy after transplantation. Furthermore, patients undergoing chronic immunosuppression are at higher risk of infection.

Only few data have been published on TIPS after LT and its role in LT recipients is largely undefined. The aim of our retrospective analysis was to critically scrutinize the indications, efficacy and safety of TIPS placement in liver recipients at our center.

MATERIALS AND METHODS

Between November 1996 and December 2005, a total of 11 TIPSs were placed in 10 liver recipients at Innsbruck Medical University Hospital, which represents 5% of all 217 TIPS procedures carried out during this time period. One of the patients received a TIPS before and after retransplantation. The mean age of the six male and four female patients was 56.8 (37-71) years. The underlying liver diseases were hepatitis C virus (HCV) cirrhosis ($n = 4$), alcoholic liver disease ($n = 2$), primary biliary cirrhosis ($n = 2$), hemochromatosis ($n = 1$) and autoimmune hepatitis ($n = 1$).

Patients underwent full-sized deceased donor LT, three of them using a piggyback technique, and the remaining patients by replacement of the retrohepatic vena cava. The mean time interval between LT and TIPS was 29.7 mo (range: 3.8-158 mo). In four patients, recurrent HCV cirrhosis was present at the time of TIPS implantation, five had ductopenic rejection, and one had portal vein thrombosis.

Therapy-refractory ascites was the indication for TIPS in seven patients, resistant hydrothorax in two, and bleeding from colonic varices in one. Ascites and hydrothorax were assessed by ultrasound and chest X-ray, respectively.

All four patients with recurrent HCV presented with decompensated cirrhosis at the time of TIPS implantation. One patient was in Child-Turcotte-Pugh class B and three were in class C. The median model for end-stage liver disease (MELD) score for all patients was 20 (12-35).

The TIPS procedure used for LT recipients did not differ from that for non-transplanted patients, as described previously^[6,7].

The immunosuppressive regimen at the time of the TIPS procedure consisted of calcineurin inhibitors,

alone ($n = 1$) or in combination with steroids ($n = 2$), mycophenolate mofetil (MMF; $n = 3$), or an mTOR-inhibitor ($n = 1$). An mTOR-inhibitor was used with MMF in one patient or with low-dose steroids in three patients.

Variables were compared using Student's *t* test, and $P < 0.05$ was considered statistically significant. Kaplan-Meier plots were calculated using SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

TIPS were placed in all patients without any procedural complications. One patient with pre-existing atrial fibrillation developed cardiac failure during the procedure but responded to specific treatment.

The mean portosystemic pressure gradient was reduced from 12.5 (8-22) mmHg to 8.7 (5-14) mmHg after the procedure. Although pressure gradients below 12 mmHg were found in three patients with refractory ascites and one with hydrothorax, the TIPS procedure was continued, with the aim of further decreasing the final pressure gradients (around 5 mmHg), in order to improve the clinical condition.

Regarding patients with refractory ascites, complete resolution of ascites was achieved in three and a partial response in two patients, whereas no response was seen in two others. TIPS failed to improve the condition in both patients with hydrothorax. After TIPS implantation, no more bleeding was seen in the patient who suffered from colonic variceal hemorrhage.

Seven out of 10 patients developed TIPS-related hepatic encephalopathy, which necessitated TIPS reduction in two patients with a later closure in another. In the other patients, encephalopathy was successfully treated with standard medical therapy. One patient developed TIPS dysfunction, which was corrected by dilatation.

Only one patient in our cohort, who underwent retransplantation, survived long-term. All other patients died, mainly from sepsis associated with multiorgan failure. The median survival time of all patients was 3.3 mo (range 0.4-20 mo; Figure 1).

The course of all 10 patients is summarized in Table 1. Although TIPS was able to reduce ascites in patients 1 and 2, both died at 1 and 3 mo after TIPS placement because of HCV recurrence, with sepsis and multiorgan failure. Both patients presented with a high MELD score of 22 and 26, respectively.

The third patient with therapy-refractory ascites first responded well to TIPS. Four months later, however, she developed massive bleeding in the upper gastrointestinal tract and lungs because of severe coagulopathy, secondary to graft failure associated with ductopenic rejection, and died.

No improvement in ascites was seen in the fourth patient. Eight months after TIPS implantation, the patient underwent retransplantation for recurrent HCV cirrhosis. A few months after retransplantation, he again developed a rapidly progressive HCV recurrence, with

Table 1 Summary of clinical data and outcomes

Patient no.	Age, sex	Cause of liver disease	Transplant pathology before TIPS	Indication for TIPS	MELD score at TIPS	CPC at TIPS	Time from transplantation to TIPS (mo)	Encephalopathy post-TIPS	Follow-up after TIPS
1	71, F	Hepatitis C	HCV recurrence	Ascites	22	C	15	Yes	Died 1 mo
2	59, M	Hepatitis C	HCV recurrence	Ascites	26	C	16	Yes	Died 3 mo
3	37, F	Primary biliary cirrhosis	Vanishing bile duct syndrome	Ascites	18	B	4	Yes	Died 4 mo
4	56, M	Hepatitis C	HCV recurrence	Ascites	15	C	158	Yes	ReLT 8 mo, died after ReLT, see below
	59, M	Hepatitis C	HCV recurrence	Ascites	13	C	18	Yes	TIPS reduction day 11 and 16; died at 2 mo
5	52, M	Fatty liver cirrhosis	Vanishing bile duct syndrome	Ascites	35	C	10	No	ReLT 3 mo; alive 32 mo
6	51, F	Hepatitis C	HCV recurrence	Ascites	19	B	61	No	Died 3 mo
7	71, M	Primary biliary cirrhosis	Thrombosis of the portal vein at the anastomosis	Ascites	12		4	No	TIPS revision 3 mo; died 12 mo
8	62, M	Hemochromatosis	Vanishing bile duct syndrome	Hydrothorax	21	C	17	Yes	Died 1.5 mo
9	68, M	Fatty liver cirrhosis	Vanishing bile duct syndrome	Hydrothorax/Ascites	30	B	11	Yes	Died 11 d
10	38, F	Autoimmune hepatitis	Vanishing bile duct syndrome + thrombosis of the portal vein	Bleeding	¹	B	8	Yes	TIPS reduction 2.5 mo; died 19 mo

¹MELD score calculation not possible due to incomplete laboratory data. ReLT: Liver retransplantation.

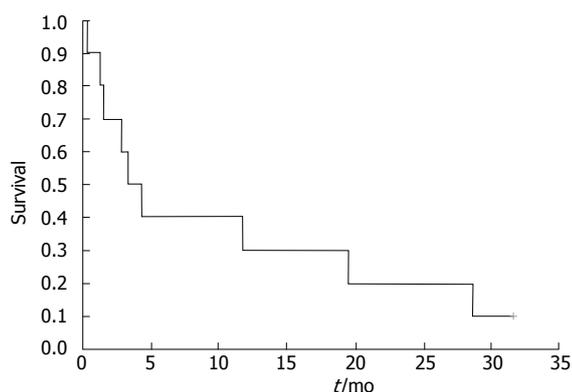


Figure 1 Kaplan-Meier plot of patients' overall survival after TIPS implantation.

massive ascites that was resistant to diuretic therapy. Therefore, 18 mo after retransplantation, a TIPS was placed again. Eleven days after placement, a reduction in the TIPS, with subsequent complete closure was necessary because of severe hepatic encephalopathy. The patient died 2 mo after the second shunt placement from sepsis with multiorgan failure.

In the fifth patient, TIPS implantation resulted in complete resolution of ascites. Three months after TIPS placement, the patient developed ductopenic rejection in association with renal failure, and underwent combined liver and kidney transplantation. Thirty-two months later, the patient is doing well with stable graft function.

The sixth patient with TIPS placement for ascites did not respond to the procedure and died 3 mo later from HCV recurrence.

Ascites further deteriorated in patient seven after TIPS placement. Shunt occlusion was suspected and the patient was given another TIPS 3 mo later, which

led to a reduction of portosystemic pressure from 19 to 12 mmHg, and disappearance of ascites. The patient, however, died 1 year after TIPS insertion from cholangitis secondary to multiple ischemic-type intrahepatic biliary strictures.

TIPS placement did not result in any improvement in the two patients with hydrothorax (patients 8 and 9). One of them died at 6 wk and the other at 11 d after TIPS, as a result of sepsis.

The patient with colonic bleeding as indication for TIPS placement developed severe encephalopathy at 2.5 mo after the procedure, which required a reduction in TIPS. The portal pressure increased from 5 to 9 mmHg, and encephalopathy improved thereafter. Although there was no recurrence of bleeding, the patient died 19 mo after TIPS because of chronic rejection.

DISCUSSION

TIPS is an established therapeutic modality for the management of complications of portal hypertension, in particular, therapy-refractory ascites or pleural effusion, as well as variceal bleeding resistant to endoscopic treatment. However, there is much less experience with TIPS after LT^[11-15]. Although the indications for TIPS should be essentially the same as in patients without LT, certain specific points need to be considered.

At our center, the indications for TIPS placement did not differ between the transplant and non-transplant patients, with therapy-refractory ascites being the main indication also in LT recipients. Therapy-refractory hydrothorax and variceal bleeding, not manageable by endoscopic means, were less frequent indications. Our analysis showed that, in principle, TIPS was technically feasible in patients with portal hypertensive

complications after LT, and was efficacious in the majority of patients. Complete resolution was achieved in three out of eight patients, and a partial response in two further patients with therapy-refractory ascites. In addition, severe variceal hemorrhage in one patient did not recur after TIPS. No improvement, however, was seen in both patients with severe hepatic hydrothorax. Similar response rates have been reported in four previously published series, including a small study of 6-12 patients^[11-14]. In these studies, improvement or complete resolution of ascites was achieved in 50%-90% of patients, and only two out of 10 patients with variceal hemorrhage experience recurrent bleeding.

The success rate of TIPS placement for managing ascites and variceal bleeding in LT was comparable with that in non-transplanted patients at our department. In the present cohort, complete resolution or significant reduction in the amount of ascites was achieved in about 70% of patients, and recurrence of bleeding was prevented in about 77% of patients (data not shown).

With an average survival of 3.3 mo, the survival in our series was extremely poor. This might mainly have been the result of the advanced stage of liver disease and the already poor prognosis of our patients at the time of TIPS placement, and not by the intervention itself. TIPS should serve as a bridge to a possible liver retransplantation. As we had only little experience with TIPS in LT recipients, this intervention was indicated with great caution. As a consequence, TIPS was placed in LT patients as the last therapeutic option, after all conservative modalities had failed. Several studies have shown that survival rates of TIPS patients with advanced disease are markedly poorer than those in earlier stages of liver disease^[4,16,17]. In fact, almost all of our patients presented with an advanced stage of graft dysfunction and high MELD scores (median score of 20). The MELD score was originally developed for patients undergoing TIPS^[18], and then slightly modified to predict survival of patients with liver cirrhosis in general^[19]. In our analysis, there was a trend towards a higher MELD score being associated with poor survival. The correlation was not statistically significant, most probably as a result of the small number of patients.

It is well known that the natural course of recurrent HCV infection is more aggressive and leads more rapidly to cirrhosis of the allograft and graft failure than HCV infections in non-transplanted patients. Subsequently, the long-term outcome of HCV-positive patients after LT is worse compared to those with other indications for transplantation^[20-23]. It has been shown that the prognosis of HCV patients is poor after decompensation, with a median survival of less than 1 year^[20]. All of our four patients with recurrent hepatitis C infection presented with decompensated cirrhosis (Child-Pugh stage B or C and MELD scores between 13 and 26).

Chronic rejection with progressive loss of bile ducts inevitably leads to irreversible loss of the allograft^[24], with liver retransplantation rates of 50%-90%^[25,26]. Prognosis is especially poor in patients with bilirubin values > 10 mg/dL^[27]. In our study, all three patients with chronic

rejection presented with a bilirubin above this level.

Therefore, an advanced stage of graft dysfunction caused by recurrent HCV cirrhosis and chronic ductopenic rejection might have been responsible for the poor survival rate in our patients. In addition, the number of liver retransplantations, the only potentially curative therapy for these patients, was lower in our series compared to other studies. Only two of our patients underwent liver retransplantation, whereas the retransplantation rate was 50% in the largest series of Amesur and co-workers^[13]. Three of our patients died while being on the waiting list for a second LT, which suggests that retransplantation should be considered as early as possible when graft decompensation occurs.

The most frequent cause of death ($n = 5$) in our cohort was sepsis associated with multiorgan failure. Thus, two interacting factors were responsible for the frequency of sepsis in our patients. Patients with impaired liver function or recurrent cirrhosis frequently develop bacterial infections, which lead to death in 30%-50% of cases^[28,29]. The risk of infection is further increased by chronic immunosuppression. Therefore, we recommend prophylactic antimicrobial therapy following TIPS placement.

The altered anatomy of the hepatic vessels that results from LT should be kept in mind before the TIPS procedure. The two most frequently used techniques are the replacement of the retrohepatic vena cava and the piggyback-type of transplantation. Previous studies have shown that there are no difficulties in TIPS placement with either of these procedures. In contrast, in patients with cava-cava liver transplantation, probing for the hepatic and portal veins in the recipient's organ might be difficult^[11-14]. No technical problems were encountered in our series, in which, three had undergone the piggyback-type of transplantation and the remaining patients had replacement of the retrohepatic vena cava. This indicates that the anatomical situation in these patients creates no problems for the TIPS procedure.

Only one patient (10%) developed dysfunction of the TIPS, which was managed successfully by TIPS dilatation. In contrast, the rate of TIPS revision in our non-transplanted patients was markedly higher at 35% ($P = 0.059$). This low rate of TIPS dysfunction in the LT group might be attributed to the fact that immunosuppressive therapy can lead to reduced intima proliferation^[11], but can also be ascribed to the very short survival of these patients.

Noticeable in our LT recipients was a low pre-interventional mean portosystemic pressure gradient of 12.5 mmHg, which suggests that ascites and hydrothorax post-transplant may not be as well-correlated with portal pressure as in the pre-transplant phase. Other factors beside the portosystemic pressure gradient, such as renal function, may play a major role in the efficacy of TIPS in LT transplant recipients. Chronic renal dysfunction is a common complication in transplant recipients, especially if calcineurin inhibitors are used^[30]. In our cohort, both patients with hepatic hydrothorax presented with markedly impaired renal function (glomerular filtration

rate < 20 and 45 mL/min per 1.73 m², respectively), which may explain their non response to TIPS treatment, although the postinterventional pressure gradients could be successfully lowered to 10 and 5 mmHg, respectively.

In the ascites group, only three of seven patients showed normal renal function, and in two of these, complete resolution of ascites was achieved. No statistically significant correlation was found between the reduction of the portal pressure gradient and response to TIPS.

Hepatic encephalopathy developed in 70% of our patients. A similar rate of encephalopathy was also described in a previous study, and is markedly higher than the incidence reported for non-transplant TIPS recipients^[4,11]. We could not find a relation between final pressure gradients and the development of encephalopathy. Mild pre-existing hepatic encephalopathy was found in two patients, and in both, a deterioration of encephalopathy was noticed after TIPS creation. Therefore, we conclude that the main reasons for the high occurrence of hepatic encephalopathy in this cohort might be the advanced stage of disease in these patients, as well as the potentially neurotoxic effects of immunosuppressive drugs, in particular the calcineurin inhibitors.

In summary, our study showed that the TIPS procedure in LT recipients was feasible without technical difficulties. Indications for TIPS seemed not to differ from those of the non-transplanted TIPS group. The TIPS procedure was efficacious in the management of therapy-refractory ascites and severe variceal bleeding unresponsive to endoscopy, in the majority of patients. TIPS did not appear to be useful in patients with hepatic hydrothorax. However, the outcome was very disappointing. The low survival rate shows that, in LT patients with an already advanced stage of graft dysfunction, TIPS does not improve prognosis. Similar to the recent work of Kim and co-workers^[15], we conclude that TIPS may not be useful in most transplant patients with an advanced graft disease. It may have its place in treating some vascular problems after LT. Otherwise retransplantation remains the only possibility to improve the survival of patients with portal hypertensive complications after LT.

ACKNOWLEDGMENTS

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COMMENTS

Background

Transjugular intrahepatic portosystemic shunt (TIPS) is a well-established therapeutic procedure in patients suffering from complications of portal hypertension, such as ascites or variceal bleeding. In rare cases, portal hypertension develops in liver transplant (LT) recipients. The use of TIPS in this special patient cohort has been discussed in the literature, but only few cases have been reported so far.

Research frontiers

In this study, the authors report a series of TIPS placements in 10 LT recipients

because of ascites, hydrothorax and variceal bleeding. In the majority of patients, at least a partial response could be achieved, however, the outcome of these patients was poor with a median survival of 3.3 mo.

Innovations and breakthroughs

Portal hypertension is a rare but severe complication after LT and leads to graft loss in the majority of patients. The pathophysiology is not well understood and the best therapeutic modality for these patients remains to be defined. TIPS placement has been discussed. This paper shows that TIPS placement is not efficacious in this cohort.

Applications

Results from this study will help to refine the post-transplant care of patients and should encourage physicians to consider retransplantation as the only effective treatment in LT patients with portal hypertensive complications.

Terminology

TIPS is an interventional technique for the creation of an intrahepatic decompressive shunt between a branch of the portal vein and the main hepatic vein, using expandable metallic stents. This leads to a decrease in portosystemic pressure gradient and has become an established therapy for patients with therapy-refractory ascites and unresponsive variceal bleeding.

Peer review

This is a well written report on a single institution's experience on the use of TIPS after LT for the treatment of portal hypertension recurrence related complications. Although the series is small, the paper gives a clear message to the readers about a selected topic in liver transplantation.

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Efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy: A prospective, randomized study

Thawatchai Akaraviputh, Charay Leelouhapong, Varut Lohsiriwat, Somkiat Aroonpruksakul

Thawatchai Akaraviputh, Charay Leelouhapong, Varut Lohsiriwat, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Somkiat Aroonpruksakul, Department of Anesthesiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Author contributions: Akaraviputh T and Leelouhapong C performed most of the study; Aroonpruksakul S and Lohsiriwat V provided analytical tools and were also involved in editing the manuscript; Aroonpruksakul S and Lohsiriwat V co-ordinated and provided the collection of all the material in addition to providing academic support for this work; Akaraviputh T and Leelouhapong C designed the study and wrote the manuscript. Supported by Faculty of Medicine Siriraj Hospital Research Project Grant

Correspondence to: Dr. Thawatchai Akaraviputh, MD, med. (Hamburg), Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Prannok Road, Bangkok 10700, Thailand. sitak@mahidol.ac.th

Telephone: +66-2-4198005 Fax: +66-2-4121370

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Abstract

AIM: To determine the efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy.

METHODS: A prospective, double-blind, randomized, placebo-controlled study was conducted on 70 patients who underwent elective laparoscopic cholecystectomy under general anesthesia at Siriraj Hospital, Bangkok, from January 2006 to December 2007. Patients were randomized to receive either 20 mg parecoxib infusion 30 min before induction of anesthesia and at 12 h after the first dose (treatment group), or normal saline infusion, in the same schedule, as a placebo (control group). The degree of the postoperative pain was assessed every 3 h in the first 24 h after surgery, and then every 12 h the following day, using a visual analog scale. The consumption of analgesics was also recorded.

RESULTS: There were 40 patients in the treatment group, and 30 patients in the control group. The pain scores at each time point, and analgesic consumption did not differ between the two groups. However,

there were fewer patients in the treatment group than placebo group who required opioid infusion within the first 24 h (60% vs 37%, $P = 0.053$).

CONCLUSION: Perioperative administration of parecoxib provided no significant effect on postoperative pain relief after laparoscopic cholecystectomy. However, preoperative infusion 20 mg parecoxib could significantly reduce the postoperative opioid consumption.

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Key words: Laparoscopic cholecystectomy; Parecoxib; Postoperative pain

Peer reviewer: Keiji Hirata, MD, Surgery 1, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Akaraviputh T, Leelouhapong C, Lohsiriwat V, Aroonpruksakul S. Efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy: A prospective, randomized study. *World J Gastroenterol* 2009; 15(16): 2005-2008 Available from: URL: <http://www.wjgnet.com/1007-9327/15/2005.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.2005>

INTRODUCTION

Preemptive analgesia has become a popular adjunct to conventional postoperative pain control. The concept of preemptive analgesia is based on the hypothesis that the most effective way to eliminate or reduce postoperative pain is to prevent nociceptive input from afferent stimuli to the central nervous system so that central nervous system hyperexcitability does not occur^[1]. Clinically, this strategy predicts not only less pain during the initial postoperative period but also a reduced intensity of pain during the days after the procedure^[2]. A variety of preoperative or preemptive analgesic regimens have been used such as intravenous administration of opioids or non-steroidal anti-inflammatory drugs (NSAIDs), local anesthetic infiltration, nerve block, and epidural block^[3].

Recent research indicates that cyclooxygenase-2 (COX-2) inhibitors, a selective class of NSAIDs, could

play an important role in perioperative pain management by reducing the inflammatory response in the periphery, modulating nociceptors, and attenuating central sensitization^[4]. The COX-2 inhibitors provide effective pain control, in addition to a lesser degree of platelet dysfunction and gastrointestinal toxicity compared to nonselective NSAIDs. Reuben *et al*^[5] reported that patients receiving perioperative oral COX-2 inhibitors experienced less postoperative pain, required fewer analgesic drugs, and had a greater range of motion after total knee arthroplasty than those receiving placebo.

The injectable COX-2 inhibitor, parecoxib, could play an important role in pain control in the perioperative and immediate postoperative period, especially in those who cannot take an oral analgesic such as a patient about to undergo intraabdominal surgery under general anesthesia. Laparoscopic cholecystectomy is the most common laparoscopic procedure performed in a general surgical unit worldwide, and accounts for about two-thirds of total laparoscopic operations in the authors' unit. However, the efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy remains controversial^[6-11].

The aim of this study was to determine the efficacy of perioperative parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy in a university hospital.

MATERIALS AND METHODS

After obtaining approval from our Institutional Ethics Committee, a prospective double-blind, randomized, controlled study was conducted at the Department of Surgery, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand, involving 70 consecutive ASA class I-III patients who underwent elective laparoscopic cholecystectomy from January 2006 to December 2007. All patients gave written informed consent.

Patients were excluded from the study for one of the following reasons: age under 18, hypersensitivity to NSAIDs, or conversion to open cholecystectomy. Patients were randomized into one of two groups by opening a sealed envelope in the operating theater: the treatment group received 20 mg parecoxib infusion 30 min before induction of anesthesia and at 12 h after the first dose; the control group received normal saline infusion as a placebo in a similar time schedule. All patients underwent laparoscopic cholecystectomy under a balanced general anesthetic technique, using fentanyl for premedication and during the intraoperative period. The procedures were performed by an experienced laparoscopist.

The degree of the postoperative pain was assessed every 3 h in the first 24 h after surgery and then every 12 h the following day, using a visual analog scale (0 = no pain, 10 = worst possible pain), by nursing staff who were unaware of the perioperative intervention. A standard postoperative analgesic regimen was administered to all patients. This consisted of intravenous pethidine 1 mg/kg prn for patients with a pain score = 5, every 3 h during the first 24 h after the operation, or until oral analgesics could be taken. The consumption of analgesics was also recorded.

Table 1 Comparison of patients' characteristics, indication for surgery and operative details between treatment group and control group (mean \pm SD) *n* (%)

	Treatment group (<i>n</i> = 40)	Control group (<i>n</i> = 30)	<i>P</i> -value
Age (yr)	57.6 \pm 12.7	56.2 \pm 15.1	0.67
Female	25 (63)	16 (53)	0.44
Co-morbid diseases	24 (60)	15 (50)	0.41
Indication for surgery			0.26
Symptomatic gallstone	28 (70)	23 (77)	
Common bile duct stone	6 (15)	6 (20)	
Gallstone induced pancreatitis	6 (15)	1 (3)	
Operative time (min)	63.7 \pm 36.9	58.1 \pm 31.5	0.51

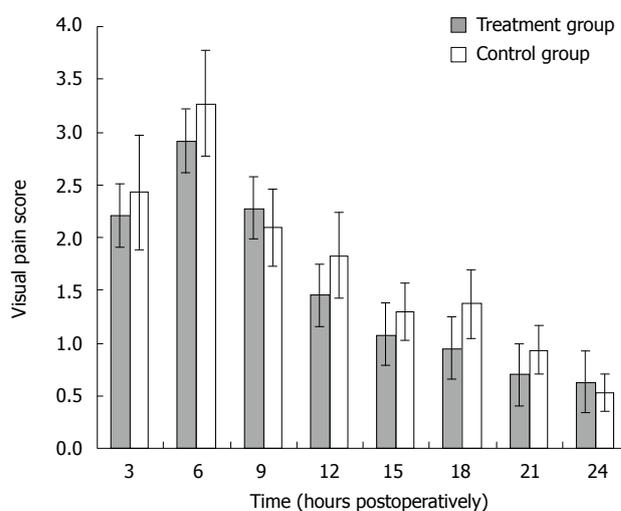


Figure 1 Average visual pain score (0 = no pain, 10 = worst possible pain) at 3, 6, 9, 12, 15, 18, 21 and 24 h after surgery in the treatment group and control group.

All data were prepared and compiled using SPSS software. Mean and SD were assessed. The Kolmogorov-Smirnov test was used to test for the pattern of data distribution. The student's unpaired *t*-test was used to compare data between the two groups when the data were in a normal distribution pattern. The Mann-Whitney *U* test was used to compare data between the two groups when the data were in a non-normal distribution. *P* < 0.05 was considered statistically significant.

RESULTS

There were 40 patients in the treatment group, and 30 patients in the control group. The patient's characteristics, indication for surgery and operative details between the treatment group and control group were well matched (Table 1). The pain scores at each time point did not significantly differ between the two groups (Figure 1). However, there were fewer patients in the treatment group than in the placebo group who required opioid infusion within the first 24 h (60% *vs* 37%, *P* = 0.053).

DISCUSSION

Parecoxib, the first injectable COX-2 inhibitor, was

introduced into clinical practice in 2001. It was found that preoperative administration of parecoxib was more effective than postoperative administration for postoperative pain relief in patients undergoing elective general surgical procedures such as appendectomy, open cholecystectomy and hernioplasty^[12]. Parecoxib can be injected intravenously or intramuscularly with good patient tolerance. The lack of platelet inhibition allows COX-2 inhibitors such as parecoxib to be administered preoperatively. Parecoxib is now increasingly used in ambulatory or day-case surgery because it reduces opioid consumption, improves pain scores, and results in earlier hospital discharge and return to normal function^[13].

In the present study, perioperative administration of parecoxib provided no significant effect on postoperative pain relief after laparoscopic cholecystectomy. One possible explanation for our observation is that administration of a total of 40 mg parecoxib is not an optimal dose for perioperative pain control. Puolakka *et al.*^[8] found that a single dose of 80 mg parecoxib resulted in the least pain intensity after laparoscopic cholecystectomy compared with 40 mg parecoxib or placebo. However, preoperative infusion of 40 mg parecoxib could significantly reduce the postoperative opioid requirement, and the incidence of opioid-related adverse effects if oral COX-2 inhibitors have been taken in the early postoperative period^[9]. There is evidence that the administration of preoperative intravenous parecoxib followed by oral COX-2 inhibitors after laparoscopic cholecystectomy resulted in a shorter length of stay in the postoperative anesthesia care unit, a better quality of postoperative recovery, and a faster return to normal activity, with greater patient satisfaction^[10].

It is also possible that pain after laparoscopic cholecystectomy has several components such as incisional and visceral pain; the latter type of pain seems to be more resistant to the analgesic effect of NSAIDs^[8]. Therefore, systemic administration of COX-2 inhibitors alone is relatively ineffective. Several investigators have suggested that intraperitoneal administration of local anesthetics could improve postoperative pain control by means of attenuation of the visceral pain. Jabbour-Khoury *et al.*^[14] reported that intraperitoneal spray of an aliquot of bupivacaine and NSAIDs, or intraperitoneal spray of local anesthetics following by intravenous NSAIDs resulted in significantly lower abdominal pain scores and incidence of vomiting after laparoscopic cholecystectomy, compared to the non-treatment group. Meanwhile, Elhakim *et al.*^[15] revealed that a combination of intraperitoneal lidocaine and tenoxicam provided better analgesia on movement, and faster return of bowel function compared with intraperitoneal lidocaine and intravenous tenoxicam after laparoscopic cholecystectomy^[15].

In conclusion, perioperative administration of intravenous parecoxib provided no significant effect on postoperative pain relief after laparoscopic cholecystectomy. However, preoperative infusion of 20 mg parecoxib could significantly reduce the postoperative opioid consumption.

COMMENTS

Background

Preemptive analgesia has become a popular adjunct to conventional postoperative pain control. The concept is based on the hypothesis that the most effective way to reduce postoperative pain is to prevent nociceptive input from afferent stimuli to the central nervous system so that central nervous system hyperexcitability does not occur.

Research frontiers

The injectable Cyclooxygenase-2 (COX-2) inhibitor, parecoxib, could play an important role in perioperative pain control and is increasingly used in day-case surgery because it reduces opioid consumption, improves pain scores, and results in earlier discharge and return to normal function.

Innovations and breakthroughs

This study determined the efficacy of perioperative 20 mg parecoxib injection on postoperative pain relief after laparoscopic cholecystectomy.

Applications

Perioperative administration of intravenous 20 mg parecoxib provided no significant effect on postoperative pain relief, but could significantly reduce the postoperative opioid consumption after laparoscopic cholecystectomy.

Terminology

COX-2 inhibitors, a selective class of non-steroidal anti-inflammatory drugs (NSAIDs), could play an important role in perioperative pain management by reducing the inflammatory response in the periphery, modulating nociceptors, and attenuating central sensitization. The COX-2 inhibitors provide effective pain control, in addition to a lesser degree of platelet dysfunction and gastrointestinal toxicity compared to nonselective NSAIDs.

Peer review

This is a well written manuscript with concise data. The concept of perioperative COX-2 infusion can make GI surgeons pay much attention.

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Effect of Lianshu preparation on lipopolysaccharide-induced diarrhea in rats

Jun Liu, Rong Wan, Xuan-Fu Xu, Xing-Peng Wang, Wen-Juan Yang, Yu-Jing Xia, Hua Liu, Qian-Lin Yan, De-Xin Yan, Chuan-Yong Guo

Jun Liu, Rong Wan, Xuan-Fu Xu, Xing-Peng Wang, Wen-Juan Yang, Yu-Jing Xia, Hua Liu, Chuan-Yong Guo, Department of Gastroenterology, Tenth People's Hospital of Tongji University, Shanghai 200072, China

Qian-Lin Yan, Department of Chinese Medicine, Tenth People's Hospital of Tongji University, Shanghai 200072, China

De-Xin Yan, Institute of Chinese Medicine of Tongji University, Shanghai 200092, China

Author contributions: Liu J and Wan R contributed equally to this work; Liu J, Wan R and Xu XF designed the research; Liu J, Xu XF, Yang WJ, Xia YJ, Liu H performed the research; Wang XP, Guo CY contributed to the analytic tools; Yan QL, Yan DX contributed to the foundation; Liu J wrote the paper.

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Correspondence to: Dr. Rong Wan, Department of Gastroenterology, Tenth People's Hospital of Tongji University, Shanghai 200072, China. wanrong1970@163.com

Telephone: +86-21-65975768 Fax: +86-21-65975768

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Abstract

AIM: To investigate the effect of Lianshu preparation on lipopolysaccharide (LPS)-induced diarrhea in rats.

METHODS: A diarrhea model was established in Sprague Dawley rats *via* injection of 1 mL of 30 mg/kg LPS. A total of 40 rats were randomly divided into normal group, LPS group, LPS + Lianshu group, LPS + berberine group ($n = 10$ in each group). Their intestinal mucosal barrier and frequency of diarrhea were observed. Levels of glucose, serum Na^+ , K^+ , Cl^- and hematocrit, plasma nitrogen monoxide (NO), diamine oxidase (DAO), and D (-)-lactate were measured. The number of IgA+ plasma cells in small intestine was detected and SIgA levels in the intestinal fluid were measured. The antipyretic activity of Lianshu preparation in rats was evaluated using Brewer's yeast-induced pyrexia (10 mL/kg of 20% aqueous suspension). Acetaminophen (250 mg/kg, intragastric administration, *bid*) was used as a standard drug for comparison. Temperature was recorded 1 h before and 6 h after Brewer's yeast injection. Finally, small intestinal

transmission in mice treated with Lianshu was detected after intraperitoneal injection of methyl prostigmin (2 mg/kg). Atropine (10 g/kg) was used as a control. The ink content in intestine was determined and the total length of intestine was measured.

RESULTS: The frequency of diarrhea was higher in LPS group than in LPS + Lianshu group and LPS + berberine group (36.70 ± 5.23 vs 28.50 ± 4.06 and 32.70 ± 9.30 respectively, $P < 0.01$), and lower in LPS + Lianshu group than in LPS + berberine group ($P = 0.03$). The levels of Na^+ , glucose, Cl^- , K^+ were significantly lower in LPS + Lianshu group than in LPS + berberine group (140.35 ± 3.19 mmol/L vs 131.99 ± 4.86 mmol/L, 8.49 ± 1.84 mmol/L vs 6.54 ± 2.30 mmol/L, 106.29 ± 4.41 mmol/L vs 102.5 ± 1.39 mmol/L, 5.08 ± 0.66 mmol/L vs 4.32 ± 0.62 mmol/L respectively, $P < 0.05$). The level of hematocrit was lower in LPS + Lianshu group than in LPS + berberine group ($0.50\% \pm 0.07\%$ vs $0.59\% \pm 0.10\%$ respectively, $P < 0.05$). The plasma levels of NO, DAO and D (-)-lactate were higher in LPS group than in normal group (79.74 ± 7.39 $\mu\text{mol/L}$ vs 24.94 ± 3.38 $\mu\text{mol/L}$, 2.48 ± 0.42 $\mu\text{g/mL}$ vs 0.82 ± 0.33 $\mu\text{g/mL}$, 5.63 ± 0.85 $\mu\text{g/mL}$ vs 2.01 ± 0.32 $\mu\text{g/mL}$ respectively, $P < 0.01$), and lower in LPS + Lianshu group than in LPS + berberine group (48.59 ± 4.70 $\mu\text{mol/L}$ vs 51.56 ± 8.38 $\mu\text{mol/L}$, 1.43 ± 0.53 $\mu\text{g/mL}$ vs 1.81 ± 0.42 $\mu\text{g/mL}$, 4.00 ± 0.54 $\mu\text{g/mL}$ vs 4.88 ± 0.77 $\mu\text{g/mL}$ respectively, $P < 0.05$). The morphology of the intestinal mucosa showed destroyed villi in LPS group and atrophied intestinal mucosa in other groups. The pathological intestinal mucosal changes were less in LPS + Lianshu group than in LPS group. The number of IgA+ plasma cells and amount of SIgA were higher in LPS + Lianshu group than in LPS group ($1.16 \pm 0.19/\mu\text{m}^2$ vs $1.09 \pm 0.28/\mu\text{m}^2$, $P = 0.026$; 0.59 ± 0.12 mg/L vs 0.15 ± 0.19 mg/L respectively, $P = 0.000$). Lianshu had counteractive effects on yeast-induced pyrexia and enterokinesia in rats.

CONCLUSION: Lianshu preparation has therapeutic effects on LPS-induced diarrhea and enterokinesia in rats.

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Key words: Lianshu preparation; Lipopolysaccharide; Diarrhea; Nitrogen monoxide; D-lactate

Peer reviewer: Ke-Ji Chen, Professor, Xiyuan Hospital, Chinese Traditional Medicine University, Beijing 100091, China

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INTRODUCTION

Infectious diarrhea is often caused by Gram-negative bacteria such as *Escherichia coli*. These organisms contain lipopolysaccharide (LPS). Antibiotics developed in the 1940s have saved millions of people's life. However, because of the widespread and inappropriate use of antibiotics, some bacterial strains become antibiotic-resistant. Antibiotic-resistant bacteria and side effects of antibiotics severely threaten the general welfare and health of people. Folk herbal drugs have been extensively studied in recent years for the treatment of acute diarrhea. This study was to investigate the effect of Lianshu preparation on LPS-induced diarrhea in rats.

MATERIALS AND METHODS

Animals

Eighty adult female and male Sprague-Dawley rats, weighing 230-280 g (Experimental Animal Breeding Center, Fudan University), and thirty 5-wk-old female and male Kunming mice, weighing 18-20 g, were used in this study following the guidelines of the Animal Care Committee of Institute of Chinese Medicine, Tongji University. The animals were housed at $22 \pm 2^\circ\text{C}$ with a humidity of $55\% \pm 5\%$ in a 12-h light/dark cycle for at least 1 wk prior to use, with free access to laboratory chow and tap water.

Lianshu preparation

Coptis chinensis, *atractylodis*, *pulsatillae* and *portulacae*, provided by Leiyunshang Company, were treated twice at 100°C for 2 h, divided into 1:5:5 proportions, then treated at 100°C for 1 h, filtered and concentrated to a relative density of 1.25 (60°C), and finally, desiccated. The effective constituents of Lianshu preparation were determined by Ley's Company (Shanghai, China). Lipopolysaccharide from *Escherichia coli* serotype 0128: B12 was purchased from Sigma Aldrich (St Louis, MO, USA). Nitrogen monoxide (NO) assay kit was provided by Jiancheng Bionic Research Center (Nanjing, China). Diamine oxidase (DAO) standard preparation, O-dianisidine, horseradish peroxidase, 1,5-pentanediamine-di-hydrochloride, D-lactate standard preparation, NAD- aminoacetic acid, and D-lactic dehydrogenase were purchased from Sigma Aldrich (St Louis, MO, USA). Anti-rat IgA mAb was purchased

from Santa Cruz Company (USA) and SIgA assay kit was purchased from Bethyl Labs Inc. (USA).

Detection of inhibitory effect of Lianshu preparation on LPS-induced diarrhea in rats

Rats were randomly divided into normal group, LPS group, LPS + Lianshu group, LPS + berberine group ($n = 10$ in each group). Before exposure to LPS, rats in normal and LPS groups were treated with distilled water, rats in LPS + Lianshu group were given Lianshu preparation (1.8 g/kg), rats in LPS + berberine group received berberine (0.2 g/kg) twice a day for 3 d. The rats were allowed to have water alone for 12 h. One hour after the last administration of drugs, rats in all groups apart from those in the normal group were treated with LPS (30 mg/kg).

Detection of diarrhea frequency before specimen collection

After treated with LPS, rats in each cage had a filter paper couch. Filter paper was changed once an hour for 4 h. The frequency of diarrhea was determined by counting the number of feces deposits on the filter paper.

Measurement of erythrocyt, blood glucose, and serum Na^+ , K^+ , Cl^- levels

Four hours later, 8 mL of blood was drawn from the abdominal aorta of each rat after anesthetized with 10 mg/kg intraperitoneal pentobarbital. Of the 8 mL blood, 2 mL was treated with heparin for erythrocyt analysis, 2 mL was centrifuged at $3000 \times g$ for 15 min at 4°C . Blood glucose and serum Na^+ , K^+ , Cl^- levels were measured.

Measurement of plasma NO, DAO and D (-)-lactate levels

Four milliliters of blood was centrifuged at $3000 \times g$ for 15 min at 4°C . Plasma was collected to measure NO at 550 nm as previously described^[1]. DAO and D (-)-lactate levels were measured by spectrophotometry at 436 nm^[2] and enzyme-linked ultraviolet spectrophotometry^[3], respectively. The absorbance at 340 nm was recorded.

Observation of morphologic changes in intestinal mucosa

After humane killing, a 5-cm long section of ileum 10 cm below the Treitz ligament was removed, dissected longitudinally, fixed in 100 mL/L formalin, embedded in paraffin, stained with hematoxylin and eosin, and observed under a light microscope.

Detection of IgA+ plasma cells in rat small intestine

ABC immunohistochemistry staining of tissue paraffin sections was performed. The brown stained IgA + plasma cells were detected and analyzed using medical image analysis software (Taimeng Technology Co. Chengdu, China).

Determination of SIgA in small intestine

A small intestine segment about 5 cm long, 15 cm

Table 1 Serum electrolyte levels in different groups (mean \pm SD)

Group	Sodium (mmol/L)	Glucose (mmol/L)	Chlorine (mmol/L)	Kalium (mmol/L)
Normal	143.46 \pm 3.34	8.37 \pm 1.06	102.03 \pm 2.82 ^c	5.37 \pm 0.96
LPS	102.48 \pm 3.67 ^b	4.99 \pm 1.23 ^b	98.40 \pm 1.42 ^a	3.38 \pm 0.29 ^b
LPS + Lianshu	140.35 \pm 3.19 ^d	8.49 \pm 1.84 ^d	106.29 \pm 4.41 ^{b,d}	5.08 \pm 0.66 ^d
LPS + berberine	131.99 \pm 4.86 ^{b,d,f}	6.54 \pm 2.30 ^{a,e}	102.5 \pm 1.39 ^{d,e}	4.32 \pm 0.62 ^{b,d,e}

^a $P < 0.05$, ^b $P < 0.01$ vs normal group; ^c $P < 0.05$, ^d $P < 0.01$ vs LPS group; ^e $P < 0.05$, ^f $P < 0.01$ vs LPS + Lianshu group.

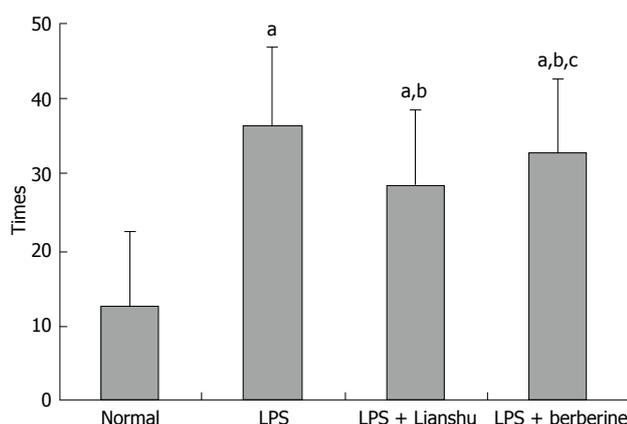


Figure 1 Frequency of diarrhea in different groups. ^a $P < 0.01$ vs normal group; ^b $P < 0.01$ vs LPS group; ^c $P < 0.05$ vs LPS + Lianshu group.

below the Treitz ligament, was removed. Intestinal fluid was collected by passing 5 mL PBS and 0.02% sodium into the small intestine. The first intestinal washing removed 85%-90% of total IgA, and the variability of samples was less than 5%. The washed out material was centrifuged at $3000 \times g$ for 10 min at 4°C. The supernatant was harvested and stored at -70°C. Total IgA was determined by ELISA.

Assay of antipyretic activity of Lianshu preparation

Antipyretic activity of Lianshu preparation in rats was evaluated using Brewer's yeast-induced pyrexia. Forty rats were divided into 4 groups. Rats in control group were given sodium chloride (2 mL) and rats in experimental groups were given Lianshu preparation (1.8 g/kg), twice a day before yeast was injected. Acetaminophen (250 mg/kg, *bid*) was used as a standard drug to compare the antipyretic action of Lianshu preparation. One hour after the last administration of drugs, fever was induced by hypodermic injection of a 20% suspension of 10 mL/kg Brewer's yeast and rectal temperature was recorded 1 h before and 6 h after injection of Brewer's yeast. The antipyretic activity of Lianshu preparation in rats was assayed.

Detection of effect of Lianshu preparation on small intestine transmission in mice

Thirty Kunming mice, after 12 h fasting, were divided into sodium chloride group, Lianshu preparation group and atropine group ($n = 10$). Mice in the sodium chloride group received 0.5 mL sodium chloride by intragastric administration, twice a day for 1 d. Mice in the Lianshu preparation group received 1.8 g/kg Lianshu preparation,

twice a day for 1 d. Mice in the atropine treatment group received 10 g/kg intraperitoneal atropine, twice a day for 1 d. All mice were given 2 mg/kg methyl prostigmin followed by 0.1 mL ink after 10 min, and killed by cervical vertebral dislocation with their belly cut open to collect intestines. The effect of Lianshu preparation on small intestinal transmission in mice was detected.

Statistical analysis

Data were expressed as mean \pm SE and analyzed by analysis of variance and Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

General health of rats

No diarrhea and loose stools occurred in rats of the normal group. The fur of rats was smooth and the rats were active. Rats in the other groups that received LPS drank more water and had loose stools. The fur of rats was fluffy, and the rats were affected by lassitude. The respiration of rats was rapid.

Frequency of diarrhea after injection of LPS

The frequency of diarrhea in LPS group, LPS + Lianshu group and LPS + berberine group was increased compared to normal group (36.70 ± 5.23 , 28.50 ± 4.06 , 32.70 ± 9.30 vs 12.40 ± 3.20 respectively, $P < 0.01$), significantly increased in LPS + Lianshu group and LPS + berberine group compared with normal group ($P < 0.01$), and decreased in LPS + Lianshu group compared with LPS + berberine group ($P = 0.03$) (Figure 1).

Glucose, serum electrolyte and hematocrit levels in rats

The levels of glucose, Na⁺, Cl⁻, and K⁺ were significantly lower in LPS + Lianshu group than in LPS + berberine group (140.35 ± 3.19 mmol/L vs 131.99 ± 4.86 mmol/L, 8.49 ± 1.84 mmol/L vs 6.54 ± 2.30 mmol/L, 106.29 ± 4.41 mmol/L vs 102.5 ± 1.39 mmol/L, 5.08 ± 0.66 mmol/L vs 4.32 ± 0.62 mmol/L respectively, $P < 0.05$), while the hematocrit level was lower in LPS + Lianshu group than in LPS + berberine group ($0.50\% \pm 0.07\%$ vs $0.59\% \pm 0.10\%$, $P < 0.05$, Table 1, Figure 2).

Plasma NO and DAO levels in rats

The plasma NO and DAO levels were higher in LPS group than in normal group ($P < 0.01$) and lower in LPS + Lianshu group than in LPS + berberine group ($P < 0.05$, Table 2).

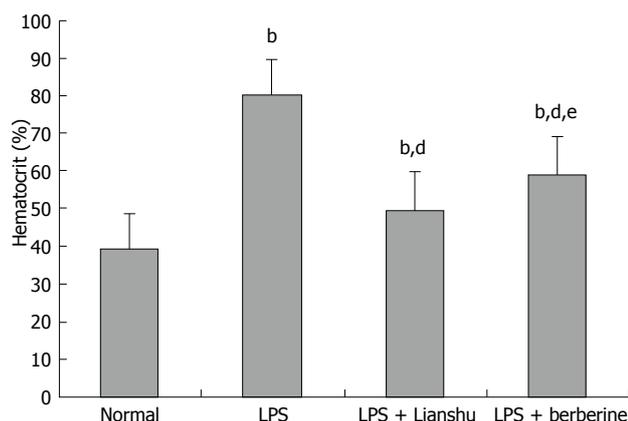


Figure 2 Hematocrit in different groups. ^b $P < 0.01$ vs normal group; ^a $P < 0.01$ vs LPS group; ^c $P < 0.05$ vs LPS + Lianshu group.

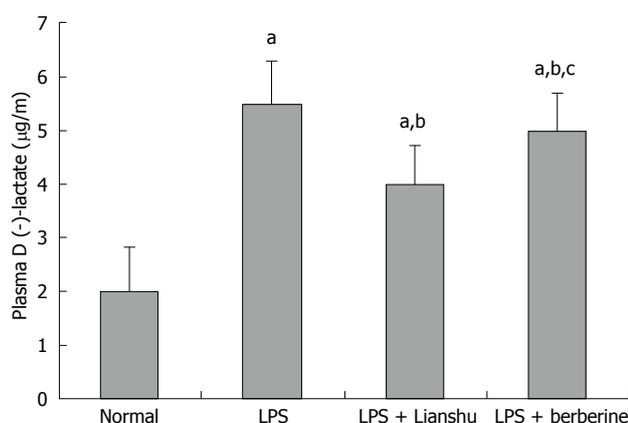


Figure 3 Comparison of plasma D (-)-lactate in different groups. ^a $P < 0.01$ vs normal group; ^b $P < 0.01$ vs LPS group; ^c $P < 0.05$ vs LPS + Lianshu group.

Plasma D (-)-lactate levels in rats

Plasma D (-)-lactate level was higher in LPS group, LPS + Lianshu group and LPS + berberine group than in normal group (5.36 ± 0.85 , 4.00 ± 0.54 , 4.88 ± 0.77 vs 2.01 ± 0.32 respectively, $P < 0.01$), and lower in LPS + Lianshu group, LPS + berberine group than in LPS group and LPS + berberine group ($P < 0.01$, Figure 3).

Morphological changes in intestinal mucosa

The villi of intestinal mucosa maintained their integrity and the shape of epithelial cells remained unchanged in the normal group. The villi were destroyed in the LPS group. Intestinal mucosal atrophy and inflammatory reaction were detectable in the other groups (Figure 4).

Staining and number of IgA+ plasma cells in intestinal mucosa

IgA+ cells, round or elliptical in shape, were distributed in the intestinal villi, lamina propria and intestinal glands. The endochylema was stained brown (Figure 5).

Number of IgA+ cells and SIgA in intestinal fluid

The number of IgA+ cells and amount of SIgA in intestinal fluid were less in LPS group than in normal group ($P < 0.01$) and higher in LPS + Lianshu

Table 2 Plasma NO and DAO levels in different groups (mean \pm SD)

Group	No ($\mu\text{mol/L}$)	DAO ($\mu\text{mol/mL}$)
Normal	24.94 ± 3.38	0.82 ± 0.33
LPS	79.74 ± 7.39^b	2.48 ± 0.42^b
LPS + Lianshu	$48.59 \pm 4.70^{b,d}$	$1.43 \pm 0.53^{b,d}$
LPS + berberine	$51.56 \pm 8.38^{b,d,f}$	$1.81 \pm 0.42^{b,d,e}$

^b $P < 0.01$ vs normal group; ^d $P < 0.01$ vs LPS group; ^e $P < 0.05$, ^f $P < 0.01$ vs LPS + Lianshu group.

Table 3 Number of IgA+ cells and SIgA in intestinal fluid of different groups (mean \pm SD)

Group	IgA+ cells ($/\mu\text{m}^2$)	SIgA (mg/L)
Normal	0.94 ± 0.21	0.50 ± 0.09
LPS	0.73 ± 0.22^a	0.22 ± 0.66^b
LPS + Lianshu	$1.16 \pm 0.19^{b,d}$	0.59 ± 0.12^d
LPS + berberine	$1.09 \pm 0.28^{a,d,e}$	$0.15 \pm 0.19^{b,c,e}$

^a $P < 0.05$, ^b $P < 0.01$ vs normal group; ^c $P < 0.05$, ^d $P < 0.01$ vs LPS group; ^e $P < 0.05$ vs LPS + Lianshu group.

group than in LPS + berberine group ($P = 0.05$, Table 3).

Counteractive effect of Lianshu preparation on yeast-induced pyrexia in rats

The temperature of rats in yeast group and sodium chloride group was similar 1 h after treatment with Lianshu preparation and higher in yeast group than in sodium chloride group 2-5 h after treatment with Lianshu preparation ($P < 0.05$). The temperature was not significantly changed in yeast + Lianshu group compared to yeast group 1 h or 2 h after treatment with Lianshu preparation. The temperature in yeast + Lianshu group decreased 3 h after treatment with Lianshu preparation ($P = 0.025$), and was almost identical to that in the normal group 4 h after treatment with Lianshu preparation. The temperature was lower in yeast + acetaminophen group than in the sodium chloride group 2 h after treatment with Lianshu preparation. Yeast + acetaminophen exhibited their effect 1 h earlier than yeast + Lianshu preparation (Table 4).

Counteractive effect of neostigmine on enterokinesia in mice

The rate of small intestinal peristalsis for mice after treatment with Lianshu preparation and atropine was different from that in mice after treatment with sodium chloride ($55.20\% \pm 10.16\%$ and $39.96\% \pm 8.57\%$ vs $65.10\% \pm 10.93\%$ respectively, $P < 0.05$, Figure 6).

DISCUSSION

A group of Japanese workers reported that parenteral LPS can lead to proliferation of gastrointestinal luminal bacteria in mice^[4]. Subsequent work established that this enhancement of bacterial growth is secondary to

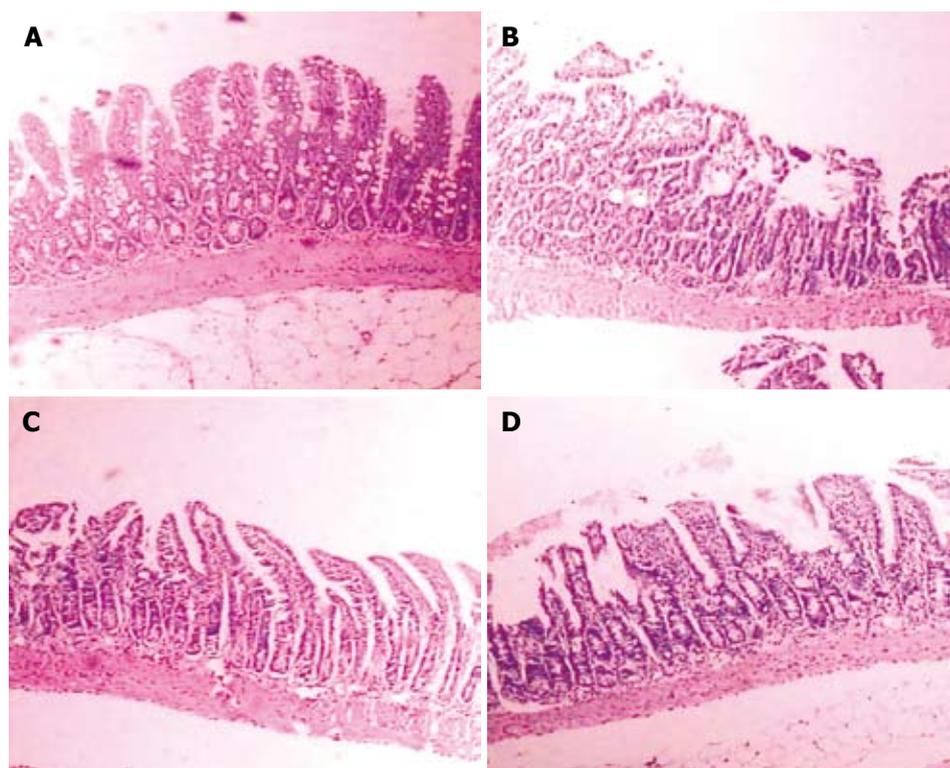


Figure 4 Optical microscopic observation of ileum mucosa of rats (HE, $\times 100$). A: Normal group; B: LPS group; C: LPS + Lianshu group; D: LPS + berberine group.

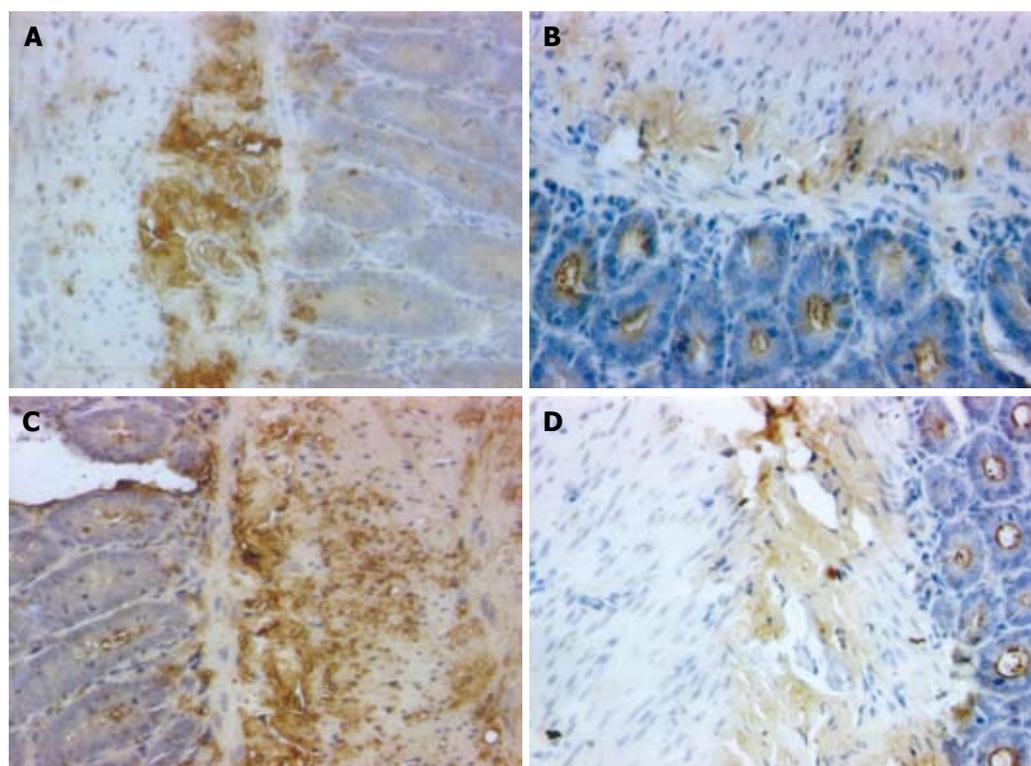


Figure 5 Staining and number of IgA+ plasma cells in intestinal mucosa ($\times 200$). A: Normal group; B: LPS group; C: LPS + LianShu group; D: LPS + berberine group.

fluid exudation, since the growth of bacteria could be suppressed by enteral unabsorbable antibiotics like neomycin^[5]. Subcutaneous LPS from Gram-negative bacteria induces intestinal diarrhea characterized by destruction of the intestinal mucosa barricade^[6-9].

In the present study, the frequency of diarrhea and gastrointestinal transit (GIT) were detected, and the levels of NO, DAO and D (-)-lactate were measured in mice after treatment with intraperitoneal LPS. Lianshu

preparation could effectively prevent diarrhea by inhibiting enterokinesia. Generally, NO can protect and repair gastrointestinal mucosa. Endogenous NO has therapeutic effects on hypoxia, inflammation and damage. However, excess NO is probably one of the most important mediators that induce blood poisoning, septic shock and multi-organ dysfunction^[10]. When stimulated by LPS, NO is excessively expressed and induces multi-organ functional lesions in stomach and intestine^[11-13].

Table 4 Counteractive effects of yeast on pyrexia in different groups ($\Delta^{\circ}\text{C}$) (mean \pm SD)

Groups	1 h	2 h	3 h	4 h	5 h	6 h
Normal	0.58 \pm 0.28	0.24 \pm 0.71	0.20 \pm 0.83	0.47 \pm 0.30	0.17 \pm 0.51	0.27 \pm 0.65
Yeast	0.14 \pm 0.94	0.75 \pm 0.28 ^b	1.06 \pm 0.28 ^b	1.16 \pm 0.39 ^b	1.69 \pm 0.43 ^b	1.46 \pm 0.59 ^b
Yeast + Lianshu	0.30 \pm 0.38	0.58 \pm 0.38	0.44 \pm 0.56 ^c	0.42 \pm 0.29 ^d	1.01 \pm 0.43 ^c	0.50 \pm 0.44 ^d
Yeast + acetaminophen	0.11 \pm 0.73	0.21 \pm 0.39 ^c	0.37 \pm 0.53 ^c	0.66 \pm 0.50 ^d	0.74 \pm 0.97 ^{a,d}	0.53 \pm 0.98 ^d

^a $P < 0.05$, ^b $P < 0.01$ vs normal group; ^c $P < 0.05$, ^d $P < 0.01$ vs yeast group.

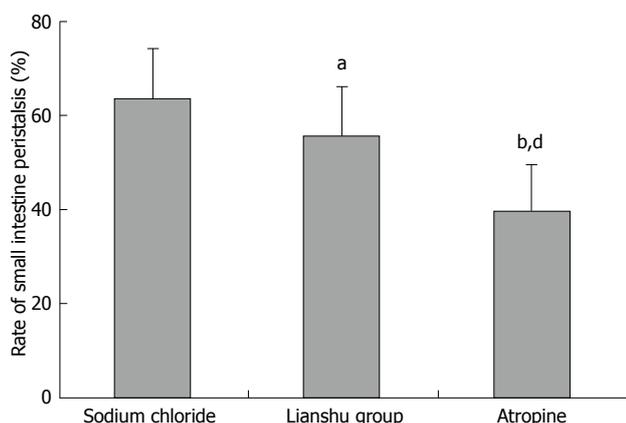


Figure 6 Counteractive effects of neostigmine on enterokinesia of mice. ^a $P < 0.05$, ^b $P < 0.01$ vs sodium chloride group; ^d $P < 0.01$ vs Lianshu group.

In this study, Lianshu preparation prevented enteritis necroticans by inhibiting the production of excess NO. DAO is an ideal index of intestinal mucosal structure and function. The activity of DAO is closely related with villi, nucleic acid and protein synthesis in mucosal cells. When intestinal mucosal epidermis is damaged, DAO is released into blood, indicating that DAO in blood reflects the destruction of the intestinal mucosal epithelial cell layer and intestinal mucosa barricade^[14,15]. In our study, DAO was localized mostly in the epithelial cell layer of mucous membrane as previously described^[16], and Lianshu preparation could effectively decrease DAO induced by LPS. However, its mechanism of action remains unclear. In addition, the function of the intestinal mucosa barricade could also be evaluated by measuring D (-)-lactate in plasma^[17-19]. D (-)-lactate is a product of bacterial metabolism and schizolysis. However, mammals cannot produce D (-)-lactate^[20]. D (-)-lactate cumulation in plasma reflects membrane permeability and barrier function of the intestinal mucosa^[21,22]. In our study, Lianshu preparation could reduce D (-)-lactate in plasma ($P < 0.01$) by protecting the barrier function of the intestinal mucosal and suppressing the overgrowth of harmful bacteria in the intestinal tract.

IgA is the main immunoglobulin in the mucosal immune system^[23]. Polymeric IgA antibodies produced by plasma cells in the lamina propria of the intestinal tract bind to polymeric immunoglobulin receptors at the base of the epithelium. The complex of IgA and polymeric immunoglobulin receptors undergoes endocytosis and vesicular transport to the apical surface of enterocytes, and is secreted into the lumen. Generally,

plasma cells are derived from B cells. It was reported that B cells are able to switch from IgM expression to IgA expression by Th2 cytokine activity in Peyer's patches of the lamina propria and mesenteric lymph nodes, and then return to lamina propria of the intestinal tract *via* systemic circulation^[24-26]. LPS, a mouse B-cell mitogen, induces B cell multiplication^[26] and differentiation into plasma cells. LPS also activates macrophages to promote the production of cytokines such as interleukin (IL)-1, IL-6 and nitric oxide, which might regulate immunoglobulin production. Therefore IgA inhibits diarrhea caused by viruses^[27-29]. Moreover, LPS might activate IgA production in the intestine^[30]. However, in our study, the number of IgA+ cells and SIgA in the intestinal fluid was less in the LPS group than in normal group, and greater in the LPS + Lianshu group than in the LPS group, suggesting that high dose LPS leads to severe destruction of intestinal mucosa.

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COMMENTS

Background

Infectious diarrhea is often caused by Gram-negative bacteria such as *Escherichia coli*. These organisms contain lipopolysaccharide (LPS). It is the important task of modern medicines to control infectious diarrhea. Antibiotics developed in the 1940s have saved millions of people's lives. However, because of the widespread and inappropriate use of antibiotics, some bacterial strains become antibiotic-resistant. Folk herbal drugs have been studied for the treatment of acute diarrhea.

Research frontiers

The method to treat diarrhea is mainly to kill bacteria or viruses. Lianshu preparation could destroy bacteria, prevent diarrhea, and protect the intestinal mucosa.

Innovations and breakthroughs

Lianshu preparation, comprised of non-antibiotic botanic components, has been used in the treatment of infectious diarrhea with a satisfactory effect. Lianshu preparation could effectively prevent diarrhea, relieve electrolyte disturbances and protect immune barriers. Its effect is better than berberine. In addition, Lianshu preparation has counteractive effects on pyrexia.

Applications

Lianshu preparation can be used as a natural medicine in treatment of infectious diarrhea with pyrexia. Chinese herbal drugs are effective against both chronic and acute diseases.

Peer review

The authors confirmed, in their study, that Lianshu preparation was effective against LPS-induced diarrhea, its effect was better than berberine, suggesting that it can be used as a natural medicine in treatment of infectious diarrhea.

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BRIEF ARTICLES

High level of ezrin expression in colorectal cancer tissues is closely related to tumor malignancy

Hong-Jian Wang, Jin-Shui Zhu, Qiang Zhang, Qun Sun, Hua Guo

Hong-Jian Wang, Jin-Shui Zhu, Qiang Zhang, Qun Sun, Hua Guo, Department of Gastroenterology, The Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200233, China

Author contributions: Wang HJ wrote the paper; Zhu JS designed the research; Wang HJ and Guo H performed the research; Zhang Q and Sun Q provided new reagent and analytic tools and analyzed data.

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Correspondence to: Jin-Shui Zhu, Professor, Department of Gastroenterology, The Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200233, China. zhujs1803@hotmail.com

Telephone: +86-21-64369181 Fax: +86-21-64837019

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Dukes stage (88.46% vs 50.00%, $P < 0.01$; 94.28% vs 51.11%, $P < 0.01$; 94.28% vs 51.11%, $P < 0.01$).

CONCLUSION: Ezrin expression is obviously higher in colorectal cancer tissues than in normal colorectal mucosa tissues, and the high level of ezrin expression is closely related to the colorectal cancer invasion and metastasis process.

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Key words: Colorectal cancer; Ezrin; Malignant tumor; Invasion; Metastasis; Immunohistochemistry

Peer reviewers: Alain L Servin, PhD, Faculty of Pharmacy, French National Institute of Health and Medical Research, Unit 756, Rue J.-B. Clément, F-92229 Châtenay-Malabry, France; Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

Abstract

AIM: To investigate the ezrin expression in normal colorectal mucosa and colorectal cancer tissues, and study the correlation between ezrin expression in colorectal cancer tissues and tumor invasion and metastasis.

METHODS: Eighty paraffin-embedded cancer tissue samples were selected from primary colorectal adenocarcinoma. Twenty-eight patients had well-differentiated, 22 had moderately differentiated and 30 had poorly differentiated adenocarcinoma. Forty-five patients and 35 patients had lymph node metastasis. Forty-five patients were of Dukes A to B stage, and 35 were of C to D stage. Another 22 paraffin-embedded tissue blocks of normal colorectal epithelium (> 5 cm away from the edge of the tumor) were selected as the control group. All patients with colorectal cancer were treated surgically and diagnosed histologically, without preoperative chemotherapy or radiotherapy. The immunohistochemistry was used to detect the ezrin expression in paraffin-embedded normal colorectal mucosa tissues and colorectal cancer tissue samples.

RESULTS: Ezrin expression in colorectal cancer was significantly higher than in normal colorectal mucosa (75.00% vs 9.09%, $P < 0.01$), and there was a close relationship between ezrin expression and the degree of tumor differentiation, lymph node metastasis and

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INTRODUCTION

Ezrin belongs to the ezrin/radixin/moesin (ERM) protein family, which act as membrane organizers and linkers between the plasma membrane and cytoskeleton^[1,2]. Ezrin is mainly expressed on the cell surface to maintain the polarity of endothelial cells^[3]. Recent studies have found that, through regulating adhesion molecules and signal transduction pathways, ezrin is involved in cell-cell and cell-matrix interactions, and might play an important role in the process of tumor cell invasion and metastasis^[4]. Overexpression of ezrin protein is correlated with the metastatic potential of several cancers^[5-8], and a high level of ezrin protein expression can induce conversion of a variety of cell lines, as well as abnormal hyperplasia^[9]. Tumor cell lines with stronger metastatic abilities are usually accompanied by overexpression of ezrin^[10]. Through testing the expression of ezrin protein in normal colonic mucosa

and colorectal cancer tissues, we aimed to establish the relationship between ezrin expression and clinical parameters, evaluate its molecular action mechanisms in the process of colorectal cancer carcinogenesis, invasion and metastasis, and provide the evidence for clinical prognosis and suitable adjuvant therapy.

MATERIALS AND METHODS

Patients and their pathological samples

The immunohistochemistry was performed in paraffin-embedded tissue samples. Eighty colorectal adenocarcinoma patients diagnosed by postoperative pathology were investigated. There were 44 male and 36 female patients, whose ages ranged from 31 to 80 years, with an average age of 55.5 years. Histologically, 28 patients had well-differentiated, 22 had moderately differentiated, and 30 had poorly differentiated adenocarcinoma. Forty-five patients were without and 35 patients had lymph node metastasis. Forty-five patients were of Dukes A to B stage, and 35 were of C to D stage. Another 22 paraffin-embedded tissue blocks of normal colorectal epithelium (> 5 cm away from the edge of the tumor) was the control group.

Drugs and reagents

Mouse anti-human ezrin mAb was purchased from Fujian Maixin Biotechnology Development Co. Ltd, and SP kit DAB from Beijing Zhong Shan Jinqiao Biotechnology Development Co. Ltd. Experiments were performed following the instructions of the manufacturers. PBS (0.01 mmol/L) was used to replace the first antibody as a negative control, while the normal colorectal mucosa was a positive control.

Result judgment

Each stained slide was assessed and given a score according to the classification standard of Mathew *et al*^[11]: score 0, no expression; score 1, < 50% of cells staining positive expression or less; score 2, \geq 50% of cells staining positive expression. Score 0-1 was recorded as negative, and score 2 recorded as positive.

Statistical analyses

SPSS for Windows version 11.0 was used for statistical analyses. The χ^2 test was used in the analysis of the relationship between ezrin and colorectal cancer clinicopathological parameters. $P \leq 0.05$ was considered as a significant difference.

RESULTS

The positive expression of ezrin in colorectal cancer was significantly higher than that in normal colorectal mucosa (Figure 1A-E). The positive rate of ezrin protein in normal colorectal mucosa was 9.09% (2/22) and 75.00% (60/80) in colorectal cancer tissues. There were significant differences between the two groups (75.00% *vs* 9.09%, $P < 0.01$), as shown in Table 1.

Table 1 Ezrin expression in different colorectal tissues *n* (%)

Group	<i>n</i>	Positive expression (%)
Normal colorectal mucosa	22	2 (9.09) ^b
Colorectal cancer tissues	80	60 (75.00)

^b $P < 0.01$ *vs* colorectal cancer tissues.

Table 2 Relationship between ezrin expression in colorectal cancer tissues and clinicopathological parameters *n* (%)

Clinicopathological parameters	<i>n</i>	Ezrin positive expression (%)
Well-differentiated	28	14 (50.00) ^b
Moderately and poorly differentiated	52	46 (88.46)
Lymph node metastasis	35	33 (94.28) ^d
Without lymph node metastasis	45	27 (51.11)
Dukes A to B stage	45	27 (51.11) ^e
Dukes C to D stage	35	33 (94.28)

There was a close relationship between ezrin expression and the degree of tumor differentiation, lymph node metastasis and Dukes stage. There were significant differences between the well-differentiated and the moderately and poorly differentiated groups (^b $P < 0.01$); lymph node metastasis group *vs* group without lymph node metastasis (^d $P < 0.01$); Dukes A to B stage *vs* Dukes C to D stage (^e $P < 0.01$).

Relationship between ezrin expression in colorectal cancer tissues and clinicopathological parameters

There was a close relationship between ezrin expression and the degree of tumor differentiation, lymph node metastasis and Dukes stage (88.46% *vs* 50.00%, $P < 0.01$; 94.28% *vs* 51.11%, $P < 0.01$; 94.28% *vs* 51.11%, $P < 0.01$), as shown in Table 2.

DISCUSSION

Ezrin protein expression in specific cell membrane regions is mainly involved in the connection between the epithelial cell cytoskeleton and the cell membrane, through membrane surface signaling molecules and some transmembrane signal transduction pathway. It participates in the regulation of cell survival, adhesion, proliferation and migration processes. Recent studies have found that ezrin protein may play an important role in the tumorigenesis, development, invasion and metastasis process, probably through regulating adhesion molecules and participating in cell signal transduction, and other channels in the tumor^[12-17]. Ezrin protein is an indispensable factor for tumor cell metastasis of osteosarcoma^[18], breast cancer^[19], nasopharyngeal carcinoma^[20], and prostate cancer^[21]. In addition, in malignant tumor tissues, there are also changes in subcellular localization of ezrin expression. Moilanen *et al*^[22] found that ezrin expression in normal ovarian epithelial cells is a kind of cell polarity expression, and that ezrin expression in malignant ovarian tumor cells is more diffusive, with a different degree of tumor cell differentiation, and the location and intensity of ezrin expression in cells is quite different. Therefore, we speculate that ezrin subcellular localization in normal

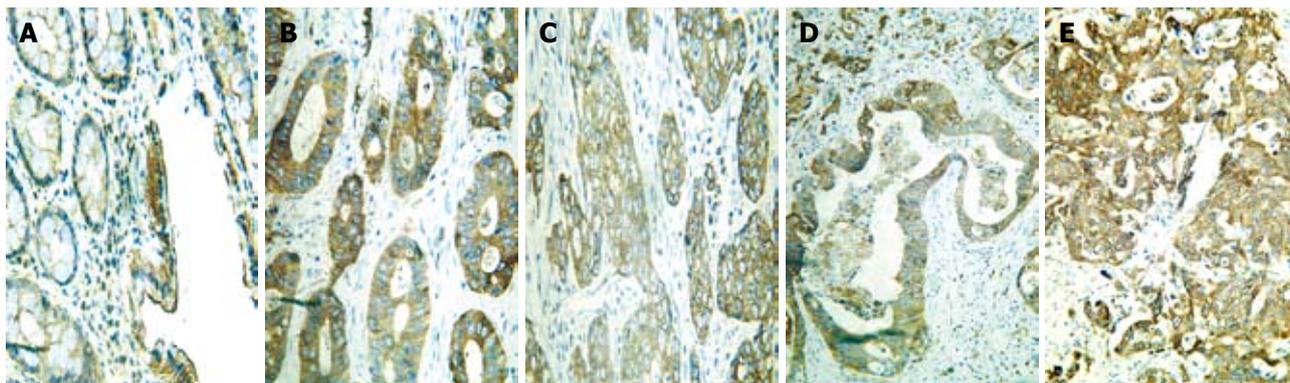


Figure 1 Ezrin expression (HE, × 400). A: Normal colorectal mucosa; B: Well-differentiated adenocarcinoma; C: Poorly differentiated adenocarcinoma; D: Adenocarcinoma without lymph node metastasis; E: Adenocarcinoma with lymph node metastasis.

cells forms the foundation of various physiological functions and cell structure. Abnormal ezrin expression or distribution will also lead to abnormal cell structure and physiological function, and accordingly, these abnormal changes participate in the occurrence, development, invasion and metastasis of malignant tumors.

The role of ezrin in tumor progression is very important and deserves much attention. Recent studies have found that ezrin is a key factor in Fas-mediated apoptosis^[23], in the P-gp1-mediated multidrug resistance of cancers, and in cannibalism of metastatic tumors^[24]. The active ezrin C-terminal is connected with the actin cytoskeleton, and the N-terminal is connected with cell adhesion molecules such as E-cadherin, and CD44^[25,26], etc. Ezrin participates in regulating cell-cell and cell-extracellular matrix adhesion, thus influencing tumor cell invasion and other biological behavior^[27-30]. CD44 is a cellular membrane receptor which can specially recognize hyaluronic acid and collagen, and regulate cell-cell and cell-extracellular matrix adhesion. Some studies have found that ezrin, CD44 and CD44 variants could make up a compound that is co-expressed in the tumor cells^[1]. Pujuguet *et al*^[31] have found that ezrin can regulate E-cadherin expression in the cell membrane through Rho protein, thereby regulating cell adhesion. At the same time, ezrin also has regulating function in the E-cadherin membrane localization, and activated ezrin can make the E-cadherin protein aggregate in the cell, thereby undermining the cell-to-cell contact and intercellular adhesive ability, and the overexpression of ezrin in the tissues also has the same function of weakening the intercellular adhesion^[32]. Through activation of RhoA and the MAPK pathway, ezrin can promote the cell adhesion plaque formation, thereby promoting the adhesive function between the tumor cells and other cells, as well as stoma cells^[33]. Therefore, we believe, through participation in the formation of the cell adhesion plaque, cytoskeletal connections and cell surface compounds assembly, and other biological functions, ezrin protein mediates and regulates cell-cell and cell-extracellular matrix adhesion, and is also involved in the malignant tumor invasion and metastasis process. This study showed that, the overexpression of ezrin in colorectal cancer tissues may

be involved in cancer invasion and metastasis. The studies on the correlation between ezrin protein and cancer might help us further reveal the tumor invasion and metastasis mechanism, and find the targets for inhibiting tumor metastasis, or indicators that forecasts the prognosis of patients with tumors.

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COMMENTS

Background

Ezrin belongs to the ezrin/radixin/moesin (ERM) protein family, which act as membrane organizers and linkers between the plasma membrane and cytoskeleton. Ample evidence has indicated that ezrin is regarded as a metastatic determinant and a key component in tumor progression and metastasis; however, its role in the process of colorectal cancer growth and metastasis is not clearly understood.

Research frontiers

Recent studies have found that a high level of ezrin protein expression can induce a variety of cell line conversions, as well as abnormal hyperplasia. Ezrin is a key factor in Fas-mediated apoptosis, the P-gp1-mediated multidrug resistance of cancers, and cannibalism of metastatic tumors.

Applications

This preliminary study about ezrin in colorectal cancer growth and metastasis may pave the way for further clinical studies on colorectal cancer dissemination and metastasis.

Terminology

Ezrin belongs to the ERM protein family, which are expressed in specific cell membrane regions, and acts as membrane organizers and linkers between the plasma membrane and epithelial cell cytoskeleton.

Peer review

This study is interesting, and discusses the ezrin expression in normal colorectal mucosa and colorectal cancer tissues, and the clinical relevance of ezrin expression to tumor invasion and metastasis. The study was carefully performed and the data and conclusions drawn are sound.

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BRIEF ARTICLES

Overexpression of DNA methyltransferase 1 and its biological significance in primary hepatocellular carcinoma

Hong Fan, Zhu-Jiang Zhao, Jian Cheng, Xian-Wei Su, Qing-Xiang Wu, Yun-Feng Shan

Hong Fan, Zhu-Jiang Zhao, Xian-Wei Su, Qing-Xiang Wu, Key Laboratory of Developmental Genes and Human Diseases, Ministry of Education, Southeast University, Nanjing 210009, Jiangsu Province, China; Department of Genetics and Developmental Biology, the School of Basic Medical Sciences, Southeast University, Nanjing 210009, Jiangsu Province, China
Jian Cheng, Prenatal Diagnosis Center, Nanjing maternity and Child Health Care Hospital, Nanjing 210004, Jiangsu Province, China

Yun-Feng Shan, Key Lab of Enteric Pathogenic Microbiology Ministry of Health, Jiangsu Centers for Diseases Prevention and Control, Nanjing 210009, Jiangsu Province, China

Author contributions: Fan H, Zhao ZJ performed the majority of experiments; Cheng J and Wu QX helped to perform the IHC; Su XW performed the statistical analysis of case information; Shan YF did experiments on cultured cell lines; Fan H designed the study and wrote the manuscript.

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Correspondence to: Hong Fan, PhD, Key Laboratory of Developmental Genes and Human Diseases, Ministry of Education, Southeast University, Nanjing 210009, Jiangsu Province, China. fanh@seu.edu.cn

Telephone: +86-25-83272314 Fax: +86-25-84463798

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Abstract

AIM: To explore the relationship between DNA methyltransferase 1 (DNMT1) and hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) and its biological significance in primary HCC.

METHODS: We carried out an immunohistochemical examination of DNMT1 in both HCC and paired non-neoplastic liver tissues from Chinese subjects. DNMT1 mRNA was further examined in HCC cell lines by real-time PCR. We inhibited DNMT1 using siRNA and detected the effect of depletion of DNMT1 on cell proliferation ability and cell apoptosis in the HCC cell line SMMC-7721.

RESULTS: DNMT1 protein expression was increased in HCCs compared to histologically normal non-neoplastic liver tissues and the incidence of DNMT1 immunoreactivity in HCCs correlated significantly with poor tumor differentiation ($P = 0.014$). There were

more cases with DNMT1 overexpression in HCC with HBV (42.85%) than in HCC without HBV (28.57%). However, no significant difference in DNMT1 expression was found in HBV-positive and HBV-negative cases in the Chinese HCC group. There was a trend that DNMT1 RNA expression increased more in HCC cell lines than in pericarcinoma cell lines and normal liver cell lines. In addition, we inhibited DNMT1 using siRNA in the SMMC-7721 HCC cell line and found depletion of DNMT1 suppressed cells growth independent of expression of proliferating cell nuclear antigen (PCNA), even in HCC cell lines where DNMT1 was stably decreased.

CONCLUSION: The findings implied that DNMT1 plays a key role in HBV-related hepatocellular tumorigenesis. Depletion of DNMT1 mediates growth suppression in SMMC-7721 cells.

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Key words: DNA methyltransferase 1; Hepatitis B virus-related hepatocellular carcinoma; RNAi; Cell proliferation; Apoptosis

Peer reviewer: Sharon DeMorrow, Assistant Professor, Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

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INTRODUCTION

DNA methylation plays an important role in transcriptional regulation, chromatin remodeling and genomic stability^[1-3]. It is catalyzed in mammalian cells by a family of highly related DNA methyltransferases (DNMTs) that use S-adenosylmethionine as the methyl donor^[4]. Alteration of DNA methylation is one of the most consistent epigenetic changes in human cancers and is involved even in the early and precancerous

stages of human carcinogenesis^[5,6]. Overexpression of DNMT1 has been detected in several human cancers^[7-11] and showed a relatively significant correlation with tumorigenesis, but not in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). HCC is a devastating disease with a very poor prognosis, and is the fourth or fifth largest cause of cancer-related death worldwide^[12,13]. More than 85% of Chinese HCC patients have HBV infection history. Data on hepatocarcinogenesis in other countries showed that DNMT1 mRNA and protein expression were significantly higher in HCCs^[14,15]. Therefore, DNMT1 may play an important role during hepatocarcinogenesis. However, the relationship between HBV infection and DNMT1 in HCC has still to be elucidated, especially in Chinese subjects. In the present study, we examined the expression of DNMT1 both in HBV-related HCC cases and cell lines from Chinese subjects. DNMT1 expression was significantly increased both in tumor tissues and HCC cell lines compared with corresponding controls. To further investigate and evaluate the possibility of DNMT1 knockdown strategies for cancer therapy, we inhibited DNMT1 using siRNA and examined the cell growth and cell proliferation ability in the HCC cell line SMMC-7721.

MATERIALS AND METHODS

Tissue specimens

We selected 42 cases of surgically resected livers from the surgical pathology files of the Department of Pathology at The Qidong County Hospital, China. The patients' clinicopathologic features are shown in Table 1. All the HCC samples were diagnosed by a single pathologist and appropriate consent was obtained. Paired samples of primary HCC and matched non-cancerous normal tissues were obtained from each patient. The pathological classification of HCC tissues was carried out and the stage of each HCC was determined according to criteria. Histological examination of noncancerous liver tissues from HCC patients revealed no remarkable findings. Thirty-five cases were associated with HBV infection, and 7 cases were negative for HBV.

Cell lines

Nine human cell lines were cultured for the study. Human hepatocellular carcinoma cell lines (BEL-7402, BEL-7404, BEL-7405, QGY-7703, QGY-7701, SMMC-7721), an immortalized human hepatocellular normal cell line (HL-7702) or a pericarcinoma (QSG-7701) cell line were cultured in RPMI-1640 (Life Technologies, Inc., Rockville, MD) containing 10% new born bovine serum in 5% CO₂ incubation at 37°C.

Preparing a vector-based siRNA construct for DNMT1

siRNAs targeting DNMT1 were designed and prepared as described previously^[16]. The siRNA sequences against DNMT1 were designed as sense and antisense oligonucleotides corresponding to nucleotide position 2620-2638 of human DNMT1 (GenBank accession No. NM001379.1). The siRNA sequence and scramble

Table 1 Clinicopathological features of hepatocellular carcinoma patients

Patients and tissue specimens	<i>n</i>
Sex	
Male	36
Female	6
Viral status	
HBs-Ag positive	35
HBs-Ag negative	7
Histology of noncancerous liver tissues	
Histologically normal	18
Liver cirrhosis	24
Tumor differentiation	
Well differentiated	0
Moderately differentiated	22
Poorly differentiated	20
Total	42

sequence were sub-cloned into the pSUPER-EGFP vector (gift from Dr. Dianqing Wu), which was identified by *Hind*III (TAKARA) and *Bgl*II (TAKARA), to be the DNMT1 siRNA construct named pMT1, with sMT1 as a control.

Transfection of DNMT1 RNAi construct to hepatocellular carcinoma cell line SMMC-7721

The human HCC cell line SMMC-7721 (No. TCHu13 Cell Bank Shanghai, China) was cultured in RPMI-1640 containing 10% new born bovine serum in 5% CO₂ incubation at 37°C. Cells were transfected with 1.5 µg of DNMT1 siRNA (pMT1) construct or sMT1 using transfectamineTM 2000 reagent (Invitrogen), and selected with 0.4 mg/mL genetincin (Life Technologies). SMMC-7721 cells were transfected with pMT1 and named 7721-MT1 cell lines or transfected with sMT1 and named 7721-sMT1 as a control.

Immunohistochemistry

Four micrometre thick sections of formalin-fixed, paraffin embedded tissue specimens from all 42 patients were deparaffinized and dehydrated. For antigen retrieval, the sections were heated for 10 min at 120°C in an autoclave, and nonspecific reactions were blocked with 5% normal horse serum. All sections were incubated with specific primary antibodies that recognized DNMT1 (goat polyclonal antibody, dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA). We previously confirmed the specificity of the goat anti-human DNMT1 polyclonal antibody by Western blotting analysis: an immunoreactive band of 193.5 kDa, corresponding to the molecular mass of DNMT1, was detected in human cancer cells, but no nonspecific bands were detected with this antibody^[16]. All primary antibody incubations were conducted at 4°C overnight and were followed by incubation with biotinylated secondary antibodies (anti-goat IgG, anti-mouse IgG, dilution 1:200; QIAGEN Laboratories) at room temperature for 30 min. The sections were then treated with Vectastain Elite ABC reagent (Vector Laboratories). All sections were counterstained with hematoxylin. For negative

control preparations, the primary antibody was omitted from the reaction sequence.

Real time PCR to detect mRNAs of DNMT1

Total RNA was purified from a normal liver cell line, a cell line established from pericarcinoma tissue and HCC cell lines with TRIzol (Invitrogen). The first-strand cDNA was synthesized from 2 µg total RNA using Oligo (dT) 18 primer and SuperScript II reverse transcription kit (Life Technologies). A PCR reaction was performed in a 50 µL volume with 5U polymerase (TAKARA) and cDNA samples equivalent to 1 ng of RNA. SYBR green with 20000 dilutions was included in each reaction for relative quantification in the ABI 7300 sequence detection system (Applied Biosystems). To normalize the input load of cDNA among samples, *β-actin* was quantified and used as an endogenous standard. The relative level of expression of each *DNMT1* among different cell lines was then calculated accordingly (ABI PRISM 7300 Sequence Detection System, USA). The primers used for PCR were as follows: *DNMT1*: sense primer, 5'-CCGAGTTGGTGATGGTGTGATC-3'; antisense primer, 5'-AGGTTGATGTCTGCGTGGTAGC-3'. *β-actin*: 5'-AAAGACCTGTACGCCAACAC-3'; antisense primer, 5'-GTCATACTCCTGCTTGCTGAT-3'. *PDCD4*: sense primer 5'-TGGATGAAAGGGCATTGAGA-3'; antisense primer, 5'-AGCCTTCCCCTCCAATGCTA-3'.

Western blotting analysis

Cells were grown and harvested at 80%-85% confluency, and cellular proteins were extracted with lysis buffer containing 0.5% NP-40, 150 mmol/L NaCl, and 1 mmol/L EDTA in 50 mmol/L Tris-HCl at pH 7.5, supplemented with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). The protein concentration of each extract was quantified by BCA assay (Pierce, Rockford, IL). Two to forty microliter of total protein was electrophoresed on 7%-15% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (PVDF, Amersham) electrophoretically. After blocking with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, membranes were incubated with goat anti-DNMT1 (Santa Cruz Biotechnology), proliferating cell nuclear antigen (PCNA) (mouse monoclonal antibody, dilution 1:200, Lexington, KY) or mouse monoclonal anti-actin (Sigma) antibodies. The membranes were then developed with peroxidase-labeled antibodies (Amersham Pharmacia, Piscataway, NJ) by Super Signal chemiluminescence substrate (Pierce, Rockford, IL). Actin protein levels were used as a control for equal protein loading.

In vitro analysis of cell growth

Cell proliferation of transiently transfected SMMC-7721 cells was measured by trypan blue dye cell count assay. Cells were cultured in triplicate in 12-well plates at a concentration of 3×10^4 cells per well. Cells were collected at 1, 3, 5 and 7 d and exposed to trypan blue (Sigma), and nonviable cells took up the dye. Both viable

(unstained) and nonviable (stained) cells were counted, and the relative survival rate (%) of each siRNA treatment was then calculated.

Flow cytometric (FCM) analysis

Stably transfected SMMC 7721 cells were washed, resuspended in staining buffer, and examined by ApoAlert Annexin V Apoptosis kit (BD Biosciences) and PI according to the manufacturer's instructions. Stained cells were analyzed by FACS (FACScalibur, BD Biosciences).

RESULTS

Immunohistochemical analysis of DNMT1 in pericancerous liver tissues and HCCs

Immunoreactivity for DNMT1 was detected in the nuclei and cytoplasm, but not in the cell membranes, of cancer cells (Figure 1). To discriminate definitely positive cases from cases with a leaky background level signal, if more than 30% of the cells in a tissue sample exhibited nuclear and cytoplasm staining the sample was considered to show positive immunoreactivity. Nuclear and cytoplasmic DNMT1 immunoreactivity was detected and overexpressed in 40.48% of patients. The incidence of DNMT1 immunoreactivity in carcinoma correlated significantly with poor tumor differentiation (Table 2, $P = 0.014$, χ^2 test). The incidence of DNMT1 immunoreactivity was significantly higher in HCCs than in pericancerous liver tissues. DNMT1 protein overexpression was not significantly associated with other parameters relating to cancer aggressiveness, such as the depth of invasion, vascular involvement, or lymph node metastasis. In HBV-related HCC cases, 70% had different degrees of hepatocirrhosis. To investigate whether there was an HBV-induced increase in DNMT1 expression in HCC, we evaluated the relationship between HBV infection and up-regulated DNMT1. There was higher expression of DNMT1 in HCC with HBV than in HCC without HBV. However, there was no significant correlation between the incidence of DNMT1 immunoreactivity in HCCs and the patients' viral status (HBs-Ag-positive, HBs-Ag-negative) by statistical analysis.

Expression of DNMT1 in nine cell lines

In order to determine whether the expression of DNMT1 was different between HCC cell lines and normal hepatocellular cell lines which were cultured under the same conditions, real-time RT-PCR was carried out in ABI 7300. DNMT1 was increased more in most HCC cell lines, especially in BEL-7405, BEL-7402 and SMMC-7721, than in a normal cell line and a pericarcinoma cell line. There were a trend that *DNMT1* increased more in HCC cell lines than in a normal liver cell line and a pericarcinoma cell line. In contrast to the low expression level of DNMT1 in the pericarcinoma cell line, the levels of DNMT1 in most HCC cell lines were increased more than 2-fold (Figure 2).

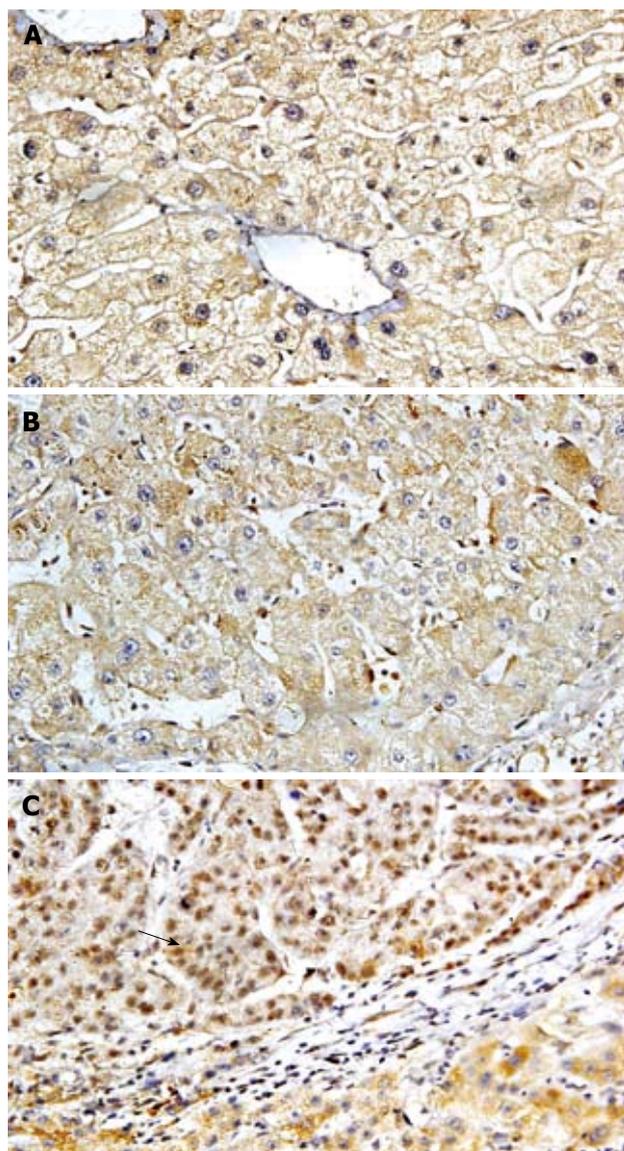


Figure 1 DNMT1 expression was analyzed in pericancerous liver tissues and HCCs by immunohistochemistry (x 100). A: The expression of DNMT1 was detected in cirrhotic noncancerous liver tissue obtained from HCC patients; B: The expression of DNMT1 was detected in well differentiated HCC; C: The expression of DNMT1 was detected in poorly differentiated HCC. The arrow is to show DNMT1 antibody staining in cell nuclei.

Decrease in DNMT1 suppresses growth of human hepatocellular carcinoma cells in vitro

We analyzed the proliferation ability of SMMC-7721 cells transfected with DNMT1 siRNA constructs and its control by FCM and trypan blue assay. The resulting RNA interference had a negative effect on SMMC-7721 growth (Figure 3). This growth suppression was noticeable two days after transfection, and the effect was more prominent in DNMT1 knockdown cells than at seven days after transfection (Figure 3A). For the determination of DNMT1 protein expression in construct pMT1 transiently transfected SMMC 7721 cells, β -actin was employed for adjustment of DNMT1 expression data to protein content. PCNA, an established cell proliferation marker and the DNA replication factor to which DNMT1 binds, was employed to evaluate cell proliferation. Comparison with PCNA expression levels

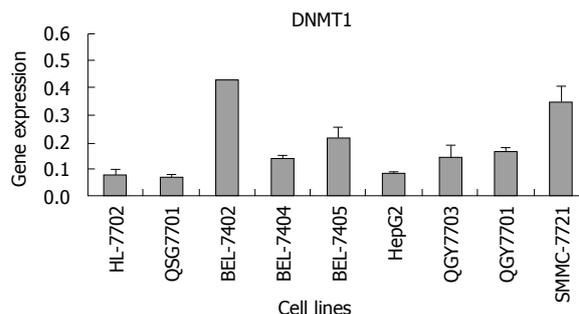


Figure 2 DNMT1 mRNA expression levels analysis in HCC cell lines by quantitative real-time PCR. DNMT1 mRNA expression levels were normalized according to the β -actin mRNA level of the same cell line. DNMT1 was increased more in most hepatocellular carcinoma cell lines, especially in BEL-7402, BBEL-7405 and SMMC-7721, than in the normal cell line HL-7702 and pericarcinoma cell line QSG-7701.

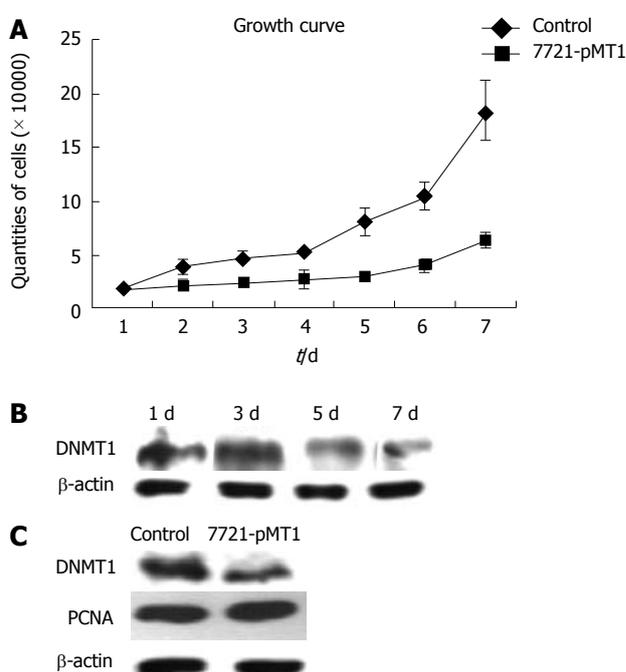


Figure 3 Inhibition of DNMT1 affects cell growth in HCC cell lines. Expression of endogenous DNMT1 protein in 7721-MT1 cells transfected with DNMT1 RNAi construct were decreased more than 80%. A: The SMMC-7721 cells were transfected transiently with DNMT1 siRNA and analyzed for their survival by trypan blue staining assay. The assays were performed in triplicates and the data are the mean \pm SE; B: Western blotting analysis of DNMT1 expression in cells transfected transiently with DNMT1 siRNA for 1, 3, 5, 7 d; C: The SMMC-7721 cells were transfected stably with DNMT1 siRNA for 2 mo and the inhibition of DNMT1 and expression of PCNA were evaluated.

revealed that the changes in DNMT1 expression were independent of the cell proliferation status (Figure 3B). The data showed that DNMT1 levels started to decrease on day 3 and was maintained until seven days after transfection with cell growth inhibition. However, there were no significant changes in PCNA expression, even in a stably decreased DNMT1 HCC cell line (Figure 3C).

DNMT1 knockdown induced cell apoptosis in SMMC-7721 cell line

Cell apoptosis detection was performed by flow cytometric analysis as described above. The data from

Table 2 Relationship between DNMT1 and pathological change in patients with HCC *n* (%), χ^2 test

	Subtotal	DNMT1 expression (P/OR: up-regulated/normal + down-regulated)			P	OR (95% CI)
		Up-regulated	Normal	Down-regulated		
Total or subtotal	42 (100.00)	17 (40.48)	13 (30.95)	12 (28.57)		
Age (yr)					0.54	1.49 (0.41-5.35)
> 50	15 (35.71)	7 (46.67)	4 (26.67)	4 (26.67)		
< 50	27 (64.29)	10 (37.04)	9 (33.33)	8 (29.63)		
Size of tumor (cm)					0.23	0.45 (0.12-1.67)
> 5	17 (40.48)	5 (29.41)	6 (35.29)	6 (35.29)		
< 5	25 (59.52)	12 (48.00)	7 (28.00)	6 (24.00)		
Histological differentiation					0.014	0.20 (0.05-0.75)
Moderate	22 (52.38)	5 (22.72)	9 (40.91)	8 (36.37)		
Poor	20 (47.62)	12 (60.00)	4 (20.00)	4 (20.00)		
HBsAg					0.169	4.5 (0.829-24.4)
Positive	35 (83.33)	15 (42.86)	12 (34.28)	8 (22.86)		
Negative	7 (16.67)	2 (28.57)	1 (14.29)	4 (57.14)		
Hepatocirrhosis					0.083	3.57 (0.81-15.71)
With	24 (57.14)	10 (41.67)	10 (41.67)	4 (16.67)		
Without	18 (42.86)	3 (16.67)	7 (38.89)	8 (44.44)		

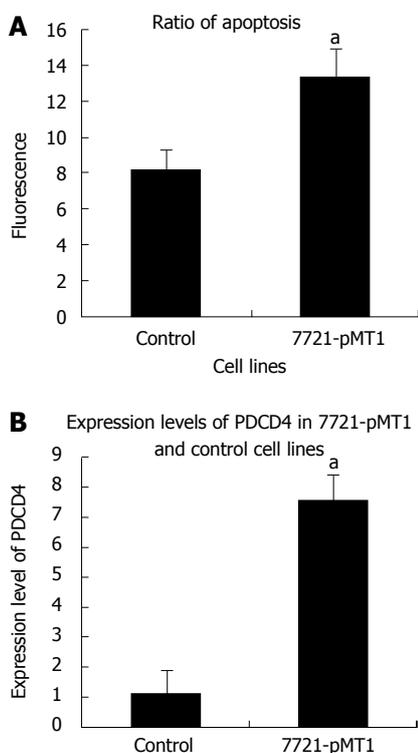


Figure 4 Knock-down DNMT1 induces cell apoptosis in HCC cell lines. A: The SMMC-7721 cells were transfected stably with DNMT1 siRNA for 2 mo and apoptosis was detected by FACS; B: Knock-down DNMT1 induced apoptosis gene PDCD4 expression in 7721-MT1 cell line compared with control cell line by quantitative real-time PCR. *Show the significant difference between 7721-pMT1 and control.

the present study indicated that DNMT1 knockdown induced cell apoptosis. The apoptotic rate increased from $8.78\% \pm 0.44\%$ to $14.24\% \pm 0.12\%$. These results indicated that DNMT1 knockdown may induce HCC cell apoptosis (Figure 4). The apoptosis gene *PDCD4* was induced by DNMT1 siRNA in SMMC-7721.

DISCUSSION

HCC is well known to develop through the stages

of dysplasia and early HCC in a background of chronic liver diseases, including chronic hepatitis and liver cirrhosis. Increased DNMT1 mRNA expression has been reported in a number of human cancers^[17-19]. DNMT1 may play an important role during hepatocarcinogenesis even at the precancerous stage^[9,20]. Although DNMT1 expression and its role were evaluated in different populations, information from Chinese subjects was still unclear. HCC is one of the most lethal and prevalent cancers in China, where HBV is one of the main attributable risk factors^[4,21]. To study the significance of aberrant DNMT1 expression during hepatocarcinogenesis in a Chinese population, we used an immunohistochemical technique to directly evaluate DNMT1 protein expression in noncancerous liver tissues and HCCs, and real-time PCR to evaluate DNMT1 expression in HCC cell lines and a normal liver cell line.

After all tumors were analyzed, HBV infection was present in 83.3% of the samples, indicating that our study cohort is representative of the subset of HCC arising from HBV infection, which is the most considerable risk factor for HCC in studied cases. In the present study, more than 80% of nonneoplastic liver tissues with either chronic hepatitis or liver cirrhosis showed variable nuclear and cytoplasm immunopositivity for DNMT1. DNMT1 immunoreactivity was certainly detected in HCCs, as it was in other cancers^[22-24]. DNMT1 overexpression was detected in 40.48% of the patients and the incidence of DNMT1 immunoreactivity was significantly higher in HCC than in pericancerous liver tissues. Specifically, the incidence of DNMT1 immunoreactivity in HCCs correlated significantly with poor tumor differentiation. The above evidence indicates that increased DNMT1 protein expression may play a role in the malignant progression of HCCs in Chinese subjects. Alternatively, in virus-associated cancers, viral proteins have been shown to disturb the host DNA methylation system by up-regulating DNMT activities, thereby increasing tumor susceptibility^[25].

Park *et al.*^[26] showed that HBx promoted specific regional hypermethylation of tumor suppressor genes and genome-wide hypomethylation by transcriptional regulation of DNMTs. HBx expression elevates overall intracellular DNMT activity by inducing DNMT1 and DNMT3A. HBx promotes epigenetic abnormalities by modulating the expression of DNMTs immediately after HBV infection, thus epigenetically accelerating hepatocarcinogenesis. In this study, we evaluated DNMT1 expression in HBV-positive cases and HBV-negative cases. No significant relationship was found between HBV infection and DNMT1 up-regulation. These data suggested there is a different mechanism of HBV affected by DNMTs expression in diverse ethnic populations although additional studies with larger sample sizes are required to confirm our findings.

According to the results of both immuno-histochemistry and real time RT-PCR, progressive increases in DNMT1 expression may be accompanied by hepatocarcinogenesis from the precancerous stage to the malignant progression of HCC. Immunohistochemical analysis of DNMT1 in biopsy specimens obtained for diagnostic purposes and/or surgically resected materials may show that DNMT1 is a biologic predictor of both HCC recurrence and poor prognosis in HCC patients. To address whether DNMT1 overexpression plays an important role in hepatocellular carcinogenesis in Chinese subjects and to evaluate DNMT1 knockdown strategies for cancer therapy, it is necessary to deplete DNMT1 in the HCC. To avoid the problem of DNMT1 siRNA not being sufficient to inhibit cell growth due to the short-term inhibition of DNMT1 expression, we employed the RNAi technique to knockdown DNMT1 expression in an HCC cell line and assessed tumor cell growth in transiently transfected and stably expressed DNMT1 siRNA cell lines, respectively. Fortunately, we observed that depletion of DNA methyltransferase 1 mediates growth suppression in the HCC cell line SMMC-7721. In order to explore whether this inhibition of DNA replication reflects a distinct alteration in cell cycle kinetics, similar to the DNA damage checkpoints that trigger arrest at distinct phases of the cell cycle^[27], we detected PCNA in the treated cells, and found there were no significant changes in PCNA expression in treated cells and controls. However, DNMT1 knockdown inhibited cell growth and induced cell apoptosis in the SMMC-7721 cell line. With a view to the results of cDNA microarray^[16], PDCD4, an over-expression apoptosis gene, may contribute to the apoptosis rate in SMMC-7721 cells treated by siRNA. PDCD4 is a proapoptotic molecule involved in TGF-beta1-induced apoptosis in human HCC cells, and a possible tumor suppressor in hepatocarcinogenesis^[28-30]. These results provide a rationale for the development of a DNMT1-targeted strategy as an effective epigenetic cancer therapy.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fourth or fifth largest cause of cancer-

related death worldwide. The aberration of DNA methylation (DNMT), which is catalyzed by DNA methyltransferases, is common in HCC. DNMT1 is the main and maintenance methyltransferase in mammals. DNMT1 may play an important role during hepatocarcinogenesis. However, the relationship between abnormal DNMT1 expression and hepatocellular carcinogenesis is still to be elucidated, especially in a Chinese population.

Research frontiers

Overexpression of DNMTs is a common event in nearly all malignancies, and DNMT1 may play an important role during hepatocarcinogenesis. In the present study, the authors demonstrated that the overexpression of DNMT1 is correlated significantly with poor tumor differentiation in HCC. Inhibition of DNMT1 through siRNA affected the proliferation ability of an HCC cell line.

Innovations and breakthroughs

Recently, most reports focus on the overexpression of DNMT1 and DNA methylation in tumors. However, abnormal expression of DNMT1 may be involved in tumorigenesis, especially in HBV-related HCC. RNA interference is a direct and efficient way to suppress the target gene in a gene function study. This is the first study to report that inhibition of DNMT1 *via* RNAi suppressed cell proliferation and induced cell apoptosis in an HCC cell line.

Applications

This study implied that DNMT1 maybe considered as a target for HCC therapy. The study also gives a general and better understanding of tumor cell biology and the potential epigenetic mechanism of hepatocellular carcinogenesis.

Terminology

DNMTs are crucial components of DNA methylation. DNMT1 is the major and best-known DNMT in somatic cells. DNMT1 overexpression correlated significantly with poorer tumor differentiation, but not with the phenotype of the cancer cells, in several tumors. DNMT1 protein might have a more direct and immediate effect on the state of cellular growth and transformation.

Peer review

The manuscript by Hong *et al* shows an increased expression of DNMT1 in hepatocellular carcinoma tissue samples and cell lines compared to controls. The increase was correlated with the degree of differentiation. Knockdown of DNMT1 expression in HCC cell lines decreased the number of cells at the end of the experiment. This did not correlate with decreased proliferating cell nuclear antigen (PCNA) expression but was associated with an increase in apoptosis and the expression of apoptosis-related gene PDCD4. For the most part the data is convincing and the experiments are well carried out.

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Synchronous incidental gastrointestinal stromal and epithelial malignant tumors

Yan-Jun Liu, Zhou Yang, Lang-Song Hao, Lin Xia, Qian-Bin Jia, Xiao-Ting Wu

Yan-Jun Liu, Lang-Song Hao, Lin Xia, Qian-Bin Jia, Xiao-Ting Wu, Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Zhou Yang, Department of General Surgery, Sichuan Provincial People's Hospital, Chengdu 610041, Sichuan Province, China

Author contributions: Liu YJ, Wu XT and Hao LS designed the study; Liu YJ, Yang Z and Xia L performed the study; Liu YJ, Yang Z and Jia QB analyzed the data; Liu YJ and Wu XT wrote the manuscript.

Correspondence to: Dr. Xiao-Ting Wu, Professor, Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. xiaoting.wu1@gmail.com

Telephone: +86-28-81812493 Fax: +86-28-85218256

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CONCLUSION: Incidental GIST may occur synchronously with other tumors and has a high prevalence in males. Surgery is its best treatment modality.

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Key words: Gastrointestinal stromal tumor; Multitumor; Synchronous tumor

Peer reviewers: Giuseppe Brisinda, MD, Department of Surgery, Catholic School of Medicine "Agostino Gemelli", Largo Agostino Gemelli 8-00168 Rome, Italy; Kazunari Murakami, Professor, Department of General Medicine, Oita University, 1-1 Idaigaoka, Hasama, Oita 879-5593, Japan

Liu YJ, Yang Z, Hao LS, Xia L, Jia QB, Wu XT. Synchronous incidental gastrointestinal stromal and epithelial malignant tumors. *World J Gastroenterol* 2009; 15(16): 2027-2031 Available from: URL: <http://www.wjgnet.com/1007-9327/15/2027.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.2027>

Abstract

AIM: To investigate the incidence of incidental gastrointestinal stromal tumor (GIST) and its etiopathogenesis.

METHODS: From January 1, 2000 to December 31, 2007, 13 804 cases of gastrointestinal epithelial malignant tumor (EMT) and 521 cases of pancreatic adenocarcinoma (PAC) were successfully treated with surgery at the Department of General Surgery and the Department of Thoracic Surgery, West China Hospital, Sichuan University, China. The clinical and pathologic data of 311 cases of primary GIST, including 257 cases with clinical GIST and 54 cases of incidental GIST were analyzed.

RESULTS: Of the 311 patients, 54 had incidental GIST, accounting for 17.4%. Of these tumors, 27 were found in 1.13% patients with esophageal squamous cell carcinoma (ESCC), 22 in 0.53% patients with gastric adenocarcinoma (GAC), 2 in 0.38% patients with PAC, 2 in 0.03% patients with colorectal adenocarcinoma, and 1 in one patient with GAC accompanying ESCC, respectively. Patients with incidental GIST presented symptoms indistinguishable from those with EMT. All incidental GIST lesions were small in size, and the majority had a low mitotic activity while only 1.9% (5/257) of clinical GIST lesions had a high risk.

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of gastrointestinal (GI) tract, probably arising from precursor interstitial cells of Cajal. Significant advances have been made in symptomatic GIST in the last two decades^[1,2]. However, little is known about the incidental GIST detected during examinations or surgery for other reasons. Its clinicopathologic characteristics are unclear. Many cases of synchronous or asynchronous GIST with other tumors have been reported as single cases^[3-6]. We discovered 54 cases of incidental GIST during surgery for epithelial malignant tumor (EMT). This study was to investigate the incidence of incidental GIST and its etiopathogenesis.

MATERIALS AND METHODS

Patients

From January 1, 2000 to December 31, 2007, 13 804 cases of gastrointestinal EMT and 521 cases of pancreatic adenocarcinoma (PAC) were successfully treated with surgery at the Department of General

Table 1 Location of 54 incidental GIST lesions and their corresponding EMT

EMT	Patients (n)	Median age	Gender (M/F)	Incidental GIST site (No. of patients)							
				Gastric cardia	Gastric fundus	Gastric body	Gastric antrum	Esophagus	Terminal ileum	Colon	Omentum
GAC	22	64.5 (45-79)	19/3	1	7	13	1	-	-	-	-
ESCC	27	63 (44-77)	24/3	1	3	19	1	2	-	-	1
GAC + ESCC	1	79	1/0	-	-	1	-	-	-	-	-
CRA	2	57.5 (54-61)	2/0	-	-	-	-	-	1	1	-
PAC	2	67.5 (65-70)	2/0	-	1	1	-	-	-	-	-
Total	54	63 (44-79)	48/6	2	11	34	2	2	1	1	1

GIST: Gastrointestinal stromal tumor; EMT: Epithelial malignant tumor; GAC: Gastric adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; CRA: Colorectal adenocarcinoma; PAC: Pancreatic adenocarcinoma.

Surgery and the Department of Thoracic Surgery, West China Hospital, Sichuan University, China. Gastrointestinal EMT cases included 2382 cases of esophageal squamous cell carcinoma (ESCC), 35 cases of esophageal adenocarcinoma (EAC), 4168 cases of gastric adenocarcinoma (GAC), 329 cases of small intestinal adenocarcinoma (SAC), and 6890 cases of colorectal adenocarcinoma (CRA). During this period, 311 cases of primary GIST (121 females, 190 males) were identified in our center, including 257 cases of clinical GIST and 54 cases of incidental GIST.

Methods

Hospital records of patients with incidental GIST were reviewed. Each patient was followed up by telephone or mail. Histopathologic features of primary GIST were evaluated by two experienced pathologists, blinded to their respective findings and patient outcomes, at the Department of Pathology, West China Hospital. The largest diameter of tumor was recorded. In patients with multiple GIST lesions, only the largest GIST lesion was included in pathological analysis. The risk category for GIST was defined by assessing the tumor size and mitotic count following the consensus guidelines of the National Institutes of Health-(NIH-NCI) workshop^[7]. In addition to the assessment of CD117 in tumor cells, reactions with CD34, SMA, and S-100 proteins were also studied. Immunohistochemical examination of these proteins was performed on tumor tissues embedded in paraffin with DAKO (Glostrup, Denmark) antibodies according to the manufacturer's instructions.

Statistical analysis

Categorical variables were compared by χ^2 test or by Fisher's exact test where applicable. Survival analysis was performed using the Kaplan-Meier method. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Of the 311 patients, 54 had incidental GIST, accounting for 17.4%. Among these tumors, 27 were found in 1.13% patients with ESCC, 22 in 0.53% patients with GAC, 2 in 0.38% patients with PAC, 2 in 0.03% patients with CAC, and 1 in one patient with GAC accompanying ESCC,

respectively.

The median age of the 54 cases of incidental GIST was 63 years (range, 44-79 years). Interestingly, 48 of them (88.9%) were males, and 6 (11.1%) were females ($P < 0.001$). The patients presented symptoms of EMT without specific clinical manifestations indicative of GIST. Among the 54 patients, only a submucous lesion in gastric fundus, 2.5 cm in diameter, was preoperatively detected in 1 patient with GAC by gastroscopy, and a single-lesion was postoperatively detected in 4 patients by specimen examination. A total of 58 incidental GIST lesions were discovered in the 54 patients, including 51 single-lesions, 2 double-lesions, and 1 triple-lesion. A total of 90.7% incidental GIST lesions occurred in stomach, 3.6% in esophagus, 1.9% in terminal ileum, 1.9% in colon and 1.9% in omentum, respectively. The most common sites were the gastric fundus and body. In our series, 4 cases with a unique coexistence style (esophageal GIST + ESCC: 2, gastric GIST + ESCC + GAC: 1, colonic GIST + CRA: 1) have not been reported previously. The location of 54 incidental GIST lesions and their corresponding EMT lesions are shown in Table 1.

Of the incidental GIST lesions, 37 (68.5%) were of spindle-cell morphology, 9 (16.7%) epithelioid morphology, and 8 (14.8%) a mixed histological type. Immunohistochemical staining showed that 50 cases (92.6%) and 52 cases (96.3%) of incidental GIST were positive for CD117 and CD34, respectively. None of them was proven to have a metastasis of GIST, while 29 cases were confirmed with metastasis derived from EMT. Incidental GIST was small in size. The majority (90.7%) had a low mitotic activity and a very low risk, while only 1.9% cases of clinical GIST had a very low risk ($P < 0.001$), and 38.5% had a high risk with a marked mitotic activity (Table 2).

All the GAC patients received radical excision (distal gastrectomy for 3, proximal gastrectomy for 2, total gastrectomy for 12, esophagogastrectomy for 5). All the ESCC patients including the patient with triple tumors underwent esophagogastrectomy. The two PAC patients underwent duodenopancreatectomy and distal pancreatectomy, respectively, with local gastrectomy. Right and left hemicolectomy was performed for the two CRA patients, respectively. Thirty-four out of the 54 patients received either adjuvant chemotherapy and/or radiotherapy after operation. None of them received oral Imatinib mesylate (Glivec) treatment. On September

Table 2 Distribution of gender, age, tumor site, tumor size, and risk in 311 patients with GIST

GIST	Patients (n)	Gender (M/F)	Median age in yr (range)	Tumor site (No. of patients)	Tumor size (cm)			Risk patients, n (%)
					Median	Mean	Range	
Incidental GISTs	54	48/6	63 (44-79)	Gastric (49), esophagus(2), ileum(1), colon (1), omentum (1)	0.8	0.9	0.2-2.5	VL: 49 (90.7); L: 5 (9.3)
Clinical GISTs	257	142/115	57 (22-87)	Gastric (147), duodenum (10), jejunum-ileum (57), colon (25), rectum (3), anal canal (3), mesenterium (6), omentum (4), pancreatic (2)	7.5	6.2	1.5-30.0	VL: 5 (1.9); L: 86 (33.5); Int: 67 (26.1); H: 99 (38.5)
Total	311	190/121	61 (22-87)	Gastric (196), esophagus(2), duodenum (10), jejunum-ileum (58), colon (26), rectum (3) anal canal (3), mesenterium (6), omentum (5), pancreas (2)	6.3	5.5	0.2-30.0	VL: 54 (17.4); L: 91 (29.3); Int: 67 (21.5); H: 99 (31.8)

Risk was determined as previously described^[7]. VL: Very low risk; L: Low risk; Int: Intermediate risk; H: High risk.

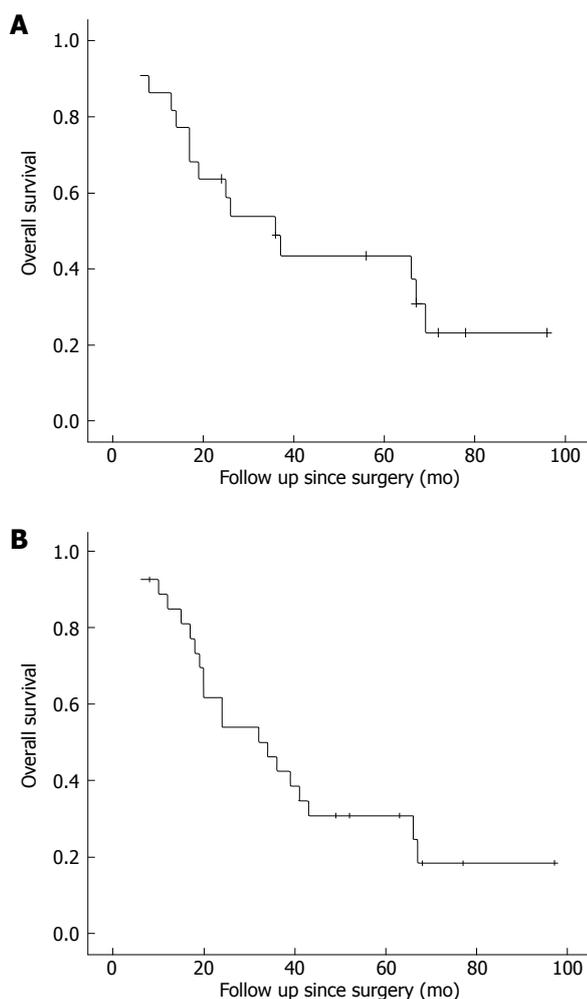


Figure 1 Kaplan-Meier survival curves. A: 22 patients with GIST accompanying GAC; B: 27 patients with GIST accompanying ESCC.

1, 2008, four of the patients were alive while 50 died of recurrence or distal metastasis of other malignancies. The remaining two patients died of other causes. Recurrent GIST was not found during the survival period of all dead patients, and the follow-up time of the remaining four. The overall 5-year survival rate of the 22 patients with GAC and incidental GIST was 31.8%, with a median survival time of 36 mo (Figure 1A). The 5-year survival rate of the 27 patients with ESCC and incidental GIST was 22.2%, with a median survival time of 32 mo (Figure 1B). The average survival time of the

two PAC and two CRA patients was 26 mo and 52 mo, respectively, and the survival time of the patients with triple tumors was 47 mo.

DISCUSSION

In our series, incidental GIST occurred simultaneously with EMT in 17.4% (54/311) of the GIST patients, which is higher than the reported incidence (14%)^[8]. However, assessment of the actual incidence of incidental GIST with EMT is difficult, because the data are only based on patients who have been surgically treated, whereas EMT patients managed with non-surgical measures are unaccounted for. Moreover, during examination or surgery, identification of GIST is incidental rather than intentional, and many lesions are missed as a result.

Notably, in addition to those with EMT, many synchronous and asynchronous cases of GIST with non-epithelial tumors have been reported, such as osteosarcoma, Burkitt's lymphoma, plasmocytoma, neuroblastoma, somatostatinoma, chronic lymphatic leukemia, lipoma and ectopic pancreas^[4,9-13]. Synchronous incidental GIST and non-tumorous diseases have been reported, such as ulcerative colitis, Meckel's diverticulum, rapidly progressive glomerulonephritis, HIV carriers, and Crohn's disease^[5,14-17]. Sanchez *et al*^[18] reported that incidental gastric GIST is found in 0.8% of patients undergoing laparoscopic Roux-en-Y gastric bypass surgery for obesity. Kawanowa *et al*^[19] showed that microscopic GIST can be found in 35% of stomach-resected patients with gastric cancer. It has been shown that microscopic GIST can be found in 10% of patients undergoing surgery for esophageal carcinoma^[20]. Especially, incidental GIST has also been detected in 0.2% of all autopsies, accounting for 10% of all patients with primary GIST^[21]. These findings suggest that incidental GIST may occur synchronously with other diseases more frequently than expected, and the incidence of incidental GIST might be much higher than that of clinical GIST.

Particular attention has been paid to clinical GIST because of its striking symptoms such as gastrointestinal bleeding, pain, dyspepsia, abdominal mass and obstruction^[22,23]. On the contrary, incidental GIST may emerge asymptotically, and even if symptomatically, the symptoms may often be vague and nonspecific^[18]. In our study, all the 54 patients presented symptoms

indistinguishable from those of EMT, which might have been overlooked because of the progressing symptoms of EMT such as severe dysphagia, weight loss, abdominal pain and anemia. The size of incidental GIST was small, and the majority (90.7%) of them had a very low risk. Also, only a few reports are available on incidental GIST with a high risk^[21,24,25]. In this study, only 1.9% of clinical GIST lesions had a very low risk, and 38.5% had a high risk, indicating that GIST is malignant. Perhaps, incidental GIST might have emerged later than EMT, or their development may have been depressed by EMT through mechanisms which are yet to be studied.

Generally, the preoperative detection rate of incidental tumors is very low. In this study, except for two patients with PAC, the other patients received endoscopic examinations preoperatively, yet only one GIST lesion was found. Difficulty in detecting the lesion might be attributed to its small size and intramural location. Incidental GIST, if detected at CT or MRI, is often mistaken for metastatic lymph nodes derived from EMT. Therefore, radiological examination is minimally helpful for its diagnosis. As a result, the endoscopist and surgeon should take the major responsibility of detecting incidental GIST. Incidental GIST occurs most commonly in stomach, esophagus, small bowel, colon and omentum. Consistent with the reported findings^[19], incidental GIST was observed in gastric fundus and body in the present study. Careful assessment of the hotspot (i.e. the upper portion of stomach) is important for both endoscopist and surgeon.

Interestingly, we found that there was a significant difference of the incidence of incidental GIST in male and female patients. Because of the unclear pathogenesis of incidental GIST, we cannot explain this finding. Further studies are needed on the gene expression in primary tumor cells from male and female patients and signal transduction may also provide us with some clues to this question.

In the absence of prospective control studies, whether resection of incidental GIST lesions helps to improve the quality of life and/or the survival rate of EMT patients remains unclear. There are two major concerns for incidental GIST if missed during operation for other tumors. First, residual GIST lesions may progress to invasive disease and cause intestinal obstruction and/or life-threatening gastrointestinal hemorrhage because the malignant potential is unpredictable based on gross appearance alone. Second, a residual incidental GIST may be mistaken for the relapse or metastasis of a previously removed neoplasm, which may result in inappropriate treatment of patients in follow-up after operation. Therefore, an en bloc resection with other tumors or an additional local resection with adequate margins has been recommended by surgeons^[6,18,25]. Making surgeons aware of this will help to correct surgical procedures, and ultimately improve the quality of life and avoid inappropriate treatment of patients during follow-up after operation.

Common carcinogenic agents, which result in a simultaneous proliferation of different cell lines (epithelial

and stromal cells), may be involved in the development of incidental GIST as a mere coincidence. In this study, males with primary GIST were more likely to have a synchronous tumor than females ($P < 0.001$). Synchronous tumors may have a high prevalence in males. Simultaneous neoplastic proliferation of epithelial and stromal cells might be stimulated by the same carcinogenic factors, such as *Helicobacter pylori* infections, germline mutations, and exposure to ionizing radiation^[6,24,26,27]. To clarify possible common carcinogenic agents against synchronous tumors, further studies are needed.

In conclusion, incidental GIST coexists with EMT at a higher incidence than expected. Surgeons are advised to be alert against possible primary GIST accompanying other tumors.

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COMMENTS

Background

Gastrointestinal stromal tumor (GIST) is one of the most common tumors in gastrointestinal (GI) tract, probably arising from precursor cells that serve as a pacemaker to trigger gut contraction. It may exist alone with clinical manifestations or coexist with other diseases. The former is usually diagnosed by its clinical presentations and called clinical GIST, while the latter is usually found during examination or surgery for other diseases and called incidental GIST.

Research frontiers

Clinical GIST has been extensively studied in the past twenty years. Many cases of GIST existing alone or coexisting with other diseases have been reported, but GIST coexisting with other GI tumors has only been reported as single cases. It is necessary to conduct a comprehensive study with a large sample size to determine its incidence and features.

Innovations and breakthroughs

For the first time, the authors report an extensive study on incidental GIST coexisting with other GI tumors. This study revealed some important and interesting information regarding incidental GIST coexisting with other GI tumors. Firstly, they found that incidental GIST coexisted most frequently with esophageal and gastric tumor (1.13% and 0.53% respectively), and least with colorectal tumor (0.03%). Secondly, the majority of clinical GISTs had a moderate or a high risk. In contrast, the majority of incidental GISTs had a very low risk. Thirdly, the incidence of incidental GIST was significantly higher in male than in female patients (88.9% vs 11.1%). Finally, this study also provided the statistics for age, survival time and prognosis of studied patients and outlined the other features of incidental GIST, such as the number of lesions, lesion location and cellular morphology, etc.

Applications

The incidence of incidental GIST coexisting with other GI tumors is much higher than expected. However, without specific manifestations, preoperative detection of incidental GIST is difficult. Residual GIST lesions may progress to invasive diseases, cause intestinal obstruction and/or life-threatening gastrointestinal hemorrhage. In addition, residual incidental GIST may be mistaken for the relapse or metastasis of previously removed tumors, resulting in inappropriate treatment of patients during follow-up after operation. A careful inspection for GIST is highly recommended during surgery for GI tumors.

Terminology

GIST is one of the tumors in the GI tract, probably arising from precursor cells that serve as a pacemaker to trigger gut contraction. GI epithelial malignant tumor (EMT) refers to a tumor arising from the surface cells of the GI tract.

Peer review

This article is the first report to present the incidence of incidental GIST accompanying gastrointestinal EMT. In this study, the authors evaluated the

incidental GIST and its clinical significances. The title of the paper reflects the major contents of the article. The abstract gives a clear delineation of the research background. Results and discussion are well organized. The conclusion is reliable and valuable.

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BRIEF ARTICLES

Effect of 5-FU on modulation of disarrangement of immune-associated cytokines in experimental acute pancreatitis

Xiao-Li Chen, Sang-Zhu Ciren, Hui Zhang, Li-Geng Duan, Alexander J Wesley

Xiao-Li Chen, Sang-Zhu Ciren, Hui Zhang, Li-Geng Duan, Department of General Surgery, The Huaxi Hospital, Medical Center, University of Sichuan, Chengdu 610041, China

Alexander J Wesley, Department of Surgery, Transplantation Division, University of Cincinnati Medical Center, Cincinnati OH 45267-0558, United States

Author contributions: Chen XL designed this study, coordinated the experiment and was the primary writer of this manuscript; Ciren SZ, Zhang H, Duan LG performed the experiment and took part in writing the manuscript; Wesley AJ was the tutor of this study and revised the manuscript.

Correspondence to: Xiao-Li Chen, MD, Department of General Surgery, The Huaxi Hospital, Huaxi Medical Center, University of Sichuan, Wainan guoxuexiang 34#, Chengdu 610041, China. zean_z@yahoo.com.cn

Telephone: +86-28-85422868 Fax: +86-28-85534151

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Abstract

AIM: To investigate the effects of 5-Fluorouracil (5-FU) on modulation of pro-inflammatory and anti-inflammatory cytokines in acute pancreatitis and the mechanism of it in the treatment of acute pancreatitis.

METHODS: Male Sprague Dawley rats were assigned to 3 Groups: Group A, sham operated rats as controls ($n = 7$); Group B, acute pancreatitis induced by ductal injection with 5% sodium cholate at a volume of 1.0 mL/kg without any other treatment; Group C, after the pancreatitis was induced as in Group B, the rats were injected intravenously with 5-FU 40 mg/kg. The animals in Groups B and C were killed at 2, 6 and 24 h after operation ($n = 7$), and blood samples were taken for measurement of tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6) (by bioassay), and interleukin-10 (IL-10), transforming growth factor- β (TGF- β) (by ELISA). The wet weight of pancreatic tissue, serum amylase levels and white blood cells were also measured.

RESULTS: Four rats in Group B and one in Group C died after pancreatitis was induced. Both pro-inflammatory cytokines (TNF- α , IL-1, IL-6) at the 2 and 6 h period and the anti-inflammatory cytokines (IL-10, TGF- β) at 24 h increased significantly ($P < 0.05$) in rats of Group B. After treatment with 5-FU, TNF- α , IL-1, and

IL-6 in serum of rats of Group C were inhibited at 2 and 6 h after operation ($P < 0.05$), and IL-10, TGF- β were inhibited at 24 h compared to Group B ($P < 0.05$). Obvious improvements in the severity of the acute pancreatitis, including the amylase levels, wet weight of pancreatic tissue and neutrophil counts, were also observed after treatment with 5-FU.

CONCLUSION: 5-FU is an anti-metabolic and immunosuppressive agent which can minimize the abnormal immune cytokine response and relieve the pathophysiological disorders associated with experimental acute pancreatitis.

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Key words: Pancreatitis; Cytokines; Systemic inflammatory response syndrome; 5-Fluorouracil

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INTRODUCTION

5-Fluorouracil (5-FU) has been used in the treatment of acute pancreatitis both experimentally and clinically since 1970^[1-3]. Several animal experiments of pancreatitis treated with 5-FU have shown very promising results, especially for a decrease in amylase and trypsin levels and improvement of survival rates^[1,2]. It has been reported that prolongation of pancreatic allograft survival and protection from pancreatitis in dog pancreas allografts occur after pretreatment with 5-FU^[4]. A prospective controlled clinical study was carried out in 1983, which showed that treatment with 5-FU was of some benefit in the modulation of clinical pancreatitis^[5]. Clinical studies conducted in Russia documented that both the mortality and the length of hospital stay were reduced after treatment with 5-FU^[6-8]. In China, administration of 5-FU

has been considered as an adjuvant therapy of acute pancreatitis. More than one thousand patients with acute pancreatitis have received the treatment of 5-FU each year in China, with many reports showing some beneficial results^[9-12]. While there are many studies focusing on clinical observation of 5-FU treatment, research involving the mechanisms is sparse, but many investigators felt that the effect of 5-FU treatment for pancreatitis was derived from inhibiting the activities of pancreatic enzymes^[1-3,5,9,10]. Recently, it has been increasingly clear that disarrangement of the immune system during acute pancreatitis is the determining factor in the pathophysiological process^[13-19]. Considering that abnormal inflammation-associated cytokines (pro- and anti-inflammatory cytokines) present a primary index of disarrangement of immune function during acute pancreatitis^[20-24], we designed this animal experiment to investigate the inhibiting effect of 5-FU on the inflammatory cytokines (TNF- α , IL-1, IL-6) and anti-inflammatory cytokines (IL-10, TGF- β) in acute pancreatitis and the relationship between the level of cytokines in serum and degree of acute pancreatitis.

MATERIALS AND METHODS

Materials

Sodium cholate was purchased from Sigma. Rat TGF- β and IL-10 EIA kits were purchased from R & D Co., USA. Reagents and instruments for measurement of TNF- α , IL-1, IL-6 were supplied by the Immunology Department, Medical Center, Sichuan University. Sprague Dawley (SD) rats were purchased from the Experimental Animal Center, Sichuan University, China.

Animals and pancreatitis model

SD rats (male, 10-12 wk-old, weighing 200-250 g) were fasted but allowed to drink water freely for 16 h before the experiment. They were allocated randomly into three Groups: Group A ($n = 7$), sham operation, with the same laparotomy under general anesthesia as Group B and sham intubation of the cholo-pancreatic duct but without any drug injection. These rats were killed 2 h later. In Group B, the acute pancreatitis Group, SD rats were injected with 5% sodium cholate into the cholo-pancreatic duct at a volume of 1.0 mL/kg using a mid-line laparotomy under general anesthesia and strict aseptic conditions to establish acute pancreatitis; Group C, acute pancreatitis with treatment of 5-FU. After pancreatitis was induced as in Group B, the rats were injected intravenously 40 min later with 5-FU 40 mg/kg. (This dosage is equal to 10-15 mg/kg in humans based on body surface). All the animals in Groups B and C were resuscitated post-operatively with 0.9% sodium chloride, subcutaneously at 6 mL/kg per hour. The surviving animals in Groups B and C were killed at 2, 6 and 24 h after operation ($n = 7$). Blood samples were taken for measurement of TNF- α , IL-1, IL-6, IL-10, and TGF- β . The wet weight of pancreatic tissue (the index of pancreatic edema), serum amylase levels and

the total and differential count of leukocytes, were also measured and recorded.

All the measurements of cytokines were done in the Department of Immunology, Medical Center, Sichuan University. IL-1, IL-6 and TNF- α were measured by bioassay according to Lederer, Kimura and Heo's methods^[25-27]. IL-10 and TGF- β were measured by EIA according to the manufacturer's instructions. Amylase and white blood cells levels were tested by the Clinical Laboratory, Medical Center, Sichuan University.

Statistical analysis

We used the analysis of variance for continuous variables to detect variation among Groups with the same time (version 9.0 SAS Institute, Inc, Cary, NC). Statistical significance was regarded as $P < 0.05$. All reported P values are 2 sided. Continuous variables were described as mean \pm SD unless stated.

RESULTS

There were 4 deaths in Group B at the 4, 6, 8, 15 h time points after pancreatitis was induced, and one rat died in Group C at 12 h.

In Group A, TNF- α , IL-1, IL-6, IL-10, and TGF- β in the serum of rats were detected as basic concentrations because of tissue injury resulting from sham operation. After acute pancreatitis was induced in Group B, the concentrations of pre-inflammatory cytokines such as TNF- α , IL-1 and IL-6 in the serum of rats increased rapidly. At the 2, 6, and 24 h periods, TNF- α , IL-1, IL-6 in Group B and Group C were significantly higher than that of Group A ($P < 0.05$). After pancreatitis was treated with 5-FU, the concentrations of IL-1 and IL-6 in serum of rats in Group C were significantly lower than those of Group B at 2, 6 h periods after operation ($P < 0.05$). At 6 h after operation the concentration of TNF- α in serum of Group C was also lower than that of Group B ($P < 0.05$). But at the time point 24 h, TNF- α , IL-1, IL-6 in Groups B and C still maintained a higher level and there was no significant difference between these two Group (Table 1). We presume that this is due to 5-FU being quickly catabolised in the body so that its regulating action disappeared swiftly and was not maintained up to 24 h. Serum IL-10 and TGF- β were significant higher in Group B and Group C than in Group A at the 24 h period ($P < 0.05$). At 24 h after operation, compared to Group B, the concentrations of IL-10 and TGF- β in serum in rats of Group C were decreased significantly ($P < 0.05$) (Table 2). When we collected the samples of pancreas after rats were sacrificed, we found that samples of pancreas in Group C were more obviously swollen and congested than those of Groups A and C. Since some doctors have investigated histopathological change of pancreas in detail in similar animal experiments with 5-FU, here we only chose the wet weight of pancreas (index of pancreatic edema) and serum amylase as indexes of severity of acute pancreatitis. In the control Group, the weight of pancreatic tissue was 0.5 ± 0.09 g. At 2, 6, 24 h

Table 1 Change of level of pro-inflammatory cytokines in serum

Group	IL-1 (ng/mL)	IL-6 (IU/mL)	TNF (IU/mL)
A	0.28 ± 0.06	34.5 ± 6.40	11.82 ± 1.87
B (2 h)	1.02 ± 0.12 ¹	98.83 ± 12.43 ¹	43.67 ± 5.72 ¹
(6 h)	1.13 ± 0.17 ¹	101.0 ± 15.07 ¹	48.67 ± 5.32 ¹
(24 h)	1.15 ± 0.13 ¹	127.17 ± 13.91 ¹	55.33 ± 12.79 ¹
C (2 h)	0.80 ± 0.07 ^{1,2}	76.33 ± 7.42 ^{1,2}	35.33 ± 4.50 ¹
(6 h)	0.70 ± 0.06 ^{1,2}	74.33 ± 11.02 ^{1,2}	31.17 ± 4.54 ^{1,2}
(24 h)	1.02 ± 0.18 ¹	112.67 ± 20.06 ¹	42.33 ± 11.64 ¹

A: Sham operation Group without acute pancreatitis and drug injection; B: Acute pancreatitis Group; C: Acute pancreatitis with 5-FU group. 2 h, 6 h, 24 h: 2 h, 6 h, 24 h after operation. ¹Compared to sham operation Group (Group A), $P < 0.05$; ²Compared to pancreatitis Group (Group B), $P < 0.05$.

Table 2 Change of level of anti-inflammatory cytokines in serum

Group	IL-10 (pg/mL)	TGFβ (pg/mL)
A	22.05 ± 14.87	66.40 ± 13.20
B (2 h)	36.52 ± 9.76	64.58 ± 10.56
(6 h)	37.75 ± 6.54	72.87 ± 18.34
(24 h)	68.13 ± 19.90 ¹	103.77 ± 28.95 ¹
C (2 h)	28.82 ± 6.63	61.15 ± 30.31
(6 h)	45.5 ± 4.72 ¹	80.27 ± 19.83
(24 h)	24.0 ± 7.86 ²	68.52 ± 11.51 ²

¹Compared to sham operation Group (Group A), $P < 0.05$; ²Compared to pancreatitis Group (Group B), $P < 0.05$.

after acute pancreatitis being induced, the wet weights of pancreatic tissue in Group B were 1.63 ± 0.54 g, 1.85 ± 0.25 g and 1.53 ± 0.13 g, respectively; but at 2, 6, 24 h after treatment with 5-FU in Group C, the wet weights of pancreatic tissue were 0.87 ± 0.22 g, 0.58 ± 0.24 g and 0.88 ± 0.13 g, respectively. There was a significant difference between the acute pancreatitis Group (Group B) and the treatment with 5-FU Group (Group C) at all time periods ($P < 0.05$). In the control Group, the concentration of serum amylase was 374.2 ± 92.84 U/L. At 24 h after operation, the concentration of serum amylase was 1817.25 ± 459.35 U/L, but after treatment with 5-FU the concentration was 797.4 ± 225.9 U/L at the same time period. The concentration of serum amylase in the acute pancreatitis Group (Group B) was higher than that in the treatment with 5-FU Group (Group C), and it reached statistical significance ($P < 0.05$). In the control Group, leukocyte count in the peripheral blood of the rats was $(6.59 \pm 2.59) \times 10^9$ /L. At 24 h after experiment, the leukocyte counts in the Groups A and B were $(6.93 \pm 0.67) \times 10^9$ /L and $(6.1 \pm 1.44) \times 10^9$ /L respectively and there was no significant difference between the two Groups. Percentage of neutrophils in rats in Group A was $0.35\% \pm 0.09\%$. In Group B, the percentage of neutrophils was increased significantly at each time period. After treatment of 5-FU, the percentage of neutrophils in Group C decreased compared to Group B, but it did not reach statistical significance until after the 2 h period (Table 3).

Table 3 Change of index of severity of acute pancreatitis

Group	Amylase (U/L)	W.Weight of P (g)	W.B.C ($\times 10^9$ /L) ²	Neutrophils (%)
A	374.20 ± 92.84	0.51 ± 0.90	6.69 ± 2.59	0.35 ± 0.09
B (2 h)	371.25 ± 86.28	1.63 ± 0.54 ¹	7.59 ± 2.25	0.74 ± 0.38 ¹
(6 h)	508.83 ± 344.82	1.85 ± 0.25 ¹	9.46 ± 2.16	0.75 ± 0.08 ¹
(24 h)	1817.25 ± 459.35 ¹	1.30 ± 0.13 ¹	6.93 ± 0.67	0.62 ± 0.11 ¹
C (2 h)	352.2 ± 118.15	0.87 ± 0.22 ²	7.90 ± 2.33	0.48 ± 0.09 ²
(6 h)	434.4 ± 138.35	0.68 ± 0.24 ²	10.72 ± 1.45	0.69 ± 0.06
(24 h)	794.40 ± 225.99 ^{1,2}	0.88 ± 0.13 ^{1,2}	6.10 ± 1.44	0.53 ± 0.10

W.Weight of P: wet weight of pancreatic tissue. ¹Compared to sham operation Group (Group A), $P < 0.05$; ²Compared to pancreatitis Group (Group B), $P < 0.05$.

DISCUSSION

More and more studies have been reported to support the theory that the severity of acute pancreatitis largely depends on the degree of secondary disarrangement of inflammatory mediators^[13-16]. Damage caused by trypsin at the initial stage of acute pancreatitis is an event that triggers the systemic inflammatory response syndrome (SIRS)^[28]. The pathological process of SIRS results in the clinical manifestation and damage to other distant organs in acute pancreatitis^[29,30]. If SIRS persists and anti-inflammatory cytokines are not adequate to suppress this response, SIRS may lead to clinical sepsis and the multiple organ dysfunction syndrome, which could account for one of the main causes of death in severe pancreatitis^[31,32]. Along with the production and release of large amounts of pro-inflammatory cytokines in acute pancreatitis, anti-inflammatory cytokines (IL-10, TGF-β, IL-4, IL-13) and other immunosuppressive factors (PGE2, glucocorticosteroids) start to be synthesized and released. This process could be helpful to restrain SIRS and restore the balance between the inflammatory and anti-inflammatory responses. However, when the anti-inflammatory cytokines and other immunosuppressive factors became predominant in severe acute pancreatitis, these mediators will inhibit the immunity against pathogens, especially inhibiting the cellular immune function, and this will result in the so-called compensatory anti-inflammatory response syndrome (CARS), a secondary immunological deficiency syndrome^[33-36]. CARS seems to be related to the systemic infection and pancreatic abscess which develop during severe acute pancreatitis^[37-39]. In the present experiment, after pancreatitis was induced in animals in Group B, pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, increased promptly, and 24 h later, the anti-inflammatory cytokines IL-10 and TGF-β increased sequentially. These results indicate that there is a pro-inflammatory process (SIRS) followed by an anti-inflammatory process in acute pancreatitis. It is thus suggested that the strategy for acute pancreatitis should not only include modulation of SIRS, but also prevention of CARS.

With this knowledge, the mechanism of treatment of

acute pancreatitis with 5-FU should be evaluated further. Previously, it was thought that inhibition of exocrine secretion of the pancreas was a fundamental mechanism of treatment of acute pancreatitis with 5-FU^[1,2,9]. 5-FU traditionally is classified as an anti-metabolic agent. 5-FU is a derivant of pyrimidine, and interferes with the synthesis of DNA and RNA both in normal cells and tumor cells. 5-FU also inhibits the synthesis of protein. Essentially, 5-FU can serve as a proteinase inhibitor and exert general action throughout the whole process of acute pancreatitis. 5-FU decreases the synthesis and secretion of pancreatic enzymes. Thus, it can alleviate the damage to pancreatic tissues by auto-digestion at the initial stage. This function has been confirmed previously^[1-4]. Results from the present experiment provide evidence that 5-FU can reduce inflammation-associated cytokines. We also presume that 5-FU inhibits proteinases produced by leukocytes, which is thought a powerful factor in development of MODS. In this study, after the treatment of acute pancreatitis with 5-FU, the activity of inflammation-related cytokines was inhibited. Meanwhile, the level of serum amylase and the weight of pancreatic tissue, factors that reflect the severity of pancreatic injury and pathological lesions, were improved significantly. This indicated that 5-FU could improve the severity of acute pancreatitis by means of modulation of disarrangement of inflammation.

We cannot recommend that 5-FU could be a definite therapy for acute pancreatitis based only on the results of this experiment. We know that there is much difference between animal experiments and clinical practice with regard to results of medical research. Deterioration of acute pancreatitis is never due to simple inflammatory processes, many factors may be involved including secondary infection, derangement of blood circulation, even genetic predisposition so that clinical effects of 5-FU on acute pancreatitis need to be validated by large scale, prospective controlled studies. But we do think that 5-FU may be a candidate for treatment of SIRS based on results of this experiment. After the immunopathogenesis of sepsis following surgical disease was elucidated, many biological products were introduced for use against the pre-inflammatory cytokines^[40-43] and the prevention of SIRS, such as anti-endotoxin antibodies^[44], anti-TNF- α antibodies^[45,46], IL-1 receptor antagonists^[47-49] and monoclonal anti-interleukin 8 antibody^[50]. None of these interventions have been shown to improve the prognosis of sepsis, possibly because many patients were already in a state in which anti-inflammatory responses dominated^[51,52]. Because inflammation plays an important role in the defense against pathogenic microbes and reparation of injured tissue, there is a possibility of infection becoming lethal by excessive anti-inflammatory therapy. In our study, elevated TGF- β and IL-10 levels in an animal model of acute pancreatitis predicted the potential tendency of immunodepression. We think that the decrease of both pro-inflammatory cytokines in addition to the decrease of anti-inflammatory cytokines after treatment with 5-FU may offer a rational strategy for treatment of SIRS. As observed above, 5-FU has multiple

actions and biphasic regulation for the disarrangement of immunity in acute pancreatitis. Compared with the effect of single inflammatory cytokine blockers, treatment with 5-FU for SIRS and CARS in surgical disease may be the more effective method. Moreover, immunoregulation with 5-FU displayed in this experiment opens a new possible pathway towards the search for therapy of surgical systemic inflammatory response syndrome.

COMMENTS

Background

5-Fluorouracil (5-FU) has been used in the treatment of acute pancreatitis both experimentally and clinically since 1970, but the mechanisms of the therapeutic effect of 5-FU are not clear, and it has been considered an adjuvant therapy of acute pancreatitis. Recently, it has been increasingly clear that disarrangement of the immune system during acute pancreatitis is the determining factor in the pathophysiologic process. Abnormal inflammation-associated cytokines (pro- and anti-inflammatory cytokines) present a primary index of disarrangement of immune function during acute pancreatitis and lead to sepsis. Sepsis revealed as self-destructive inflammatory reaction remains a puzzle worldwide with respect to its pathological mechanism and corresponding preventive and therapeutic strategies for the clinicians.

Research frontiers

The hotspots of sepsis therapy research have focused on the modification of the inflammatory factors existing in sepsis, ever since the basis of sepsis injuries were revealed as self-destructive inflammatory reactions. Although with some frustrations, research is still focused on the immune regulation.

Innovations and breakthroughs

The authors designed an acute pancreatitis animal model to investigate the inhibiting effect of 5-FU on the inflammatory cytokines (TNF- α , IL-1, IL-6) and anti-inflammatory cytokines (IL-10, TGF- β) in acute pancreatitis and the relationship between the level of cytokines in serum and degree of acute pancreatitis. The experiments obtained encouraging results that the 5-FU, as an immunosuppressive agent, could be effective because of its regulation of immunity. Previously it was thought that inhibition of exocrine secretion of pancreas was a fundamental mechanism of treatment of acute pancreatitis with 5-FU. This trial reveals the immunoregulatory effect of 5-FU in the therapy of acute pancreatitis. The majority of research in the last 20 years on sepsis focused on the blocking agents of inflammatory factors, which failed in clinical trials. According to their trial, 5-FU has multiple actions and biphasic regulation for disarrangement of immunity in acute pancreatitis. Compared with the effects of single inflammatory cytokine blockers, treatment with 5-FU for SIRS and CARS in surgical disease may be the more effective method.

Applications

This trial reveals the potential of 5-FU treatment against acute pancreatitis and sepsis in the clinic. 5-FU is cheaper and safer as a typical immunosuppressive agent, and has been a familiar therapy compared to new medicines.

Terminology

5-FU is one of the first pyrimidine antagonists to be synthesized as an antimetabolite; 5-FU *in vivo* is transformed enzymatically into 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), which covalently binds and inhibits thymidylate synthase (TS) and interferes with the synthesis of nucleic acids and prevents the cell from making DNA. Another bio-transformed form of 5-FU, 5-fluorouridine-5'-triphosphate (FUTP), also incorporates itself into RNA and disrupts biological activity of RNA and protein. Systemic inflammatory response syndrome (SIRS) is the self-destructive, severe systemically inflammatory reaction of the body responding to invasion of pathogen, trauma or ischemia.

Peer review

This is an innovative study in which authors analyze the regulative effect of 5-FU on inflammatory reaction in rats. The results are encouraging and suggest that 5-FU is a potential therapeutic substance that could be used in acute pancreatitis, and sepsis caused by other etiologic factor.

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CASE REPORT

Hepatic failure caused by plasma cell infiltration in multiple myeloma

Fadi E Rahhal, Robert R Schade, Asha Nayak, Teresa A Coleman

Fadi E Rahhal, Robert R Schade, Section of Gastroenterology, Department of Medicine, Medical College of Georgia, School of Medicine, Augusta, GA 30912, United States

Asha Nayak, Teresa A Coleman, Section of Hematology/Oncology, Department of Medicine, Medical College of Georgia, School of Medicine, Augusta, GA 30912, United States

Author contributions: Rahhal FE, Schade RR, Nayak A and Coleman TA contributed equally to this work.

Correspondence to: Fadi E Rahhal, MD, Section of Gastroenterology, Department of Medicine, Medical College of Georgia, 1120 15th street, Augusta, Georgia 30912, United States. fadirahhal@yahoo.com

Telephone: +1-706-7212238 Fax: +1-706-7210331

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Abstract

Although plasma cell infiltration is not rare in autopsy of patients with multiple myeloma (MM), it is very rarely detected in living patients. This is because MM rarely causes significant liver dysfunction that requires further evaluation. A 49-year-old man presented with acute renal failure and was diagnosed with kappa light chain MM stage II B. Thalidomide and dexamethasone were initiated. The patient developed a continuous increase in bilirubin that led to severe cholestasis. A liver biopsy revealed plasma cell infiltration. He then rapidly progressed to liver failure and died. Treatment options are limited in MM with significant liver dysfunction. Despite new drug therapies in MM, those patients with rapidly progressive liver failure appear to have a dismal outcome.

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Key words: Hepatic failure; Multiple myeloma; Cell infiltration

Peer reviewer: Melanie Maria Deutsch, PhD, Department of Internal Medicine, Hippocraton General Hospital, Vasilissis Sofias 114, 11527, Athens, Greece

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INTRODUCTION

Plasma cell infiltration of the liver can be detected in up to 45% of patients with multiple myeloma (MM) at autopsy^[1,2]. However, only rare cases have been reported of massive plasma cell infiltration of the liver that leads to non-obstructive cholestasis with progression to liver failure^[3-10]. Here, we report a patient with MM with biopsy-proven plasma cell involvement of the liver. The patient died of rapidly progressive liver failure despite treatment for MM.

CASE REPORT

A 49-year-old black man presented with acute renal failure with a serum creatinine of 13 mg/dL. He was found to have circulating plasma cells in the blood and was diagnosed with kappa light chain MM by a bone marrow biopsy, which revealed 90% plasma cells with kappa light chain restriction. Free kappa light chains were noted in the serum. Cytogenetics of the bone marrow plasma cells revealed an abnormal hypodiploid clone with deletions of chromosomes 4, 13, 16, 17 and 21. A skeletal survey was negative for lytic bone lesions. The serum hemoglobin was 10 g/dL and the serum calcium was normal. Liver enzymes were normal at presentation. The physical examination was remarkable only for cachexia. Therapy with thalidomide (200 mg/d) and high dose dexamethasone (40 mg on days 1-4, 7-11, 14-17 and 21-24) was initiated. After receiving three cycles over a 3-mo period, the patient had clearance of circulating plasma cells on the peripheral blood smear but no response on repeat bone marrow biopsy. His therapy was switched to bortezomib.

One month after the initiation of thalidomide and dexamethasone, he started developing a gradual increase in total and direct bilirubin from a normal baseline. Right-upper-quadrant ultrasound revealed an enlarged liver with a largest dimension of 23 cm; however, no mass lesions were noted. The parenchymal echogenicity was within normal limits. Abdominal computed tomography performed with oral and IV contrast agents revealed a diffusely enlarged liver with no mass lesions and no evidence of intra- or extrahepatic ductal dilatation. After three cycles of thalidomide and dexamethasone therapy, the total bilirubin had increased to 3.9 mg/dL.

One week after switching to bortezomib, the patient

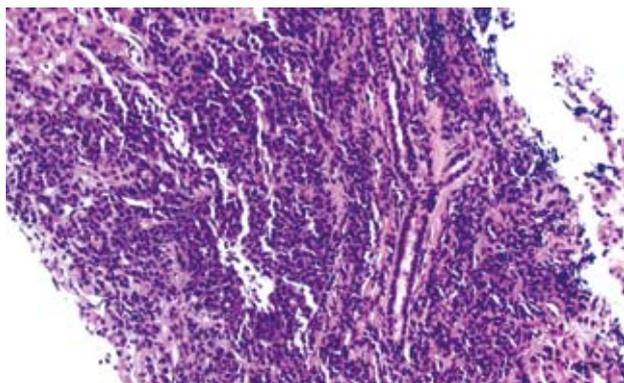


Figure 1 HE liver biopsy showing massive plasma cell infiltration (HE).

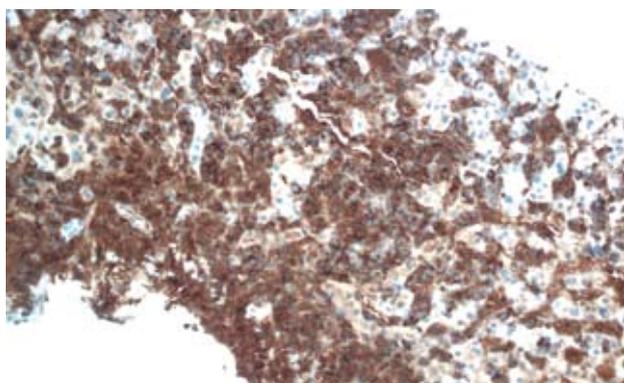


Figure 2 Positive kappa light chain stain on liver biopsy.

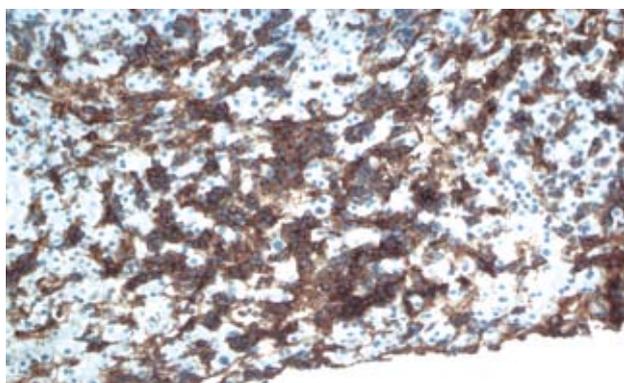


Figure 3 Positive CD138 (syndecan-1), a plasma cell marker. CD138 is expressed on plasma cells, including the malignant plasma cells of MM and some lymphomas.

was admitted to the hospital for further evaluation after the total bilirubin acutely increased to 9.4 mg/dL. The remainder of the liver function tests revealed aspartate aminotransferase of 41 U/L, alanine aminotransferase of 38 IU/L, lactate dehydrogenase of 306 IU/L, and gamma-glutamyltransferase of 286 IU/L. The direct bilirubin was 5.8 mg/dL. The physical examination was remarkable for frank jaundice and hepatomegaly. A repeat ultrasound of the abdomen showed significant liver enlargement up to 26 cm over a period of 2 mo with no evidence of biliary obstruction. A hepatobiliary imino-diacetic acid scan revealed delayed hepatic uptake consistent with severe

hepatocellular dysfunction. Hepatitis serology for hepatitis A/B/C, and antinuclear and antineutrophil cytoplasmic antibodies was negative. Cytomegalovirus serology was also negative. A transjugular liver biopsy was performed to rule out drug toxicity. It demonstrated massive infiltration of the liver parenchyma with plasma cells (Figure 1). The immunological profile of the liver biopsy revealed diffuse kappa light chain restriction that was similar to that in the bone marrow biopsy, which supported the diagnosis of MM with hepatic infiltration (Figures 2 and 3). Flow cytometry of the liver biopsy revealed plasma cells with CD38/CD138 co-expression and kappa light chain restriction. No evidence of amyloidosis was evident on immunostaining. During his 2 wk of hospitalization, the patient rapidly developed hepatic failure, characterized by coagulopathy, hyperbilirubinemia, encephalopathy and ascites. He was discharged to hospice care and expired 1 wk later.

DISCUSSION

Pathologic liver involvement in patients with MM has been reported in up to 45% of patients^[1,2]. An autopsy series of 128 cases with MM done by Perez-Soler *et al*^[3] showed diffuse infiltration of the liver by plasma cells in 10 of 21 patients with liver tissue involvement. Thomas *et al*^[4] reviewed 64 cases of MM, including autopsy reports and prior medical records; 58% of patients had hepatomegaly, defined as percussible dullness of more than 12 cm or a liver edge palpable 4 cm below the right costal margin, and 25% had splenomegaly. Jaundice was reported in nine patients (14%) and serum bilirubin values ranged from 3.2 to 17.3 mg/dL. Only six patients (9%) had a completely normal liver on pathological examination, while 40% had plasma cell involvement of the liver in the form of plasmacytoma or diffuse sinusoidal infiltration. Both these two autopsy series excluded cases with plasma cell leukemia^[3,4].

The histological pattern of liver involvement in MM can be in the form of light chain deposition disease, extramedullary plasmacytoma, amyloidosis, or a diffuse infiltrative pattern. Massive liver involvement can be either from tumor-forming plasmacytomas or diffuse sinusoidal flooding^[4]. The latter can consist of sinusoidal flooding by plasma cells of varying degrees of differentiation with little or no propensity to destroy liver parenchyma^[5]. Some of these patients may present with non-obstructive jaundice and show elevations in alkaline phosphatase from plasma cell infiltration, as did our patient^[3]. Only two prior cases of severe cholestasis associated with hepatic failure have been reported^[6,7]. Radiographic imaging is unable to detect this diffuse infiltrative pattern and a liver biopsy is essential to make a diagnosis.

Plasmacytomas (nodular forming pathology) are less common. This entity can be detected radiographically as space-occupying lesions in the liver or in the head of the pancreas, and may be associated with biliary obstruction^[8-10].

Amyloidosis manifested as tissue deposition of clonal light-chain fibrils is seen in 15% of the general MM patient population. Liver involvement has been reported

in MM patients as well as those with primary systemic amyloidosis^[11]. Elevation in alkaline phosphatase is more common and liver enzymes are often normal or mildly elevated. Obstructive jaundice occurs rarely and only a few cases have been reported, with some associated with hepatic failure^[12-18].

The clinical significance of liver involvement in MM is uncertain. Treatment of MM with hepatic involvement requires systemic therapy. Successful treatment with combination chemotherapy or steroids alone has been reported^[6,19,20]. Talamo *et al*^[21] have reviewed the medical records of 24 patients with MM involvement of the gastrointestinal (GI) system as documented by tissue biopsy, 11 of whom had hepatic involvement. GI involvement at the time of initial diagnosis of MM was less common than later in the disease course.

The treatment of MM patients with significant liver dysfunction is challenging because of the inability to deliver most chemotherapeutic agents in the setting of liver failure. Substantial dose reductions are required to administer anthracyclines and vincristine in the setting of liver failure. Steroids, and the newer agents thalidomide and bortezomib have been administered in these settings, although thalidomide has been cited in a single case report as causing fulminant hepatic dysfunction, and liver toxicity has been reported rarely with bortezomib^[22,23].

Stem cell transplantation (SCT) is now the standard first-line therapy for myeloma patients who respond to chemotherapy. Patients are analyzed to evaluate the role of treatment with high-dose chemotherapy with autologous or allogeneic SCT, and its impact on survival^[21]. It has been found that GI involvement is common during relapse after SCT. Plasmablastic morphology was common (Bartl grade III) in 29% of cases with GI involvement. Monosomy 13, which is one of the most powerful negative prognostic factors, was observed in 46% of the cases, while it is present in 15% of the general MM patient population. SCT is effective in inducing remissions but relapse is common.

In conclusion, there are no classical clinical manifestations of liver infiltration in MM. The initial presentation can be subtle, but then very rapidly progressive, as in our case. The number of clinically reported cases of liver involvement with MM is small, therefore, it is difficult to ascertain the prognosis of this clinical presentation of the disease or its response to therapy. We suspect that the prognosis is poor because of the limited number of chemotherapeutic agents that can be administered to patients with severe liver dysfunction. The optimal approach in managing these cases can only be standardized after studying a larger number of patients.

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Chemical ablation of the gallbladder using alcohol in cholecystitis after palliative biliary stenting

Tae Hoon Lee, Sang-Heum Park, Sang Pil Kim, Ji-Young Park, Chang Kyun Lee, Il-Kwun Chung, Hong Soo Kim, Sun-Joo Kim

Tae Hoon Lee, Sang-Heum Park, Sang Pil Kim, Ji-Young Park, Chang Kyun Lee, Il-Kwun Chung, Hong Soo Kim, Sun-Joo Kim, Division of Gastroenterology, Department of Internal Medicine, Soonchunhyang University College of Medicine, Cheonan Hospital, 23-20 Bongmyung-dong, Cheonan 330-721, South Korea

Author contributions: Lee TH, Chung IK, and Park SH contributed the design of the study; Lee TH performed all of the procedures described in the paper; Kim SP, Park JY, Lee CK and Kim HS provided the clinical advice; Park SH and Kim SJ edited the manuscript; Lee TH wrote the paper.

Correspondence to: Tae Hoon Lee, MD, Division of Gastroenterology, Department of Internal Medicine, Soonchunhyang University College of Medicine, Cheonan Hospital, 23-20 Bongmyung-dong, Cheonan 330-721, South Korea. thlee9@lycos.co.kr

Telephone: +82-41-5703675 Fax: +82-41-5745762

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Abstract

Chemical ablation of the gallbladder is effective in patients at high risk of complications after surgery. Percutaneous gallbladder drainage is an effective treatment for cholecystitis; however, when the drain tube cannot be removed because of recurrent symptoms, retaining it can cause problems. An 82-year-old woman presented with cholecystitis and cholangitis caused by biliary stent occlusion and suspected tumor invasion of the cystic duct. We present successful chemical ablation of the gallbladder using pure alcohol, through a percutaneous gallbladder drainage tube, in a patient who developed intractable cholecystitis with obstruction of the cystic duct after receiving a biliary stent. Our results suggest that chemical ablation therapy is an effective alternative to surgical therapy for intractable cholecystitis.

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Key words: Percutaneous cholecystostomy; Cholecystitis; Biliary stenting; Alcohol; Chemical therapy

Peer reviewer: Nahum Méndez-Sánchez, MD, PhD, Departments of Biomedical Research, Gastroenterology & Liver Unit, Medica Sur Clinic & Foundation, Puente de Piedra 150, Col. Toriello Guerra, Tlalpan 14050, México City, México

Lee TH, Park SH, Kim SP, Park JY, Lee CK, Chung IK, Kim HS, Kim SJ. Chemical ablation of the gallbladder using alcohol in cholecystitis after palliative biliary stenting. *World J Gastroenterol* 2009; 15(16): 2041-2043 Available from: URL: <http://www.wjgnet.com/1007-9327/15/2041.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.2041>

INTRODUCTION

Tumor obstruction of the cystic duct is a known risk factor for the development of cholecystitis following biliary stent placement. Percutaneous cholecystostomy is an effective treatment for such cholecystitis^[1,2]. However, recurrent cholecystitis or retractable symptoms may be troublesome. Recently, chemical ablation of the gallbladder has been shown to be effective in patients at high risk for complications after surgery^[3]. Absolute alcohol or 95% ethanol causes necrosis and fibrosis in the gallbladder epithelium, which reduces the gallbladder to a shrunken fibrous remnant^[4].

However, until now there have been few human studies of which sclerosants are safe and feasible, and for how long the sclerosant has to be in contact with the mucosa.

In this report, we describe the successful chemical ablation of the gallbladder in a patient who developed intractable cholecystitis with obstruction of the cystic duct, after undergoing palliative stenting for the management of a malignant biliary obstruction.

CASE REPORT

An 82-year-old woman presented with gradually aggravated right upper-abdominal pain after undergoing biliary stent implantation for the palliative management of a cholangiocarcinoma 2 wk previously. Upon presentation, clinical examination revealed severe tenderness of the right upper abdomen without rebound tenderness. Laboratory tests revealed the following: white blood cell count, $14.380 \times 10^9/L$ (normal $4.0-10.8 \times 10^9/L$); total bilirubin, 5.4 g/dL (normal 0.1-1.0 g/dL); aspartate aminotransferase, 118 IU/L (normal < 40 IU/L); alanine aminotransferase, 118 IU/L (normal < 40 IU/L); alkaline phosphatase, 118 IU/L (normal 39-117 IU/L); and carbohydrate antigen 19-9, 98 U/mL (normal $<$



Figure 1 Abdominal CT scan revealing a markedly enlarged and distended gallbladder with a thickened wall.

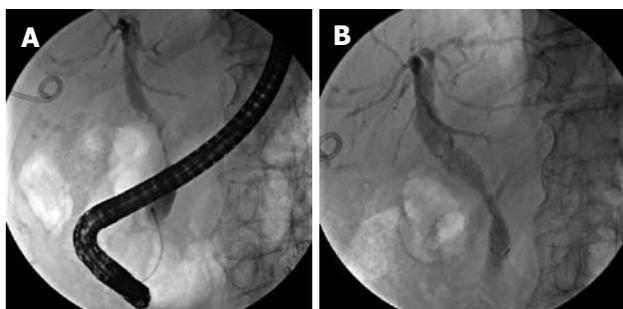


Figure 2 Cholangiographic findings. A: Endoscopic retrograde cholangiopancreatography showed a severe irregular segmental stricture at the mid-CBD, without visualization of the cystic duct; B: A covered metal stent, 60 mm in length, was implanted in the narrowed CBD.

34 U/mL). Abdominal computed tomography (CT) revealed a markedly enlarged and distended gallbladder with a thickened wall (Figure 1). Clinically, both cholecystitis and cholangitis were suspected, based on CT and laboratory data. Following decompression of the gallbladder via percutaneous cholecystostomy, endoscopy using a duodenoscope (TJF 240; Olympus, Tokyo, Japan) was performed, which showed a completely occluded plastic biliary stent. The occluded stent was subsequently removed. Cholangiography showed a severe irregular segmental stricture at the mid common bile duct (CBD), without visualization of the cystic duct, a finding that indicated cystic duct occlusion caused by tumor invasion (Figure 2A).

For the management of cholecystitis and malignant stricture, the percutaneous drainage tube was left in place and a covered metal biliary stent (Niti-S; Taewoong Medical Co., Ltd., Seoul, Korea), 60 mm in length, was implanted through the peroral route (Figure 2B). Seven days later, the percutaneous cholecystostomy was draining less than 50 mL/d; therefore, removal of the drain tube was attempted. However, the patient complained of recurrent abdominal pain and discomfort whenever the drain tube was closed, and the amount of fluid draining continued at a rate of > 40 mL/d. Consequently, removal of the drain tube failed. As a result of the patient's advanced age and her refusal of palliative cholecystectomy, medical ablation of the

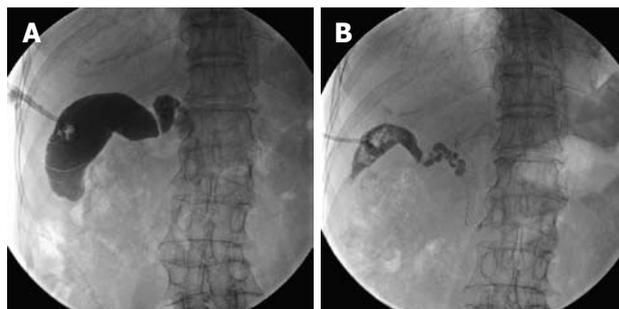


Figure 3 Chemical ablation therapy. A: Before chemical ablation of the gallbladder, the gallbladder volume was measured by infusing contrast medium through the drain tube; the contrast medium did not pass into the CBD; B: Three weeks later (after a total of three chemical ablation sessions), cholecystography showed a marked collapse in the lumen of the gallbladder.

gallbladder was considered to be a good option for treating the symptoms and to allow the removal of the percutaneous drain tube.

After informed consent from the patient and approval by the ethics committee of our hospital, 99% absolute ethanol was used as a sclerosant for chemical ablation of the gallbladder. The volume of the gallbladder was measured by filling it with contrast medium, followed by aspiration (Figure 3A). Absolute ethanol, 1-2 mL less than the volume of the gallbladder, was infused into the gallbladder through the drain tube. Initially, a total of 55 mL of ethanol was infused and the drainage tube was closed. Then, the patient changed positions every 10 min for a total of 30 min, and the sclerosant was drained. Cholecystography was performed 1 wk after the first chemical ablation, and it showed a decrease in the size of the gallbladder. The same method was repeated twice more in weekly sessions with 40 and 25 mL of ethanol, respectively. During the final week, a small amount of bright yellow fluid (< 10 mL/d) was draining, and cholecystography showed a marked collapse in the lumen of the gallbladder (Figure 3B). During each procedure, the patient's vital signs were closely monitored. The patient had no abdominal pain or other complications related to the procedure. The cholecystostomy drain tube was removed, and there were no complications in the following 8 wk, during which she remained under outpatient observation.

DISCUSSION

Endoscopic insertion of biliary stents is a well-established palliative treatment for obstructive jaundice caused by unresectable malignant disease^[5-7]. As a result of increased use, complications such as cholangitis, cholecystitis, pancreatitis, stent migration, and stent occlusion are being reported increasingly^[8]. In particular, cholecystitis has been reported in 1.9%-12% of stent insertion cases^[9,10]. For several reasons, obstruction of the cystic duct by a tumor is a risk factor for the development of cholecystitis following biliary stent placement^[2].

Although cholecystectomy is a safe and effective treatment in patients with cholecystitis, the morbidity

and mortality of this operation increases considerably in the elderly and unfit patients who often have concomitant diseases. Percutaneous gallbladder drainage or aspiration, transpapillary gallbladder drainage, and endoscopic-ultrasound-guided gallbladder drainage have been reported for the management of cholecystitis after stent placement or for cystic duct invasion by a tumor^[1,2,11]. However, in cases such as those reported here, when the drain tube cannot be removed because of recurrent symptoms, retaining it causes problems for the patient, and its experimental removal may cause other complications.

Chemical ablation of the gallbladder may be a useful alternative to cholecystectomy in high-risk patients or in those who refuse surgery. Recently, experimental studies on chemical ablation of the gallbladder *in vitro* and *in vivo* have demonstrated that many sclerosants, including 95% ethanol, 3% sodium tetradecyl sulfate, 5% tetracycline, and 5% trifluoroacetic acid, ablate gallbladder mucosa^[3,12-14]. Oh *et al*^[15] used 99.9% ethanol for the chemical ablation of cystic tumors of the pancreas. Xu *et al*^[3] reported that minicholecystostomy followed by chemical ablation of the gallbladder was safe and effective. In that study, 95% ethanol was in contact with the gallbladder mucosa for 30 min every 4 h, for a total of eight times after occlusion of the cystic duct. A suitable chemical for gallbladder mucosal ablation must be safe, effective, and require brief contact time with the mucosa. However, there have been few human studies to determine which sclerosants are feasible and the duration for which the sclerosant must be in contact with the mucosa. Some studies have reported complications, including mucocele, gallbladder hydrops, abscess formation, and perforation; however, there have been no serious, life-threatening complications^[3,4,12-14].

In our case, we used absolute ethanol as a chemical sclerosant. In animal and human studies, alcohol has been found to be safe and has resulted in few complications; however, it requires more treatments of longer duration than other sclerosants. More studies are needed to determine which sclerosants are suitable, how often they need to be applied and at what interval, and the contact duration required for chemical ablation. Absolute ethanol is a safe sclerosant and procedure-related complications did not develop during the procedure or follow-up period. Especially in the case of cystic duct obstruction by tumors, this method is easy and feasible, and is not affected by the presence of additional biliary stents. However, if the cystic duct is patent, the use of chemical agents is restricted and another approach is necessary.

In summary, following development of cholecystitis after stent placement, with tumor obstruction of the cystic duct, or in patients with recurring symptoms,

chemical ablation using absolute ethanol may be an alternative to percutaneous cholecystostomy or surgical cholecystectomy. Further investigation of this technique and the identification of suitable sclerosants are necessary, and long-term follow-up should be conducted.

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S- Editor Li LF L- Editor Kerr C E- Editor Yin DH

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Alexandra A Alexopoulou, MD

2nd Department of Internal Medicine, University of Athens, Medical School, Hippokraton General Hosp, 40 Konstantinoupolos St, 16342 Hilioupolois Athens, Greece

Chi-Hin Cho, B.Pharm, PhD, Professor of Pharmacology & Chairman

Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

Dr. Olivier Detry

Department of Abdominal Surgery and Transplantation, University of Liège, CHU Sart Tilman B35, B-4000 Liège, Belgium

Dr. Deborah L Diamond

Department of Microbiology, University of Washington, Box 358070, Seattle, WA 98195-8070, United States

Keiji Hirata, MD

Surgery 1, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Dr. Jörg C Hoffmann, MA, Priv, Doz, MD, Chief of the Medizinischen Klinik I,

MIT Schwerpunkt Gastroenterologie, Diabetologie, Rheumatologie und Onkologie, St. Marien- und St. Annastiftskrankenhaus, Salzburger Straße 15, D67067 Ludwigshafen, Germany

Toru Ishikawa, MD

Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Dr. Ralf Jakobs, Professor,

Director of the Department of Medicine C (Gastroenterology), Klinikum Ludwigshafen, Bremsersstrasse 79, 67063 Ludwigshafen, Germany

Juan-Ramón Larrubia, PhD

Gastroenterology Unit and Liver Research Unit., Guadalajara

University Hospital, Donante de Sangre s/n, 19002 Guadalajara, Spain

John M Luk, Associate Professor

Department of Surgery, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong, China

Didier Merlin, PhD, Associate Professor

Department of Medicine Division of Digestive Diseases, Emory University, 615 Michael Street, Atlanta, GA 30322, United States

Carlos J Pirola, PhD, FAHA

Instituto de Investigaciones Medicas A Lanari, Combatientes de Malvinas 3150, Buenos Aires-1427, Argentina

Ramesh Roop Rai, MD, DM (Gastro.), Professor & Head

Department of Gastroenterology & Hepatology, S.M.S. Medical College & Hospital, Jaipur 302019, (Rajasthan), India

Dr. Richard A Rippe

Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Riina Salupere, MD, PhD

Division of Endocrinology and Gastroenterology, University of Tartu, L.Puusepa street 6, Tartu 51014, Estonia

Ala Sharara, MD, FACP Associate Professor of Medicine, Head

Division of Gastroenterology, Director, Endoscopy Unit, American University of Beirut Medical Center, Associate Consulting Professor, Duke University Medical Center, PO Box 11-0236, Riad El Solh 110 72020, Beirut, Lebanon

Dr. Karel van Erpecum

Department of Gastroenterology and Hepatology, University Hospital Utrecht, PO Box 855003508 GA, Utrecht, The Netherlands

Siegfried Wagner, Professor

Medizinische Klinik II, Klinikum Deggendorf, Perlasberger Str. 41, Deggendorf 94469, Germany

George Y Wu, Professor

Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, 263 Farmington Ave, Farmington, CT 06030, United States

Harry HX Xia, PhD, MD

Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936-1080, United States

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/>

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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.apdwcgress.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



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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.

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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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Za-zhi* 1999; **7**: 285-287

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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 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]

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- 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]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary 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. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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