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EDITORIAL

Microsatellite instability and MLH1 promoter hypermethylation in colorectal cancer

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Abstract

Colorectal cancer (CRC) is caused by a series of genetic or epigenetic changes, and in the last decade there has been an increased awareness that there are multiple forms of colorectal cancer that develop through different pathways. Microsatellite instability is involved in the genesis of about 15% of sporadic colorectal cancers and most of hereditary nonpolyposis cancers. Tumors with a high frequency of microsatellite instability tend to be diploid, to possess a mucinous histology, and to have a surrounding lymphoid reaction. They are more prevalent in the proximal colon and have a fast pass from polyp to cancer. Nevertheless, they are associated with longer survival than stage-matched tumors with microsatellite stability. Resistance of colorectal cancers with a high frequency of microsatellite instability to 5-fluorouracil-based chemotherapy is well established. Silencing the MLH1 gene expression by its promoter methylation stops the formation of MLH1 protein, and prevents the normal activation of the DNA repair gene. This is an important cause for genomic instability and cell proliferation to the point of colorectal cancer formation. Better knowledge of this process will have a huge impact on colorectal cancer management, prevention, treatment and prognosis.

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Key words: MLH1; Methylation; Colorectal cancer; Microsatellite instability; CpG island methylator phenotype; Chromosomal instability

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Colorectal cancer (CRC) is caused by a series of genetic or epigenetic changes, and in the last decade there has been an increased awareness that there are multiple forms of CRC that develop through different pathways. The common mechanism (more than 50% of sporadic CRCs) is that of chromosomal instability (CIN)[1-3]. The second pathway (35% of sporadic CRCs) is caused by epigenetic inactivation of tumor suppressor genes. Because of the mechanism involved in this, it is called the CpG island methylator phenotype (CIMP). A third pathway is caused by failure of the DNA mismatch repair system, and these tumors have a characteristic signature mutation called microsatellite instability (MSI), MSI-high (MSI-H), MSI-low (MSI-L) or MSI-stable (MSS). Recently, Jass classified CRCs according to CpG island methylator phenotype and MSI status into 5 new types: (1) CIMP-high, MSI-H, BRAF mutation; (2) CIMP-high, MSI-L or MSS, BRAF mutation; (3) CIMP-low, MSS or MSI-L, KRAS mutation; (4) CIMP-negative, MSS; (5) CIMP-negative, MSI-H (Lynch Syndrome)[4]. There were clinical, morphological and prognostic factors characteristic of every type, thus Jess concluded that this approach will have a tremendous impact on management, prevention and treatment of CRC.

Microsatellite instability is involved in the genesis of about 15% of sporadic CRCs and most of hereditary nonpolyposis CRCs (HNPCC)[5-7]. The multiple errors in repetitive DNA sequences (microsatellites) result from a failure of the DNA mismatch repair (MMR) system to edit errors made during DNA replication[8]. The DNA MMR system is inactivated either by hypermethylation of the promoter, which silences gene transcription of hMLH1 (epigenetic phenomenon; sporadic CRC), or because of germ-like mutations in MMR genes MLH1, MSH2, MSH6 and others (genetic phenomenon, HNPCC)[9,10]. Recently, Hitchins and colleagues described an inheritance of a cancer-associated MLH1 germ-line epimutation[11]. They found evidence that MLH1 promoter hypermethylation of one allele was transmitted from a mother to her son but was erased in his spermatozoa, thus the allele had reverted to the normal active state.

Tumors with a high frequency of MSI-H tend to be diploid, to possess a mucinous histology, and to have a surrounding lymphoid reaction. They are more prevalent in the proximal colon and have a fast pass from polyp to cancer. Nevertheless, they are associated with longer survival than stage-matched MSI-L tumors or MSS tumors[12-18]. It is still unclear if this favorable prognosis is attributable to the inherently lower aggressiveness of MSI-H tumors or their greater sensitivity to chemotherapy

other than 5-fluorouracil (5-FU).

Resistance of MSI-H CRC to 5-FU based chemotherapy is well established[19]. A significant overall survival benefit was noted in patients with CRC who were treated with 5-FU based chemotherapy compared with those who were not. However, within the MSI-H group, there was no difference in survival by 5-FU treatment, whereas in the MSS group, the authors noted a significant difference in survival between patients given 5-FU and untreated patients. These findings indicate that the type of genomic instability within a colorectal tumor might dictate patient response to 5-FU based chemotherapy[20]. Ribic and co-investigators[21] used specimens from patients with stage Ⅱ or Ⅲ CRC who were previously enrolled in prospective, randomized trials of 5-FU based chemotherapy. Among the patients who had not received adjuvant chemotherapy, those with MSI-H tumors had longer overall survival and higher rates of 5-year disease-free survival than patients with MSI-L or MSS tumors. MSI-H status in patients who did not receive 5-FU based adjuvant chemotherapy was significantly associated with a better survival. However, among the group with MSI-H tumors, treatment was associated with a worse outcome for both stage Ⅱ and Ⅲ cancers.

Recently, an association between JC virus and CRC has been reported[22]. The virus may act by stabilizing β-catenin, facilitating its entrance to the cell nucleus and triggering proliferation and cancer formation. Another possibility is initiation of hMLH1 methylation, since association between methylation and exposure to carcinogens such as viruses has been observed[23].

In summary, MLH1 promoter hypermethylation is an important event, silencing the MLH1 gene expression and preventing the formation of MLH1 protein and normal activation of the DNA repair gene. This induces genomic instability and cell proliferation to the point of CRC formation. Better knowledge of this process will have a significant impact on CRC management, prevention, treatment and prognosis.

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REVIEW

A comparative review of HLA associations with hepatitis B and C viral infections across global populations

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Abstract

Hepatitis B (HBV) and hepatitis C (HCV) viral infection or co-infection leads to risk of development of chronic infection, cirrhosis and hepatocellular carcinoma (HCC). Immigration and globalization have added to the challenges of public health concerns regarding chronic HBV and HCV infections worldwide. The aim of this study is to review existing global literature across ethnic populations on HBV and HCV related human leukocyte antigen (HLA) associations in relation to susceptibility, viral persistence and treatment. Extensive literature search was conducted to explore the HLA associations in HBV and HCV infections reported across global populations over the past decade to understand the knowledge status, weaknesses and strengths of this information in different ethnic populations. HLA DR13 is consistently associated with HBV clearance globally. HLADRB1*11/*12 alleles and DQB1*0301 are associated with HBV persistence but with HCV clearance worldwide. Consistent association of DRB1*03 and *07 is observed with HCV susceptibility and non-responsiveness to HBV vaccination across the population. HLA DR13 is protective for vertical HBV and HCV transmission in Chinese and Italian neonates, but different alleles are associated with their susceptibility in these populations. HLA class Ⅰ molecule interactions with Killer cell immunoglobulin like receptors (KIR) of natural killer (NK) cells modulate HCV infection outcome *via* regulating immune regulatory cells and molecules. HLA associations with HBV vaccination, interferon therapy in HBV and HCV, and with extra hepatic manifestations of viral hepatitis are also discussed. Systematic studies in compliance with global regulatory standards are required to identify the HLA specific viral epitope, stage specific T cell populations interacting with different HLA alleles during disease progression and viral clearance of chronic HBV or HCV infections among different ethnic populations. These studies would facilitate stage specific therapeutic strategies for clearance of HBV and HCV infections or co-infections across global populations and aid in identification of HBV-HCV combined vaccine. HLA associations of chronic HBV or HCV development with confounding host factors including alcohol, drug abuse, insulin resistance, age and gender are lacking and warrant detailed investigation across global populations.

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Key words: Human leukocyte antigen; HBV persistence; HCV persistence; Interferon response to HBV and HCV; HBV vaccination response

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INTRODUCTION

Hepatitis B (HBV) together with hepatitis C (HCV) accounts for 75% of liver diseases and are regarded a major threat to public health worldwide. Hepatitis B and C co-infections have raised major concern in HIV, transplant and other immunosuppressed patients. Intravenous drug abuse is currently the main risk but nosocomial infection is also of concern for HBV and HCV infection. Three independent factors seem to be associated with fibrosis: age, daily alcohol consumption and male gender. Over two billion people are expected to be infected with HBV during their lifetime and about 350 million are estimated to be chronic carriers[1]. Chronic carriers develop life-threatening liver cirrhosis or HCC. Hepatitis C infected patients have greater chances to develop chronic hepatitis and liver cancer. About 54% to 86% of infected individuals develop chronic manifestation, and females and children tend to have lower rate of chronicity. It is estimated that about 200 million people around the world are HCV infected. 4.1 million (1.6%) Americans have been infected with HCV, of whom 80% are chronically infected[2].

The mechanism of HBV and HCV pathogenesis remains elusive. Host genetic factors are proposed to be governing the pathology of disease progression or

regression along with the viral and environmental factors. Interplay of HLA restricted T lymphocytes, antibody-secreting B-lymphocytes, NK cells and cytokines, conditions the immune response to viral infections. Effective presentation of viral antigens to CD4+ T cells and CD8+ T cells by HLA class Ⅱ and class Ⅰ molecules respectively, is the key regulation of optimum immune response against viral infection, and further dictates viral clearance or persistence. Inconsistency is observed in response to HBV vaccination and interferon treatment following HBV and HCV infection. Varied response to vaccination is implicated to specific combinations of polymorphic HLA class Ⅱ alleles that influence the capacity of HLA class Ⅱ molecules to bind and present antigen to CD4+ cells that augment antibody production and cytotoxic T lymphocytes activation. Numerous clinical and laboratory investigations have identified several immunogenetic factors involved in conditioning viral hepatitis. Gene polymorphisms and heterozygosity at HLA loci enable HLA molecules to present a wide array of antigens and diversify the properties of HLA molecules with respect to antigen binding and presentation[3].

HLA associations with respect to HBV and HCV infection susceptibility, protection, disease severity, interferon treatment response and response to vaccination have been intensively investigated across the global populations. We have attempted to review the published literature on HLA associations with HBV and HCV infections with the purpose of identifying common HLA allele association with hepatitis B and C. Convergence of this information would provide but not be limited to (a) insight into the immunopathogenesis of HBV-HCV co-infections and superinfections, (b) identification of genetic markers to predict the course of infection/treatment response and (c) enable designing of combined vaccine strategies for viral hepatitis B and C.

HLA ALLELE ASSOCIATIONS WITH HEPATITIS B INFECTION IN GLOBAL POPULATIONS

Viral persistence and viral clearance

Viral clearance (acute infection) or persistence (chronic infection) is conditioned by immune response mechanisms regulated by HLA molecules. HLA associations with susceptibility and protection from persistent HBV infection, though robust, are inconsistent even within the same population. HLA allele associations with HBV clearance and persistence in global population are outlined in Tables 1 and 2.

HLA Class Ⅰ allele associations: susceptibility to HBV infection and chronicity is attributed to HLA A*0206 allele in Taiwanese, B35 in Chinese[4], B18, B35, B40, Cw3 allele in Russian, A3 and B18 in Kazakhs[5] B8-Cw-7 haplotype in Senegalese[6], and HLA A*01-B*08-DRB1*03, B-44-Cw1601 and B*44-Cw*0501 haplotype in American Caucasians[7] (Table 1). Also, HLAB8 in European Caucasians is associated with non-response to HBV vaccination (Table 3) indicating inefficient immune response against HBV antigen in HLA B8 carriers[7].

HLA B61 in Taiwanese[4], A*26 in Japanese[8] and A*0301 in American Caucasians[7] are protective (Table 2). HLA A2 and A11 are protective alleles for HBV chronicity in Russians and Kazakhs respectively[5]. HLA A-24 and Cw1 are related to lower risk for chronic manifestations of HBV infection in Turkey[9].

HLA Class Ⅱ allele associations: HLA class Ⅱ alleles HLA DRB1*13 and HLA DRB1*11/*12 are consistently associated with viral clearance and viral persistence of HBV infection respectively in major populations (Table 2). In contrast HLA DRB1*11/*12 alleles are associated with HBV clearance in Chinese[10,11] and HLA DRB1*13 is reported as a susceptibility gene for chronic HBV infection in Turkish populations[9]. HLA DR7 and DRB1*15 are also protective for HBV infection in Chinese but associated with chronic infection in Turkish and Indian populations respectively[9,12-14]. HLA DR15 is also reported to be associated with responsiveness to HBsAg vaccine in European Caucasian populations (Table 4). These variations observed in HLA allele associations are confounding and suggest influence of other genes on the effect of these alleles on disease outcome.

HLA DR13 is associated with protection from vertical transmission of HBV and HCV in Chinese and Italian infants[15-17] and with HBV clearance across the population (Table 2). The protectiveness of HLA DR13 is proposed to be either due to proficient antigen presentation by DR13 molecules or linked polymorphisms in neighboring immune regulatory gene.

Hepatitis B viral persistence and disease chronicity is associated with HLA DQA1*0501 and HLA DQB1*0301 in Chinese and African Americans[10,18] and with HLA DR9 in Chinese and Koreans[11,19]. In Chinese populations HLA DR3 is associated with HCV persistence and vertical transmission[10,15]. Pellegris *et al*[20]studied HBV and HCV related HCC in Italian subjects, 73 with liver cirrhosis and 32 without liver cirrhosis. They have suggested the possibility of HLA DQ1 allele association with susceptibility to liver cell destruction and HLA DR3 with absence of cell destruction in HCC subjects showing liver cirrhosis and no cirrhosis respectively.

HLA Class Ⅲ allele associations: Polymorphisms in cytokine genes influence outcome of HBV infection. In Chinese populations tumor necrosis factor-alpha (TNF)alpha-857GG is associated with self-limiting infection while TNF-alpha-857 CC and -238G/A polymorphism[21,22] with chronic HBV infection. Polymorphism at position -238 G/A in TNF alpha is associated with chronic HBV infection and defective viral clearance[23] in German Caucasians. The carriers of the -592A allele in IL-10 promoter and -308G/-238G haplotype homozygotes in the TNF-alpha promoter region have higher risk of persistent HBV infection in Koreans[24]. In Italian patients the genotype -308G/G and haplotype TCGG (-T1031, -C863, -G308, -G238) are associated with an unfavorable prognosis in those with chronic HBV infection[25]. TNF-alpha promoter polymorphism -308A is common in Iranian population but is not associated with hepatitis B disease chronicity[26]. IL18 polymorphism at position -137G is associated with chronic HBV infection while -137C is protective in Chinese. Polymorphism at

Table 1 Association of HLA alleles with Chronic HBV infection across global population

HLA	Effect	Country and cases	Healthy controls	Recovered	Chronic infection	Reference
HLA Ⅰ						
HLA A*0206	VP	Taiwan (Taiwanese Aborigines)	34	229	138	[4]
HLA B-35	VP	Taiwan (Han Chinese)	98	324	98	[4]
HLA A*2	VP/Disease severity	Japan				[8]
HLA B18, B35, B40, Cw3	Susceptibility to chronic HBV infection	Russia				[5]
HLA A3, B18	Susceptibility to chronic HBV infection	Kazakh				[5]
HLA: B8-Cw7 haplotype	Liver cancer associated with HBV infection	Senegal	96		98	[6]
HLA B*8	VP	USA (Caucasians)		342	194	[7]
HLA A*01-B*08-DRB1*03, B*44-Cw*1601 and B*44-Cw*0501 haplotype	VP	USA (Caucasians)		342	194	[7]
HLA Ⅱ						
HLA DRB1*1202#	VP	Taiwan (Han Chinese)	98	324	98	[4]
HLA DQA1*0302	VP	China		148 (R), 212 (AC)	207	[113]
HLA DRB1*0301, DQA1*0501, DQB1*0301	VP	China	106	30	54	[10]
HLA DR9, DQ9	VP	China		56	30	[11]
HLA-DRB1*06, DRB1*08, and DRB1*16	VP	China	200		72	[14]
HLA DR9	VP	Korea (ESRD patients)	946	243	83	[19]
HLA DRB1*11# and DRB1*15	VP	India	100		26	[12]
HLA DQA1 *0501, DQB1 *0301	VP	USA (African American)		60	31	[18]
HLA DR3	Susceptibility to HBV infection & vertical transmission	China (Pregnant women)	40		78	[15]
HLA-DRB1*1201/1202#	Susceptibility to liver cirrhosis	China (Cirrhosis subjects from Hubei area)	108		116	[13]
HLA B13, B8, DR7, DR13, DQ3	Susceptibility to chronic HBV infection	Turkey	50		50	[9]
HLA DQA1 0501, DQB1 0301, DRB1 1102# haplotype	VP	USA (African American)		60	31	[18]

VP: viral persistence/chronic infection; AC: Asymptomatic carriers; Common VP alleles: DQB1*0301, #: DRB1*11/12; ESRD: End stage renal disease; R: Recovered; HBV Hepatitis B virus.

position -137C may be associated with higher production of IL18 that augments IFN-gamma production and thus better immune response against HBV infection[27]. Genetic ability to produce low levels of IFN-gamma is related to susceptibility for chronic HBV infection[28]. Polymorphism of IL10 promoter at position -819T/C and -592A/C is related to chronic infection[29]. Decreased IL10 production owing to -819T and -592A allele contributes to the asymptomatic carrier state and thus is relevant for disease progression in Japanese[30]. Frodsham *et al*[31]have identified an HLA class Ⅱ cytokine receptor gene cluster as a major susceptibility locus for HBV infection *via* genome analysis in Gambian populations. Polymorphism at the type Ⅰ IFN receptor gene, IFN-AR2, and the IL-10RB gene is associated with viral clearance. TNF-alpha polymorphism at position -244G/A is not present in American Caucasian and Egyptian hepatitis B patients but is present in Egyptian healthy individuals suggesting its protective role[32].

Response to HBV vaccination

Variegated antibody response following standard HBV vaccination in otherwise healthy individuals is observed, with 5%-10% showing no response. It is suggested that non-responsiveness may also be associated with antigen-specific HLA determined deficiency in the T cell repertoire and not only with defective antigen presentation or HLA class Ⅱ affinity for hepatitis surface antigen (HbsAg) derived peptide[33]. Clinical evidence suggests strong association of HLA genes with response to HBV vaccination in the global population, which is outlined in Tables 3,4 and 5. Martinetti *et al*[34] explored HBV vaccination response in neonates to identify the genetic predisposing factors influencing the response.

HLA class Ⅰ allele associations: HLA class Ⅰ and class Ⅱ molecules are synergistically involved in the immune response to recombinant HbsAg, with a stronger association of HLA DRB1 locus[35]. HLA class Ⅰ loci (A, B and C) association with vaccine responsiveness is not clear and is considered mainly due to its strong linkage disequilibrium with HLA DR locus. Non-responsiveness to HbsAg vaccination is reported to be associated with HLA A1, B15, and B40 in Indians of Asian origin, HLA A1, A2, B8, and B7 in Caucasians and HLA B54 in Chinese. Responsiveness is associated with HLA A11 in Indians of Asian origin and A10, A1 and B7 in Caucasians (Tables 3 and 4).

HLA class Ⅱ allele associations: HLA DR alleles in several populations are major determinants of response to HBV vaccination. Clinical studies have supported HLA DR3 and DR7 association with non-responsiveness to HBsAg vaccination (Table 3). HLA haplotype

Table 2 Association of HLA allele with protection from chronic HBV infection across global population

HLA	Effect	Country and cases	NC	R	CI	Reference
HLA I HLA B61	Early HBeAg seroconversion	Taiwan (chronically infected children)			81	[114]
HLA A*26	No disease progression in HBV carriers	Japan				[8]
HLA A-24 and Cw1	Low risk for HBV related chronic disease	Turkey	50		50	[9]
HLA A2	VC	Russia				[5]
HLA A11	VC	Kazakh				[5]
HLA A*0301	VC	USA (Caucasians)		342	194	[7]
HLA II						
HLA DRB1*4001	VC	Taiwan (Taiwanese Aborigines)	98	324	98	[4]
HLA DRB1*0406, DRB1*0701	VC	Taiwan (Han Chinese)	98	324	98	[4]
HLA DQA1*0301, DQA1*0102	VC/AC	China		148 (R) & 212 (AC)	207	[113]
HLA DRB1*1101/ *1104, DQA1*0301	VC	China	106	30	54	[10]
HLA DR12 (DRB1*1201)	VC	China		56	30	[11]
HLA-DRB1*07	VC/Protection from HBV infection.	China	200		72	[14]
HLA DR6 (DRB1*13)	VC	Korea (ESRD patients)	946	243	83	[19]
HLA DRB1*13	VC	India	100		26	[12]
HLA DRB1*1301 and DRB1*1302	VC	Gambia (children & Adult men)	891 & 25	218 & 195	185 & 40	[110]
HLA DRB1*1301/1302	VC	Spain	24	11	38	[115]
HLA DRB1*1301/1302	VC	Germany	101		117	[116]
HLA DR13 (DRB1*13)	VC	Germany	208	33	32	[117]
HLA DQB1*0503	Early HBeAg seroconversion	Taiwan (Chronically infected children)			81	[114]
HLA DR13	Resistance to HBV infection & vertical transmission	China (Pregnant women)	40		78	[15]
HLA-DRB1*1501/1502	Protection from liver cirrhosis	China (Cirrhosis subjects from Hubei area)	108		116	[13]
HLA DR5	No disease progression in HBV carriers	Japan				[8]

VC: Viral clearance/self limiting infection, AC: Asymptomatic carriers; Common VC alleles: DRB1*13; ESRD: End Stage Renal Disease; R: Recovered; HBV: Hepatitis B virus.

Bw54CREG, C4RFLP (6.5 kb + 12.0 kb)-DR4-DRw53-DQw4, (DQA1*0301-DQB1*0401) is in increased frequency in Japanese non-responders (NR) while HLA group DR1, DRw6, DQw1 are in low frequency in the non-responders[36] to HbsAg vaccine. HLA DR3, DR4, DR7, DR13 (DRB1*1302), DR14, DR16 is associated with non-responsiveness and HLA DR1, DR5, DR11, DR13 (DRB1*1301) and DR15 with responsiveness (Tables 3 and 4) to HBV vaccination across the population. HLA DR alleles associations were not reported in Indian (Asian) populations. Higher frequency of HLA DR4, DR7 and DR14 is associated with non-responsiveness and HLA DR1 with responsiveness in Japanese, Chinese and Caucasian populations (Tables 3 and 4). HLA DR10 and DR51 association with HBV vaccine responsiveness is reported only in Indian (Asian) population[37]. HLA DRB1*13 alleles carriers are hyper responsive to anti measles vaccine and HLA DR7 with non-responsiveness, resembling the pattern of response for recombinant HbsAg vaccine[38].

HLA DP alleles -DPB1*1101 with non-responsiveness, DPB1*0401 with responsiveness- are significant in determining the HBV vaccination response[39] in Belgian populations. HLA DQ2 (DQB1*02) is predominantly associated with non-responsiveness to HBV vaccine in Indian (Asian) and Caucasian populations (Table 3). HLA DQB1*0202, DQB1*0502, DQB1*0604 and DQA1*0102 alleles are associated with non-responsiveness and DQB1*0301, DQB1*0501, DQB1*0603, DQA1*0101 and 0103 with responsiveness in different European populations to this vaccine. Despite robust association in wide population HLA DQ2 did not qualify to be a non-responder marker[40] for HBV vaccine in European populations. However it was suggested to be an indicator for sub-optimal response to HBV vaccination in homozygous subjects.

HLA class III allele associations: HLA class III complement allele C4AQ0 is strongly associated with non-responsiveness to anti HBV vaccination[41]. It may contribute to inefficient complement activation and thus failure of B cells to secrete anti-HB immunoglobulin[34,42]. Complement protein production is linked to HLA haplotype[43]. Other HLA class III genes including BfF, Cw4, Cw2, and C4A6 are associated with responsiveness to HBV vaccination in the Caucasian population (Table 4). Kramer *et al*[44] studied the TCR/CD3 density in non-responders and responders following vaccination in end stage renal disease (ESRD) patients. In these patients lower density of TCR/CD3 was reported in responders carrying C4A*6 and BfF alleles in comparison to non-carriers. Low TCR/CD3 density is related with non-responsiveness and is found associated with HLA A1-B8-DR3 haplotype. The HLA DR3 (associated with HBV vaccine non responsiveness) positive responders lacked C4A*6 and BfF alleles. HLA A1, C4A*6 and Bf*F alleles

Table 3 HLA allele association with Non-responsiveness to HBsAg vaccine across global population

HLA	Effect	Country	Vaccinees	Rs	NRs	Reference
HLA I HLA B54	Non- responsiveness	China	Healthy vaccinees	30	29	[118]
HLA A*0602, A*1101 and B*35	Non-responsiveness/ antibody production	Japan	Healthy medical students			[35]
HLA A1, A10, B15, B40.	Non responsiveness	India	Healthy volunteers	15 of 87 Rs	15	[37]
HLA A10, CW4	Non-responsiveness	Turkey	Health care workers		12	[119]
HLA A1, B8	Non-responsiveness	Germany, Poland	ESRD Patients on hemodialysis	119	34	[44,46]
HLA B8	Non-responsiveness	Belgium	Homosexual vaccinees	39	9	[120]
HLA A2	Non-responsiveness	Spain	Hemodialysis patients			[121]
HLA II HLA DR7##	Non- responsiveness	China	Healthy vaccinees	30	29	[118]
HLA DRB1*07##	Non-responsiveness	China (Han)	Healthy vaccinees	145	118	[122]
HLA DR14, DR52	Non- responsiveness	Chinese (Taiwan)	Non responder vaccinees		26	[123]
HLA DRB1*0405, DRB1*1101, DR4	Non-responsiveness/ antibody production	Japan	Healthy medical students			[35]
HLA DQ2$	Non responsiveness	India	Healthy volunteers	15 of 87	15	[37]
HLA DR7#	Non-responsiveness	Turkey	Health care workers		12	[119]
HLA DR3, DQ2$	Non-responsiveness	Germany, Poland	ESRD Patients on hemodialysis	119	34	[44,46]
HLA DR3	Non-responsiveness	Belgium	Homosexual vaccinees	39	9	[120]
HLA DRB1*07#, *DQB1*02$, and DPB1*1101	Non-responsiveness	Belgium	Vaccinees	134	117	[39]
HLA DRB1*03, DRB1*14	Non-responsiveness	France	Hemodialysis patients	301	114	[124]
HLA DRB1*1302, DQB1*0604, DQA1*0102	Non-responsiveness	Sweden	Healthy vaccinees	69	53	[48]
HLA DRB1*0701#, DQB1*0202$	Non-responsiveness	UK	Vaccinees	117	86	[125]
HLA DRB1*0701#, DQB1*0202$	Non-responsiveness	UK	HBsAg Non responders			[45]
HLA DQB1*02$	Non-responsiveness	Italy	Neonates	76	49LR, 43SR 19TNR	[34]
HLA DRB1*1601, DQB1*0502, DQA1*0102 Haplotype	Non-responsiveness	Slovenia	Health care workers	60	36	[126]
HLA DR3, DR7#, DQ2$	Non-responsiveness	Spain	Hemodialysis patients			[121]
HLA DRB1*3, DRB1*7, DRB1*14x	Non-responsiveness	Germany	Healthy adults & Infant vaccinees	53 & 56	73 & 62	[127]
HLA-B8, SC01, DR3 and HLA B44-FC31, DR7#	Non-responsiveness	USA	Healthcare worker		20	[33,49]
HLA DRB1*07#	Non-responsiveness	USA	Healthy vaccinees	85	79	[128]
HLA III HLA C4A*6, C4A*Q0##, Bf*F, Bf*S07	Non-responsiveness	Germany, Poland	ESRD Patients on hemodialysis	119	34	[46]
HLA C4AQ0##	Non-responsiveness	Italy	Neonates	40	26LR, 21SR, 10TNR	[41]
HLA C4AQ0##	Non-responsiveness	Italy	Neonates	76	49LR, 43SR 19TNR	[34]
HLA C4AQ0##	Non-responsiveness	Germany	Healthy vaccinees	53	73	[42]

Rs: Responders; NRs: Non-responders; ESRD: End stage renal disease; LR: Low responders, SR: Slow responders; TNR: Total non-responders. Common HLA alleles $: DQ2, #: DR7, ##: C4AQ0, DR3.

are associated with non-responders as well as responders but occur within different haplotypes in non-responders and responders for HBV vaccination.

Homozygous individuals for non-responder haplotype are strongly non-responsive to HBV vaccination when compared to the heterozygous state[45]. Homozygotes for HLA A1, B8, DR3, and DQ2 alleles are found exclusively in non-responders while heterozygotes are mostly non-responders[46] for HBV vaccination.

Non-uniformity observed in HLA association may be due to interaction between HLA factors within a haplotype. In Belgian populations HLA DPB1*0201 is associated with non-responsiveness to HBV vaccine when it occurs with haplotype DRB1*0701/DRB4*0101-DQB1*020*[39]. Also interaction with non-HLA genes in addition to HLA genes may influence responsiveness to HBV vaccination[47]. Presence of certain HLA genes in both responder and non-responder subjects suggests a role for other genes (cytokines, T cell receptors etc) in influencing the response to anti Hepatitis B vaccination and HLA alleles may be markers for such association[48]. Several susceptibility genes for immunoglobulin deficiency associated with haplotype HLA-B8, SC01, and DR3 are found in higher frequency in HBV vaccine non-responders[33,49]. Low levels of IL2 cause hypo-response to HBV vaccination[50]. IL10 ACC haplotype is associated with lower production of IL10 and thus favor strong humoral immune response to HbsAg[51].

HLA ALLELE ASSOCIATIONS WITH HEPATITIS C INFECTION IN GLOBAL POPULATIONS

Viral persistence and viral clearance

Activation of T helper (TH) 1 response is associated with self-resolving viral infection and that of TH2 with chronicity. The reactivation mechanism of T cells contributing to activation of particular TH CD4+ cells during HCV infection is not understood. Since negative finding by Vitte *et al*[52] in Caucasians, several HLA alleles and haplotypes have been found associated with susceptibility and resistance to hepatitis C infection, progression to liver damage and cirrhosis, development of hepatocellular carcinoma and response to interferon

Table 4 HLA association with responsiveness to HBsAg vaccine across global population

HLA	Effect	Country	Vaccinees	Rs	NRs	Reference
HLA Ⅰ, HLA A1, B7, B12	Responsiveness (Low)	Germany Poland	ESRD Patients on hemodialysis	119	34	[46]
HLA A1, A19, B5, B27, Cw2, Cw4	Responsiveness (High)	Germany Poland	ESRD Patients on hemodialysis	119	34	[46]
HLA Ⅱ, HLA DRB1*02	Responsive	China (Han)	Healthy vaccinees	145	118	[122]
HLA DRB1*0101, DRB1*08032, DQA1, DQB1, DPA1 and DPB1	Responsiveness	Japan	Healthy medical students			[35]
HLA DR4	Responsiveness (Low)	Germany, Poland	ESRD Patients on hemodialysis	119	34	[46]
HLA DRB1*010, DR5, DPB1*040, DQB1*0301, DQB1*0501.	Responsiveness	Belgium	Vaccinees	134	117	[39]
HLA DRB1*01, *DRB1*15# and DRB1*16	Responsiveness	France	Hemodialysis patients	301	114	[124]
HLA DRB1*1301, *DR15#, DQA1*0103, DQA1*0102, DQB1*0603,	Responsiveness	Sweden	Healthy vaccinees	69	53	[48]
HLA DRB1*11, DQB1*0301	Responsiveness	Italy	Neonates	76	49LR, 43SR 19TNR	[34]
HLA DRB1*1 DRB1*13, *DRB1*15#	Responsiveness	Germany	Healthy adults & infant vaccinees	109	135	[127]
HLA Ⅲ C4A*6, C4B*2, Bf*F	Responsiveness (High)	Germany Poland	ESRD Patients on hemodialysis	119	34	[46]

Rs: Responders; NRs: Non-responders, ESRD: End stage Renal Disease, LR: Low responders, SR: Slow responders, TNR-total non-responders common HLA alleles: DRB1*01, #: DR15.

Table 5 Association of HLA haplotypes with response to HbsAg vaccination across global population

HAPLOTYPE	Effect	Country	Vaccinees	Rs	NRs	References
HLA DR4, 1122 (DRB1*0401-22, 1122)-DR53 (DRB4*0101101, 0102/3)-DQB4 (DQB1*04)	Non-responsiveness	China				[129]
HLA DR14-DR52	Non-responsiveness	Chinese (Taiwan)	Non-responder vaccinees		33	[123]
HLA A1, B8, Bfs, C4AQ0, C4B1, DR3, DQ2 and HLA A1, B8, BfF, C4A6, C4B2, DR3, DQ2	Non-responsiveness	Germany, Poland	ESRD Patients on hemodialysis	34	119	[46]
HLA DRB4*0101-DRB1*0301/DRB3* *0101 DQB1*0202 and DPB1*0201 DRB1*0701/DRB4*0101, DQB1*020*	Non-responsiveness	Belgium	Vaccinees	134	117	[39]
HLA DQB1*0604, DQA1*0102, DRB1*1302 haplotype	Non-responsiveness	Sweden	Healthy vaccinees	69	53	[48]
HLA C4A*Q0, DRB1*0301, DQB1*02	Non-responsiveness	Italy	Neonates	40	26LR, 21SR 10TNR	[41]
HLA C4AQ0, DQB1*02	Non-responsiveness	Italy	Neonates	76	49LR, 43SR 19TNR	[34]
HLA B44-DRB1*0701-DQB1*0202	Non-responsiveness	UK	Vaccinees	117	86	[125]
HLA DRB1*1601, DQB1*0502, DQA1*0102 haplotype	Non-responsiveness	Slovenia	Health care workers	60	36	[126]
HLA B8-DR3	Non-responsiveness	Germany	Healthy adult & infant vaccinees	109	135	[127]
HLA DQB1*0603, DQA1*0102, DR15 and HLA DQB1*0603, DQA1*0103, DRB1*1301 haplotype	Responsiveness	Sweden	Healthy vaccinees	69	53	[48]
HLA DRB1*11, DQB1*0301	Responsiveness	Italy	Neonates	76	49LR, 43SR 19TNR	[34]

ESRD: End stage renal disease. Rs: Responders; NRs: Non responders; LR Low responders; SR: Slow responders, TNR: Total non-responders.

therapy in several populations. These are outlined in Tables 6, 7 and 8. Associations with HLA class Ⅱ genes are reported more often than HLA class Ⅰ. HLA class Ⅰ allelic diversity is suggested to have little influence on fibrosis and disease severity associated with chronic HCV infections[53].

HLA class Ⅰ allele associations: Susceptibility to HCV infections is associated with A*19 in Saudi people[54] and with HLA A*28, A*29, B*14, DR7 in Egyptians[55]. HLA A11-C*04 is associated with HCV persistence in Ireland[56]. HLA B-35 and B8 are strongly associated with chronic HCV infection, with HLA B-35 being positively related to chronic viral infection regardless of viral type[5,57]. In American whites, HCV persistence is associated with HLA Cw*04 and HLA B53, homozygosity of HLA Cw*04 have stronger effect on persistence than single copy of the allele[58].

Protection from HCV infection is associated with HLA-B51, -B52, -B55, -B56, -B61, B70, -Cw1, -Cw3, and -Cw4 in Japanese populations[59]. In Ireland HLA B27 and A*03 have strong association with self-resolving HCV infection[60]. Spontaneous HCV clearance is associated with HLA B-27 in German women cohorts[61] and HLA A*1101, B57 and Cw0102 are reported to be associated with viral clearance in American whites[58]. HLA C allele association with HCV clearance is found in Japanese (Cw1, Cw4)[59], Russian (Cw4)[5] and American populations (Cw0102)[58] (Table 6). HLA Cw*0602 protects from

Table 6 Association of HLA alleles with susceptibility to viral persistence &chronic HCV infection across global population

HLA	Effect	Country	NC	R	Chronicinfection		Reference
					AC	CLD/CHC	
HLA I HLA-B61, Cw3,	VP/HCV infection	Japan	293			60	[69]
HLA B54	VP with CLD	Japan	916		33	97	[68]
HLA B55, -B56, B70	VP	Japan	172			113	[59]
HLA A3, B-35, B-46	VP	Korea	206			137	[109]
HLA A28, A29, B14	HCV infection	Egypt					[55]
HLA A-19	HCV infection	Saudi people.	122			146	[54]
HLA-A10, HLA-B35, HLA-B40 and HLA-Cw3	VP-CLD	Russia					[5]
HLA-A30, B35, B41, Cw2, A1-B35, A9-B8	VP-CLD-LC	Russia				107	[57]
HLA B8, B18	VP-CHC	Ireland		86		141	[60]
HLA C*04	VP	Ireland (Whites)		86		139	[56]
HLA B14	VP & active hepatitis C	Italy	489			117	[130]
HLA B18	Susceptibility to CLD	Spain	116		48	93	[131]
HLA-A*2301 and HLA-Cw*04	VP	USA		231		444	[58]
HLA-Cw*07,	Risk factor for vertical infection	Italy (infants born to HCV+ mothers)	44 uninfected infants born to HCV+ mothers			21	[16]
HLA II HLA DR4, DQB1*0401 DQB1*0402	VP/ HCV infection	Japan	293			60	[69]
HLA DRB1*0405, DQB1*0401	VP with CLD	Japan	916		33	97	[68]
HLA DRB1*0405, DQB1*0401	VP with LC	Japan	1216		50	67	[70]
HLA DQB1*0503	VP with LC	Japan	201		43	60	[132]
HLA DRB1*0301#, DQB1*0201, DQB1*0502	VP/CHC	Thailand	140	43	21	36	[133]
HLA, DRB1*0803, DQB1*0601 and DQB1*0604	VP	Korea	206			137	[109]
HLA DRB1*0301#	VP	Egypt (Hemophilic and HCV-, HCC+ patients)	15 Healthy & 25 HCV-		10 HCV+	15 (HCV-HCC+)	[134]
HLA DR7	HCV infection	Egypt					[55]
HLA-DRB1*0701, DRB1*15, DRB4*0101	Viral persistence	UK (European)		85		170	[135]
HLA DQB1*0201	VP-CHC	Ireland		86		141	[60]
HLA DRB1*0701 (HCV 1b)	VP	Ireland (females receiving HCV 1b contaminated AntiD immunoglobulin)		84		72	[136]
HLA DRB1*1001, DRB1*1101	VP/CLD	Italy	179		41	99	[137]
HLA DQB1*0502	VP-CLD	Italy	200	35	42	107	[138]
HLA DR14, DR17	VP-CLD	Italy	70		34	39	[139]
HLA DRB1*0301	VP-CHC	Germany	101			105	[140]
HLA DRB1*07	VP-CLD	German & North Europeans	2045			99	[94]
HLA DR B1*13 and DRB1*14	Susceptibility to infection	German & North Europeans	2045			99	[94]
HLA DR3#	Susceptibility to chronic disease	Spain	116		48	93	[131]
HLA DRB1*13 and DRB1*07	Necro inflammatory activity during infection	Poland				134	[95]
HLA DRB1*13 allele	VP	Poland				134	[95]
DRB1*03# and DQB1*0201(male gender)	CLD/LC	France				233	[141]
HLA G*010401, -DRB1*0701, -DRB1*1401 and homozygosity for HLA-G 14bp deletion	Risk factor for vertical infection	Italy(infants born to HCV+ mothers)	44 uninfected infants born to HCV+ mothers			21	[16]
HLA DRB*4001	High viral load	Taiwan					[142]
HLA III MICA-A4	Susceptibility to CLD	Spain	116		48	93	[131]

VP: Viral persistence/chronic infection; CHC: Chronic hepatitis C; CLD: Chronic liver disease; NC: Normal control; R: Recovered; AC: Asymptomatic carriers; LC: Liver cirrhosis. Common VP alleles: # DRB1*03, DRB1*0701.

vertical HCV transmission in infants while HLA CW*07 is the susceptibility allele[16] for HCV.

NK cells mediate direct killing of infected and transformed cells contributing to viral clearance and protection from tumor development. This killer activity is under the control of HLA class I molecule interaction with inhibitory and activation receptors-killer cell immunoglobulin like receptors (KIR) of NK cells. HLA C molecules interact with KIRs of NK cells and modulate their cell killer activity. Activation receptor KIRDL3 and HLA-C1C1 ligand interact directly to influence viral clearance in HCV infection[62]. KIRDL3 associated protection is not found in individuals lacking HLA-C1C1 allele[62]. HLA C2C2 interaction with NK cell receptor is associated with viral persistence in Spanish populations[63]. KIR3DS1-HLA Bw4180 genotype is associated with protection from development of HCC in HCV carriers[64]. HLA Cw7 inhibitory interaction with NK cells is proposed

Table 7 Association of HLA alleles with Protection from HCV infection and viral clearance

HLA I	Effect	Country	NC	R	Chronic infection		Reference
					AC	CHC/CLD	
HLA I HLA-B51, -B52, B61, -Cw1, Cw3, and Cw4	VC	Japan	172			113	[59]
HLA A2	AC	Japan	172			113	[59]
B50 (21)	VC	Egypt					[55]
HLA-B8,	Protection from infection	Saudi people	122			146	[54]
HLA-Cw4	Protection from chronic infection	Russia					[5]
HLA A*03,B*07,B*27,Cw*01	VC/Protection from chronic infection	Ireland		86		141	[60]
HLA-A*1101,HLA-B*57 and HLA-Cw*0102	Viral clearance	USA		231		444	[58]
HLA-Cw*0602	Protection against vertical infection	Italy (infants born to HCV+ ve mothers)	44 uninfected infants born to HCV+ mothers			21	[16]
HLA Bw4180/KIR3DS1	HCV carriers	Spain	116		51	47 (LC), 54 (HCC)	[64]
HLA-C1/KIR2DL3	VC/protection from infection	UK		352		685	[62]
HLA A*34,B*56	Low viral load	Taiwan					[142]
HLA II HLA-DR9- DQB1*0301 and DQB1*0303	VC/protection from HCV infection	Japan	293			60	[69]
HLA DRB1*1302, DRB1*1101## and DQB1*0604 alleles	AC/no CLD	Japan	916		33	97	[68]
HLA DRB1*12(1201/1202), DQBI*0301, DRB3*03	AC/no CLD	Japan	201		43	60	[132]
HLA DRB1*0901,DQB1*0303	AC-no LC	Japan	1216		50	67	[70]
HLA DRB1*04, DRB1*0701, DQA1*0201,DQB1*0301	VC	Thailand	140	43	21	36	[133]
HLA DRB1*0301, DQA1*0501 and DQB1*0201	VC/protection from HCV infection	Korea	206			137	[109]
HLA DRB1*0101##	VC	Egypt (Hemophilic and HCV- HCC+ ve patients)	15 Healthy & 25 HCV-		10 HCV+	15 HCV- HCC+	[134]
HLA DRI and DR3	VC/Protection from infection	Saudi people	122			146	[54]
HLA DRB1*11#	VC/Protection from infection	Turkey	43			49	[143]
HLA DQA1*03 and DQB1*0302	VC/Protection from chronic infection	N.European whites	177			104	[144]
HLA-DRB1*0301,DRB1*1101#, DRB1*1201# and HLA-DQB1*0301	Viral clearance	UK (European)		85		170	[135]
HLA DRB1*04, DQA1*03 and DQB1*0301	VC/Protection from chronic infection	UK	134	49		55	[145]
HLA DQB1*0302	Protection from infection	UK	134	49		55	[145]
HLA DRB1*01##. (HCV 1b)	VC	Ireland (females who received HCV 1b contaminated AntiD immunoglobulin)		84		72	[136]
HLA DRB1*0101##, DRB1*0401, DRB1*15	VC/Protection from chronic infection	Ireland		86		141	[60]
HLA DRB1*0101##	Viral clearance	Ireland		73		84	[78, 146]
HLA DR5#	Protection from chronic hepatitis C	Italy	489			117	[130]
HLA II HLA DRB1*1601, DQB1*0502	Protection from HCV infection	Sardinia (Thalassemia major for transfusion)	606 healthy & 30 HCV- patients			116	[147]
HLA DRB1*1104, and DRB3*03	Protection from chronic manifestation /carries	Italy	179		41	99	[137]
HLA DRB1*1104,DQB1*0301	VC	Italy	200	35	42	107	[138]
HLA DR 11	VC/Protection from infection	Italy	70		34	29	[139]
HLA DQB1*0301	Protection from HCV related HCC	Italy	144			29	[148]
HLA DRB1*1301 and DQA1*0103	Protection from chronic HCV infection	Germany	101			105	[140]
HLA-DRB1*15011	Viral clearance/ Protection	Germany		21		49	[149]
HLA-DRB1*11(DR5) and HLA-DQB1*03(DQ3)	Protection from CLD	Germany	501			108	[150]

HLA-DR11	AC/Protection from CLD	Spain	116		48	93	[131]
HLA DQB1*0301	Protection from chronic infection	Poland	103			129	[108]
HLA DRB1*11	Mild liver damage	Poland				134	[95]
HLA DQB1*0301 and DRB1*1101	VC	France	800	25		103	[74]
HLA DQB1*0301 and female sex	VC	France	800	63		282	[75]
HLA DRB1*11	Protection from progression of liver disease	France				233	[141]
HLA DRB1*11 (female association)	AC/less severity of chronic hepatitis	France			83	233	[76]
HLA DQB1*0301	VC (strongly in Black subjects)	USA		200		374	[77]
HLA DRB1*0101, DQB1*0501	VC (in white subjects)	USA		200		374	[77]
HLA DR13	Protection against vertical infection	Italy (infants born to HCV+ ve mothers)		17 (serum reverted)		18	[17]
HLA-DQB1*06, -G*0105N, DRB1*1104 and -DRB1*1302 alleles	Protection against vertical infection	Italy (infants born to HCV+ ve mothers)	44 uninfected infants born to HCV+ mothers			21	[16]
HLA DRB1*1502	Low viral load	Taiwan					[142]

VC: Viral clearance; CHC: Chronic hepatitis C; CLD: Chronic liver disease; NC: Normal control; R: Recovered; LC: Liver cirrhosis; AC: Asymptomatic carriers. Common HLA alleles #: DR5 (DRB1*11, DRB1*12 allele), ##: DRB1*0101, DQB1*0301.

Table 8 HLA Haplotype association with HCV viral clearance and persistence across global population

HLA	Effect	Country	NC	R	Chronic infection		Reference
					AC	CLC/CHC	
Viral persistence							
HLA Cw3- DR4-DQB1*0401 or *0402, and HLA-B61-DR4 -DQB1*0401 or 0402	VP/chronic infection	Japan	293			60	[69]
HLA B54-DRB1*0405-DQB1*0401 haplotype	VP-CLD	Japan	916		33	97	[68]
HLA DRB1*0405-DQB1*0401 haplotype	VP with LC	Japan	1216		50	67	[70]
HLA DRB1*0301, DQA1*0501, DQB1*0201	VP	Thailand	140	43	21	36	[133]
A*01-B*08-Cw*07-DRB1*03011-DQB1*0201	VP-CHC	Ireland		86		141	[60]
HLA DRB1*15-DQB1*0602	High viral load/increased risk for disease severity	Ireland (viremic females)				57	[71]
HLA A*11, C*04	VP	Ireland (Whites)		86		139	[56]
HLA DQA1*0201-DQB1*0201	Susceptibility to Chronic hepatitis C	Italy	179		41	99	[151]
HLA DR3/MICA-A4/B18	Susceptibility to chronic disease	Spain	116		48	93	[131]
HLA DRB1*0701-DQA1*0201-DQB1*02 and DRB1*1501-DQA1*01-DQB1*0602	VP-CLD	Poland	103			129	[108]
HLA DRB1*0301 -DQB1*0201	VP	USA					[77]
HLA-Cw*04-B*53	VP	USA		231		444	[58]
Viral clearance							
HLA B44-DRB1*1302-DQB1*0604 and DRB1*1302-DQB1*0604	AC/no progression to CLD	Japan	916		33	97	[68]
HLA DRB1*0901-DQB1*0303	AC-no LC	Japan	1216		50	67	[70]
HLA A*03-B*07-DRB1*15-DQB1*0602 and A*02-B*27-Cw*01-DRB1*0101-DQB1*0501	VC/Protection from chronic infection	Ireland		86		141	[60]
DRB1*0701 and DQB1*02	Stable viral load/slow disease progression	Ireland (viremic females)				57	[71]
HLA DRB1*1104, DQA1*0501, DQB1*0301 haplotype	Protection from Chronic hepatitis	Italy	179		41	99	[151]
HLA DRB1*1104, DQB1*0301	VC	Italy	200	35	42	107	[138]
HLA DRB1*0101 -DQB1*0501 haplotype	Viral clearance (in white subjects)	USA		200		374	[77]

VP: Viral persistence; VC: Viral clearance; CHC: Chronic hepatitis C; CLD: Chronic liver disease; NC: Normal control; R: Recovered; AC: Asymptomatic carriers; LC: Liver cirrhosis.

to be associated with HCC in Italian patients[20]. It is notable that HLA B8-Cw7 haplotype is associated with HBV associated liver cancer[6]. In an in vitro study, HLA E is shown to inhibit NK cell mediated lysis of HCV infected cells via interaction with NK cell receptor NKG2A, contributing to HCV persistence[65]. HLA E expression on hepatocytes is stabilized by recognition of HCV core protein by HLA A2 molecules[65]. Increased expression of inhibitory receptor CD94/NKG2A on NK cells was found in chronic hepatitis C patients and is suggested to contribute to immune resistance towards HCV infection and HCV mediated cellular transformation via modulating dendritic cell function and cytokine secretion[66,67].

HLA class Ⅱ allele associations: HLA DRB1*0405 and DQB1*0401/0402 alleles are associated with viral persistence and chronic HCV infection in Japanese[68-70].

Also, DRB1*0405-DQB1*0401/0402 haplotype[59] and HLA B54-DRB1*0405-DQB1*0401 haplotype are associated with HCV induced liver injury[68]. In Thailand and most European Caucasians HLA DRB1*0301, DQB1*0201 and DQA1*0201 are found associated with chronic HCV infection and HLA DRB1*0701 is associated with disease severity throughout the European populations (Table 6). In Irish viremic females increased risk for HCV infection related disease severity and high viral load was associated with HLA DRB1*15-DQB1*0602[71]. In Italy HLA DQB1*0502 and DR14 and DR 17 are associated with risk to liver cirrhosis and progressive liver damage respectively (Table 6).

HLA DRB1*11 alleles and DQB1*0301 is consistently associated with decreased disease severity of Hepatitis C worldwide (Table 7)[72,73]. In French populations DQB1*0301 is associated with viral clearance in females and DRB1*11 with protection from disease progression in males[74,75] for hepatitis C infection. In contrast Reno *et al*[76] found association of DRB1*11 with HCV clearance in the French female gender. HLA DQB1*0301 is linked to HCV clearance in American populations showing stronger association with African-Americans[77]. Carriers of HLA DR11 and DQB1*0301 alleles may present the HCV epitopes more efficiently to CD4+T cells than others and thus show efficient viral clearance[73]. Viral clearance is associated with DRB1*0101 and DQB10501 in American Caucasians and with DRB1*0101 in Irish, Saudi, and Egyptian populations (Table 7). HLA DRB1*0101 allele appears to have better HCV specific T cell response[78]. DRB1*0701-DQB1*02 haplotype is associated with low HCV viral load in Ireland[71]. HLA DR7 is associated with hepatitis C disease severity in several populations and also with HBV infection, except for in Thailand where it is associated with HCV viral clearance along with HLA DQA*0201. HLA DR13 is protective for vertical transmission in infants born to HCV+ ve Italian mothers[16,17]. In infants born to infected mothers HLA DRB1*1104 is associated with seroreversion and DRB1*1101 with viral persistence[79] for HCV infection. In Japanese and the DRB1*0901-DQB1*0303 haplotype is related to protection from cirrhosis in hepatitis C[70] and HLA B44-DRB1*1302-DQB1*0604 and DRB1*1302-DRB1*0604 with asymptomatic carrier phenotype[68].

HLA class Ⅲ allele associations: HLA class Ⅲ and cytokine genes have been reported in influencing HCV infection outcome. HLA DR3/MICA-A*4, B*18 is increased in patients with HCC but absent in HCV carriers[64]. Single Nucleotide Polymorphism (SNP)-863A in TNF alpha gene is associated with HCV clearance in African American patients, while wild-type haplotype -863C/-308G is associated with viral persistence in the same[80]. TNF alpha-308 G is found associated with HBV persistent infection in Chinese and Koreans but its status with American populations for HBV is not known. IL10 ATA haplotype possibly influences HCV clearance in Italians[81]. Positive association is reported between TNF alpha 238.2 promoter variant and chronic active hepatitis B and C in Germans[23]. In Indian (Asian) populations the TNF-beta (A/A) allele is indicated to be associated with disease progression[82].

HLA ALLELE ASSOCIATIONS WITH RESPONSE TO INTERFERON THERAPY IN HEPATITIS B AND C VIRAL INFECTIONS

Interferon treatment is currently the most adopted therapy for chronic hepatitis patients. It upregulates the expression of HLA DR, CD80 and ICAM- Ⅰ molecules on dendritic cells and thus augmenting the immune response[83]. Interferon treatment for chronic HBV and HCV infection is successful only in one third of patients. HLA DR locus appears to be a prominent immunogenetic factor influencing interferon treatment response. HLA association in independent studies on Chinese populations has reported HLA DRB1*14 and DQA1*0501 and DQB1*0301 to be associated with responsiveness in chronic hepatitis B. DRB1*07 allele carriers are prevalent among chronic hepatitis B patients and associated with non-responsiveness to anti HBV vaccination and IFN-alpha treatment (Tables 1, 3 and 9).

In Chinese populations DRB1*04 is associated with non-responsiveness to interferon therapy in HCV as well as HBV infected subjects (Table 9)[84,85]. HLA DRB1*04 is associated with male non-responders while HLADRB1*07 is reported for female responsiveness in Chinese patients with chronic HCV infection[84]. Japanese HLA allele associations with response to interferon treatment is inconsistent (Table 9) and involve HLA class Ⅰ and class Ⅱ alleles for hepatitis C. HLA A24-B54-DR4 haplotype and HLA B54 allele are predictors of poor response to IFN therapy in Japanese hepatitis C subjects[86]. High serum level of IL10 is also suspected to result in poor response to interferon treatment in chronic HCV cases[47]. Low serum levels of IL10 are associated with responsiveness to HBV vaccination[51].

In Spanish populations HLA B44 is associated with responsiveness to interferon + ribavirin therapy but not when interferon therapy is given alone in hepatitis C cases. Also, HLA class Ⅱ does not influence response to interferon treatment in this population[87]. HLA DRB1*0404 is associated with responsiveness in the HCV infected Caucasian population[88]. Piekarsha *et al*[89] did not find any association of HLA DRB1* alleles with response to Interferon alpha 2b treatment of Polish HCV subjects, but in other populations these alleles are reported to influence the response (Table 9). HLA DRB1*07 is associated with non-responsiveness for HBV and for HCV infected subjects from France, but is associated with responsiveness in HCV cases from Poland and Germany and females subjects from China (Table 9).

HLA allele association with interferon treatment response seems to differ for chronic HBV and HCV infections in global populations. This can be attributed to differences in the antigenic determinants of these two different viruses and consequently differences in the HLA molecules involved. However associations of same HLA allele for hepatitis B as well as C with respect to therapeutic response or non-response, though in different population, warrants explanation. HLA typing for interferon treatment receiving cohorts in different population would give an insight towards the mechanism and immune interactions

Table 9 HLA association with response to interferon therapy in chronic hepatitis (B and C) patients across global population

HLA	Effect	Country	R	NR	References
Hepatitis B					
HLA DQA1*0501 and DQB1*0301	Responsiveness	China	32	28	[85]
HLA-DRB1*14	Responsiveness	China	11	24	[14]
HLA DRB1*04,DQA1*0303	Non-responsiveness	China	32	28	[85]
HLA-DRB1*07 (HBV type c)	Non-responsiveness	China	7		[152]
HLA-DQB1*07	Low response	China	11	24	[14]
Hepatitis C					
Non responsiveness					
HLA I HLA B54	Non responsiveness	Japan	20	47	[86]
HLA II , HLA DR9	Non responsiveness	Japan			[153]
HLA DR6	Non responsiveness	Japan	21	32	[154]
HLA DRB1*04 (males and HCV1b)	Non responsiveness	China	10	11	[84]
HLA B1*13	Non responsiveness	Turkey			[155]
DRB1*07	Non responsiveness	France	50	120	[75]
Responsiveness					
HLA I HLA B55, B62 and Cw3	Responsiveness	Japan	54	118	[156]
HLA B51, CW1	Responsiveness	Japan			[153]
HLA B44	Responsiveness to Interfron + Ribavirin	Spain	53	52	[87]
HLA II HLA DRB*10101	Responsiveness	Japan	32	106	[157]
HLA DR6	Responsiveness	Japan	21	32	[154]
HLA DRB1*07 (females and HCV 2b)	Responsiveness	China	10	11	[84]
HLA DR2	Responsiveness	Egypt	25	30	[158]
DQB1*06(HCV genotype 1)	Responsiveness	France	50	120	[75]
HLA DRB1*0404	Responsiveness	Canada	6	64	[88]
Haplotypes					
HLA A-24-B54-DR4	Non responsiveness	Japan	20	47	[86]
HLA B7-DRB10101	Responsiveness	Japan	32	106	[157]
DRB1*0701-DQA1*0201-DQB1*02	Responsiveness	Poland	29	26	[108]

R: Responders to Interferon therapy; NR: Non-responders to interferon therapy.

involved. Interferon therapy involves adverse effects; HLA studies will help identifying responder/no responder markers that would help select patients suitable for interferon therapy.

HLA ASSOCIATIONS WITH VERTICAL TRANSMISSION OF HEPATITIS B AND HEPATITIS C VIRAL INFECTIONS

Vertical transmission from[92] infected mothers is a major contributor to increased HCV and HBV infection. Genetic factors in neonates surely influence the outcome of vertical transmission. Liu *et al*[15] reported HLA DR3 carriers to be at risk of vertical transmission of HBV and DR13 carriers to be resistant in Chinese populations. In Italian subjects, HLA DR13 (DRB1*1302) and DRB1*1104 were reported to be protective in neonates for vertical transmission[16,17,79]. HLA DRB1*1104 is associated with seroreversion in infants born to infected mothers but DRB1*1101 is associated with viral persistence in such infants. The difference in association related to sub allelic differences could to be due to glycine/valine dimorphism at position 86 of the antigen binding sites of the DR B1* molecule. HLA DRB1*86GG was associated with HCV infected children while DRB1*86VV homozygotes were seroconverts[79]. HLA-DQB1*06, -G*0105N, -Cw*0602, DRB1*1104 and -DRB1*1302 alleles are protective while HLA-Cw*07, -G*010401, -DRB1*0701, -DRB1*1401 and homozygosity for HLA-G 14bp deletion are risk factors for HCV vertical transmission in Italian children[16].

HLA ASSOCIATIONS WITH THE EXTRA-HEPATIC MANIFESTATIONS OF HEPATITIS B AND HEPATITIS C VIRAL INFECTIONS

HLA associations have been described for extra-hepatic co-morbidities triggered by HCV and HBV infection. HLA DQB1*0603 is associated with development of HBV associated membranous nephropathy in South African black children[90]. HLA DRB1*11 and HLA DRB1*1302, DQB1*0404, DQB1*0604 are associated with susceptibility and protection respectively to HBV related glomerulonephritis in Korea[91]. Sebastiani *et al*[92] proposed involvement of HLA DR6 in extra hepatic manifestation of HCV related diseases. HCV associated oral lichen planus was reported in HLA DR6 carriers (DRB1*13/*14 alleles)[93]. HLA DRB1*13 alleles are reported to be associated with disease severity in chronic HCV infection in European populations[94,95]. Chronic HCV infection also shows association between DQB1*11 and DR3 with formation of cryoglobulins[72]. HLA studies in this aspect would allow finding of HLA marker for extra hepatic manifestations of hepatic viral infections. Such studies may help prevent post transplantation associated liver damage in recipients. A recent study has suggested donor–recipient mismatch at HLA DRB1 locus and HLA B14 to be responsible for severe fibrosis development in the recipient[96]. The knowledge of HLA gene marker

association with risk for HBV/HCV related disease progression/cirrhosis may be helpful while seeking donor-recipient HLA match before transplantation.

Czaja *et al*[97] have suggested that genetic predisposition, facilitated by viral infection may trigger autoimmune hepatitis (AIH). Similarities in HLA associations in viral hepatitis and AIH are observed. In European and North American Caucasians HLA DRB1*0301 is strongly associated with AIH Ⅰ[97], and in most European population DRB1*0301 is associated with HCV persistence (Table 6). HLA DRB1*0405 is a susceptible allele for AIH Ⅰ as well as HCV viral persistence in Japanese[68,70,97]. In Germans DRB1*07 is a risk factor for AIH Ⅱ, and is also found associated with HCV viral persistence[94,97]. Such associations require an extensive study of HLA association in AIH and viral hepatitis patients.

HLA ALLELE ASSOCIATIONS WITH HEPATITIS A, D AND E VIRAL INFECTIONS

HLA restricted virus specific T cells play an essential role in Hepatitis A related hepatocellular injury, however limited information about the host HLA association in HAV infection is available. HLA-A9 was suggested to be the susceptibility factor in Brazil during an outbreak of hepatitis A infection[98]. HLA-B27[99] and HLA A1, B8, DR3[100] associations with fulminant hepatitis A infection in HCV infected patients is reported. Hepatitis A viral infection (HAV) is suspected to trigger AIH in genetically susceptible patients carrying HLA DRB1*0401[101]. Strong association of DRB1*1301 haplotype with protracted forms of HAV infection that further developed to AIH in pediatric patients is reported[102]. Extensive investigation of HAV infected individuals worldwide will contribute to the limited information regarding its HLA association and its relation to HLA associations of HBV and HCV infections.

HLA association with Hepatitis D and E is not known. Hepatitis E causes acute infections, and Hepatitis D is associated with Hepatitis B infection. A high risk of chronic delta (HDV) infection in Russians is associated with HLA-B8 and HLA-B35, and in Kazakhs with HLA-B35 and HLA-D40[5].

DISCUSSION

Extensive allele diversity is observed in HLA associations with susceptibility and protection regarding HCV and HBV infections and disease progression in different global ethnic populations. HLA loci diversity due to racial admixture, environment and selection pressure and by inherent polymorphic nature results in allelic variation in different ethnic groups, correspondingly we get different HLA associations with disease in different populations. Thus association of disease outcome with HLA alleles appears to depend upon the ethnicity of the infected individual and therefore is inconsistent across the populations despite being robust within an ethnic group.

The specific HLA associations with HBV and HCV infections are different, agreeing to their differences in viral properties and disease pathogenesis, with few exceptions where they share few HLA loci. This could be due to linkage disequilibrium of HLA alleles with disease-associated genes or shared HLA restricted HCV and HBV T cell viral epitopes. It is intriguing that HLA associations are oppositely directed and indicate interactions with other unidentified factors in influencing the HLA mediated immune signaling. It is also observed that HBV and HCV infections tend to suppress each other. HBV and HDV can suppress HCV infection and HCV and HDV can have negative effect on HBV. HCV super infection is seen to reduce HBsAg expression and promote its clearance[103].

HLA DR9 is protective for HCV infection in Japanese[69,70] but is a susceptible factor for chronic HBV in Koreans and Chinese[11,19] (Tables 1 and 6). In a comparative study of HBV and HCV infected subjects, MICA (*) 015 is associated with viral clearance in case of HCV infection but with chronic infection in case of HBV[104]. HLA Class Ⅱ HLA DRB1*11 and DQB1*0301 are protective for HCV infections, but are also associated with chronic HBV infection. Also, HLA DQB1*0301 is associated with responsiveness to interferon therapy in chronic HBV infection[85] and also to anti HBsAg vaccination[39]. Interferon treatment may activate antigen presentation by aiding generation of more DQB1*0301 responding cytototxic T lymphocytes and thus a protective response[85]. Constantini *et al*[105] did not find any association between HCV clearance and alpha interferon therapy with IL1, IL10 and TNF alpha genes. This warrants further investigation with other HLA class Ⅲ genes to know the influence of interferon and a protective association of HLA DQB1*0301 allele in HCV clearance. Cytokine genes should also be investigated for any synergistic association with any of these HLA alleles in HBV infection. Involvement of the same alleles with different outcomes in response to viral infection in different population is intriguing. Collaborative studies involving well-characterized cohorts from different populations are needed.

The difference in influence of HLA DRB1*11 and DQB1*0303 in HCV and HBV infection outcome could be due to variation in viral specific antigen presentation and thus differences in immune responses. Investigating the molecular mechanisms involving HLA DR*11 and DQ*3 associated viral clearance in HCV would provide better understanding of this aspect. Identifying HLA DR*11 and DQ* 3 restricted viral HCV epitopes for CD8+ cells would allow finding or designing similar HBV specific epitopes. This can further be used therapeutically to enhance viral clearance in HLA DR*11 and DQ*3 carrying HBV infected subjects. Investigating involvement of shared epitopes for HBV/HCV may open up opportunities for generating combined HBV-HCV vaccine in the future.

Immunodominant antigenic epitopes have been identified in HCV and they could be investigated for HLA restricted stage specific presentation to develop HLA specific vaccine and peptide-based immunotherapies[106]. HLA B27 restricted HCV specific CD8+ epitope recovered from female subjects has been identified[61]. Such CD8+ specific HLA restricted peptides have been proposed as vaccine and have been shown effective against

human metapneumovirus infection and disease in mice[107].

Since immunization at present is the most effective means of combating HBV infection, non-responders always remain at risk of getting infected. Identifying non-responding immunogenetic marker could help protect non-responders by giving them alternative protective therapy and would also permit avoiding administering vaccine to identified non-responding groups in a given population. Designing HLA restricted epitope based anti-HBV vaccine is an alternative for non-responding groups.

Association of HLA DR13 alleles is protective in both HCV and HBV infections in several populations (Tables 2 and 7) and is proposed to be involved in enhancing immune response to various diseases[17]. In contrast HCV disease susceptibility and severity is linked to DR13 in German and Polish populations[94,95], though other reports from Germany suggest its protective role (Table 7). It is also protective in vertical transmission of HBV and HCV infection (Tables 2 and 7). Recognizing HLA DR13 restricted HBV and HCV T cell epitopes could lead to identification of similar/common antigenic determinants and open possibilities for designing a combined anti HBV-HCV vaccine.

HLA DRB1*07 is positively associated with severe disease outcome in both HBV and HCV infections (Tables 1 and 6). This allele is related to non-responsiveness to anti-HBV vaccination and interferon treatment in subjects with chronic HBV and HCV infections (Table 9), but is reported with responsiveness to interferon treatment in individuals with chronic HCV infection from China and Poland[84,108]. HLA B35 and B40 is associated with chronic HBV and HCV infection in Russian populations[5,57], and the authors suggest chronic hepatitis to be associated with HLA B*35, independent of virus type. HLA B35 involvement is reported with viral persistence of HCV in Koreans[109], and of HBV in Han Chinese[4]. HLA B8 is associated with viral persistence and chronicity for both HBV and HCV infections in Caucasian populations (Tables 1 and 6). HLA Cw*07 is a risk factor for vertical infection in Italians[16] and is associated with HBV related liver cancer in Senegalese[6]. Such associations suggest inability of these alleles to mount efficient immune responses for viral clearance irrespective of the viral type and warrants elaborate studies concentrating these alleles in several populations, to identify predisposing genetic markers for disease severity.

Studies focusing on viral hepatitis in the United States (US) in American populations have suggested differences in HLA allele association for Caucasians and African-Americans. HBV viral persistence is associated with HLA A*01-B*08-DRB1*03, B*44-Cw*1601 and B844-Cw*0501 haplotype in Caucasian Americans while HLA DQA1*0501, DQB1*0301 and DRB1*1102 haplotype in African Americans[7,18]. HLA A*0301 and HLA DRB1*1302 is associated with HBV clearance in Caucasian Americans, the later being associated with HBV viral clearance in Gambian populations[7,110]. HCV persistence is associated with HLA A*2301 in Caucasian Americans and with the HLA-Cw*04 allele in both Caucasian and African American populations. Also, two copies of HLA-Cw*04 showed stronger persistence than one copy of the allele[58]. Viral clearance showed stronger association with HLA A*1101 and B*57 alleles in both Caucasians and African Americans but HLA Cw*0102 showed stronger association with viral clearance only in Caucasians[58]. Another study by Thio *et al*[77] reported HLA DQB1*0301 to be associated with viral clearance showing stronger associations in African Americans than Caucasian Americans. Also HLA DRB1*0101 and DQB1*0501 showed association with HCV viral clearance, but only in Caucasian Americans. No information is available on HLA associations with interferon treatment response and racial differences in US American populations which needs investigation.

Variations in the immune response to viral insult appear to be genetically inherited. Reports on HLA gene associations with viral infections are inconsistent and contradictory. Several factors including (1) alteration in antigen binding affinity, (2) ineffective antigen presentation, (3) ineffective interaction of Ag-MHC and TCRs, (4) absence of specific T cells, (5) cytokine deficiency and (6) inadequate complement activation may contribute to defective/inefficient HLA mediated immune response mechanisms and various disease manifestations. Indirect associations of HLA in disease pathology have also been proposed. Circulating autoantibodies against HLA Class II and class I molecules have been found, and may represent autoimmune response against HLA molecules that may induce HCV related chronic liver damage[111]. Complement abnormality has been associated with several liver diseases.

Polymorphism of TNF alpha and IL10 promoter has been implicated to influence HBV and HCV infections suggesting a vital role for cytokines in disease outcome. Genes outside the major histocompatibility complex also play a major role in determining the disease/treatment outcome of viral hepatitis. Altered expression of CD45 isoforms influence HCV outcome in humans and transgenic mice studies[112]. NK stimulating and inhibitory signaling receptor and HLA interaction influence viral persistence or clearance. Association of viral persistence and clearance is also reported with chemokine receptors[72].

Due to (1) the highly polymorphic nature of HLA genes, (2) diversity observed in genetic interactions and (3) complexity of immune response, it has not been possible so far to "Tag" any allele or loci as a potential candidate for a particular disease or disease outcome. However clinical evidence vividly indicates such associations. Thus, it is prudent to argue that response to viral hepatitis infection is immunogenetically governed by multiple candidate genes (including HLA and non HLA genes) acting either independently or in association. HLA genes could be directly involved or may be closely linked to genetic markers for true susceptibility, protection and treatment response genes.

CONCLUSION

Racial diversity, variations in the study design, methodology and complex immune-regulatory mechanisms make it difficult to find consistent association of HLA alleles with a given HBV or HCV disease even in the same ethnic group of the global population.

The host-virus interaction resulting in acute or chronic

viral infection depends on cellular immune responses that are regulated by the host's HLA type and HLA restricted viral escape mutants. Much research is required for involving either the CD8 antigenic epitope or NK cell (involving KIR ligands) based HLA associations in individuals undergoing acute *vs* chronic infections in different ethnic populations. Thus, novel strategies are needed to combat the escape mechanisms utilized by viruses in different ethnic populations.

Limited numbers of studies across various ethnic populations have been conducted in relation to HBV vaccination outcome or various therapeutic outcomes against HBV or HCV infections. Thus, global network studies will be useful for HLA disease associations in different ethnic global populations in relation to various therapeutic or HBV vaccination outcome and resulting in viral clearance. Information on HLA association with disease outcome holds immense promise for designing host specific therapeutic strategies. HLA studies involving collaborative analysis of several immune regulatory host genes including T cell repertoire variables in cohorts from around the globe are needed to answer the puzzle regarding various host related immunogenetic factors in conditions resulting in varying outcomes of HBV or HCV hepatitis infection or their co-infections.

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GASTRIC CANCER

Synergistic effect of oxymatrine and angiogenesis inhibitor NM-3 on modulating apoptosis in human gastric cancer cells

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Abstract

AIM: To investigate the synergistic effect of oxymatrine (OM) and angiogenesis inhibitor NM-3 on modulating apoptosis in human gastric cancer cell lines SGC-7901, MKN-45, MKN-74.

METHODS: Human gastric cancer lines SGC-7901, MKN-45, MKN-74 were treated with OM in the absence and presence of NM-3. The inhibitory rates were detected by MTT assay. Synergistic effect of OM and NM-3 on the growth of survivin, bcl-2, bax and *p*53 in SGC-7901 cells were examined by semiquantitative RT-PCR and Western blotting, and their growth inhibitory effects were also observed on SGC-7901 tumor xenograft in nude mice.

RESULTS: OM combined with NM-3 exhibited a synergistic inhibitory effect on the growth of SGC-7901, MKN-45 and MKN-74 cells in a time-dependent manner. Twenty-four hours after treatment with OM, NM-3 alone and their combination, mRNA expression of survivin and bcl-2 in SGC-7901 cells decreased, *p*53 mRNA expression increased. OM (4 g/L) combined with NM-3 significantly increased the expression of *p*53 mRNA and decreased the expression of survivin and bcl-2 compared with either agent alone (193% ± 34% *vs* 129% ± 12%; 44% ± 18% *vs* 92% ± 18%; 36 ± 17% *vs* 93% ± 23%, $P < 0.05$). Western blotting showed that the synergistic effect of OM and NM-3 on protein translation of survivin, bcl-2 and *p*53 was in accordance with their mRNAs. Furthermore, OM/NM-3 combination obviously exhibited antitumor growth effect in xenografted human gastric cancer cells SGC-7901 compared with either agent alone.

CONCLUSION: OM combined with NM-3 has synergistic inhibitory effects on human gastric cancer cells *in vitro* and can suppress the growth of xenografted human gastric cancer cells SGC-7901 *in vivo*.

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Key words: NM-3 compound; Oxymatrine; Gastric cancer; Apoptosis

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INTRODUCTION

NM-3 is a novel angiogenesis inhibitor isolated from a culture filtrate of streptoverticillium eurocidicum[1-3]. Studies indicated that NM-3 is an effective inhibitor of angiogenesis[4]. Subsequent work has shown that NM-3 also exhibits direct cytotoxic effects on endothelial and carcinoma cells[5]. Moreover, NM-3 has been found to potentiate the therapeutic effects of certain chemotherapeutic agents, including 5-fluorouracil, paclitaxel, and cyclophosphamide[6]. Our previous study showed that NM-3 combined with carboplatin can suppress the growth of human gastric cancer SGC-7901 cells xenografted in nude mice[7], suggesting that NM-3 combined with carboplatin could not only increase anticancer effect but also aggravate toxicity. In this study, we selected oxymatrine (OM) to substitute carboplatin for its lower toxic side-effect and its ability to kill human cancer cells[8-10]. The purpose of this study was to investigate whether OM combined with NM-3 has synergistic inhibitory effects on human gastric cancer cells and growth of xenografted human gastric cancer cells SGC-7901.

MATERIALS AND METHODS

Medicine and reagents

NM-3 supplied by Professor Robert (New York University) was dissolved in PBS sterilized at a stock concentration of 1 mg/mL. For *in vivo* experiments, NM-3 (10 mg/kg per d) was administered *via* i.p. injection. OM was purchased

from Shanghai Huamei Corporation (China). MTT purchased from Sigma (USA) was dissolved in 0.01 mol/L PBS. TRIzol reagent was from Gibico BRL Co (Germany). M-MuLV reverse transcription-polymerase chain reaction kit was from TaKaRa (Japan). One-step RNA PCR kit was obtained from AMV TaKaRa (Japan). Survivin and *p*53 polyclonal antibodies were purchased from Santa Cruz Co (USA). Bcl-2 and bax antibodies were obtained from San Francisco (CA). PCR primers were synthesized in Shanghai Sangon (China).

Tumor cells

Gastric cancer cell lines SGC-7901 and MKN-45 and MKN-74 were purchased from Wuhan University and maintained at 37℃ in DMEM supplemented with 10% FCS and 2 mmol/L glutamine in a humidified incubator containing 50 mL/L CO_2 until they were amplified to a certain concentration 6×10^6/mL.

Animals

Thirty-two 6-7 wk old BALB/C-nu/nu mice weighing 18-22 g were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (SPF, NO.SCXK033).

Cell culture and growth assay

Cell growth was assessed by MTT assay. A total of 5×10^3 cells were seeded into each well of 96-well culture plates in RPMI 1640 medium for 24 h, treated with scalar concentrations of OM in the absence or presence of 10 mg/L NM-3, and incubated for 24, 48, 72 and 96 h respectively. After incubation, 10 μL of MTT solution (5 g/L) was added to each well, and the plates were then incubated for 3 h at 37℃. The growth medium was then replaced with 150 μL of dimethyl sulfoxide (Wako) per well, and the absorbance at A490 was measured using Titertek Multiscan. IC50 with SAS statistical software.

Semiquantitative RT-PCR

One μg of total RNA from each sample was resuspended in a 25 μL final volume of reaction buffer, containing 10 × one-step RNA PCR buffer, 25 mmol/L $MgCl_2$, 10mmol/L dNTP mixture, 20 U RNase inhibitor, 2.5U AMV RTase XL, 2.5 U AMV-Optimized Taq, 20 μmol/L forward primer, 20 μmol/L reverse primer, and RNase-free dH_2O. Following primers were for survivin (349 bp): 5'-GGA CCA CCG CAT CTC TAC AT-3', 3'-CC TTT GAC GCT TCT TTC ACG-5; bcl-2 (292 bp): 5'-TGC ACC TGA CGC CCT TCA C-3', 3'-G ACA CTA AAG AGG ACC GAC AGA-5'; bax (364 bp): 5'-ACC AAG AAG CTG AGC GAG TGT C-3', 3'-C CGT CTG GCA CTG GTA GAA ACA-5'; *p*53 (396): 5'-CAG CCA AGT CTG TGA CTT GCA CGT AC-3', 3'-CT ACT GTC TTT GTG AAA AGC TGT ATC-5'; β-actin(542 bp): 5'-GCTCCGGCATGTGCAA-3', 3'-TGATGGAGTACTTCTAGGA-5'.

The PCR conditions were at 94℃ for 5 min, 1 cycle; at 94℃ for 30 s, at 59℃ (survivin, bcl-2, *p*53, β-actin) or at 56℃ (bax)for 30 s, at 72℃ for 45 s, 35 (survivin, bcl-2, *p*53, β-actin) or 30 (bax) circles; at 72℃ for 7 min, 1 circle. The PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and revealed by a gel documentation system (FR-200, Shanghai Fu Ri Bio Co. China). The content of cDNA was semi-quantitatively normalized with housekeeping gene β-actin as the internal control.

Western blotting

Gastric cancer SGC-7901 cells were seeded into 250 mL flasks, grown to confluence and incubated in the presence of NM-3 either alone or in combination with OM (1, 2, 4 g/L) for 24 h. Cells were then harvested and lysed in a modified protein lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 135 mmol/L NaCl, 1 mmol/L EDTA, 10mmol/L CHAPS, 10 mg/L aprotinin, 0.02 mmol/L APMSF), and the protein concentration of the lysates was measured. Total cell protein extracts (15 μg/lane) were separated by SDS-PAGE using ready gels (Bio-Rad Laboratories, Milan, Italy) and electrophoretically transferred onto PVDF membranes. The membranes were blocked with 5% dried non-fat milk in PBS buffer and 0.05% Tween20. The filters were then incubated with primary antibodies against survivin, bcl-2, bax and *p*53. Each of these antibodies was diluted according to the manufacturer's instructions. Membranes were then washed with PBS-T buffer (PBS with 0.1% Tween 20) and incubated with the appropriate secondary antibodies. Immunoreactive proteins were visualized with enhanced chemiluminescence (Amersham International, Buckinghamshire, UK).

Tumor xenograft studies

Suspensions of tumor cells SGC-7901 ($5\text{-}10 \times 10^6$ viable cells/mouse) were implanted into the bilateral flank region of BALB/c-nu/nu mice as previously described[7]. When the tumors grew to 50-100 mm^3, the animals were randomly divide into four groups (n = 9). The animals were pair matched so that the median tumor volume for each group was similar: control group: NM-3 (10 mg/kg per day) *via* i.p. injection thrice per week; treatment groups: OM (1, 2, 4 g/L and NM-3 (10 mg/kg per day) via i.p. injection thrice per week. The tumor volumes were estimated by $V = ab^2/2$, where a and b are tumor length and width, respectively. To evaluate the antitumor effects of OM and NM-3, tumor size and body were measured thrice per week.

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was performed using Chi-square test , Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Synergistic growth inhibitory effect of OM and NM-3 on SGC-7901, MKN-45 and MKN-74 cells

We analyzed the synergistic growth inhibitory effects of OM and NM-3 on the gastric cancer cell lines SGC-7901, MKN-45 and MKN-74. MTT assays were performed to examine the growth inhibitory effect of OM or NM-3 alone (Figure 1A) and OM in combination with NM-3 (Figure 1B). Each cell line was treated with 1, 2 and 4 g/L

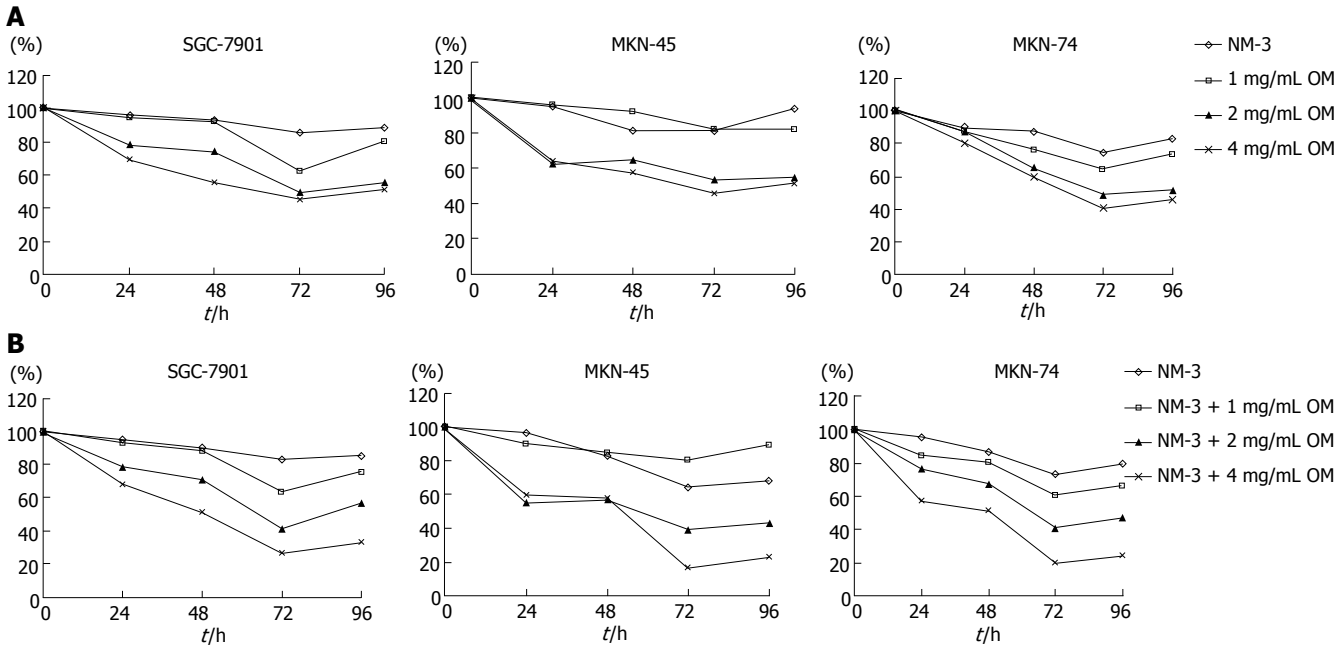

Figure 1 Growth inhibitory effects of OM alone and in combination with NM-3 on 3 gastric cancer cell lines, SGC-7901, MKN-45 and MNK-74. **A**: MTT assays were performed to examine the growth inhibitory effect of OM alone. Clear growth inhibition was observed in a time- and dose- dependent manner in each of the gastric cancer cell lines; **B:** Growth inhibitory effects of OM combined with NM-3. The concentrations of OM used are indicated for each symbol.

Table 1 NM-3 IC_{50} values for gastric cancer cell lines after treatment with NM-3 alone and in combination with OM (mean ± SD, g/L)

	NM-3 alone	NM-3 plus OM	Fold
SGC-7901	321.21 ± 51.21	132.84 ± 21.13	2.54
MKN-45	245.56 ± 31.34	66.07 ± 18.21	4.13
MKN-74	225.92 ± 33.64	71.75 ± 20.11	3.38

OM, either alone or in combination with NM-3. The time-course effects were observed 24, 48, 72 and 96 h after treatment. MTT assays were performed to analyze the inhibitory effects of the drugs at each time point, which were compared to the non-treated control cells. Treatment with OM alone and combined OM/NM-3 inhibited the growth of three gastric cancer cell lines in a time- and dose- dependent manner.

The OM IC50 levels were found to be 2.51, 3.02 and 2.32 mg/mL for SGC-7901, MKN-45 and MKN-74, respectively. To determine the potent effects of OM on NM-3 activity, the growth inhibitory effects of NM-3 alone or in combination with OM on three gastric cancer cell lines were evaluated. After treatments the IC50 of NM-3 for each cell line was compared with that of NM-3 in combination with 4 g/L OM (Table 1). These values were significantly reduced by 2.54-, 4.13- and 3.38-fold in each case, suggesting that OM in combination with NM-3 had synergistic effects on growth of gastric cancer cells.

Changes in expression of survivin, bcl-2,bax and p53

To define the mechanisms of OM and NM-3 in inducing apoptosis, we first studied the changes in mRNA expression of survivin, bcl-2, bax and *p*53. The mRNA and protein expression levels in SGC-7901 cells were analyzed by RT-PCR and Western blotting. After treatment with NM-3 or with OM (1, 2, 4 g/L) for 24 h, both cDNA and proteins in survivin, bcl-2, bax and *p*53 in SGC-7901 cells were evaluated.

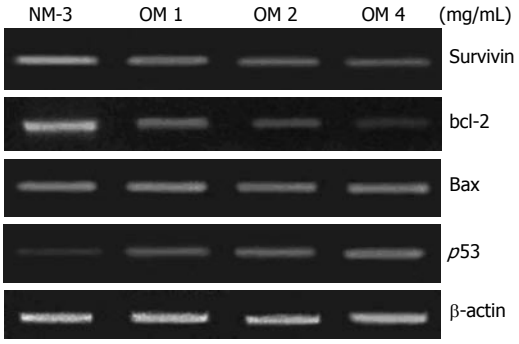

Figure 2 After 24 h treatment identification of differentially expressed survivin, bcl-2, bax, *p*53, β-actin genes in human gastric cancer line SGC-7901 by means of semiquantitative RT-PCR. NM-3 signifies NM-3 alone; OM 1 mg/mL signifies OM 1 mg/mL combined with NM-3; OM 2 mg/mL signifies OM 2 mg/mL combined with NM-3; OM 4 mg/mL signifies OM 4 mg/mL combined with NM-3.

As shown in Figures 2 and 3, treatment with OM (1 mg/mL, 2 mg/mL, 4 mg/mL) in combination with NM-3 increased the mRNA levels in bax and *p*53 and decreased the survivin and bcl-2 levels in a dose-dependent manner. Combined 4 mg/mL OM and NM-3 significantly increased *p*53 mRNA expression (193% ± 34% *vs* 129% ± 12%, $P < 0.05$) and significantly decreased survivin and bcl-2 mRNA expression compared to NM-3 alone (44% ± 18% *vs* 92% ± 18%, 36% ± 17% *vs* 93% ± 23%, $P < 0.05$) compared to NM-3 alone.

The expression of *p*53 protein 24 h after treatment with increased dose of OM was 2.8-fold higher in OM treatment group than in NM-3 treatment group.

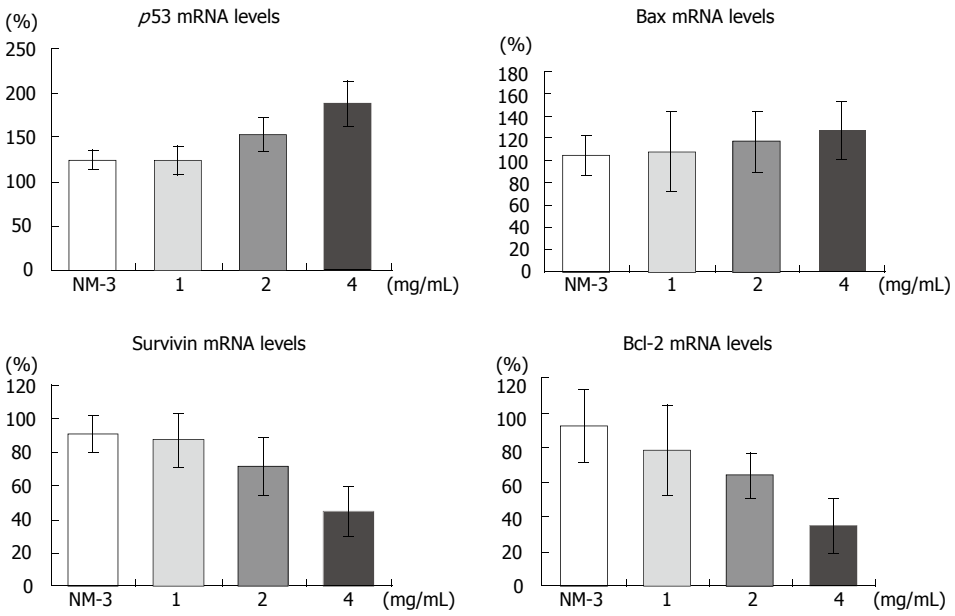

Figure 3 Differentially expressed mRNA histograms of survivin, bcl-2, bax, *p*53, β-actin genes in human gastric cancer line SGC-7901 after 24 h treatment. There are significant differences in expression of survivin bcl-2 and *p*53 mRNA between the NM-3 and NM-3 combined OM 4 mg/mL groups ($P < 0.05$).

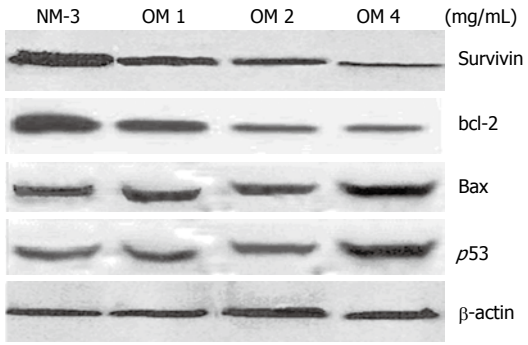

Figure 4 Western blotting analysis showing expression of survivin bcl-2 bax and *p*53 in human gastric cancer line SGC-7901 after 24 h treatment. NM-3 signifies NM-3 alone; OM 1 mg/mL signifies OM 1 mg/mL combined with NM-3; OM 2 mg/mL signifies OM 2 mg/mL combined with NM-3; OM 4 mg/mL signifies OM 4 mg/mL combined with NM-3.

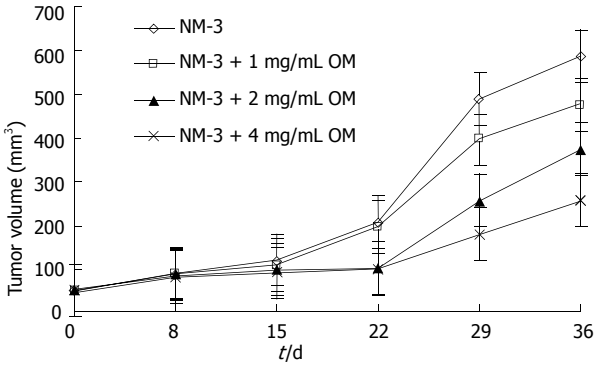

Figure 5 Curve lines of mean tumor volume (mm^3) of nude mice with xenografted human gastric cancer in different groups, effects of NM-3 alone and 1 mg/mL, 2 mg/mL, 4 mg/mL OM combined with NM-3 on gastric tumor volume in 36 d.

Survivin expression decreased in a dose-dependent manner. Moreover, the expression of bax protein was slightly increased after treatment with NM-3 alone or in combination with OM. There was a significant difference in *p*53, survivin and bcl-2 expression between the two groups treated with OM (4 mg/mL) in combination with NM-3 or with NM-3 alone ($P < 0.05$). These findings strongly suggest ed that synergism between OM and NM-3 induced apoptosis in more gastric cancer cells than NM-3 alone (Figure 4).

In vivo effects of NM-3 and NM-3 in combination with OM on human tumor xenografts

To assess the potential synergistic effects of OM and NM-3 *in vivo*, human tumor xenograft experiments were performed. We examined the growth inhibitory effects of NM-3 alone and in combination with OM on SGC-7901cells transplanted into nude mice. The resulting growth curves for gastric cancer xenografts during various treatment schedules are shown in Figure 5.

NM-3 alone (10 mg/kg per day) or in combination with OM showed a tumor growth inhibitory rate of 9.5% ± 3.1%, 12.3% ± 3.5%, 40.6% ± 9.7% and 52.8% ± 12.4% on d 15, and 18.8% ± 4.8%, 28.6% ± 8.4%, 53.4% ± 13.5% and 75% ± 18.5% on d 36, respectively. OM in combination with NM-3 had stronger effect than NM-3 alone on tumor growth ($P < 0.05$). These results further suggested that combined OM and NM-3 had synergistic effects on tumor growth. In addition, none of the treated animals in our experiments showed weight loss due to drug toxicity, demonstrating that combined OM and NM-3 was effective against gastric cancer and could be used as a new therapeutic tool for treatments of tumors. Furthermore, no increase in toxicity was observed after combined treatment.

DISCUSSION

When NM-3 is combined with other chemotherapeutic agents, it could effectively inhibit the growth of tumor[6]. In our previous studies we used NM-3 (10-40 mg/kg) in combination with carboplatin to treat nude mice xenografted with SGC-7901 cell lines, and found that

some mice became emaciated for diarrhea and anorexia in early stage and some died[7]. The death rate was higher than 16% in groups treated with NM-3 (greater than 20 mg/kg). However, all the mice treated NM-3 (10 mg/kg) showed less severe reaction. We substituted OM for carboplatin because OM can kill human cancer cells with less toxic side-effects. It was recently reported that OM induces apoptosis of human hepatoma SMMC-7721 cells and human colorectal tumor SW1116 cells[11,8].

In this study, we analyzed the effects of OM and OM in combination with NM-3 on proliferation of human gastric cancer lines *in vitro*. OM alone and in combination with NM-3 inhibited the growth of each gastric cancer cell line in a time- and -dependent manner. Moreover, their striking synergistic effects were observed in three gastric cancer cells with NM-3 IC50 values being obviously decreased when combined with OM. Furthermore, in nude mice with SGC-7901 xenografts, treatment with OM and NM-3 exhibited significant synergistic antitumor effects. Our present studies showed that when OM was used in combination with NM-3, a significant reduction in xenograft volumes was observed without an apparent increase in toxicity, demonstrating that combined OM and NM-3 is more effective against gastric cancer.

To further discuss the exact mechanism of apoptosis induced by OM and NM-3, we detected the changes in survivin, bcl-2, bax and *p*53 genes by semiquantitive PCR. The result showed that OM in combination with NM-3 could down-regulate the expression of survivin, bcl-2 and up-regulate the expression of *p*53 mRNA. We also validated the similar results from Western blotting, showing that the inhibitory effect of OM in combination with NM-3 is associated with their ability to induce apoptosis by regulating the transcription and translation of survivin, bcl-2 and *p*53 genes.

Cell apoptosis is induced and controlled by many complicated factors, such as expression changes of correlative apoptosis genes and blockage of cell cycle. Survivin belongs to the inhibitory apoptosis protein family and is a good marker of most cancer cells[12,13]. Various strategies to target survivin in cancer treatment are currently under investigation with promising results in vivo and *in vitro*[14-22]. Bcl-2 is a central apoptotic suppression gene and overexpresses in most immortal and cancer cells[23]. Most therapeutic agents act by blocking the function of anti-death bcl-2 proteins or by enhancing the activities of pro-death proteins such as bax[24]. Once the brake provided by anti-death bcl-2 members is removed, the pro-death protein bax oligomerizes at the mitochondrial membrane leading to release of apoptogenic factors into the cytosol, an event initiating a deadly proteolytic cascade[25].

P53 is a multifunctional protein with multiple modifications and biochemical properties. *p*53 has the ability to bind to specific DNA sequences and functions as a potent transcription factor, that trans-activates and trans-represses specific genes involved in controlling cell cycle and apoptosis[26]. Activation of *p*53 is associated with induction of p21 expression, an event regulated in part by *p*53-mediated trans-activation. When p21 cells down-regulate Cdk2 and arrest in G1-S-phase[27,28], *p*53 also acts as a tumor suppression protein opposite to bcl-2. Recent studies indicate that conventional anticancer drugs can perform their functions modulating bcl-2 protein expression, whereas this modulation seems ultimately to lead to the presence of *p*53[29,30].

In this study, we focused on the synergistic effects of OM and NM-3 on human gastric tumor cells and evaluated their apoptosis regulation *in vitro* and *in vivo*. The data show that OM in combination with NM-3 inhibits growth and proliferation of tumor cells, and induces apoptosis of human gastric cancer cells, which is associated with down-regulation of surviving and bcl-2 expression and up-regulation of *p*53 expression in SGC-7901 cells. In conclusion, our present study strongly indicates that combined OM and NM-3 is synergistically effective against gastric carcinomas.

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GASTRIC CANCER

Association of serum vascular endothelial growth factor-C and lymphatic vessel density with lymph node metastasis and prognosis of patients with gastric cancer

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Abstract

AIM: To investigate whether serum vascular endothelial growth factor-C (SVEGF-C), VEGF-C, and lymphatic vessel density (LVD) in tumor tissues are related to lymph node metastasis (LNM) and prognosis in gastric cancer.

METHODS: SVEGF-C levels of 80 gastric cancer patients and 20 healthy donors were examined using ELISA. VEGF-C expression and LVD were examined using immunohistochemical staining. Kaplan-Meier survival analysis was performed to determine their influence on the prognosis of the patients.

RESULTS: The SVEGF-C level in gastric cancer patients (595.9 ± 201.0 ng/L) was significantly higher (P = 0.000) than controls (360.0 ± 97.4 ng/L). Both SVEGF-C and LVD were significantly higher in poorly differentiated adenocarcinomas, T3 and T4, LNM, distant metastasis, and pTNM groups Ⅲ and IV (P = 0.000). The sensitivity and specificity of SVEGF-C for predicting LNM were 82.8% and 81.8%, respectively (cut-off = 542.5 ng/L). The positive expression rate of VEGF-C was significantly higher in cancerous than in normal tissues (65% *vs* 20%; P = 0.001). VEGF-C expression up-regulation was significantly related to differentiation, depth of invasion, LNM, distant metastasis, and pTNM stage (P = 0.000). LVD was 10.7 ± 3.1/200 HP in the experimental group *vs* 4.9 ± 1.3/200 HP in controls (P = 0.000); LVD in cancerous tissues with and without LNM was 12.0 ± 2.7/200 HP *vs* 7.6 ± 0.5/200 HP, respectively (P = 0.000). SVEGF-C and LVD were significantly higher in VEGF-C positive than in negative patients (P = 0.000); SVEGF-C level was related to LVD (P = 0.000). Kaplan-Meier survival analysis factors predicating poor prognosis were: SVEGF-C level (P = 0.001), VEGF-C expression and LVD (both P = 0.000).

CONCLUSION: SVEGF-C level, VEGF-C and LVD are related to LNM and poor prognosis of patients with gastric cancer. SVEGF-C may be a biomarker for LNM in gastric cancer.

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Key words: Gastric cancer; Serum VEGF-C; Lymphoangiogenesis; Lymph node metastasis; Survival

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INTRODUCTION

Vascular endothelial growth factor (VEGF) is involved in the neoangiogenesis of solid tumors[1,2]. VEGF-C is a member of the VEGF family that can induce lymphangiogenesis, a critical factor in the progression of many malignant tumors[3-6]. Although the mechanism of lymphangiogenesis and the role of the new lymphatic vessels remain unclear, experimental studies indicate that lymphangiogenic factor VEGF-C may stimulate lymphangiogenesis[7-9]. Several reports have also demonstrated a relationship between VEGF-C expression and lymph node metastasis (LNM) in many cancers, including gastric cancer, oral squamous cell cancer, squamous cell cancer of the esophagus, and non-small cell lung cancer[10-13]. Kitadai *et al*[14] reported that podoplanin is a specific antibody for lymphatic vessel density (LVD), suggesting that LVD correlates with the clinicopathological factors of patients with gastric cancer. Our research has also shown that podoplanin can be used to identify LVD and lymphangiogenesis is related to LNM of colorectal cancer[15].

In a study of patients with lung cancer, Tamura *et al* reported that circulating SVEGF-C levels may provide

additional information to distinguish between the absence and presence of LNM[16]. Other studies have shown that the SVEGF-C level was higher in patients with cervical cancer than in healthy controls, however, the higher levels did not correlate with LNM[17,18]. In patients with non-small cell lung cancer, both the assay of SVEGF-C, matrix metalloproteinase-9, VEGF and CT examination has been shown to be a clinically useful indictor to predict the presence of LNM[19, 20].

To date, few studies have demonstrated whether SVEGF-C level, VEGF-C expression and LVD in tumor tissues indicate the presence of LNM and can predict survival of patients with gastric cancer. In this study, we investigated the circulating level of SVEGF-C, VEGF-C expression and LVD to determine whether they indicate prognosis in patients with gastric cancer.

MATERIALS AND METHODS

Patients and tumor samples

From October 2002 to October 2003, 80 patients (54 men and 26 women) with a mean age of 57.1 years (± 16.3 SD; range 19-87 years) underwent radical gastrectomy for gastric cancer in the third and first affiliated hospitals of Sun Yat-Sen University. The paraffin embedded specimens of these 80 gastric cancers and 20 normal gastric tissue specimens were collected. Of the 80 gastric cancer specimens, 9 were fungating, 61 ulcerative, and 10 infiltrating; 30 were well differentiated adenocarcinomas, 6 moderately differentiated, and 44 poorly differentiated. The invasive depths were: 7T1, 29T2, 36T3 and 8 T4. LNM was found in 58 cases, and 11 patients had distant metastasis. The patients were staged according to surgical and pathological findings using the UICC TNM classification: 19 in stage Ⅰ, 13 in stage Ⅱ, 36 in stage Ⅲ, and 12 in stage Ⅳ. Seventy-eight patients followed up for at least 8 mo (mean 37.1 mo; range 8-49 mo) either by telephone or mail. Informed consent was obtained from all of the patients. Twenty patients with benign gastric disease cases served as a control group.

Blood SVEGF-C level assay with ELISA

Peripheral venous blood samples were collected according to standard hospital procedure. Serum tubes were centrifuged at 3500 r/min for 10 min at 4℃ and then were separated and stored at -70℃. The SVEGF-C level was determined using a quantitative sandwich enzyme immunoassay technique (Bender MedSystems, MedSystems Diagnosis, Vienna, Austria) according to the manufacturer's guidelines. The system uses a solid phase monoclonal antibody and an enzyme-linked polyclonal antibody against recombinant human VEGF-C. For each analysis 100 μL of serum was used. All analyses and calibrations were performed in duplicate. The recombinant human VEGF-C standards were measured to form a standard curve. Results were expressed in ng/L. The coefficient of variation of interassay determinations reported by the manufacturer was < 5.0%. The limits of sensitivity of the VEGF-C assay were 46.9 ng/L.

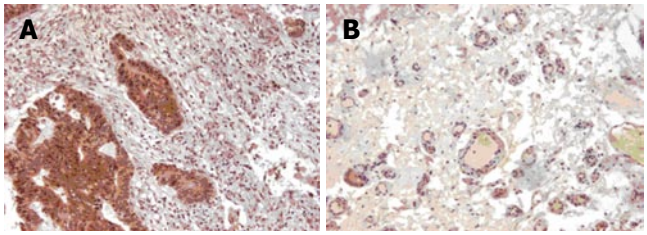

Figure 1 VEGF-C (**A**) and podoplanin (**B**) expression in gastric cancer (S-P, × 200).

VEGF-C and podoplanin expression

Specimens were fixed in a 10% formaldehyde solution and embedded in paraffin. Sections 4 μm thick were cut and mounted on glass slides. Immunohistochemical staining was performed using the avidin-biotin method. The podoplanin antibody, rat anti-VEGF-C monoclonal antibody, rat SP and DAB kits were provided by Beijing Zhongshan Biotechnology Co., LTD.

The formalin-fixed, paraffin-embedded 4 μm tissue sections were deparaffinized with xylene, dehydrated in ethanol, and incubated with 3% hydrogen peroxidase for 5 min. After being washed with phosphate buffered saline (PBS), tissue sections were incubated in 10% normal bovine serum for 20 min, followed by an overnight incubation with podoplanin (1:100) or anti-VEGF-C (1:150) antibody. Biotinylated goat antimouse immunoglobulins were used as secondary antibodies. Peroxidase-conjugated avidin was used in a dilution of 1:500. Finally, 0.2 g/L diaminobenzidine and 10 mL/L hydrogen peroxide in PBS were used as the substrate. Specimens positive for podoplanin and VEGF-C were used as positive controls and with the first antibody substituted by PBS as negative controls.

Brown granules in the cytoplasm of a tumor cell were considered indicative of a positive cell. For the evaluation of VEGF-C expression, immunostaining was classified into two groups, corresponding to the percentage of immunoreactive cells. The cut-off point to distinguish negative from positive expression was 30% (Figure 1A). LVD was determined based on brown staining of endothelial cells with podoplanin under a 200-fold light microscope field. Any single brown stained cell or cluster of endothelial cells that was clearly separated from the adjacent microvessel, was considered a lymphatic vessel (Figure 1B). A mean lymphatic vessel of 4 different hot regions was regarded as the LVD of the specimen. Two pathologists who were blinded to data did the observation. The average of microvessels with FVIIIRag staining in 10 hot regions was calculated as MVD.

Statistical analysis

The Chi-square test or Fisher exact test was used to compare the categorical variables. The Student *t* test was used to analyze continuous variables. Pearson's rank correlation test was used to determine the relationship between SVEGF-C level and LVD. The cut-off value of SVEGF-C level to determine LNM was established according to ROC curve. Kaplan-Meier survival analysis was used to estimate survival time and the long-rank

Table 1 Relationship between SVEGF-C level, expressions of VEGF-C and LVD and pathological features of gastric carcinoma mean ± SD

Pathological characteristics	*n*	SVEGF-C (ng/L)	VEGF-C		LVD(/200HP)
			+	-	
Differentiation degree					
G1+G2	36	427.9 ± 130.0	9	27	7.9 ± 0.9
G3	44	733.4 ± 130.0[c]	41	3[a]	13.1 ± 2.2[c]
Depth of invasion					
T1+T2	36	536.1 ± 227.4	14	32	9.8 ± 2.8
T3+T4	44	644.9 ± 161.5[a]	36	8[c]	11.5 ± 3.2[b]
LNM					
Yes	58	650.9 ± 198.6	46	12	12.0 ± 2.7
No	22	451.0 ± 115.5[c]	4	18[c]	7.6 ± 0.5[c]
Distant metastasis					
Yes	11	834.3 ± 80.0	10	1	14.0 ± 1.2
No	69	558.0 ± 187.0[c]	40	29[c]	10.2 ± 3.0[c]
pTNM stage					
Ⅰ + Ⅱ	32	412.5 ± 130.0	7	25	7.7 ± 0.9
Ⅲ + Ⅳ	48	718.2 ± 134.4[c]	43	5[c]	12.7 ± 2.4[c]

[a]$P < 0.05$ *vs* differentiation degree of G1 + G2 group and invasion depth of T1 + T2 group; [b]$P < 0.01$ *vs* invasion depth of T1 + T2 group; [c]$P < 0.001$ *vs* differentiation degree of G1 + G2 group, invasion depth of T1 + T2 group, LNM group, distant metastasis group and pTNM stage of Ⅰ + Ⅱ group.

test was used to compare the differences in it. Statistical analyses were performed using the SPSS software (SPSS 11.5, SPSS Inc., Chicago, IL, USA). Results were considered statistically significant at $P < 0.05$.

RESULTS

SVEGF-C level, expression of VEGF-C and LVD in gastric cancer

The SVEGF-C level was significantly ($P = 0.000$) higher in patients with gastric cancer (595.9 ± 201.0 ng/L) than in healthy donors (360.0 ± 97.4 ng/L). With a cut-off value for SVEGF-C of 367.5 ng/L, the sensitivity and specificity for diagnosis of gastric cancer patients was 85% and 80%, respectively ($P = 0.000$). VEGF-C positive expression was significantly ($P = 0.001$) higher in gastric cancer tissue (50/80) than in normal gastric tissue (4/20), There was significantly ($P = 0.000$) more LVD in the experimental group (10.7 ± 3.1/200 HP) than in control subjects (4.9 ± 1.3/200 HP).

SVEGF-C level, VEGF-C expression, and LVD were associated with the clinicopathologic features of gastric cancer (Table 1). The level of SVEGF-C reached the highest sensitivity (82.8%) and specificity (81.8%) in diagnosis of LNM when a cut-off value of 542.5 ng/L was used ($P = 0.000$).

The SVEGF-C level was significantly ($P = 0.000$) higher in VEGF-C positive patients (675.4 ± 153.9 ng/L) than in negative patients (463.5 ± 200.4 ng/L). There was a positive correlation between SVEGF-C and LVD ($r = 0.728$, $P = 0.000$). LVD in VEGF-C positive and negative groups was 12.2 ± 2.8/200 HP and 8.3 ± 2.0/200 HP, respectively ($P = 0.000$).

Correlation between SVEGF-C level, VEGF-C and LVD and patient survival

In this study, the 3-year survival rate was 56.3%. The mean survival of patients with high (> 595.9 ng/L) SVEGF-C and low (< 595.9 ng/L) SVEGF-C was 29.1 ± 13.3 mo and 44.0 ± 4.6 mo, respectively ($P = 0.001$; Figure 2A). Patients in the high (> 10.7/200 HP) LVD group had a mean survival of 27.4 ± 12.6 mo, while the mean survival time in low (< 10.7/200 HP) LVD group was 44.7 ± 3.1 mo ($P = 0.000$; Figure 2B). The mean survival time was shorter in the VEGF-C positive expression group than in the negative expression group (33.8 ± 13.3 mo *vs* 42.6 ± 7.4 mo, $P = 0.000$; Figure 2C).

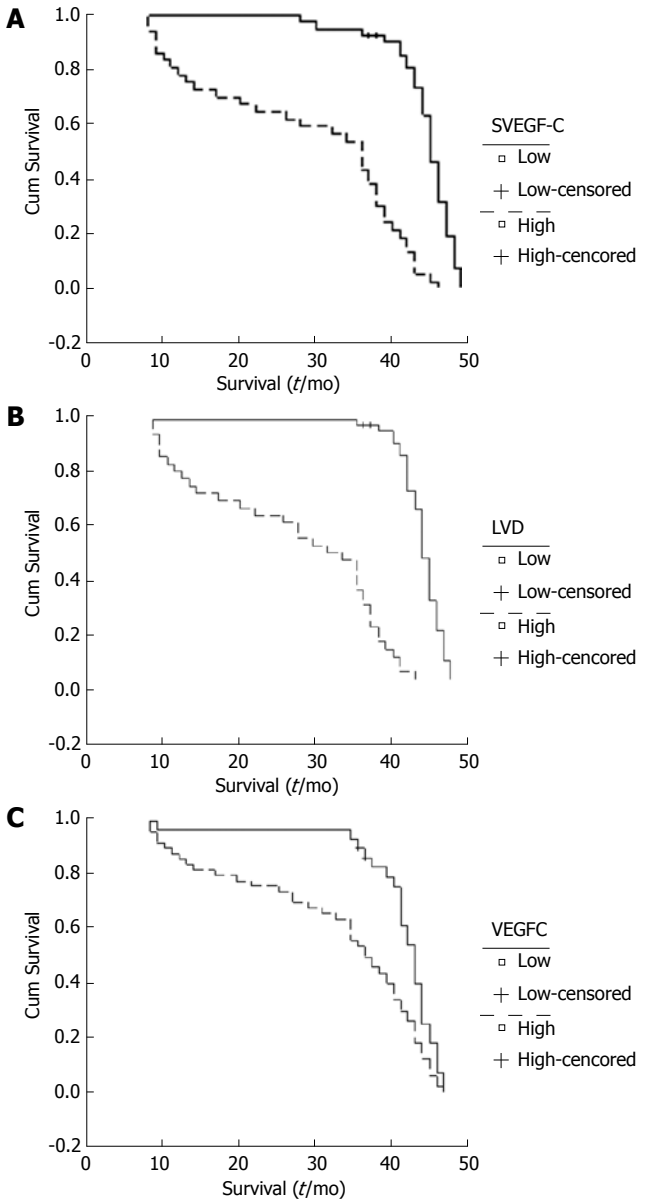

Figure 2 Kaplan-Meier survival curves in gastric cancer SVEGF-C, LVD and VEGF-C groups. **A**: SVEGF-C; **B**: LVD; **C**: VEGF-C.

DISCUSSION

VEGF-C, a member of VEGF family, is known to bind to vascular endothelial growth factor receptor-3 on lymphatic endothelial cells and promote lymphangiogenesis[1-3]. Several studies have found that in many cancers, the positive expression of VEGF-C is higher in tumor tissue than in normal tissue and higher in positive LNM groups than in negative ones[9-12]. Arinaga *et al*[13] have shown that patients

with non-small cell lung cancer and positive staining for VEGF-C had unfavorable survival rates. Our early research showed that VEGF-C expression was stronger in colorectal cancer tissue than in normal colorectal tissue and that there was a relationship between VEGF-C expression and LNM[15]. The higher rate of VEGF expression in cancerous tissue in the present study clearly demonstrates that VEGF-C over-expression exists in gastric cancer. This study also shows that VEGF-C expression is related to the clinicopathologic features of gastric cancer. These results strongly indicate that VEGF-C may promote lymph metastasis and progression of gastric cancer. VEGF-C produced from cancer cells may increase the expression of vascular endothelial growth factor receptor-3 on the lymphatic endothelial cells and promote the formation of new lymphatic vessels. The present study indicates that positive expression of VEGF-C in tumor tissue is an unfavorable factor for the survival of gastric cancer patients. This is consistent with other studies indicating that VEGF-C expression is an independent factor in the prognosis of patients with gastric cancer[21-23]. Given these data, it is reasonable to assume that a high expression of VEGF-C may be a biomarker for the development of gastric cancer and may predict unfavorable survival rates in patients with this malignancy.

In the present study, the SVEGF-C level is higher in patients with gastric cancer than in healthy controls, which is consistent with results from studies in patients with other cancers, including lung, esophageal, and colorectal cancer[16,24,25]. Tamura *et al*[16] found that the SVEGF-C level was higher in patients with non-small cell lung cancer with LNM than in the those without. Similarly, Krzystek-Korpacka *et al*[21] observed that SVEGF-C was a biomarker of LNM in esophageal cancer and Ichikura *et al* found that patients with colorectal cancer with LNM had a higher SVEGF-C level than those without LNM[22]. Another study, however, conducted in patients with advanced cervical cancer did not show a correlation between pretherapeutic levels of VEGF-C and LNM[18]. It has been suggested that circulating VEGF-C level might provide additional information to distinguish between the absence and presence of LNM in patients with cancer. We found that SVEGF-C was related to VEGF-C expression in patients with gastric cancer. Mathur *et al*[17] reported similar results in patients with cervical cancer. To the best of our knowledge, however, this is the first report identifying SVEGF-C level as a marker for LNM and an unfavorable factor for the prognosis of patients with gastric cancer.

Neogenesis of lymphatic vessel and lymphatic invasion is frequently found in the stroma of cancers, while the presence and role of intratumoral lymphatic vessels have been controversial. In an earlier study, we found that LVD was higher in cancerous colorectal tissue than in normal specimens and a significant correlation with the pathological features of the disease[15]. The present study demonstrates similar results in gastric cancer, and LVD was higher in patients with LNM than without and higher in the VEGF-C positive group than in the VEGF-C negative group. We also found a significant correlation between SVEGF-V level and LVD, and that LVD predicated a poorer prognosis of patients with gastric cancer. Previous research has already demonstrated that LVD is correlated with LNM in human gastric carcinoma[14]. Skobe *et al*[26] demonstrated the occurrence of intratumoral lymphangiogenesis within human breast cancers and Munoz-Guerra *et al*[27] documented a strong association between LVD and loco-regional recurrence in patients with oral cancer. Kitadai *et al*[14] reported that LVD in patients with gastric cancer was significantly higher in those with LNM, and the high LVD was highly correlated with VEGF-C expression, while Massi *et al*[28] reported that LVD was significantly higher in melanomas with LNM metastasis than in non-metastatic melanomas. However, there has been controversy about the number and role of LVD in malignant diseases. Brundler *et al*[29] showed that LVD was significantly reduced in adenocarcinoma, being half that in the normal stomach. In prostate adenocarcinoma, LVD was significantly lower than the peritumoral and normal prostate, and was not significantly different between patients with and without LNM[30]. Massi *et al*[28] found that peritumorous LVD was an independent variable affecting overall survival.

In summary, SVEGF-C level, VEGF-C expression and LVD in gastric cancer may be prognostic parameters to identify patients with poor outcome. Preoperative SVEGF-C level might be a useful biomarker for judging the presence of LNM in patients with gastric cancer.

COMMENTS

Background

There are few studies which demonstrated simultaneously whether serum vascular endothelial growth factor-C (SVEGF-C) level, VEGF-C expression, and lymphatic vessel density (LVD) indicate LNM and whether this influences the survival of patients with gastric cancer. The authors investigated the lymphangiogenesis factor to determine if it was related to the progression and prognosis of patients with gastric cancer.

Research frontiers

To date, the mechanism of lymphangiogenesis and role of new lymphatic vessel have been unclear. Many studies indicated that lymphangiogenic factor VEGF-C could stimulate lymphangiogenesis. Some reports indicated that LVD was related to the clinicopathological features in many cancers. It has also been reported, although controversially, that circulating SVEGF-C level may provide additional information for distinguishing between the absence and presence of LNM in patients with cancer.

Innovations and breakthroughs

In the present study, the authors examined SVEGF-C, VEGF-C expression and LVD in gastric cancer using ELISA and immunohistochemical staining. It was found that not only SVEGF-C but also VEGF-C expression and LVD in tumor tissues were related to LNM and survival of patients with gastric cancer. These three factors in gastric cancer have been investigated simultaneously for the first time.

Applications

The results of the present study show that SVEGF-C level, VEGF-C expression, and LVD in tumor tissue are related to both progression and prognosis of patients with gastric cancer. These findings may help understand, to some degree, the role of lymphangiogenesis in this malignant disease. All the three parameters could provide information on the development and prognosis of patients with gastric cancer. And preoperatively high SVEGF-C level might be a biomarker for the occurrence of LNM in gastric cancer.

Terminology

Lymphangiogenesis: the mechanisms of lymphatic vessel formation. The lymphatic vasculature is important for transporting macromolecules from the interstitial

space. The LNM of malignant tumors is perhaps based on the dissemination of tumor cells via the lymphatic vessels. Studies showed that Prox-1, VEGF-C and VEGF-A are required for the budding and sprouting of lymphatic vessels. Currently, the exact mechanism of lymphatic formation is unclear.

Peer review

Wang and coworkers looked at serum VEGF-C and lymphatic vessel density in gastric cancer, and found a positive correlation both with lymph node metastasis and poor prognosis of the patients with gastric cancer. The paper is of some interest and importance.

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BASIC RESEARCH

Cloning and expression of special F protein from human liver

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Abstract

AIM: To clone human liver special F protein and to express it in a prokaryotic system.

METHODS: Total RNA was isolated from human liver tissue and first-strand cDNA was reverse transcribed using the PCR reverse primer. Following this, cDNA of the F protein was ligated into the clone vector pUCm-T. The segment of F protein's cDNA was subcloned into the expression vector pET-15b and transformed into *E. coli* BL21 (DE3) pLyss. Isopropy-β-D-thiogalactoside (IPTG) was then used to induce expression of the target protein.

RESULTS: The cDNA clone of human liver special F protein (1134bp) was successfully produced, with the cDNA sequence being published in Gene-bank: DQ188836. We confirmed the expression of F protein by Western blot with a molecular weight of 43 kDa. The expressed protein accounted for 40% of the total protein extracted.

CONCLUSION: F protein expresses cDNA clone in a prokaryotic system, which offers a relatively simple way of producing sufficient quantities of F protein and contributes to understanding the principal biological functions of this protein.

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Key words: F protein; Liver; Gene-cloning; Protein expression; Prokaryotic system

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INTRODUCTION

F protein was first isolated from mouse liver by Fravi and Lindenmann in 1968[1]. This particular protein can be divided into F1 and F2[2]. Liver disease is the most serious disease impairing public health. Although there are many diagnostic methods to estimate the severity of injury to the liver, none has been demonstrated to be completely reliable. It is therefore necessary to develop serological test methods that can precisely predict the degree of damage to liver. F protein is mainly concentrated in hepatic cytoplasm where its expression is 1370-fold higher than in serum. However, when liver cells are damaged, F protein is released to increase its serum concentration. It is therefore possible to establish a correlation between the concentration of serum F protein and the degree of hepatocellular damage.

Using affinity-based purification techniques, Oliveira and colleagues[3,4] were the first to describe the existence of F protein in mammalian cells. F protein is a substance within the liver that identifies organ specificity and genus non-specificity. A large amount of F allelic protein exists in the liver of rats. Even though its biological function is unclear, it is thought to be responsible for immune tolerance and immune response observed in rats following transplantation[5-11].

At present, only a few laboratories have begun to elucidate the proper biological function of F protein[5,6,10]. Indeed, no database contains the cDNA sequence of human variant of F protein. Here, we describe the cloning and expression of human F-protein from cDNA prepared from human liver[12].

MATERIALS AND METHODS

Materials

Clone receptor *E. coli* DH5a was purchased from Dalian BAO and kept in our laboratory. Clone vector pUCm-T was purchased from Shanghai Shenggong Company. The expression vector pET-15b was provided by Professor Lu Yichen. AMV RNA reverse transcriptase, endonuclease *Bam*HI, *Eco*R I , *Nde*I, Taq DNA polymerase (EX -Taq premix), X-gal, isopropy-β-D-thiogalactoside (Pharmacia), DL2000 DNA marker, DL15000 DNA maker, λDNA/ *Eco*R I + *Hin*d Ⅲ were all purchased from Dalian BAO. The primers used for PCR were synthesized by Beijing AOKE. Ampicillin was purchased from Huamei and chloramphenicol was purchased from Beijing DINGGUO.

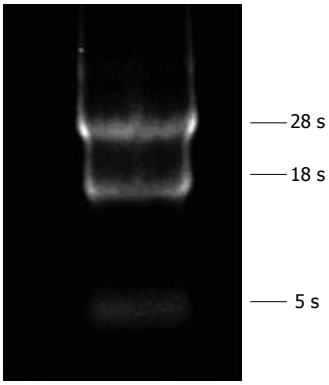

Figure 1 Total RNA isolated from liver tissue of human.

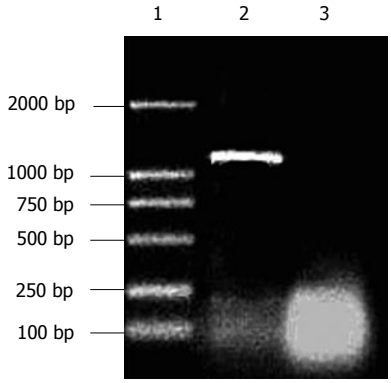

Figure 2 Gel-PAGE of PCR products. Lane 1: DL-2000 marker; lane 2: PCR reactive product; lane 3: blank control.

Isolation of human liver special F protein cDNA

Isolation and detection of total RNA from human liver: RNA was extracted from approximately 0.4 g of fresh liver tissue using 4 mL Trizol® reagent. The purity and integrity of RNA obtained were determined by agar gel electrophoresis.

RT-PCR amplification of human liver F protein cDNA: Using the cDNA sequence of rat F protein from Gene-Bank, primers were designed to amplify the cDNA of F protein by RT-PCR. The forward primer sequence is 5' P-TCC AGA CCT CAC ACCGTT-3' and the reverse primer sequence is 5'-TCC AGA CCT CAC ACC GTT-3'. Prior to amplifying the template RNA, cDNA was produced by reverse transcription at 42℃ for 60 min, and at 94℃ for 5 min. The products were then amplified for 35 cycles at 94℃ for 5 min, at 94℃ for 30 s, at 55℃ for 30 s, at 72℃ for 1.5 min, and a final extension at 72℃ for 10 min. The PCR products were then analyzed by agarose gel electrophoresis[13].

Insertion of F gene clone into T-carrier: The PCR products of F protein were dissolved in 6 μL of sterilized water under an ultra-violet lamp. DNA fragments were incorporated into the pUCm-T vector using a T4 DNA ligase at 16℃ overnight.

Transformation and identification of recombinant bacteria: Calcium chloride treatment rendered DH5a bacteria competent for uptake of the pUCm-T vector. Colonies positive for the exogenous gene were screened using the blue–white patch method, then isolated and named pUCm-T-F. Isolated colonies were digested with *Nde*I and *Bam*HI and sequenced in Shanghai Shenggong Company with ABI PRISM 377-96.

Expression, purification and detection of human liver special F protein

Construction of expression vector: Plasmid pUCm-T-F was digested using *Nde*I and *Bam*HI and ligated with the vector overnight at 16℃. Following this the recombinant plasmid was transformed into *E. coli*, BL21 (DE3) pLyss. The transformed bacteria were selected base on ampicillin and chloramphenicol resistance. Recombinant plasmids were analyzed to confirm possession of vector and termed pET15b-F.

Expression of F protein in bacterial strain: BL21 (DE3) pLyss containing plasmid pET15b-F was grown overnight in LB media at 37℃ until the OD was between 0.4 and 0.6. At this time point, IPTG (1 mmol/L) was added to the media and maintained at 37℃ for 3 h. Culture media were isolated and expressed proteins were analyzed by Western blot. The blank strain BL21 (DE3) pLyss containing no pET15b-F expression plasmid and the strain containing the blank vector pET-15b (i.e no F-protein) were used as controls.

Purity of expressed protein: Following precipitation and centrifugation the protein was purified by using a Ni-charged IDA agarose affinity chromatography column.

Detection of expressed protein SDS-PAGE: Electrophoresis and Western-blot were used to analyze the expression of all proteins obtained in this study.

RESULTS

Isolation of gene clone

Gel-PAGE of total RNA from human liver tissue: Following isolation of RNA, its integrity and purity were confirmed by agar gel electrophoresis.

As can be seen in Figure 1, lines corresponding to 28s and 18s rRNA bands gave a ratio of 2:1 for 28S and 18S, indicating that no degradation of RNA occurred.

RT-PCR: The isolated RNA was then subjected to RT-PCR with amplified DNA measured by agar gel electrophoresis. The marker used is DL-2000, each strip correlated 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp in the electrophoresis diagram.

As can be seen from Figure 2, the PCR product was approximately 1.2 kbp, similar to the size of rat F protein as published in Gene Bank.

Construction of clone vector (pUCmT-F)

The PCR products were separated on agar gel, extracted, purified and ligated with the pUCm-T vector (Figure 3). The plasmid vector was then digested using *Nde*I and *Bam*HI and detected on agarose gel in order to analyze fragment sizes (Figure 4).

The empty vector of pUCm-T was 2773 bp with PCR product being 1177 bp. Therefore, when digested with *Bam*HI, there should be a fragment of 3950 bp. From the polyclonal site of the pUCm-T vector, the *Bam*HI enzyme digestion site was 14 bp downstream of the PCR product insertion place. The *Eco*R1 site was 84 bp downstream of the PCR product insertion point. Therefore, as can be seen from Figure 4, when both *Bam*HI and *Eco*R1 were used, fragments of 2675 bp and 1275 bp could be obtained. Sequencing results: The sequencing results were against

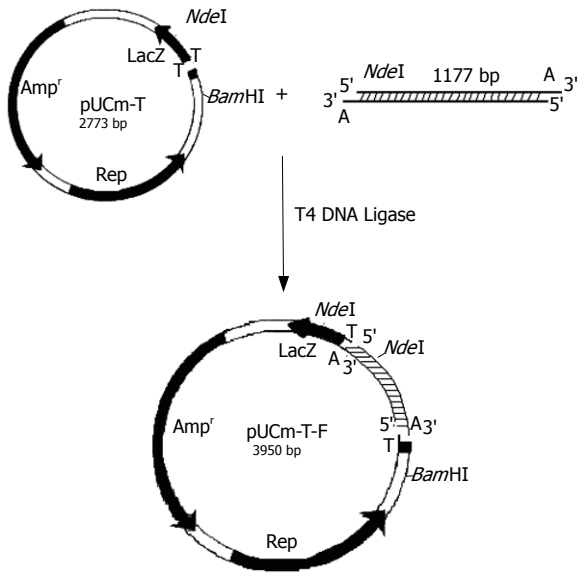

Figure 3 Construction of the clone vector.

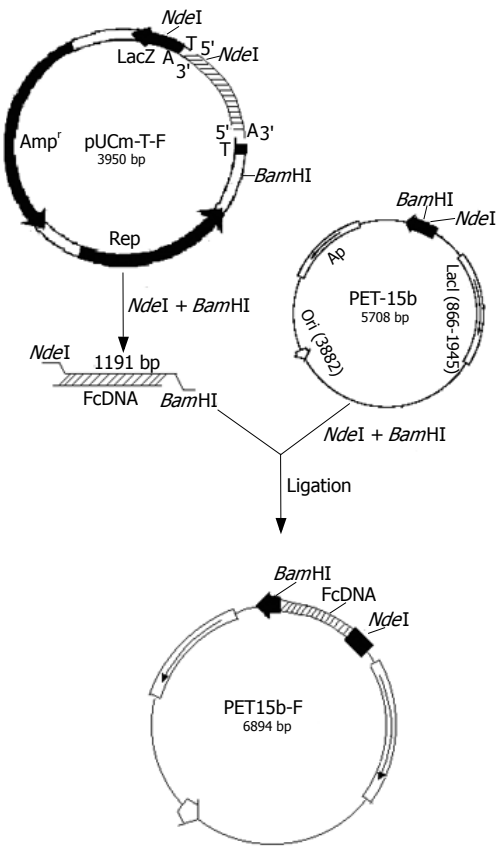

Figure 5 Construction of expression vector.

that of rat F-protein in Genebank with homology to the sequence of human F-protein, confirming the obtained cDNA clone of human liver F protein.

Expression, purification and detection of F protein

Construction and identification of expression vector: The expressing vector was digested using *Nde*I and *Bam*HI, then ligated with the cDNA fragment of F protein (1.3kb). After analysis through digestion, the expression vector was

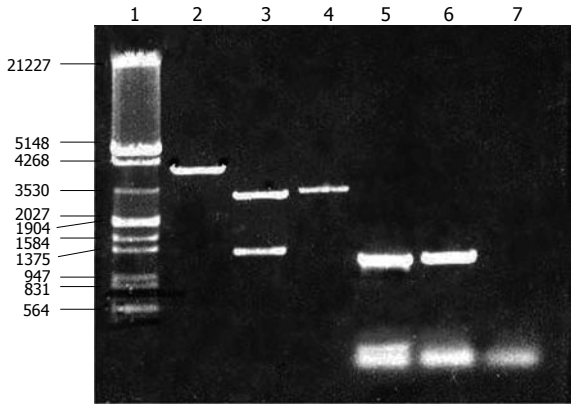

Figure 4 Restriction analysis of recombinant clone carrier pUCm-T. Lane1: Marker λDNA/*Eco*R Ⅰ + *Hind*Ⅲ; lane 2: single enzyme digestion of recombinant plasmid pUCm-T-F/*Bam*HI; lane 3: double enzyme digestion of recombinant plasmid pUCm-T-F/*Bam*HI + *Eco*R; Lane 4: free plaszmid pUCm-T after single enzyme digestion; lane 5: RT-PCR product of total mRNA from human liver; lane 6: PCR product of recombinant plasmid pUCm-T-F; lane 7: PCR product of free plasmid pUCm-T.

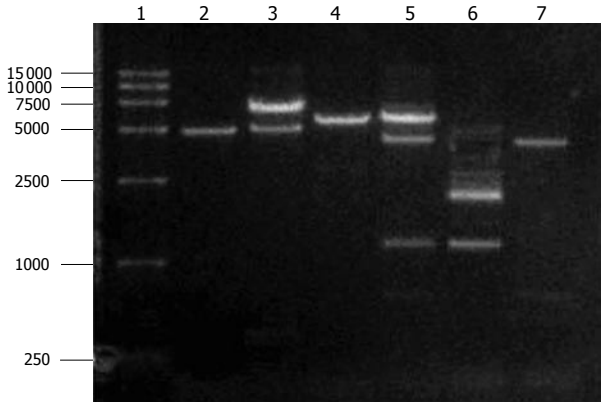

Figure 6 Digestion and identification of expression vector. Pet15b-F. Lane 1: DNA Marker (DL15000); lane 2: single enzyme digestion of helper plasmid pLysS in expressed strain (pLysS/*Hind*Ⅲ); lane 3: single enzyme digestion of expression vector pET15b-F containing the exogenous gene (pET15b-F/*Hind*Ⅲ); lane 4: double enzyme digestion of the blank vector pET-15b-pET-15b/*Bam*HI + *Nde*I); lane 5: double enzyme digestion of the expression vector pET15b-F containing the exogenous gene (pET15b-F/*Bam*HI + *Nde*I; lane 6: double enzyme digestion of cloning vector pUCm-T-F containing the exogenous gene (pUCm-T/*Bam*HI + *Nde*I; lane 7: double enzyme digestion of helper plasmid pLysS in the expressed strain (pLysS/*Bam*HI + *Nde*I).

termed pET15b-F (Figure 5).

Digestion and identification of expression vector: The expression strain BL21 (DE3) pLyss contains two plasmids, one is the expression vector pET15b-F and the other is a helper plasmid. Following isolation of these plamids from the expression strain, they were digested with *Hind*Ⅲ. Running the digestion products in an agarose gel revealed two bands (Figure 6), one (6891 bp) corresponding to the expression vector containing the exogenous gene encoding for F-protein, the other (5000 bp) to the helper plasmid pLyss. Double digestion (using *Bam*HI and *Nde*I) of the helper plasmid again yielded a single band as expected whereas double digestion of the expression vector yielded 2 bands, one corresponding to the cDNA of F protein and the other to the cloning vector.

Expression of F protein: After expression of F-protein

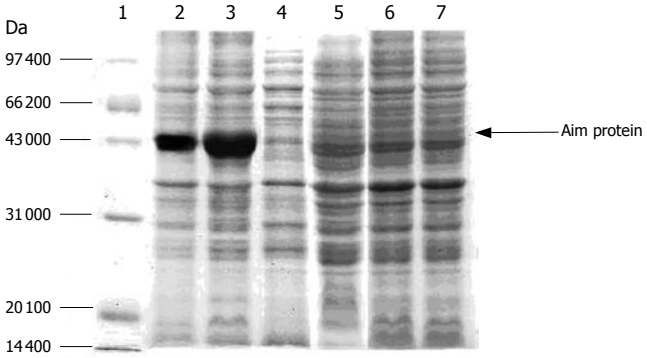

Figure 7 SDS-PAGE of pET15b-F under experimental conditions. Lane 1: low molecular weigh protein marker; lane 2: all strains of proteins after inducing the expression vector pET15b-F containing the exogenous gene by IPTG; lane 3: inclusion body precipitation by cleavage after inducing the expression strain containing the exogenous gene; lane 4: supernatant by centrifugation after inducing the expression strain containing the exogenous gene; lane 5: all proteins from strains not induced by IPTG containing the exogenous gene; lane 6: expression of proteins induced by IPTG containing no exogenous F protein gene; lane 7: expression of proteins induced by IPTG in a blank strain containing no expression vector.

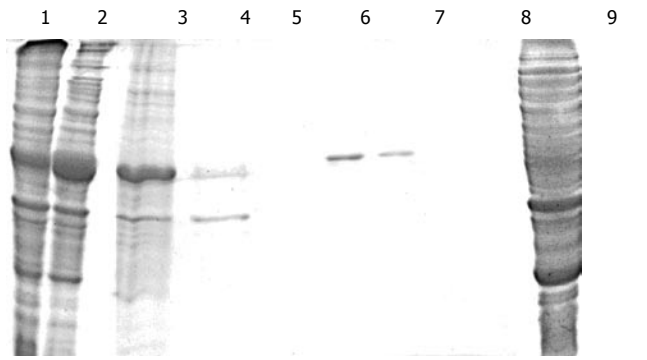

Figure 8 SDS-PAGE of target protein in purity process. Lane 1: protein induced from bacteria; lane 2: inclusion body precipitation of all bacteria after cleavage and centrifugation; lane 3: dissolved inclusion granule after preliminary purification; lane 4: rough protein eluted by affinity chromatography; lane 5: chromatography effluent before the target protein was eluted; lane 6: first pipe of aim effluent; lane 7: second pipe of aim effluent; lane 8: last pipe of aim effluent; lane 9: blank control (the blank strain containing no expression vector).

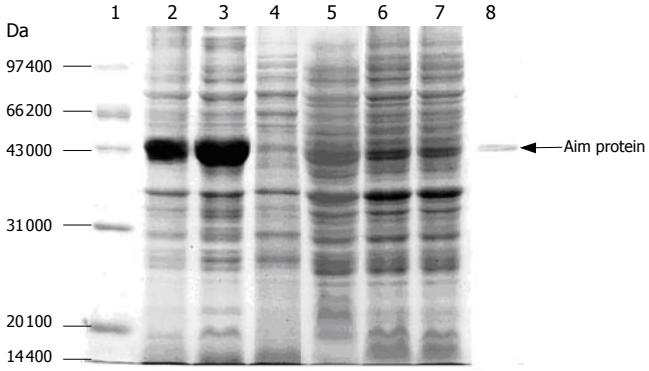

Figure 9 Electrophoresis of target protein in all expressed and purified strains Lane 1: low molecular weight marker; lane 2: whole bacteria induced protein; lane 3: inclusion body precipitation after cleavage and centrifugation; lane 4: supernate from all strains cleaved and centrifuged; lane 5: whole bacterial protein not induced; lane 6: expression strain containing induced blank vector (pET15b); lane 7: induced expression of proteins not containing the expression vector; l8: electrophoresis of the target protein taken out from the gels.

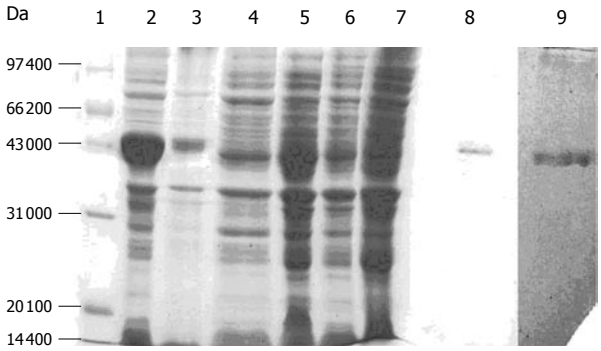

Figure 10 Western-blot of purified human liver F protein. Lane 1: low molecular weight marker; lane 2: precipitation of inclusion granule cleavage; lane 3: inclusion granule dissolved by carbamide; lane 4: supernatant of expression strain after cleavage and centrifugation; lane 5: expression of strain not induced; lane 6: protein of induced expression strain containing the blank vector (pET-15b, not containing exogenous gene F); lane 7: whole proteins of induced blank expression strain not containing the expression vector; lane 8: protein purified by a His-tag column; lane 9: Western-blot result of purified human liver F protein.

was induced using 1 mmol/L IPTG, the media were subjected to centrifugation in order to collect all secreted proteins, which were then analyzed using SDS-PAGE (Figure 7). The collected proteins were then purified using a Ni-charged IDA affinity chromatography column and the purified proteins were detected by SDS-PAGE (Figure 8).

As can be seen from Figure 8, there was a substantial amount of rough protein in the preparation prior to purification by chromatography. Following this purification step, pure F protein was obtained.

Electrophoresis of target protein in all expressed and purified strains: The purified target proteins, expression strain, blank control and protein marker were put into SDS-PAGE to detect the size of target protein by electrophoresis. Figure 9 shows 43 kDa of target F protein band.

Western blot analysis of F-protein: Western blot showed strong immunoreactivity by using a polyclonal antibody against human liver F-protein (Figure 10).

DISCUSSION

Since liver specific F protein was isolated in 1960s[1], it has been used in the diagnosis of hepatic diseases and in judging the degree of hepatocellular damage. Serum F protein has been shown to have a close correlation with the degree of hepatocellular damage[2-4], thus representing a new sensitive and specific serologic marker which could reflect the degree of hepatic damage. No age or sex-related differences have been found in serum F protein levels. Use of serum F protein as a tool to identify liver damage is of great importance[1-4,14].

The importance to study F-protein function is highlighted by reports describing it as an organ specific antigen[4]. It has been previously demonstrated that liver F protein is directly involved in the immunorejection of liver transplants in rats[10,15]. Thymus inoculation of liver F protein has been shown to alleviate the rejection of liver

transplants, demonstrating that F-protein is an important antigen against transplantation[10,15]. Due to the difficulties in separating and purifying tissue cells, the study of tissue specific antigenicity in this regard has been hindered. As studies on the expression and distribution of liver F protein are limited, it is difficult to perform a detailed analysis of its function. The Mrs of F protein have been reported to be 40 kDa-45 kDa[16], which is possibly due to the differences in techniques used to isolate F protein[16]. Due to the difficulties in obtaining sufficient amount of F protein, it is difficult to show its proper biological function.

At present there are no published studies on the clone of human liver F-protein gene. No corresponding gene sequence has been reported. Due to the similarity between the genes of rodents and humans and the fact that no published sequence of human liver F-protein is available, we used the published rodent gene sequence to design 10 pairs of forward primers and reverse primers. We subsequently applied them to the RNA isolated from human liver tissue. The cDNA obtained from the subsequent RT-PCR was then subjected to PCR amplification with products analyzed by agarose gel electrophoresis. The 10 pairs of forward primers and reverse primers are as follows:

1 F primer: 5'-CGG CAT ATG TAC TGG GAC AAA GGA CCA AAG CCT GAG AGA-3'

R primer: 5'-TCC AGA CCT CAC ACC GTT GGT CTC CAG-3'

2 F primer: 5'-CTG GAT CCG GAG GTT TGA CTA AGA TCA ATC A-3'

R primer: 5'-GCG AAT TCT GGA TCT GGG ACT TCT TCT TG-3'

3 F primer: 5'-CTG GAT CCG GTG CAC ACG GAA TAT AGC TC-3'

R primer: 5'-GCG AAT TCT TTA ATC GGG AGG GCT GGA-3'

4 F primer: 5'-TAT CAT ATG GTA CTG GGA CAA AGG ACC AAA GCC T3'

R primer: 5'-TAT GGA TCC CAT CTG GGA AGG CAT CTT TAC TCC A3'

5 F primer: 5'- CAG GCT GCC TCC TTC TAT TG-3'

R primer: 5'- CAG GTG GTC ACC CAT CTC TT-3'

6 F primer: 5'-AAG AGA TGG GTG ACC ACC TG-3'

R primer: 5'-AGC ACA GCG AAC TTC ACC TT-3'

7 F primer: 5'-GTA CTG GGA CAA AGG ACC AAA GCC-3'

R primer: 5'- GAT CTG GGA AGG CAT CTT TAC TCG –3'

8 F primer: 5'-TCC CTG GAA CAA GAG ATG G-3'

R primer: 5'-AGC ACA GCG AAC TTC ACC T-3'

9 F primer: 5'-AAG AGA TGG GTG ACC ACC TG-3'

R primer: 5'-TTC ACC TTC CCG AAT TTG TC-3'

10 F primer: 5'-TCC CTG GAA CAA AGA GAT GG3'

R primer: 5'-TTC ACC TTC CCG AAT TTG TC3'

Using these primers we found that only certain primer sequences could result in amplification. Therefore, we selected the pair of forward and reverse primers to inverse transcript and amplify human liver F-protein cDNA resulting in RT-PCR products of 1200 bp.

As the size of the cDNA is so large, after ligating the DNA with the cloning vector a certain number of base mutations occurred. In order to correct them, the following primers were designed to repair these mutations:

1 F primer: 5'-CATATCCTACTGGGACAAAG -3'

R primer: 5'-CTTGATGACATGACTGACCACCTC-3'

2 F primer: 5'-TGGTCAGTCATGTCATCAAGCAA GGG-3'

R primer: 5'-TTCCCTCAAGTGGCGGATCGTT GTG-3'

3 F primer: 5'-CGATCCGCCACTTGAGGGAACG AC-3'

R primer: 5'-GGATCCGATCTGGGAAGGCATC-3'

Finally, it was possible to isolate the PCR products with these three pairs of primers and we used the 1 up stream and the 3 down stream as the primers to amplify the whole sequence. After ligating the PCR products with the cloning vector, the sequencing results from PCR confirmed isolation of the target gene.

In order to optimize protein expression, we carried out experiments under certain conditions that may influence the expression of F protein. The optimal exogenous protein expression occurred with an *E. coli* OD_{600} value of 0.3-0.5 when *E. coli* was in a state of logarithmic growth. In this particular stage the expression of proteins could be influenced by exogenous factors that may promote protein expression. If the OD_{600} value exceeds 0.6, the growth of *E. coli* enters the telophase stage whereby a number of factors can negatively influence protein expression, including accumulation of toxic products such as organic aids, H_2O_2 and decreased pH. Essentially, bacterial reproduction decreases while their mortality increases, so the ability to express exogenous protein significantly decreases. In the exponential growth phase, protein expression is induced by IPTG at the concentration of 0.8-1.2 mmol/L. If the concentration range of IPTG exceeds 0.8-1.2 mmol/L, it promotes maximum protein expression in a period of 5 h. In summary, the optimal experimental conditions for inducing maximum expression of proteins were as follows: *E. coli* optimum concentration is OD_{600} = 0.5, Optimum concentration of inducer IPTG is 1.0 mmol/L, and Optimum time is 5 h.

Following submission of the gene sequence of human liver F protein cDNA to GENEBANK (the submission number was DQ188 836), we proceeded to blast the sequence and discovered a 99% sequence homology between human and rat liver F-protein. The bases at 179, 830 and 1133 of human liver F-protein gene sequence was G, T, A and the corresponding base sequence of rat liver F-protein gene sequence was A, C, T, respectively.

Western-blot analysis of the obtained protein revealed that its molecular weight is in agreement with the published molecular weight of human liver F-protein (approximately 43 kDa). Therefore, we could express F-protein and confirm its identity in a prokaryotic system, thus laying a foundation for subsequent studies on elucidating the proper biological function of F-protein.

In conclusion, we report the isolation of total RNA from the rat liver obtaining the intact sequence of F protein cDNA through reverse transcription. The PCR products can be cloned into pUCm-T, confirming that the cloned fragment is identical to that of the rat liver F-protein mRNA sequence. F protein is expressed in an *E.coli* expression system. Furthermore, the protein has the same immune specialty as human liver F protein.

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CLINICAL RESEARCH

Sonographic fatty liver and hepatitis B virus carrier status: Synergistic effect on liver damage in Taiwanese adults

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Abstract

AIM: To examine the epidemiology of hepatitis B virus carrier status (HBVC) and sonographic fatty liver (SFL) in Taiwanese adults, and to evaluate their possible interaction in inducing liver damage (LD). From an epidemiological viewpoint, we analyzed previous studies which indicated that fatty liver sensitizes host immune response to HBV infection and enhances liver damage.

METHODS: A cross-sectional retrospective analysis of health records including medical history, physical examination, abdominal sonogram, blood biochemistry and hepatic virological tests. We utilized the Student's *t*-test, chi-square, multivariate logistic regression and synergy index to assess risks for LD.

RESULTS: Among a total of 5406 Taiwanese adults (mean age 46.2 years, 51.5% males), the prevalence of LD, HBVC and SFL were 12.3%, 15.1% and 33.4%, respectively; 5.1% of participants had SFL plus HBVC. Multivariate logistic regression analysis demonstrated that male gender (odds ratio (OR) = 2.8, 95% confidence interval (CI): 2.3-3.5), overweight state (OR = 1.6, 95% CI: 1.3-2.0), HBVC (OR = 2.5, 95% CI: 2.0-3.1) and SFL (OR = 4.2, 95% CI: 2.2-5.3) were independently associated with LD. Synergism analysis showed that the adjusted OR for LD in adults with HBVC-alone was 3.3 (95% CI: 2.4-4.6), SFL-alone, 4.7 (95% CI: 3.7-6.1) and combined HBVC and SFL, 9.5 (95% CI: 6.8-13.3); the synergy index was 1.4 (95% CI: 1.001-2.0).

CONCLUSION: In Taiwanese adults, SFL plus HBVC have a significant synergistic association with LD.

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INTRODUCTION

Fatty liver and hepatitis B virus (HBV) infection are important causes of liver damage (LD)[1-3]. However, only limited data is available on the interaction of these two risk factors in inducing liver damage.

Fatty liver, termed histologically as hepatosteatosis, refers to an accumulation of fat in the hepatocytes. Fatty liver can be detected non-invasively by sonogram[4]. Several epidemiological studies have shown that sonographic fatty liver (SFL) has a strong positive association with the metabolic syndrome[2,5]. SFL may progress to fibrosis or cirrhosis and is a growing health problem in many developing as well as developed countries[2,5,6]. HBV infection is usually transmitted through blood or sexual contact with an infected person. Chronic HBV infection also results in serious sequelae including hepatocellular carcinoma, and continues to be an important public health problem in hyperendemic areas including several Asian countries[3].

Both SFL and HBV carrier status (HBVC) are commonly encountered by primary health care doctors in Taiwan[4,7]. According to previous epidemiological studies, coexistence of SFL and HBVC is not uncommon in clinical practice[8]. The findings that fatty liver sensitizes the host immune response to the HBV infection had been reported in some studies[9,10]; however, little is known as to whether the damaging effects of SFL and HBVC are enhanced by the co-existence of the two diseases.

We examined the epidemiology of SFL and HBVC in Taiwanese adults by analyzing retrospectively the database of a health care center in Taipei. We focused on whether a combination of these two liver disorders has a deleterious effect on the serum alanine aminotransferase levels, which is a conventional marker of liver damage and is a predictor of advanced liver disease[11,12].

Table 1 Demographic data of the total study population and the subgroups defined by HBVC[1] and SFL[2] positive or negative status mean ± SD

Characteristics	All cases (*n* = 5406)	All cases classified by HBVC (*n* = 5406)		All cases classified by SFL (*n* = 5406)	
		HBVC (-) (*n* = 4589)	HBVC (+) (*n* = 817)	SFL (-) (*n* = 3603)	SFL (+) (*n* = 1803)
Age (yr)	46.2 ± 9.4	46.3 ± 9.5	45.9 ± 8.8	45.3 ± 9.6	48.0 ± 8.7[d]
BMI (kg/m^2)	23.5 ± 3.3	23.5 ± 3.3	23.7 ± 3.4	22.3 ± 2.7	25.9 ± 3.1[d]
Waist (cm)	82.6 ± 9.2	82.5 ± 9.1	83.2 ± 9.8	79.9 ± 8.5	87.8 ± 8.3[d]
AST (U/L)	22.8 ± 9.6	22.1 ± 8.3	26.9 ± 14.0[b]	21.6 ± 9.1	25.3 ± 10.1[d]
ALT (U/L)	25.8 ± 19.9	24.3 ± 17	34 ± 30.4[b]	21.6 ± 16.5	34.0 ± 23.3[d]
Male, *n*(%)	2760 (51.1)	2271 (49.5)	489 (59.9)[b]	1598 (44.4)	1162 (64.5)[d]
Overweight, *n* (%)	2237 (41.4)	1883 (41.0)	354 (43.3)	920 (25.5)	1317 (73.0)[d]
Central obesity, *n* (%)	1038 (19.2)	883 (19.2)	155 (19.0)	545 (15.1)	493 (27.3)[d]
SFL, *n* (%)	1803 (33.4)	1526 (33.3)	277 (33.9)		
HBsAg-positive, *n* (%)	817 (15.1)			540 (15.0)	277 (15.4)
HBsAb-negative, *n* (%)	1983 (36.7)	1211 (26.4)		1313 (36.4)	670 (37.2)
LD, *n* (%)	666 (12.3)	472 (10.7)	174 (21.3)[b]	200 (5.6)	466 (25.9)[d]

HBVC: hepatitis B virus carrier status; HBVC (-)/HBVC (+), non-carrier status/hepatitis B virus (HBV) carrier status. SFL: sonographic fatty liver; SFL (-)/SFL (+), subject without SFL/subject with SFL. BMI: body mass index = Weight (kg)/Length (m)^2; ALT: alanine aminotranferease (U/L); AST: aspartate aminotransferase (U/L); HBsAg: HBV surface antigen; HBsAb: anti-HBV surface antibody. BMI > 24. Waist circumference > 90 cm for men; > 80 cm for women. LD: liver damage defined as ALT > 40 (U/L). [b]$P < 0.01$ *vs* reference subjects HBVC (-); Student's *t* test for continuous variables; χ^2 tests for categorical variables. [d]$P < 0.01$ *vs* reference subjects SFL(-); Student's *t* test for continuous variables; χ^2 tests for categorical variables.

MATERIALS AND METHODS

Subjects

The records of 5899 adult subjects, 18 to 65 years in age who had visited the Shin Kong Wu Ho-Su Memorial Hospital health center between January 2004 and December 2005 for routine periodic checkups, were collected anonymously.

Methods

A questionnaire on personal medical history, including alcohol consumption (usage more than once a week in the recent past 3 mo: yes *vs* no; yes defined as a habitual drinker), medication usage and major operations, was filled out by the examinees. A total of 493 participants were excluded from the final analysis for following reasons: habitual drinking, history of major abdominal surgery, diabetes and long-term use of corticosteroids, hormone replacement therapy, insulin replacement therapy and oral antidiabetic agents.

The physical examination data included anthropometrical evaluation and measurements of systolic and diastolic blood pressure. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured midway between the lowest rib and the iliac crest. Definition of overweight was BMI > 24 kg/m^2, and that of central obesity was waist circumference > 90 cm for men and > 80 cm for women, based on Taiwanese criteria[13].

The biochemical blood tests were obtained using the Hitachi/Roche auto analyzer model 7600 PPEE (Hitachi Corp, Tokyo, Japan). The tests included aspartate aminotransferase (AST), alanine aminotransferase (ALT), fasting plasma glucose, serum triglycerides, and total and high-density lipoprotein (HDL) cholesterol. The results of tests for metabolic syndrome (blood glucose and lipids) are not shown in the tables but were adjusted for the multivariate analysis. HBV surface antigen (HBsAg), anti-HBV surface antibody (HBsAb) and anti-HCV antibody were measured by the microparticle enzyme immunoassay (MEIA) using the Abbott Axsym System instrument (Abbott Laboratories Services Corporation, Taipei, Taiwan). LD was defined by cutoff > 40 U/L for ALT[14].

Abdominal sonographic examinations were performed using convex-type real-time electronic scanners (Toshiba SSA-340 with 3.75 MHz convex-type transducer) by four gastrointestinal specialists who were blind to the examinees' medical history and blood test results. The definition of SLF was based on a comparative assessment of image brightness relative to the kidneys, as described previously[4,15-17].

SAS software was used for statistical analysis (Version 8.0; SAS Institute, Cary, NC, USA). Student's *t* and chi-square tests were used for analyzing continuous variables and categorical variables, respectively. Multivariate logistic regression was utilized to evaluate the relationship between salient risk factors. A program developed in the SAS computer package for the calculation of synergy index (SI) was used[18]. ASI value of > 1.0 indicates that the interaction is more than an additive interaction, i.e. is a synergism.

RESULTS

The records of a total of 5406 Taiwanese adults (2760 males, 2646 females; aged from 18 to 65 years) were used for the analysis.

Table 1 summarizes the findings in the total study population. The mean ± standard deviation of the different characteristics were: age, 46.2 ± 9.4 years; BMI, 23.5 ± 3.3 kg/m^2; waist circumference, 82.6 ± 9.2 cm; AST, 22.8 ± 9.6 U/L and ALT, 25.8 ± 19.9 U/L. Central obesity was seen in 19.2% individuals while 41.15% were overweight. The prevalence rates of LD, HBVC and SFL

Table 2 Results of logistic multivariate regression analysis[1] of LD[2] and tested variables

Risk factors	All cases	
	OR	95% CI
Gender (male *vs* female)	2.8	2.3-3.5
Overweight (yes *vs* no)	1.6	1.3-2.0
Central obesity (yes *vs* no)	1.0	0.8-1.3
SFL (yes *vs* no)	4.2	3.3-5.3
HBVC (yes *vs* no)	2.5	2.0-3.1

[1]Data were adjusted for age, gender, blood pressures, sugar, lipids and hepatitis C virus infection. LD: liver damage defined as ALT > 40; ALT: alanine aminotranferease (U/L). OR: odds ratio; CI: confidence interval. BMI > 24; BMI: body mass index = Weight (kg)/Length (m)^2. Waist circumference > 90 cm for men; > 80 cm for women. SFL: sonographic fatty liver. HBVC: hepatitis B virus carrier status.

were 12.3%, 15.1% and 33.4%, respectively; 5.1% of participants (277 in 5406) were HBV carriers with SFL.

The results obtained in the subgroups categorized by HBVC and SFL are shown in Table 1. HBV carriers had significantly higher levels of AST (26.9 ± 14.0 *vs* 22.1 ± 8.3 U/L, $P < 0.001$) and ALT (34.0 ± 30.4 *vs* 24.3 ± 17.0 U/L, $P < 0.001$) compared to non-carriers. There were no significant differences between HBV carriers and non-carriers with respect to age, BMI, and waist circumference. The proportion of males was higher in HBV carriers compared to non carriers (59.9% *vs* 49.5%). Liver function tests were abnormal significantly more frequently in HBV carriers compared to non-carriers (21.3% *vs* 10.7%). No significant differences were observed between carriers and non-carries with respect to SFL (33.3% *vs* 33.9%), overweight status (41% *vs* 43.3%) and central obesity (19.2% *vs* 19%). The proportion of subjects who were HBsAb negative was 36.7% in the total population and 26.4% in non-HBV carriers.

The results of different parameters based on whether the subjects were positive or negative for SFL are shown in Table 1. The results showed significant ($P < 0.001$) differences between subjects with SFL compared to those without SFL for all the characteristics: age 45.3 ± 9.6 *vs* 48 ± 8.7 years, BMI 22.3 ± 2.7 *vs* 25.9 ± 3.1 kg/m^2, waist circumference 79.9 ± 8.5 *vs* 87.8 ± 8.3 cm, AST 21.6 ± 9.1 *vs* 25.3 ± 10.1 U/L, ALT 21.6 ± 16.5 *vs* 34 ± 23.3 U/L, overweight status 25.5% *vs* 73%, obesity 15.1% *vs* 27.3% and liver damage, 5.6% *vs* 25.9%. There were significantly more males in the subgroup with sonographic fatty liver compared to those without SFL (64.5% *vs* 44.4%; $P < 0.001$). The only exceptions were HBVC (15.0% *vs* 15.4%, $P = 0.72$) and HBsAb-negative rates (36.4% *vs* 37.2%, $P = 0.61$), which were similar in the subgroups without or with SFL.

The results of the following physical findings and metabolic tests have not been shown in the tables but were used as potential confounding factors in the multivariate analysis: systolic blood pressure 15.5 ± 2.4 kPa, diastolic pressure 9.4 ± 1.5 kPa, fasting sugar 4.9 ± 0.5 mmol/L, triglycerides 3.2 ± 2.0 mmol/L and HDL-C 1.4 ± 0.4 mmol/L. The prevalence of elevated blood pressure was 23.6%, hyperglycemia 2.7%, hypertriglyceridemia 24.8%, and hypo-HDL-cholesterolemia 21.1%.

The results of multivariate analysis and adjusted odds ratios for LD of the total population are summarized in Table 2. According to the multiple logistic regression analysis, risks factors such as male gender (OR = 2.8, 95% confidence interval (CI): 2.3-3.5), overweight state (OR = 1.6, 95% CI: 1.3-2.0), SFL (OR = 4.2, 95% CI: 2.2-5.3) and HBVC (OR = 2.5, 95% CI: 2.0-3.1) were independently associated with LD in total study population.

Table 3 displays the results obtained in the four subgroups: SFL plus HBVC, SFL-alone, HBVC-alone and the reference group which consisted of non-carriers without SFL. Adults in the SFL plus HBVC group had significantly ($P < 0.001$) higher values of the following characteristics: age 48.2 ± 8.3 *vs* 45.4 ± 9.7 years, BMI 26.1 ± 3.2 *vs* 22.3 ± 2.7 kg/m^2, waist circumference 88.9 ± 9.4 *vs* 79.9 ± 8.5 cm, AST 29.6 ± 16.4 *vs* 20.9 ± 8.1 U/L, ALT 43.3 ± 39.4 *vs* 20.3 ± 14.6 U/L, overweight state 73.3% *vs* 25.1%, central obesity 31.1% *vs* 15.5% and abnormal liver function 37.2% *vs* 4.2%. Likewise, the SFL-alone subjects, had significantly higher values compared to the reference group with respect to: age 47.9 ± 8.8 years, BMI 25.9 ± 3.0 kg/m^2, waist circumference 87.5 ± 8.0 cm, AST 24.5 ± 8.3 U/L, ALT 32.3 ± 18.5 U/L, overweight state 73.0%, central obesity 26.7% and liver damage 23.8%. Unlike the SFL-alone and SLF plus HBVC groups, the HBVC-alone subjects had significantly higher levels than the reference group only with respect to AST 25.6 ± 12.5 U/L, ALT 29.2 ± 23.3 U/L and liver damage 13.2%, whereas age, BMI, waist circumference, rates of overweight or central obesity were not significant (Table 3). There were significantly more males in the SFL-alone, HBVC-alone and SFL plus HBVC groups: 63.0%, 53.3% and 72.6% respectively, compared to the reference group, 42.8%. The proportion of HBsAb-negative subjects was similar in non-carriers with or without SFL, 26.0% *vs* 27.1%.

Table 4 shows the risk of LD in subjects with SFL or HBVC alone and those with SFL plus HBVC compared to the reference group. The multivariate-adjusted odds ratios of liver damage for adults with HBVC-alone, SFL-alone and combined SFL plus HBVC were 3.3 (95% CI: 2.4-4.6), 4.7 (95% CI: 3.7-6.1) and 9.5 (95% CI: 6.8-13.3) respectively, the synergy index of HBV plus SFL for elevated AST levels was 1.4 (95% CI: 1.006-2.0), indicating a synergist effect[18].

DISCUSSION

In the present study carried out on a Taiwanese adult population, the prevalence rates of HBVC and SFL were 15.1% and 33.4% respectively, which are similar to those reported from other hyperendemic developing and developed Asian countries[3,5,19].

Male gender and an overweight state were independently associated with liver damage as non-modifiable and theoretically modifiable risk factors. Although several workers have shown that males are vulnerable to HBV infection, hepatosteatosis and liver damage[20-24], studies on overweight subjects also show that weight reduction brings about significant improvement in the liver function tests[6]. The importance of weight control in the management of liver damage should be

Table 3 Characteristics of four subgroups classified by HBVC and SFL positive or negative status mean ± SD

Characteristics	Liver status			
	HBVC (-) SFL (-) (*n* = 3063)	HBVC (-) SFL (+) (*n* = 1526)	HBVC (+) SFL (-) (*n* = 540)	HBVC (+)SFL(+) (*n* = 277)
Age (yr)	45.4 ± 9.7	47.9 ± 8.8[b]	44.8 ± 8.8	48.2 ± 8.3[b]
BMI (kg/m^2)	22.3 ± 2.7	25.9 ± 3.0[b]	22.5 ± 2.7	26.1 ± 3.2[b]
Waist (cm)	79.9 ± 8.5	87.5 ± 8.0[b]	79.9 ± 8.5	88.9 ± 9.4[b]
AST (U/L)	20.9 ± 8.1	24.5 ± 8.3[b]	25.6 ± 12.5[b]	29.6 ± 16.4[b]
ALT (U/L)	20.3 ± 14.6	32.3 ± 18.5[b]	29.2 ± 23.3[b]	43.3 ± 39.4[b]
Male, *n*(%)	1310 (42.8)	961 (63.0)[b]	288 (53.3)[b]	201 (72.6)[b]
Overweight, *n* (%)	769 (25.1)	1114 (73.0)[b]	151 (28.0)	203 (73.3)[b]
Central obesity, *n* (%)	476 (15.5)	407 (26.7)[b]	69 (12.8)	86 (31.1)[b]
LD, *n* (%)	129 (4.2)	363 (23.8)[b]	71 (13.2)[a]	103 (37.2)[b]
HBsAb-negative, *n* (%)	797 (26.0)	414 (27.1)		

HBVC, hepatitis B virus carrier status. SFL, sonographic fatty liver. HBVC (-) SFL (-), without SFL nor HBVC; HBVC (-) SFL (+), SFL-alone; HBVC (+) SFL (-), HBVC-alone; HBVC (+) SFL (+), SFL plus HBVC. BMI, body mass index = Weight (kg)/Length (m)^2. 5ALT, alanine aminotranferease (U/L); AST, aspartate aminotransferase (U/L); HBsAb, anti-hepatitis B surface antibody. BMI > 24. Waist circumference > 90 cm for men; > 80 cm for women. LD: liver damage defined as ALT > 40 (U/L). [a]P < 0.01 and [b]P < 0.01 *vs* reference subjects HBVC (-) SFL (-); Student's t test for continuous variables; χ^2 tests for categorical variables.

Table 4 Prevalence and odds ratio of LD for single or combined states of HBVC and SFL

Liver status		Liver damage rate (%)	OR	95% CI	SI	95% CI
SFL	HBVC					
(-)	(-)	4.2	1.0	Reference	No risk	-
(-)	(+)	13.2[b]	3.3	2.4 - 4.6	One risk only	-
(+)	(-)	23.8[b]	4.7	3.7 - 6.1	One risk only	-
(+)	(+)	37.2[b]	9.5	6.8 - 13.3	1.4	1.001 - 2.0

LD: liver damage defined as ALT > 40 (U/L); ALT: alanine aminotranferease (U/L). HBVC: hepatitis B virus carrier status. SFL: sonographic fatty liver. OR: odds ratio; CI: confidence interval; data were adjusted for age, gender, blood pressures, sugar, lipids and hepatitis C virus infection. SI: synergy index, Ref [18]. [b]P < 0.01 *vs* reference subjects; data were analyzed by multivariate logistic regression analysis.

emphasized in Taiwanese adults, especially males.

Our findings support previous observations that both SFL and HBVC have a strong association with liver damage[6,25]. In addition, our study shows that SFL and HBVC produce liver injury by different mechanisms. In hepatosteatosis, the severity of liver injury is closely related to the amount of lipid accumulation in the hepatocytes[15,16]. Fat accumulation can be semi-quantitatively estimated by sonograms[5,16,26,27], thus analysis of hepatosteatosis on sonogram can determine the potential for liver injury[15,16,27-29]. By contrast, the severity of liver injury in HBV carriers depends on several viral factors including virus type and viral load[30-33]. Thus, knowledge of HBsAg carrier status alone, without information on the viral type and number viral copies, provides only limited information on the severity of liver injury[34,35]. In the present multivariable analysis (Tables 2 and 4), HBVC exhibited a significant but weaker association with liver injury than SFL, nevertheless, in future studies the impact of these risk factors on liver damage should be assessed by analysis adjusted for virus type and viral copies.

Our study provides an epidemiological analysis on the influence of combined HBVC and SFL on liver damage, and indicates a synergistic effect (Table 4). According to the widely accepted "two-hit theory" of steatohepatitis, the "first hit" is excess lipid accumulation in hepatocytes; lipids in the liver cells are sensitive to the "second hit" involving factors such as oxidative stress, oxygen-radicals, cytokines, lipid-peroxidation, which result in steatohepatitis[36-40]. It is possible that the two hits can exist simultaneously in lipid-containing hepatocytes infected with HBV.

In the present study, the "first hit", lipid accumulation detected by sonogram, showed no significant difference between HBV carriers and non-carriers (Table 3). These findings are in agreement with previous observations[41] that HBVC prevalence is not influenced by the presence of SFL, and vice versa (Table 1). Despite the insignificant association of the "first hit" regarding the coexistence of HBVC and SFL, the "second hit" should be considered as the candidate causing significant liver injury. Several studies on HBV infection and fatty liver have reported oxidative stress-related events such as the assembly of HBV viral particles[42], the HBV altered lipid peroxidation pathway and hepatocyte electron transport system by charged HBV[43-45]. It is reasonable to theorize that these oxidative stress-related events act together in inducing additional and potent oxidative stress in the hepatocytes, thus enhancing the "second hit", which promotes injury to the vulnerable lipid-containing hepatocytes, resulting in hepatitis.

In addition, from the view point of molecular biology,

both hepatosteatosis and HBV infection are associated with dysfunction of the mitochondria, which are the producers of metabolic energy, biosensor for oxidative stress, and effector for liver cell death through the process of apoptosis[46,47]. Previous studies on hepatitis B virus have shown that a viral protein, HBx protein, is found in the mitochondrion and targets mitochondrial calcium regulation, which results in mitochondrial dysfunction[48,49]. On the other hand, investigations on hepatosteatosis demonstrate that excess lipid peroxidation plays a role in triggering mitochondrial membrane permeabilization and apoptosis[50-53]. Mitochondrial dysfunction caused simultaneously by HVB infection and hepatosteatosis should be considered as an important cause of hepatocellular injury, represented by a significant increase in the frequency of abnormal liver function tests in SFL-affected HBV carriers.

Although our study shows a statistical association between HBVC, SFL and LD, a direct biochemical and biomolecular evidence for the causality and synergism awaits further investigations.

To date, besides weight reduction and physical exercise, there are few recommendations for the management of fatty liver[6,36,39]. At the same time, the use of hepatitis B vaccine has been shown to effectively reduce the incidence of new infections, and vaccination is recommended for at-risk, sexually active adult individuals[54]. In our study, 27.1% of fatty liver-affected non-HBV carriers were HBsAb-negative (Table 1). As SFL is one of the major causes of liver damage, and is an independent risk factor predicting advanced liver disease for HBV carriers[3,7], expanding vaccination recommendations to fatty liver-affected subjects could be an effective preventive strategy against advanced liver disease.

To sum up, sonographic fatty liver and HBV carrier status are the most prevalent liver problems in Taiwanese adults, with male subjects being more vulnerable to fatty liver, HBV infection and liver damage than females. Health promotion strategies such as weight control and use of HBV vaccination should be emphasized in Taiwanese adults, especially males. Since HBVC combined with SFL has a synergistic effect on liver injury, we recommend HBV vaccination for Taiwanese adults with fatty liver who do not have adequate protective antibodies.

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CLINICAL RESEARCH

Clinicopathological study of solid and pseudopapillary tumor of pancreas: Emphasis on magnetic resonance imaging findings

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Abstract

AIM: To report the clinicopathological features and magnetic resonance imaging (MRI) findings of solid and pseudopapillary tumor (SPT) of pancreas.

METHODS: From 1981 to 2005, 26 surgically treated cases of SPT were retrospectively reviewed. MRI findings of the latest 11 consecutive SPT cases were investigated.

RESULTS: There were 25 women and one man having SPT (median age: 23 year) with a median tumor size of 7.5 cm. Among them, nine patients developed solid pseudopapillary carcinoma. During the median follow-up period of 66 mo, the 5-year survival rate of the 26 SPT patients was 96.2%. Three MRI features were proposed including Type 1 image, displaying SPT with completely solid part. All SPT patients with type 1 image were detected incidentally. Type 2 image displays of SPT with solid mass hemorrhage and type 3 image with massive hemorrhage. All the eight SPT patients with type 2 and 3 images suffered abdominal pain due to hemorrhage from SPT.

CONCLUSION: SPT had a favorable survival rate irrespective of surgical procedures, malignancy, and MRI findings, however, MRI could reliably correlate with its clinicopathological features.

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Key words: Solid and pseudopapillary tumor; Pancreas; Magnetic resonance imaging

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INTRODUCTION

Solid and pseudopapillary tumor (SPT) of pancreas is a rare disease entity, occurring mainly in young women[1-4]. The pathologic features of SPT are well characterized[1,2,5]. Since 1959, over 500 cases of SPT of the pancreas have been reported in literature[3,6]. According to the World Health Organization's (WHO) classification, it is designated as tumors of the exocrine pancreas[1], It is also well known for its indolent biologic behavior. Despite its low malignant potential, proximately 15% of patients with SPT develop metastatic disease, most involving the liver or peritoneum[2]. Even in the presence of disseminated disease, the clinical course is usually favorable[1-3].

With recent advances in diagnostic modalities, including ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and fine-needle aspiration biopsy (FNAB), the tumor was more frequently reported[1,3,7,8]. CT may help to differentiate between SPTs and pancreatic cystic neoplasm, whereas MRI may help to differentiate those from islet cell tumors[7].

Here, we reviewed 26 cases of SPT treated at Chang Gung Memorial Hospital from 1981 to 2005 with respect of clinicopathological, radiological, operative findings, and outcomes. With emphasis on MRI findings, we proposed three specific MRI features to correlate this rare disease entity with its clinicopathological features more precisely.

MATERIALS AND METHODS

Subject

From 1981 to 2005, 26 consecutive patients with SPT of the pancreas underwent surgical treatment at the Department of Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan. Six of these 26 patients had been reported previously[9]. 20 new patients with SPT were included in this study.

Evaluation

Preoperative evaluation of SPT included history taking physical examinations, routine laboratory examinations, ultrasonography (US), endoscopic ultrasonography (EUS),

Table 1 Clinicopathological features of 26 SPT patients underwent surgical treatment

	n	(%)
Gender		
Male	1	3.8
Female	25	96.2
Symptom		
No symptom	5	19.2
Abdominal pain	10	38.5
Abdominal palpable mass	5	19.2
Jaundice	2	7.7
Epigastralgia	4	15.4
Tumor marker		
CEA < 5 (μg/L)	26	100
CA 19-9 < 37 (kU/L)	26	100
Tumor location		
Head	11	42.3
Body	3	11.6
Tail	6	23.1
neck and body	1	3.8
body and tail	5	19.2
Resectability		
Resectable	24	92.3
Unresectable	2	7.7
Operation method		
Whipple's operation	8	30.8
Partial pancreatectomy	3	11.6
Distal pancreatectomy	7	26.9
Enucleation of the pancreatic tumor	6	23.1
Bypass surgery	1	3.8
Biopsy only	1	3.8
Tumor behavior		
Solid-pseudopapillary neoplasm	17	56.4
Solid-pseudopapillary carcinoma	9	34.6

abdominal computed tomography (CT), and magnetic resonance imaging (MRI). The imaging features of the latest 11 consecutive SPT cases underwent MRI study by one of the authors (Tseng JH) (Magnetom Vision; Siemens Erlangen, Germany; projection technique and multislice plus maximum intensity projection) were studied. Of the 26 SPT patients discharged from the hospital after surgical treatment, all of the patients were closely followed up at regular intervals until death or until the time of this manuscript writing.

Statistical analysis

Survival was calculated and plotted using the Kaplan-Meier method from the time of surgical treatment until the writing of this manuscript. All statistical analyses were performed utilizing the SPSS computer software (Version 12.0, Chicago, IL, USA).

RESULTS

Demographic, clinicopathological features, and outcomes

Table 1 summarizes the clinicopathological features of the 26 SPT patients. There were 25 females and 1 male ranging in ages from13 to 57 (median/mean, 23.0/25.2) yrs. Abdominal pain comprises the most common symptom for SPT (38.5%). Five (19.2%) of the 26 SPT patients were detected incidentally during physical check-up. Two of 26 SPT patients developed jaundice (7.7%). The size of SPT ranged from 3.8 to 15.0 (median, 7.5) cm. All known tumor markers including CEA and CA 19-9 are within normal limit, irrespective of malignancy. Pancreatic head is the most frequent location of tumor (Table 1). Two of the 26 SPT patients developed into unresectable status due to multiple liver metastasis and carcinomatosis. Among 26 SPT patients, nine patients were classified as solid pseudopapillary carcinoma by the World Health Organization's (WHO) classification (Table 2). Four of nine solid pseudopapillary carcinoma developed duodenal invasions, while the other three developed portal vein invasions. The duration of follow-up ranged from 10.1 to 236.8 mo (median, 66.0) mo. During the follow-up period, 2 patients of 9 malignant SPT (22.2%) had distant metastasis of SPT after surgical treatment. Two of the 26 patients died of causes unrelated to the tumor or surgical procedure. One underwent Whipple's operation and died of hepatic encephalopathy after a grand mal seizure. The other one underwent bypass surgery and died of hepatic failure due to hepatitis exacerbation. All the other cases have survived until now, whether cases were benign or malignancy. The five-year survival rate of the 26 SPT patients was 96.2%.

MRI imaging features

Table 3 summarized the MRI features. MRI was used in eleven SPT patients with three specific features correlating well with pathological features. MRI had three specific features correlating well with pathological features. Three patients had type 1 image and all the three patients had incidentally found SPT.

Type 1 image means SPT with completely solid part. T1W1 image revealed homogenously hypointense and slightly hyperintense than pancreas parenchyma on T2W1 image. Strong and rapid enhancement and gradually fading pattern could be observed (Figure 1).

Type 2 image means SPT with solid mass with hemorrhage. T1W1 image revealed hypointense with heterogeneously hyperintense area. The hyperintense areas on T1W1 appeared slightly hyperintense on T2W1 image meaning hemorrhage while enhancement of capsule and gradual enhancement of solid part (Figure 2).

Type 3 image means SPT with massive hemorrhage. T1W1 revealed mainly hyperintense with intermediate and hypointense areas. The hyperintense areas on T1W1 image appeared slightly hyperintense on T2W1 image. Only capsular enhancement could be detected (Figure 3). All the eight SPT patients with type 2 and 3 images suffered abdominal pain due to hemorrhage from SPT.

DISCUSSION

As shown in our results, solid pseudopapillary tumor (SPT) of pancreas predominated in adolescent girls and young women (median/mean: 23.0/25.2 years; rang 13-57 years) and is uncommon in men (1/26; 3.8%). Although SPT of pancreas is uncommon, it has been recognized with increasing frequency in recent years, accounting for approximately 1%-2% of all exocrine pancreatic tumors[1,3]. Although literature showed no preferential localization within the pancreas, the head of pancreas is the preferential site of the occurrence of SPT in this study with an incidence rate of 42.3%[1]. The etiology

Table 2 Clinicopathological features of 9 solid pseudopapillary carcinoma patients

No.	Age (yr)/ Sex	Tumor location	Extension	Vascular invasion	Operation method	Metastasis	Metastasis (mo)	Status	Follow-up (mo)
1	36/F	Head	Duodenal wall	-	Whipple's operation	-	-	Alive	70
2[1]	34/F	Tail	-	-	Distal pancreatectomy	-	-	Alive	12
3	39/F	Head	-	-	Whipple's operation	Liver	39	Alive	58
4	22/F	Neck, body	-	PV	Partial pancreatectomy,	-	-	Alive	202
5	18/F	Head	Duodenal wall	-	Whipple's operation	-	-	Alive	145
6	16/F	Head	Duodenal wall	-	Whipple's operation	-	-	Alive	56
7	20/F	Body, tail	Extrapancreatic	-	Biopsy only	Liver, omentum Peritoneum Pelvic wall	0	Alive	75
8	28/F	Head	-	PV	Whipple's operation	-	-	DOC	66
9	19/F	Head	-	PV, SMA	Roux-en-Y cystojejunostomy	-	-	DOC	83

[1]means perineural invasion and angioinvasion; PV: Portal vein; SMA: Superior mesenteric artery; DOC: Die of other cause.

Table 3 MRI features of 11 SPT patients

No.	Type of image	Presentation	Age	G	Size (cm)	Location	T1WI	Enhancement	T2WI	H
1	Ⅰ	RUQ mass, incidentally noted	28	F	3.5	Tail	Hypointense homogenously	Gradual enhancement of solid component	Hypointense homogenously	(-)
2	Ⅰ	GI upset, incidentally noted	22	F	7.5	Body and tail	Hypointense homogenously	Gradual enhancement of solid component	Hypointense homogenously	(-)
3	Ⅰ	RUQ mass, incidentally noted	22	F	6	Head	Hypointense homogenously	Gradual enhancement of solid component	Mild hyperintense	(-)
4	Ⅱ	Abdominal pain	14	F	5	Body	Hyperintense heterogeneously	Heterogenous	Hyperintense (hyperintense areas on T1WI)	(+)
5	Ⅱ	Abdominal pain	29	F	5	Head	Hyperintense heterogeneously	Heterogenous	Hyperintense (hyperintense areas on T1WI)	(+)
6	Ⅱ	Abdominal pain	33	F	11	Tail	Hyperintense heterogeneously	Heterogenous	Hyperintense (hyperintense areas on T1WI)	(+)
7	Ⅱ	Abdominal pain	29	F	7	Head	Hyperintense heterogeneously	Heterogenous	Hyperintense (hyperintense areas on T1WI)	(+)
8	Ⅱ	Abdominal pain	23	F	7.6	Head	Hyperintense heterogeneously	Heterogenous	Hyperintense (hyperintense areas on T1WI)	(+)
9	Ⅲ	Abdominal pain	27	F	6	Tail	Hypo and hyperintense homogenously	Capsular enhancement, Gradual enhancement of solid compnent	Hyperintense (hyperintense areas on T1WI)	(+)
10	Ⅲ	Abdominal pain	36	F	7	Head	Hyperintense heterogeneously	Heterogenous	Hyperintense (hyperintense areas on T1WI)	(+)
11	Ⅲ	Abdominal pain	16	F	5	Head	Hyperintense heterogeneously	Heterogenous	Hyperintense (hyperintense areas on T1WI)	(+)

Ⅰ: Completely solid; Ⅱ: Typical; Ⅲ: Massive hemorrhage; GI: Gastrointestinal; H: Hemorrhage.

and pathogenesis of SPT is unknown[1,3,6]. Very few women developed a SPT after long-term use of hormone contraceptives[1]. The striking sex and age distribution point to genetic and hormonal factors and our previous report indicated an association with endocrine disturbances including overproduction of progesterone[1,6]. Usually, the neoplasm caused abdominal discomfort and pain (10/26; 38.5%) or they were found incidentally on routine physical examination (5/26; 19.2%). As shown in this study, jaundice is rare, even in tumor that originated from the head of the pancreas, and there is no association with endocrine function. All known tumor markers including CEA and CA 19-9 are within normal limit, irrespective of malignancy.

Regarding the role of endoscopic ultrasound (EUS) in evaluation of SPTs, Lee proposed that the characteristic sonographic findings of solid and papillary epithelia neoplasm were well-encapsulated, cystic and solid masses,

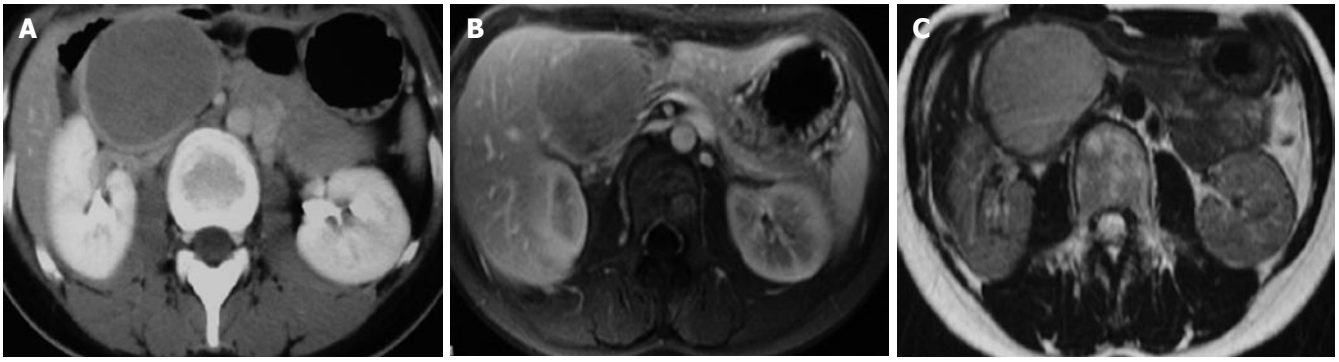

Figure 1 Completely solid pattern. **A**: Abdominal CT with contrast showed a well-defined homogeneous soft tissue mass at the pancreatic head; **B**: T1-weighted MR image with Gd-DTPA enhancement revealed a pancreatic head tumor containing homogenous low-signal-intensity with compression the confluence of splenic vein; **C**: T2-weighted MR image revealed a well-defined pancreatic head tumor containing slightly high-signal-intensity than pancreas parenchyma.

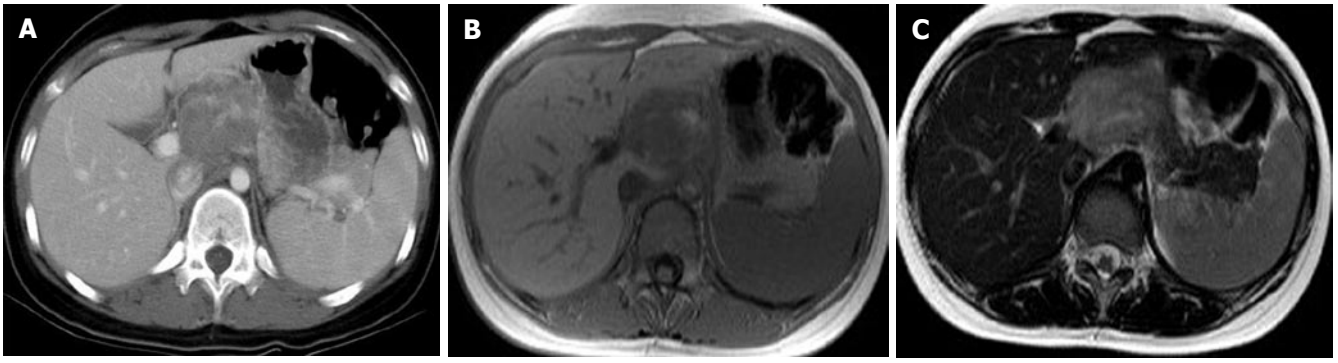

Figure 2 Typical pattern. **A**: Abdominal CT with contrast showed a well-defined heterogenous mass in the pancreatic head with part of hyperdensity; **B**: T1-weighted MR image demonstrates a pancreatic head mass with low-signal-intensity and heterogeneously high-signal-intensity area; **C**: T2-weighted MR image demonstrates a heterogenous hyperintense mass in the pancreatic head with foci hypointensities, high signal intensity means hemorrhage.

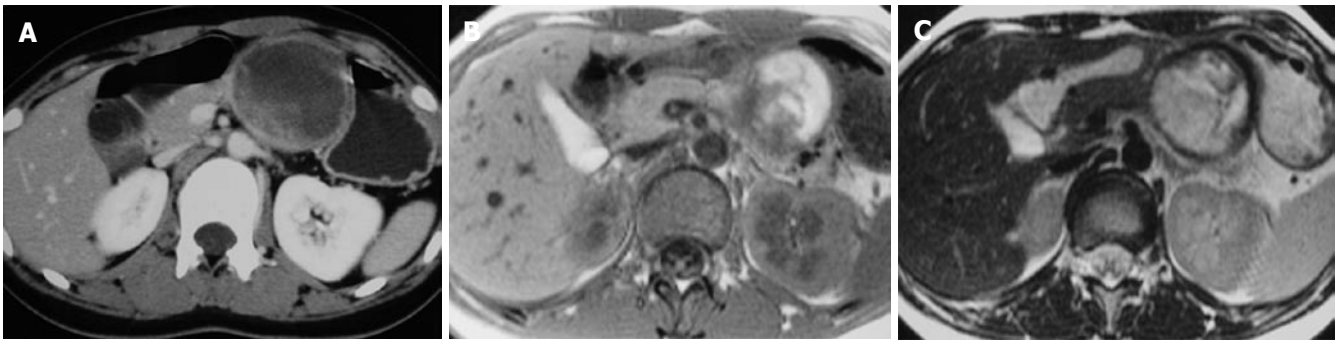

Figure 3 Massive hemorrhage pattern. **A**: Abdominal CT with contrast revealed a well-defined pancreatic body tumor containing heterogenous attenuation; **B**: T1-weighted MR image revealed a pancreatic body tumor containing revealed mainly high-signal-intensity with intermediate- and low-signal intensity area and irregular septation; **C**: T2-weighted MR image demonstrates a well-defined heterogenous slightly hyperintense mass with foci hypointense part. The high-signal-intensity area on T1W1 image appeared slightly hyperintense on T2 weighted MR image.

but sometimes the mass was seen as a pure solid-looking mass or had internal septations or calcification[10]. Fine-needle aspiration (FNA) may have played an important role in preoperative planning by helping distinguish SPTs from other pancreatic lesions with a significantly different prognosis and treatment[11]. EUS-FNA represents a diagnostic technique commonly used in adults that may be useful in identifying the rare pediatric patient with pancreatic malignancy[12]. Thus, accurate diagnosis of the special type of pancreatic tumor is obviously important. Fine-needle aspiration biopsy and cytologic analysis or excisional biopsy and histologic analysis are sometimes needed for definitive diagnosis.

Regarding radiological study, CT may help to differentiate SPT from other cystic neoplasm whereas MRI may help to differentiate SPT from islet cell tumors[7]. Regarding radiological features of SPT, MRI revealed an encapsulated mass lesion with solid and cystic component

as well as hemorrhage without obvious internal septation should arouse the suspicion of SPT of pancreas, especially in a young female patient. As shown in this study, MRI may be superior to CT in terms of correlation between radiological and clinicopathlogical findings of SPT. Furthermore, in the setting of renal insufficiency or dye allergy when contrast is contraindicated, MRI had an advantage when compared with CT. We specifically proposed three MRI features to correlate well with clinicopathological features. Type 1 image means SPT with completely solid part. T1W1 image revealed homogenously hypointense and slightly hyperintense than pancreas parenchyma on T2W1 image. Strong and rapid enhancement and gradually fading pattern could be observed. Type 2 image means SPT with solid mass with hemorrhage. T1W1 image revealed hypointense with heterogeneously hyperintense area. The hyperintense areas on T1W1 appeared slightly hyperintense on T2W1 image, meaning hemorrhage, while enhancement of capsule and gradual enhancement of sold part. Type 3 image means SPT with massive hemorrhage. T1W1 revealed mainly hyperintense with intermediate and hypointense areas. The hyperintense areas on T1W1 image appeared slightly hyperintense on T2W1 image. Only capsular enhancement could be detected.

As shown in this study, a few SPT could be found to be attached to the pancreas or even an extrapancreatic locations. Invasion of the adjacent organs or the portal vein is not uncommon. As shown in this series, few metastasizing SPTs (2/26; 7.6%) developed. Common metastatic sites are regional lymph nodes, the liver, peritoneum, and greater omentum.

Regarding prognosis, Nishihara reported SPT must be classified as lesions of uncertain malignant potential due to varying histological features[13]. As shown in this study, enucleation and partial pancreatectomy is as good as distal pancreatectomy, although surgical oncologic principles are not followed in the former. Based on the clinical follow up, there does not appear to be any difference in survival based on resection techniques. So we suggest that SPT is indolent and surgery for SPT should induce minimal functional deficit. Furthermore, the prognosis is good with 96.4% 5-year survival rate for 26 SPTs. After complete removal of SPT, more than 95% of the patients with SPT are cured. Local spread or dissemination to the peritoneal cavity has been reported due to abdominal trauma and rupture of the tumor. Even in patients who had local spread, recurrent or metastases, long disease-free survival has been recorded after initial diagnosis and resection. Only a few patients have died of metastasizing SPTs.

In conclusion, we present the clinical features and outcomes of 26 SPT patients with emphasis on MRI findings. SPT had a favorable survival irrespective of surgical procedures, malignancy, and MRI pictures. MRI could reliably correlate with its clinicopathological features with specific imaging features.

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RAPID COMMUNICATION

Appropriateness of colonoscopy: Diagnostic yield and safety in guidelines

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Abstract

AIM: To evaluate if the guidelines for the appropriateness of performing colonoscopy by American Society for Gastrointestinal Endoscopy (AGSE) and Italian Society of Digestive Endoscopy (SIED) yield a good diagnostic efficacy and do not present risks of missing important colonic pathologies in an Italian population sample.

METHODS: A total of 1017 consecutive patients (560 men and 457 women; mean age 64.4 ± 16 years) referred to an open-access endoscopy unit for colonoscopy from July 2004 to May 2006 were evaluated according to ASGE and SIED guidelines for appropriateness of performing the procedure. Diagnostic yield was defined as the percentage of relevant colonic pathologies of the total number of colonoscopies performed.

RESULTS: About 85.2% patients underwent colonoscopy that was considered appropriate based on at least one ASGE or SIED criterion, while it was considered inappropriate for 14.8% of patients. The diagnostic yield of colonoscopy was significantly higher for appropriate colonoscopies (26.94% *vs* 10.6%, $P < 0.001$) than for inappropriate colonoscopies (5.3%). There was no missed colorectal cancer following the ASGE/SIED criteria.

CONCLUSION: ASGE/SIED guidelines have shown a good diagnostic yield and the rate of missing relevant colonic pathologies seems very low. Unfortunately, the percentage of inappropriate referrals for colonoscopy in an open-access endoscopy system is still high, despite the number of papers published on the issue and the definition of international guidelines. Further steps are required to update and standardize the guidelines to increase their diffusion and to promote educational programs for general practitioners.

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Key words: Colon; Colonoscopy; Endoscopy; Guidelines

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INTRODUCTION

Colonoscopy is a widely available diagnostic modality that has replaced barium enema for colorectal examination[1]. The growing demand of gastrointestinal (GI) endoscopy in most developed countries has determined an unavoidable increase of health care costs, therefore health care systems are experiencing a cost crisis[2,3]. Colonoscopy is relatively expensive and associated with a small but definite rate of complications[2]. To maintain or enhance the quality of care in an increasingly cost-conscious environment, the ability to determine appropriateness of the care may be essential[4].

Appropriateness of procedures, such as colonoscopy, is defined by the fact that the health benefit exceeds the health risk by a sufficiently wide margin of security[5]. To define the correct use of GI lower endoscopy, the American Society for Gastrointestinal Endoscopy (ASGE) has developed indication criteria for diagnostic endoscopic procedures[6]. However, the exclusive use of these criteria cannot be considered a safe approach, since many studies reported that a significant percentage of relevant pathologies could be found even in colonoscopies defined as not appropriate by ASGE criteria[4,7].

Approximately 20% of colonoscopies in Italy were performed with indications not considered appropriate by ASGE criteria[2,3,7]. More recently, the Italian Society of Digestive Endoscopy (SIED) supplied different appropriateness criteria for colonoscopy[8] (Table 1).

The aim of the present study was to evaluate the appropriateness of performing colonoscopy in an open access Italian endoscopy unit using the ASGE/SIED guidelines and the efficacy of the above-mentioned

guidelines to detect relevant diseases (particularly neoplasms) without missing relevant pathologies.

MATERIALS AND METHODS

This prospective study was carried out from July 2004 to May 2006 in an open-access endoscopy unit in Asti, Italy. All consecutive outpatients referred to our endoscopy unit for colonoscopy by primary care physicians entered the study. A written informed consent and confidential medical information were obtained from all patients before the procedure, and the study was conducted according to the local ethical rules following the principles of the Helsinki Declaration (Edinburgh revision, 2000).

The protocol included: (1) accurate patient interview and examination, evaluation of previous diagnostic tests (fecal occult blood, barium contrast enema, anemia, etc.) and determination of the indication category specified by the ASGE/SIED guidelines with definition of appropriate or inappropriate colonoscopy before the procedure; (2) performance of colonoscopy by an endoscopist while its appropriateness was determined by another endoscopist. The study did not include those who could not complete the colonoscopy because of the presence of a neoplastic stenosis. Inpatients and asymptomatic outpatients referred for the government's colorectal cancer screening program, therapeutic and emergency colonoscopies were also excluded from the study.

Statistical analysis was performed with the Chi-square test; and values of $P < 0.05$ were considered statistically significant.

RESULTS

From July 2004 to May 2006, we performed 2245 colonoscopies on 1133 inpatients and 95 outpatients referred for the government's colorectal cancer screening program. And 172 were incomplete and excluded. As a result, a total of 1017 patients entered the study, including 560 (55.1%) men and 457 (44.9%) women. The mean age was 64.4 years with a range of 16-89 years. In this series, cecal intubation was not possible in 3.5% of cases due to neoplastic stenosis. A total of 866 colonoscopies (85.2%) were defined appropriate based on at least one ASGE or SIED criterion, while 151 (14.8%) were defined inappropriate.

The indications for appropriate colonoscopies were found in 866 patients, and are listed in Table 2. The most frequent indications were hematochezia (23.7%), presence of occult fecal blood and/or iron deficiency anemia (16.7%), and surveillance of polyps (14.8%). Indications for inappropriate colonoscopies are listed in Table 3. The most frequent inappropriate indications were surveillance of colonic polyps and colorectal carcinoma at different intervals from those recommended (32.4%), and sporadic non-specific abdominal pain or chronic abdominal pain with previous endoscopic investigations (19.9%).

The rate of clinically relevant diagnosis was significantly higher in patients whose colonoscopy was classified as appropriate (233 of 866 patients, 26.9%) than in those with inappropriate indications (16 of 151, 10.6%, $P < 0.001$). Aspecific colitis and diverticulosis were not considered as clinically relevant endoscopic findings. The most frequent pathologic findings in appropriate colonoscopies are listed in Table 4, the low grade dysplasia adenomas and colorectal cancer were more common.

Only 1 case of adenoma with high-grade dysplasia, 2 cases of adenoma with low-grade dysplasia and 5 with unretrieved polyps of less than 5 mm in size had pathologic findings in inappropriate procedures.

DISCUSSION

Over the recent years the number of colonoscopies has grown exponentially, due to the increase of the demand by population and primary care physicians. This has led to long waiting lists and unavoidable economic consequences on the management of the Health Care System, which is experiencing a very severe cost crisis in Italy. Thus, the appropriateness of colonoscopy performance can reduce overuse, improve quality of care and decrease costs[5], especially in those countries where the digestive endoscopic services function on an open access system basis. Defining strong criteria for the appropriateness of endoscopic procedures is therefore needed to face the health care demand of the population and the government to reduce costs.

ASGE has developed and published the indication categories for diagnostic colonoscopy procedures[6]. More recently, however, authors from different countries[4] have suggested that the ASGE guidelines require modifications. Colonoscopies performed following ASGE indications demonstrated clinically relevant findings only in a slightly higher prevalence than those performed without these indications (28.8% versus 20.2%), suggesting that the exclusive use of these criteria cannot be considered a safe approach[8]. Charles *et al*[9] reported that 40% of the patients undergoing colonoscopy for an ASGE approved indication have pathological results, compared to 22% of those with inappropriate indications. Similarly, Morini *et al*[10] reported a positive diagnosis of 43% for appropriate colonoscopies and 16% for inappropriate procedures.

A more recent study indicated that the proportion of patients in "not listed" category was about 16%, with a diagnostic yield of 13.4%, about three times higher than the diagnostic yield of colonoscopy with inappropriate indications. The two most frequent unlisted indications in this study were unexplained weight loss and alterations of bowel habits; the authors conclude that these unlisted indications must be included in the future version of the ASGE guidelines[11]. The same opinion has been supported by Chan[4] in a cross-sectional study performed at the University of Malaya. These indications are included in the SIED guidelines for colonoscopy. In fact, three colorectal carcinomas in our study were diagnosed in patients only complaining of recent and persistent constipation.

In fact, in our prospective study on an open access system, based on general practitioners' demand, the indications for colonoscopies according to the ASGE and SIED criteria were appropriate in 85.2% of cases and inappropriate in 14.8%, with an appropriateness higher than that reported in literature. We found very

Table 1 ASGE and SIED selection criteria for colonoscopies

ASGE	SIED
Abnormality on barium enema	Abnormality on barium enema, [1]Computerized Tomography scan, Magnetic Resonance Imaging, Ultrasonography
Haematochezia–Fecal occult blood test positivity–melena after negative upper GI endoscopy	Haematochezia–Fecal occult blood test positivity-Melena after negative upper GI endoscopy
Unexplained iron deficiency anemia	Unexplained iron deficiency anemia
Surveillance for colonic neoplasia and colonic polyps	Surveillance for colonic neoplasia and colonic polyps
Clinically significant diarrhea of unexplained origin	[1]Persistent and significant alterations of bowel habits
Surveillance of inflammatory bowel diseases patients	Surveillance of inflammatory bowel diseases patients
Evaluation of inflammatory bowel diseases of colon when colonoscopy will influence immediate management	Evaluation of inflammatory bowel diseases of colon when colonoscopy will influence immediate management
Chronic abdominal pain: once to rule out disease	Chronic abdominal pain: once to rule out disease
	[1]Unexplained weight loss

[1]Different criteria between ASGE and SIED guidelines.

Table 2 921 indications in 866 appropriate colonoscopies

ASGE/SIED guidelines	*n*	%
Haematochezia	218	23.7
Occult faecal blood presence	154	16.7
Surveillance after endoscopic polypectomy (3-5 yr intervals following adequate clearance of neoplastic polyps)	136	14.7
Persistent change in bowel habits	105	11.4
Surveillance after resection of cancer (colonoscopy to remove synchronous neoplastic lesion at or around time of curative resection of cancer followed by colonoscopy at 3 yr and 3-5 yr thereafter to detect metachronous cancer)	100	10.8
Chronic abdominal pain	57	6.2
Unexplained iron deficiency anemia	54	5.9
Family history of sporadic colorectal cancer before the age of 60: colonoscopy every 5 yr beginning at the age of 10 yr earlier than the affected relative or every 3 yr if adenoma is found	42	4.6
Abnormality on imaging	28	3.0
Unexplained weight loss	18	1.9
Chronic inflammatory bowel disease of colon, if more precise diagnosis or determination of the extent of activity of disease will influence immediate management	9	1.0
In patients with ulcerative or Crohn's pancolitis ⩾ 8 yr or left sided colitis ⩾ 15 yr every 1-2 yr with systematic biopsies to detect dysplasia		

Table 3 Indications in 151 inappropriate colonoscopies

Indications	*n*	%
Surveillance of colonic polyps out of recommended intervals (3-5 yr intervals following adequate clearance of neoplastic polyps)	49	32.4
Transitory or already endoscopically investigated unmodified chronic abdominal pain	30	19.9
Transitory change in bowel habit	21	13.9
Colorectal carcinoma surveillance out of guidelines (colonoscopy to remove synchronous neoplastic lesion at or around time of curative resection of cancer followed by colonoscopy at 3 yr and 3-5 yr thereafter to detect metachronous cancer)	20	13.2
Melena with upper gastrointestinal source already identified	9	6.0
Screening in patients with family histories of sporadic colorectal cancer before age of 60 out of guidelines (colonoscopy every 5 yr beginning at the age of 10 yr earlier than the affected relative or every 3 yr if adenoma is found)	6	4.0
Hematochezia in patients < 40 yr without previous rectal evaluation	4	2.6
Follow-up for inflammatory bowel diseases out of recommended intervals. Chronic inflammatory bowel disease of the colon, if more precise diagnosis or determination of the extent of activity of disease will influence immediate management	3	2.0
In patients with ulcerative or Crohn's pancolitis ⩾ 8 yr or left sided colitis ⩾ 15 yr every 1-2 yr with systematic biopsies to detect dysplasia		
Anal symptoms	3	2.0
Rectal incontinence	2	1.3
Abnormal serologic markers (CEA, carcino Embriogenic Antigen, Cancer Antigen 19-9)	2	1.3
Metastatic adenocarcinoma of unknown origin without colonic symptoms when it will not influence management	1	0.7
Inguinal hernia	1	0.7

few pathologic elements in the inappropriate series (10.59% in not indicated versus 25.40% in indicated), which constitutes a lower percentage than the reported percentage[2,3,7,10]. This may be due to the wider criteria applied or due to the fact that we did not consider as clinically relevant diagnosis of other diseases, such as

Table 4 Pathologic findings in appropriate colonoscopies

Pathologic findings	866 appropriate		151 inappropriate	
	n	%	*n*	%
Low grade dysplasia adenoma	92	41.8	9	6.0
Colorectal cancer	71	32.3	-	-
Undetermined polyps (unretrieved polyps size < 5 mm)	25	11.0	5	3.3
High grade dysplasia adenoma	25	11.4	2	1.3
Inflammatory bowel diseases	17	7.7	-	-
In situ adenocarcinoma	3	1.4	-	-
	233	26.9	16	10.6

diverticulosis and aspecific colitis. In our study there was no missed colorectal cancer diagnosis following the ASGE/SIED criteria and even the percentage of less relevant pathologies was very low.

However, the main issue we want to stress is that it is highly necessary to improve the diffusion of appropriateness criteria so as to give better diagnostic safety in the population and to reduce the heavy medical costs. The use of detailed and explicit appropriateness criteria for colonoscopy significantly enhances the identification of relevant lesions, especially colon cancer[12], but further studies are required[13-15], including better educational programs for general practitioners to improve patient selection for colonoscopy, thus contributing to enhancing the quality and efficiency of health care.

COMMENTS

Background

Colonoscopy is a widely available diagnostic and therapeutic procedure for which demand continues to grow. This has resulted in an increase in overall costs and waiting lists. Specific guidelines for colonoscopy have been published and periodically updated to increase the diagnostic yield of the procedure.

Research frontiers

To increase the effectiveness and safety of colonoscopy, studies have been published about the optimization, standardization and communication of international guidelines.

Innovations and breakthroughs

Many recent articles in literature focused on the comparison among different guidelines, while others pay more attention to the economic impact if strict adherence to guidelines is followed.

Applications

A further exploration of the aspects presented in this paper and other similar studies in a future perspective could lead to a better understanding of the relationship between quality of care and humans, technological and financial economic resources.

Terminology

Appropriateness of care is defined by the fact that in any given procedure, the benefits exceed the risks by a wide enough margin to make it worth providing it. The diagnostic yield of an endoscopic procedure is defined as its capacity of identifying a lesion that is potentially important to patient care.

Peer review

The selection of patients who should undergo endoscopic procedures is one of the key points of the diagnostic process, which should avoid the excessive use of invasive and expensive tests as well as the underestimation of potentially harmful diseases.

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RAPID COMMUNICATION

Role of intravenously administered hyoscine butyl bromide in retrograde terminal ileoscopy: A randomized, double-blinded, placebo-controlled trial

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Abstract

AIM: To evaluate the role of hyoscine butyl bromide in facilitating retrograde ileoscopy.

METHODS: Retrograde terminal ileoscopy was attempted in 200 consecutive patients undergoing colonoscopy. After intubation of the cecum and visualization of the ileocecal valve, hyoscine butyl bromide injection or normal saline was given intravenously to the patients in a double blind random fashion. The pulse rate and oxygen saturation were measured continuously. After completion of the procedure, endoscopists were then asked to score the ease of intubation and the ease of visualization of the terminal ileum on a visual scale of 1 to 10. The patients were also asked to score the pain after receiving hyoscine butyl bromide injection on a score of 1 to 10.

RESULTS: Terminal ileoscopy could be performed in 188 patients. The mean (SD) visual analogue score for the ease of intubation of the cecum was 7.4 (0.65) in the injection group and 5.9 (0.8) in the placebo group (P < 0.001). The mean (SD) length of ileum visualized in the injection group was 14.4 (3.3) cm and 10.4 (2.7) cm in the placebo group (P < 0.001). The mean (SD) visual analogue score for ease of visualization of the terminal ileum was 7.5 (0.69) in the injection group and 5.9 (0.7) in the placebo group (P < 0.001). The pain score experienced by the patients was 6.5 (0.7) in the injection group and 6.7 (0.69) in the placebo group (P < 0.008). Although the pulse rate increased significantly in patients receiving the drug, no statistically significant difference was noted in the oxygen saturation between the two groups either before or after administration of the drug. No complications were observed in either of the groups.

CONCLUSION: Hyoscine butyl bromide injection is a useful adjunct in helping the intubation and visualization of terminal ileum during colonoscopy.

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Key words: Ileoscopy; Colonoscopy; Colon; Ileum; Hyoscine; Tuberculosis; Inflammatory bowel disease; Cancer

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INTRODUCTION

Colonoscopy is the procedure of choice for investigating patients with diseases of the colon and terminal ileum, such as colonic adenomas or cancer, fecal occult blood loss, inflammatory bowel disease, iron deficiency anemia, colonic tuberculosis and hematochezia[1-8].

Terminal ileoscopy is an integral part of complete colonoscopy. Retrograde terminal ileoscopy has been noted to be useful in patients with inflammatory bowel disease, diarrhea, lymphoma, cytomegalovirus-induced ileitis, tuberculosis, portal hypertension and a host of other conditions involving the terminal ileum[7-21]. In a recent study, we have noted that obtaining blind biopsies from even a normal-appearing terminal ileum is useful in patients suspected of having ileocaecal tuberculosis[22]. Although terminal ileoscopy has been recommended by several authors[7-10,22], it is not routinely performed when colonoscopy is done in all patients undergoing colonoscopy[3]. A more recent study showed that routine ileoscopy is very useful and easy to perform after proper training has been imparted. Except for a study that found that hyoscine butyl bromide injection is helpful in performing ileoscopy[23], the role of hyoscine butyl bromide in facilitating retrograde terminal ileoscopy has not been evaluated. The present study was therefore planned to evaluate the role, if any, of hyoscine butyl bromide injection in performing ileoscopy at colonoscopy.

MATERIALS AND METHODS

Retrograde terminal ileoscopy was attempted in 200

Table 1 Indications for colonoscopy in two groups of patients n = 200

Indication	Group 1 (active drug)	Group 2 (placebo)	Total
Unexplained abdominal pain	55	56	111
Suspected colonic tuberculosis	14	12	26
Ulcerative colitis	11	13	24
Hematochezia	5	5	10
Fecal occult blood positive	4	6	10
Colon cancer	2	4	6
Occult gastrointestinal bleeding	2	2	4
Colonic polyp	1	2	3
Portal hypertension (colonic bleed)	2	1	3
Crohn's disease	2	1	3

Table 2 Effect of hyoscine N-butyl bromide on the vital parameters mean ± SD

	Drug	Placebo	*P*
Pulse rate before administration	85.7 (4.7)	85.6 (3.8)	NS
Pulse rate after procedure	93.2 (4.9)	86.5 (3.9)	< 0.001
Highest pulse rate achieved before ileoscopy	94	92	
Highest pulse rate achieved during ileoscopy	102	92	
Po_2 before administration	95.4 (1.5)	95 (1.3)	NS
Po_2 after administration	94.4 (1.8)	94.6 (1.7)	NS
Minimum Po_2 before ileoscopy	92	94	NS
Minimum Po_2 during ileoscopy	90	90	NS

consecutive patients undergoing colonoscopy. Colonic preparation was performed using a polyethylene glycol-electrolyte-based solution (Peglec, Tablets India, Ltd, Chennai, India).

Patients with a previous history of abdominal surgery, glaucoma, obstructive uropathy, autonomic dysfunction and allergy to hyoscine butyl bromide (Buscopan) were excluded from the study. All patients received midazolam for conscious sedation. Colonoscopy was performed using an Olympus videocolonoscope (CF 130 L, Olympus, Tokyo) without any attachment.

At colonoscopy, a careful search was made for any lesion in the colon. After reaching the cecum, attempt was made to localize the ileocecal opening. Once it was visualized, 1 mL of hyoscine butyl bromide injection (German Remedies, Mumbai) containing 20 mg of the drug or an equal volume of normal saline was administered intravenously to the patients in a double blind random fashion by a nursing assistant. Once in the terminal ileum all attempts were made to go as far as possible in the ileum. Any abnormality in mucosa of the terminal ileum was carefully recorded and biopsies were obtained from suspicious-looking lesions. The endoscopists scored the ease of intubation and the ease of visualization of the terminal ileum on a visual analogue score of 1-10. The length of terminal ileum visualized was also recorded during withdrawal of the colonoscope, when the length of the colonoscope inside the ileocecal valve was measured. The pulse rate and oxygen saturation were measured continuously using a pulse oximeter. However for the study purposes the values just before administration of hyoscine butyl bromide or saline and at the end of the procedure were taken. After the procedure the patients scored the pain they felt while the terminal ileum was intubated or visualized, on a visual analogue score of 1-10.

Statistical analysis was performed using the Student's *t*-test and chi-square test with or without Yates' correction as appropriate. All patients gave their informed consent and IRB clearance was obtained before start of the trial.

RESULTS

During the study period, 200 colonoscopies were performed. The indications for colonoscopy are given in Table 1. The cecum could be intubated in all patients. Repeat colonoscopy had to be performed in 21 (10.5%) patients due to poor preparation during the first colonoscopy.

Despite all attempts, the terminal ileum could not be intubated in 12 patients: 4 due to deformed ileocecal valve and 8 due to non-visualization of the ileocecal opening. These patients were excluded from the final analysis. Hyoscine butyl bromide injection was administered to 2 patients and normal saline to 2 patients with deformed ileocecal valve respectively. As per protocol, injections were administered only on visualization of the ileocaecal opening and therefore no injection was given to patients in whom the ileocecal valve was not visualized.

The mean (SD) age of these patients was 44.7 (10.6) years (range 25-65 years). There were 147 males and 41 females in the study.

There were 94 patients in both groups. The mean (SD) visual analogue score for the ease of intubation was 7.4 (0.65) in the injection group and 5.9 (0.8) in the placebo group. The difference between the two groups was statistically significant ($P < 0.001$). The mean (SD) length of ileum visualized was 14.4 (3.3) cm in the injection group and 10.4 (2.7) cm in the placebo group ($P < 0.001$). The mean (SD) visual analogue score for ease of visualization of the terminal ileum was 7.5 (0.69) in the injection group and 5.9 (0.7) in the placebo group ($P < 0.001$). The mean (SD) pain score experienced by the patients was 6.5 (0.7) in the injection group and 6.7 (0.69) in the placebo group. The difference between the two groups was statistically significant ($P < 0.008$).

The effect of the drug on the vital parameters is shown in Table 2. Although the pulse rate increased significantly in patients receiving the drug, no statistically significant difference was noted in the oxygen saturation between the two groups either before or after administration of the drug.

DISCUSSION

Colonoscopy remains the procedure of choice for most diseases of the large bowel[1]. Although colonoscopy is performed routinely for suspected abnormalities of the colon[1], ileoscopy is not commonly employed in the work-up of patients with ileocolonic abnormalities[3]. Despite the fact that a number of studies have demonstrated the utility of retrograde ileoscopy during colonoscopic examination of the large bowel[7-22], a recent study speculated that

perceived difficulty of ileal intubation, time constraints, and the expectation of a low diagnostic yield are the likely reasons for the resistance on the part of the endoscopists in attempting retrograde ileoscopy[3].

We did not study the success of terminal ileal intubation in the two groups as the rate of terminal ileal intubation is high in the hands of the experienced and motivated endoscopists. However, the results of the present study were encouraging and hyoscine butyl bromide injection was noted to have a salutary effect on all the parameters evaluated in the study, including the ease of terminal ileal intubation and the length of the terminal ileum visualized. This should be helpful in motivating endoscopists to perform retrograde terminal ileoscopy. Even the patients perceived the procedure to be less painful when hyoscine butyl bromide was administered. Moreover, adverse effects were not observed, although the patients receiving the drug had significant tachycardia compared to those receiving placebo (Table 2).

Injection of hyoscine butyl bromide has been utilized to achieve aperistalsis in patients undergoing ERCP. However, its role in colonoscopy is not very clear[24-28]. It should be noted that in the present study we did not use it till we achieved cecal intubation and visualized the ileocecal valve, suggesting that it should not be used to facilitate colonoscopy but only to make ileoscopy easier for the endoscopist and patients. An earlier study noted the drug is a useful adjunct in intubating the terminal ileum during routine colonoscopy[23].

Glucagon has been used in lieu of hyoscine butyl bromide for achieving aperistalsis in patients undergoing ERCP[29,30]. Glucagon has also been evaluated as an adjunct in facilitating colonoscopy[31,32]. Whether glucagon can be used in lieu of hyoscine butyl bromide in achieving ileal intubation in patients undergoing colonoscopy, remains to be studied. However, since the action of glucagon and hyoscine butyl bromide are similar, and glucagons has been used instead of hyoscine, to achieve aperistalsis during ERCP, we do not foresee any reason why glucagons cannot replace hyoscine. Although the side effects of glucagon are less, the cost is a prohibitive factor in its routine use, especially in the developing countries.

A few earlier studies have raised concern about the adverse changes in the vital parameters following administration of hyoscine butyl bromide[25,27] although not all studies observed such changes[24,26]. However, we did not observe any untoward incident or adverse changes in the vital parameters in this study, which might be due to the very short time required between administration of the drug and completion of the ileoscopy. It is important to remember that hyoscine butyl bromide or saline injection was administered only when the ileocaecal valve could be identified and the endoscopist was prepared to intubate the terminal ileum.

We have noted that retrograde terminal ileoscopy is a useful investigation in patients with ileocolonic tuberculosis[33] and can diagnose the cause of hematochezia in patients in whom an earlier colonoscopic examination fails to reveal any abnormality[34]. Obtaining blind biopsies from normal appearing cecum and terminal ileum could yield a positive diagnosis in a select group of patients suspected of having ileocecal tuberculosis[22]. The use of hyoscine butyl bromide injection would make the procedure easier and it is expected that more endoscopists would perform retrograde terminal ileoscopy while performing colonoscopy.

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RAPID COMMUNICATION

Clinical heterogeneity in autoimmune acute liver failure

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Abstract

AIM: To describe the outcome and prognosis in a cohort of patients with acute liver failure due to autoimmune hepatitis without liver transplantation.

METHODS: A retrospective trial was conducted in 11 patients with acute liver failure due to autoimmune hepatitis who attended the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubiran. Demographic, biochemical and severity indexes, and treatment and outcome were assessed.

RESULTS: Among the 11 patients, with a median age of 31 years, 72% had inflammatory response syndrome, and six patients received corticosteroids. The mortality rate within four weeks was 56%, and the one-year survival was 27%. In the survivors, severity indexes were lower and 83% received corticosteroids.

CONCLUSION: We observed a relatively high survival rate in patients with acute liver failure due to autoimmune hepatitis. This survival rate could be influenced by severity of the disease and/or use of corticosteroids.

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Key words: Autoimmune hepatitis; Acute liver failure; Corticosteroids

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INTRODUCTION

Acute liver failure (ALF) is defined as the development of hepatic necrosis with encephalopathy within eight weeks of the onset of liver disease. It is a complex multisystemic event that evolves after a catastrophic insult to the liver ending in the development of a coagulopathy and encephalopathy within a short period of time. ALF is a heterogeneous condition incorporating a range of clinical syndromes[1]. Several etiologies are involved in ALF. However, in the USA and UK, paracetamol, hepatitis A or B and seronegative are the most common ones[2]. The overall mortality varies widely from 10% to 90% in different cohorts. The higher survival rates were observed in those subjects who received liver transplantation, and nowadays ALF accounts for 5%-12% of all liver transplantations[3].

Autoimmune hepatitis (AIH) is an uncommon cause of ALF and is usually associated with type 2 autoimmune hepatitis, particularly in children[4]. In an adult population, AIH occurs in less than 10% of all cases of ALF[5], and acute AIH presents with fulminant hepatic failure and is the last cause of liver transplantation[6]. Few reports about the clinical characteristics and outcomes in this rare group of subjects with ALF are available. Clinically, it is considered a condition that is no longer responsive to corticosteroid therapy or other immunosuppressive therapies[1]. However, Miyake *et al*[7] recently demonstrated in a Japanese population a survival rate of 45% without treatment with corticosteroids. The aim of this study was to describe the clinical outcome and prognoses in a cohort of adult subjects with ALF due to AIH without liver transplantation.

MATERIALS AND METHODS

Subjects

Patients admitted to the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubiran (Mexico City) for ALF, secondary to AIH, from 1984 to 2004 were studied retrospectively. The autoimmune etiology was defined according to the biochemical and histological criteria of the International Auto-immune Hepatitis Group[8]. Diagnosis of ALF was made according to the previous published criteria[9] which include: the development of hepatic encephalopathy (≥ grade Ⅱ) within eight weeks of the onset of initial symptoms, a prothrombin activity of < 40%, and the absence of previous chronic or alcoholic liver disease. Hepatic encephalopathy was graded on the standard scale of Ⅰ-Ⅳ.

Table 1 Clinical features at time of diagnosis

Case No.	Age (yr)	IAHSS	New onset AIH	BMI (m/kg^2)	HE	SIRS	Bilirubin (mg/dL)	ALT (IU/L)	ALP (UI/L)	INR	Globulin (g/dL)	Creatinine (mg/dL)	ANA	Corticosteroids
[1,3]1	65	17	Yes	31.1	2	Yes	21.9	309	274	1.60	4.1	1.1	1:40	Yes
[1,2]2	17	17	Yes	22.0	3	Yes	15.3	358	136	1.50	2.5	0.9	1:40	Yes
[2]3	66	17	No	20.0	2	Yes	8.4	31	207	1.50	4.4	0.9	1:80	Yes
[1,2]4	28	17	Yes	30.3	2	Yes	22.9	128	262	1.90	5.7	1.1	1:80	Yes
[1,2]5	52	14	Yes	27.5	2	No	21.2	217	186	1.90	4.5	0.6	1:40	No
[1]6	17	20	Yes	22.5	2	No	7.6	414	271	1.70	7.7	0.8	1:80	Yes
7	31	18	No	19.9	4	Yes	22.0	541	138	3.60	2.9	6.6	1:80	Yes
8	25	14	No	35.0	3	Yes	26.8	23	309	1.90	3.1	2.7	1:40	No
[1,3]9	67	13	Yes	26.9	2	No	13.8	683	135	8.12	2.4	2.3	1:80	No
10	18	12	Yes	27.0	3	Yes	22.6	126	120	2.20	2.7	1.1	1:40	No
11	33	13	Yes	23.3	3	Yes	20.1	173	199	3.60	4.3	1.4	1:80	No

IAHSS: international autoimmune hepatitis scoring system; HE: hepatic encephalopathy grade; SIRS: systemic inflammatory response syndrome; ALT: alanine aminotransferase; ALP: alkaline phosphatase; INR: international normalized ratio; ANA: antinuclear antibody; BMI: body mass index. [1]Cases treated with corticosteroids, [2]Cases that survived one month, [3]Cases that survived more than three years.

Other etiologies were excluded by the absence of IgM anti-hepatitis A virus antibody, IgM anti-hepatitis B virus core antibody or hepatitis B surface antigen. In addition, drug-related disease, acute fatty liver of pregnancy, ischaemic hepatitis, Wilson's disease, malignant infiltration, cytomegalovirus, Epstein-Barr virus and herpes simplex were also excluded.

Baseline clinical characteristics such as age, gender, and body mass index were recorded; similar biochemical values, and Acute Physiology And Chronic Health Evaluation score[10], systemic inflammatory response syndrome[11] and multiple organ failure were assessed[12]. Mortality was classified as either in-hospital and out-hospital.

Statistical analysis

All statistical analyses were carried out with the SPSS/PC v 10.0 program (SPSS Inc, Chicago, IL, USA). Continuous variables were expressed in terms of median and range. Statistical significance was set at $P < 0.05$. Variables were analysed using the exact Fisher's test (two-tailed), Mann–Whitney *U* test and 95% confidence intervals.

RESULTS

Among 58 subjects with ALF, AIH was the etiology (19%) in 11 cases. All cases had onset of encephalopathy within 28 days, and all were not diagnosed previously with AIH. Ten were females (90.9%), with a median age of 31 years (range, 17-67 years) and mean body mass index of 25 ± 4 m/kg^2. Liver biopsy was performed in eight subjects to determine the diagnosis. Eight subjects (72%) had systemic inflammatory response syndrome. The baseline biochemical values were: bilirubin 18.4 ± 6.2 mg/dL, alanine aminotransferase 273 ± 219 U/L (median, 273; range, 31-683), alkaline phosphatase 203 ± 67 U/L, international normalized ratio 2.6 ± 1.95 (median, 1.9; range, 1.5-8.12) and creatinine 1.77 ± 1.72 mg/dL (median, 1.1; range, 0.6-6.6) (Table 1). The overall mortality was 54.5% (n = 6); however, only 16.6% (1/6) of these subjects received corticosteroids. Corticosteroid was used based on the initial clues that indicated AIH as a cause of ALF. Additionally, all patients were treated with maximal available supportive care according to their clinical characteristics.

In-hospital mortality was 45.4% (n = 5), the one-month survival rate was 54% (n = 6), with three subjects (27%) surviving more than one year, and the longest follow-up being six years.

When the subjects were analysed according to death/survival, differences were observed in the prevalence of severe hepatic coma, APACHE Ⅱ score, SIRS, sepsis, severe sepsis, and multiorgan failure at baseline (Table 2).

Those subjects who survived more than three years (cases 1, 2 and 9) had similar characteristics, except for age, creatinine and INR at presentation. The three subjects received corticosteroids: 1 g/d methylprednisolone i.v. for three days, followed by hydrocortisone 100 mg i.v. until enteral feeding was available, when 40 mg prednisone was administered. Cases 1 and 9 had the longest follow-up for six years, and in case 1 all drugs were withdrawn at four years of follow-up without additional liver function tests; case 2 was followed for four years, and at the last visit, treated with 50 mg/d azathioprine; and case 9 continues to be treated with prednisone.

DISCUSSION

Although liver transplantation can improve the outcome in ALF, the overall mortality without liver transplantation is still high[13]. In this study, we presented the clinical experience of one single referral hospital in subjects with AIH and ALF.

These findings are particularly interesting, because we observed a higher prevalence of AIH (about 20%) compared to other reports[7,14]. Information about AIH in ALF is scarce. Recently, Miyake *et al*[7] in a Japanese study reported the clinical characteristics in 11 subjects with fulminant AIH. Contrary to our study, their response rate to corticosteroids without transplantation was low (two of seven subjects), and an unexpectedly high survival rate was observed in those with supportive treatment.

In recent years, a better response in subjects with ALF has been observed. At least two studies showed higher survival rates (a three-week survival rate without

Table 2 Clinical features at d 1 of survivors and non-survivors

Variables	In-hospital survival (n = 6) median (range)	In-hospital non-survival (n = 5) median (range)	P
Age (yr)	52 (17-67)	29.5 (18–66)	0.43
HE grade (Ⅲ or Ⅳ), n (%)	1 (16)	4 (80)	0.10
Body mass index (m/kg^2)	26.9 (22–31.1)	25.1 (20–35)	0.39
APACHE score	8 (6–13)	20 (11–23)	0.08
Total bilirubin (mg/dL)	15.3 (7.6–21.9)	22.3 (8.4–26.8)	0.35
Alanine aminotransferase (IU/L)	358 (217–683)	127 (23–541)	0.35
INR	1.7 (1.5–8.12)	2.05 (1.5–3.6)	0.35
Creatinine (mg/dL)	0.9 (0.6–2.3)	1.25 (0.9–6.6)	0.20
Serum sodium (mEq/L)	134 (131–138)	134 (125–149)	0.35
SIRS (yes), n (%)	0	1 (20)	0.45
Sepsis (yes), n (%)	1 (16)	2 (33.2)	0.42
Severe sepsis (yes), n (%)	1 (16)	3 (60)	0.19
Septic shock (yes), n (%)	0	0	NS
Multiple organic failure (yes), n (%)	0	5 (100)	0.02
Corticosteroids (yes), n (%)	5 (83)	1 (20)	0.06

HE: hepatic encephalopathy grade; APACHE: acute physiology and chronic health evaluation; SIRS: systemic inflammatory response syndrome.

liver transplantation is 80% in the Miyake *et al*[7] study and 54% at a four-week survival in our study) compared with one initial report (a three-week survival rate without liver transplantation was approximately 20%). In fact, the mortality rate observed in all cases of ALF in our institution was 49%[15]. However, we observed a lower mortality rate at one month (only with statistic tendency) in subjects who received corticosteroids (18% *vs* 80% in those without corticosteroids), but survivors showed lower values of severity at baseline. We observed a trend of better prognosis in those subjects treated with corticosteroids. This could be explained by the fact that since this therapy is oriented to pathophysiological process involved in the etiology of ALF, only supportive management was offered due to lack of transplantation.

Unfortunately, due to the low number of subjects in reported series[16,17], it is difficult to establish more categorical conclusions about therapeutic interventions, and prospectively controlled trials using corticosteroids are unethical, particularly when the majority of subjects with acute AIH present with fulminant hepatic failure[18].

In subjects with ALF due to AIH, many pathogenic events occur; for instance, an important loss of homeostasis with multiorgan failure as the main outcome, includes an uncontrolled immune response. Although liver transplantation is considered the best treatment of choice in ALF, it is not always available in all centres, and the clinical usefulness of other non-surgical options directed to pathogenic mechanisms has not been fully explored.

Corticosteroids are considered the main treatment of AIH, but their side-effects are often significant. In the clinical scenario of acute liver failure, the study of Harry *et al*[19] demonstrated that the use of high doses of corticosteroids in subjects with hypotensive acute liver failure (none with AIH) could help reduce noradrenaline requirements after 48 h with steroid therapy. However, no differences were observed in survival, but length of intensive care unit stay was prolonged in the steroid-treated group, increasing the length of waiting time for a suitable organ donor. Unfortunately, the incidence of infection due to resistant organisms such as Candida albicans was high in this group.

In conclusion, we observed in a cohort of subjects from a clinical setting where transplantation is not feasible, a relatively high clinical heterogeneity of ALF due to AIH that showed a high survival rate. These observations may be influenced by both severity of the disease and the use of high doses of corticosteroids.

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RAPID COMMUNICATION

Prevalence and risk factors of asymptomatic hepatitis C virus infection in Egyptian children

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Abstract

AIM: To identify the prevalence, risk factors and manifestations of asymptomatic hepatitis C virus (HCV) infection in Egyptian children.

METHODS: Children at the age of 1-9 years were screened for HCV antibodies and alanine aminotransferase (ALT) levels. Every child with elevated ALT and/or detectable HCV antibodies was tested for HCV RNA by RT-PCR and compared with two negative controls for risk factors and signs and symptoms of liver disease.

RESULTS: We screened 1042 children, six of them had elevated ALT, negative HCV antibody and positive RNA, likely representing acute hepatitis C cases. Fifteen children were HCV seropositive, 5 of them were HCV RNA positive. Asymptomatic HCV infection was present in 2.02% (positive results for either HCV antibodies or HCV-RNA or both). Symptoms such as diarrhea, abdominal pain, history of fatigue and school absence because of illness and risk factors such as dental care were significantly more common among HCV positive cases than among controls. None of the HCV positive children was diagnosed as having signs of advanced liver disease upon clinical or ultrasonographic examination.

CONCLUSION: Asymptomatic HCV infection is detectable in 2.02% Egyptian children.

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Key words: Hepatitis C virus; Child; Egypt; Prevalence; Transfusion; Risk factor

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INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection is relatively low in children, with an anti-HCV prevalence rate of 0.2%-0.4% in the Western world[1]. Blood transfusion is the principal route of transmission of HCV in children[2]. Recent evidence suggests that new cases are due to perinatal (vertical) transmission[3].

Egypt has the highest prevalence of adult HCV infection in the world, averaging 15%-25% in rural communities[4-6]. The main (90%) HCV genotype is type 4[7-9]. The magnitude of HCV infection in children is not well understood, apart from subgroups in two-community based cross sectional studies[10,11]. In the first study from upper Egypt[10], 84 (3%) out of 2967 subjects under 19 years of age screened for HCV antibody using ELISA technique, were found to be positive. The overall prevalence in the 6033 screened subjects was 8.7%. In the second study from Lower Egypt[11], 178 (9%) out of the 2010 screened subjects under 19 years of age were found to be positive. The overall seroprevalence in the 3999 screened subjects was 24.3%. Analysis of risk factors was significant for male circumcision by informal health care provider[11]. Although blood transfusion, circumcision, vertical transmission, and living in a house with an infected family member are the established risk factors for HCV transmission, approximately 70% of acquired infections are due to unidentified risk factors[11]. A recent study of intrafamilial transmission showed that the incidence of offsprings to acquire HCV from anti-HCV–positive parents is slightly higher (incidence rate of 8.7/1000 per year) in the positive mother than in the anti-HCV positive father (6.6/1000)[12].

According to a study by El-Raziky *et al*[13] HCV infection is not always benign in the Egyptian children as ALT levels are elevated in half of the subjects and

histological abnormalities are detectable in three quarters of HCV-RNA positive cases. It is not clear whether identification of HCV early in childhood can alter the course of the disease due to the limitations of current antiviral therapies. Therefore long-term morbidity of the disease is a concern. The aim of the present study was to identify the prevalence and possible risk factors of asymptomatic HCV infection in Egyptian children and to detect the underlying signs and symptoms of liver disease associated with asymptomatic HCV in Egyptian children.

MATERIALS AND METHODS

Subjects

A hospital-based study of patients was carried out from January to August 2004 at the General Outpatient Clinics of Cairo University Pediatric Hospital (CUPH) which provides medical care to attending children (without reference or residence area restriction).

Inclusion criteria were attendants to the CUPH Outpatient Clinics, children at the age of 1-9 years*, children not known to have liver disease, parent's informed consent to participate in the study. Exclusion criteria were attendants to the Hepatology Clinic and the Haematology Clinic, and critically ill patients. Parents of the participants or legal guardians signed an informed consent approved by the IRB, if they fulfilled the criteria of screening. Oral consent, in the presence of a witness, was provided for illiterate participants. *We excluded participants below the age of one year due to the expected presence of passively acquired maternal anti-HCV in children of this age group. Those older than 9 years were also not included because inclusion of older children would include adolescents who might have different risk factors and enough time to follow them up when they were still in the paediatric age group.

Laboratory, physical and social assessment

Alanine aminotransferase (ALT), antibodies to hepatitis C virus (anti-HCV) (Axsym system HCV version 3.0 Abbott Diagnostics Division: Germany) were detected in all children. Any case with elevated ALT and/or positive HCV antibody was subjected to HCV RNA by an in-house reverse transcription-polymerase chain reaction (RT-PCR) on whole sera amplification of DNA by PCR[14] and asked to fill in a questionnaire about risk factors for transmission and a questionnaire about symptoms, while the mothers were blinded to results.

A full general and local abdominal clinical examination was performed on patients with positive results and 2 randomly selected matched negative controls. Abdominal ultrasonography was done with ultrasound Toshiba 250, 3.5/5.0 MHz convex probes. The socioeconomic status was calculated according to Egyptian score including education of father and mother, crowding index and sanitation of the house[15].

Sample size and analysis

The sample size was calculated and estimated to be approximately one thousand children according to the reported HCV seroprevalence ranging from 0.5%-3% in Egyptian children[10].

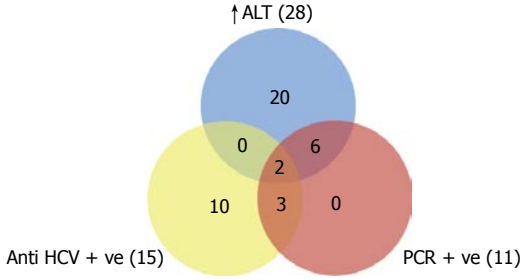

Figure 1 Abnormal laboratory results from 1042 screened children.

Univariate statistical results (mean ± SD) were used to estimate the prevalence of risk factors and liver function morbidity in the HCV positive cases and HCV negative controls. Chi-square test or Fischer's exact test (when appropriate) was used to assess the statistical significance of associations between individual risk factors and liver function morbidity with HCV infection, and Odds ratios were used to measure the strength of associations (OR > 3). $P < 0.05$ was considered statistically significant. SPSS program 12.0 was used for analysis.

RESULTS

A total of 1042 children were enrolled in this study. The results obtained from these cases were analyzed. The mean age of screened children was 3.88 ± 2.13 years. Male to female ratio was 1.1:1. Forty-one out of the 1042 children had elevated ALT and/or positive HCV antibody. Of these 41 children, 26 had elevated ALT, 13 positive HCV antibody alone and 2 both. HCV-RNA was amplified by RT-PCR revealing positive results in 11 out of the 41 children. The abnormal laboratory results of the 1042 screened children are shown in Figure 1. Among the 41 children, 6 had elevated ALT, negative HCV antibody and positive RNA, likely representing acute hepatitis C cases (0.58%). Their mean age was 2.8 years, ranging 2-4 years. The mean ALT was 231.5 IU + 262.61 IU ranging 43-633 IU. These laboratory results were tested once. Four out of six children had less than 5-fold elevation of ALT and the remaining two had more than 10 -fold elevation of ALT.

Fifteen children were HCV seropositive. Of them, 5 were HCV RNA positive, 2 had elevated ALT. Among the 28 children (2.7%) with elevated ALT, 20 were negative for both markers of HCV, 9 out of these 20 children were diagnosed as acute hepatitis A in 7 and hepatitis B in 1, and 1 developed HCV antibodies 4 mo later suggesting occult acute hepatitis C. Evidence of HCV infection was present in 21 cases (2.02%) positive for HCV antibodies and/or HCV-RNA.

Clinical data and possible risk factors for HCV acquisition were compared between the 18 children with evidence of HCV infection except for 3 who were lost in follow-up and 112 age, sex, residence and socioeconomically matched negative controls. Relevant and statistically significant clinical data are shown in Table 1. Symptoms such as diarrhea and abdominal pain,

Table 1 Comparison of clinical data obtained from HCV cases and control children

	HCV cases n = 18 (%)	Controls n = 112 (%)	P
Age at screening in years (mean ± SD)	4.00 ± 2.1	3.89 ± 2.0	NS
Male: female ratio	0.6:1	1.4:1	NS
BMI	16.61	16.76	NS
Residence			
Urban	16 (88.9)	104 (92.9)	NS
Rural	2 (11.1)	6 (5.4)	
Semi rural	0 (0)	2 (1.8)	
Socioeconomic score			
High class 20-26	2 (11.1)	8 (7.1)	NS
Middle class 15-20	4 (22.2)	36 (32.1)	
Low class 10-15	9 (50)	41 (36.6)	
Very low class less 10	3 (16.7)	27 (24.1)	
ALT folds (mean ± SD)	2.3 ± 4.1	0.4 ± 0.1	0.045
Present history			
Dark urine	4 (22.2)	14 (12.5)	NS
Diarrhea	5 (27.8)	12 (10.7)	0.046
Yellowish sclera	1 (5.6)	4 (3.6)	NS
Abdominal pain	3 (16.7)	2 (1.8)	0.002
Past history			
Jaundice	3 (16.7)	20 (17.9)	NS
School absence because of illness	3/7 (42.9)	1/48 (2.1)	0.01
Poor general health	6 (33.3)	20 (17.9)	NS

NS: P value is nonsignificant.

Table 2 Comparison of clinical data obtained from acute and chronic cases of HCV infection

	Likely acute HCV n = 4 (%)	Chronic HCV n = 14 (%)	P
Present history			
Dark urine	2 (50)	2 (14.3)	0.20
Diarrhea	2 (50)	3 (21.4)	0.53
Yellowish sclera	1 (25)	0 (0)	0.22
Abdominal pain	0 (0)	3 (21.4)	1.0
Past history			
Jaundice	0 (0)	3 (21.4)	1.0
School absence because of illness	1 (25)	2 (14.3)	0.43
Poor general health	1 (25)	5 (35.7)	1.0

history of easy fatigue and school absence because of illness were significantly more common among HCV positive cases than among controls. These relations were not significant when the clinical data were compaired between those who were considered as acute cases and others with chronic HCV (Table 2).

Risk factors are listed in Table 3. HCV cases were found to have 3.5 times of exposure to dental care than their controls. In addition, surgical interventions such as operations, stitches, catheterization, abscess drainage and cautery were not statistically significant.

Examination of the 18 cases with evidence of HCV infection revealed jaundice in 3 cases. None of the HCV positive children had splenomegaly. Ultrasonographic examination revealed evidence of periportal thickening in only one case (5.6%).

DISCUSSION

HCV can cause asymptomatic infection[16]. Children with chronic HCV infection are usually free of symptoms, frequently with normal or borderline alanine aminotransferase (ALT) values[17]. The worldwide seroprevalence of HCV ranges 0.2%-2%[18]. Egypt has the highest prevalence of adult HCV infection in the world. The natural history of HCV infection in children is ill-defined. In our study evidence of HCV infection was present in 2.02% cases, which is lower than that reported in other Egyptian studies in rural communities (3% and 9% of subjects under 19 years of age were found to be positive in two-community based studies respectively)[10,11]. In India, a hospital-based serological survey showed that the prevalence of HCV in children at the age of 0-9 year is 3.6%[16]. Anti-HCV antibodies were detected in 4.09% hospitalised Pakistani children screened for markers of HCV[19]. However, a more recent Pakistani screening showed that the seroprevalence of anti HCV antibodies in asymptomatic children (3-15 years old) is 0.58%[20]. The prevalence of hepatitis C in children of developed countries is lower (0.05%-0.4% in USA)[21]. The seroprevalence in the third national health and nutrition examination survey (NHANES Ⅲ) was 0.2% in children aged between 6 and 11 years[22].

Much higher prevalence rates are reported in adult Egyptians with 12%-24% anti-HCV seropositivity in the general population[4,5,23]. The higher rate in adults is explained by the consistent increase of seropositivity with age.

In the present primary health care-based case-control study, no definite significant risk factor for acquisition of HCV could be detected both in cases and in controls. Lu *et al*[24] reported that no statistically significant difference in risk factors could be found among HCV antibody positive and negative children.

Egyptian studies documented that analysis of risk factors is significant for male circumcision by informal health care provider[11]. Blood transfusion, circumcision, vertical transmission, and living in a house with infected family member are not reported in more than 20% individuals, but about 70% have infection without identified risk factor in subjects under 19 years of age[11]. Hyder *et al*[20] showed that multiple exposures to unsterilized needle pricks, razors or dental procedures are responsible for a higher carrier rate in low socioeconomic groups.

Our results showed that symptoms such as diarrhea, abdominal pain, history of fatigue and school absence because of illness were significantly more common among HCV positive cases than among controls. Jaundice was present in 3 cases (16.7%) and soft rounded 3 cm liver enlargement below the costal margin was found in two of them. Jara *et al*[25] reported that symptoms such as anorexia, asthenia or abdominal pain, were present in only 14% of their patients, while none of them had jaundice or extrahepatic manifestations. Bortolotti *et al*[26], who conducted a clinical study on 77 Italian and Spanish children, showed that hepatitis C in children is usually asymptomatic.

None of our HCV positive children had splenomegaly.

Table 3 Comparison of risk factors for HCV positive and negative children

Risk factor	HCV cases *n* = 18 (%)	Controls *n* = 112 (%)	*P*	Odds ratio	95% CI	
					Lower	Upper
Circumcision by non medical personnel for males and females	3/9 (33.3)	39/94 (41.5)	0.63 (NS)	0.71	0.17	2.99
Injections by non medical personnel	9/17 (52.9)	57/111 (51.4)	0.90 (NS)	1.1	0.38	2.96
History of HCV in family members	3 (16.7)	21 (19.4)	0.78 (NS)	1.2	0.32	4.55
Ear piercing for females	11/14 (78.6)	43/53 (81.1)	0.83 (NS)	1.2	0.28	5.00
Attendance to medical care	11 (61.1)	99 (88.4)	0.01 (S)	4.9	1.60	14.71
Dental care	5 (27.8)	13 (11.6)	0.07 (NS)	3.5	1.13	10.82
Blood transfusion	3 (16.7)	7 (6.3)	0.12 (NS)	3.0	0.70	12.87
Delivery at home	7 (38.9)	24 (22.0)	0.13 (NS)	2.5	0.85	7.21
Post natal care	3 (16.7)	5 (4.6)	0.09 (NS)	0.24	0.05	1.1
Non medically assisted delivery	5 (27.8)	15 (13.4)	0.16 (NS)	0.42	0.13	1.3
Traditional hair cutting	9 (50)	81 (72.3)	0.14 (NS)	ND	ND	ND
Exposed to used syringes	2 (11.1)	13 (11.6)	0.95 (NS)	0.95	0.20	4.62
Exposed to used razors	4 (22.2)	12 (10.7)	0.17 (NS)	2.381	0.67	8.41
Exposed to blood	1 (5.6)	4 (3.6)	0.43 (NS)	ND	ND	ND
Associated medical diseases	3 (16.7)	17 (15.2)	0.66 (NS)	ND	ND	ND

No urinary catheter or tattoo in cases or controls. ND: not detectable.

Ultrasonography revealed evidence of periportal thickening in only one case (5.6%). Lu *et al*[24] reported that none of their HCV children had sonographic parenchymal changes in the liver when studying an endemic area in Taiwan.

Comparing the clinical data of those considered as acute cases and others with chronic HCV (Table 2) as well as their past history; symptoms were present in acute cases but not statistically significant. Similarly according to the AAP[27], acute infections are usually clinically silent and symptoms if present, are indistinguishable from symptoms found in patients with hepatitis A or B. Infected children may complain of anorexia, malaise, fatigue, and abdominal pain during the acute phase[28]. The acute stage of hepatitis C remains clinically silent in most infected people, and only 15%-20% of individuals develop symptoms[29]. Symptoms such as low-grade fever, fatigue, appetite loss, abdominal pain, nausea, and vomiting usually occur during the sixth or seventh week after infection and resolve within a few weeks[29,30].

In our study, fifteen children were HCV seropositive, 5 of them (33.3%) were HCV RNA positive. Vogt and colleagues[31] found that seventeen years after 67 children were infected with hepatitis C virus from blood transfusions during cardiac surgery, only 37 (55%) had detectable HCV RNA. Sehgal *et al*[28] showed that most children infected with HCV have no long-term complications. Persistent infection occurs in 50%-60% of infected children even in the absence of biochemical evidence of liver disease[27]. Of these, less than 10% develop chronic hepatitis, and less than 5% develop cirrhosis[28]. Hoshiyama *et al*[32] reported that 60%-80% of children with HCV infection develop chronic hepatitis C. The spontaneous clearance of HCV RNA in those vertically infected with HCV is estimated to be around 20%[8,18,33].

The relatively low prevalence of HCV-related morbidity in Egyptian paediatric cases is consistent with the reported result in Egyptian adults[34].

In conclusion, asymptomatic HCV is detectable in 2.02% of Egyptian children. HCV RNA should be tested in children with elevated ALT even in absence of detectable HCV antibodies.

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RAPID COMMUNICATION

Cyclic vomiting syndrome in children: Experience with 181 cases from southern Iran

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Abstract

AIM: To evaluate the clinical presentation, response to prophylactic therapy and outcome of children with cyclic vomiting syndrome (CVS) in Shiraz, Iran.

METHODS: During a period of 11 years (March 1994 to March 2005), 181 consecutive children with a final diagnosis of CVS were evaluated, treated and followed in our center. Patients were randomized to receive either amitriptyline or propranolol as prophylactic treatments.

RESULTS: There were 88 boys and 93 girls with mean age of onset of symptoms of 4.9 ± 3.3 years (range, neonatal period to 14 years), the mean age at final diagnosis was 6.9 years (range, 1.5 to 14), and the mean duration between the onset of the first attack and the final diagnosis of CVS was 2 ± 1.81 years (range, 1/6 to 8). The mean duration of each attack was 4.26 days (range, from few hours to 10 d) and the mean interval between the attacks was 1.8 mo (range, 1 wk to 12 mo). The time of onset of the attacks was midnight to early morning in about 70% of cases. Amitriptyline was effective in 46 out of 81 (56%) patients ($P < 0.001$). Propranolol appeared to have a superior action and was effective in 74 out of 83 (92%) patients ($P < 0.0001$).

CONCLUSION: There is a significant lag time between the onset of clinical symptoms and the final diagnosis of CVS in our area. In patients with typical clinical presentations of CVS, who are examined by an experienced physician, invasive workup is not necessary. Propranolol appears more effective than amitriptyline for prophylactic use in children with CVS.

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Key words: Cyclic vomiting syndrome; Children; Propranolol; Amitriptyline

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INTRODUCTION

Cystic vomiting syndrome (CVS) is an idiopathic disorder, which presents with stereotypic episodes of severe, intractable non-bilious vomiting[1]. The episodes have a rapid onset, persist for several hours to days, and are separated by symptom-free intervals[1,2]. Although many consider CVS to be a migraine equivalent, little is known about its etiology or pathogenesis[2-7]. After gastroesophageal reflux (GER), CVS is the second most common cause of recurrent vomiting in children[8]. The disorder usually begins in childhood, mainly in preschool age children[9-13].

The vomiting is self-limited, and resolves spontaneously if left untreated, but severe, prolonged attacks can lead to dehydration and electrolyte disturbances and related complications. CVS has no specific diagnostic test and no specific treatment, or generally accepted management[1,2]. The diagnosis of CVS is based on a typical clinical presentation, and by exclusion of other possible causes with a similar presentation[1,2,5,14-16]. Consensus diagnostic criteria proposed in 1994 suggested four essential clinical features of cyctic vomiting syndrome[17-19]: (1) three or more recurrent episodes of vomiting; (2) varying intervals of completely normal health between episodes; (3) stereotypical episodes that are repetitive with regard to symptom onset and duration; (4) absence of laboratory or radiographic findings of an alternative diagnosis (absence of an organic cause).

Supportive criteria for the diagnosis of CVS are: self-limited nature of the attacks; accompanying symptoms of nausea, abdominal pain, headache, motion sickness, photophobia and lethargy; and with associated signs of fever, pallor, dehydration, excessive salivation and social withdrawal. In children, nausea and possibly lethargy are considered to be key diagnostic features[17]. The most controversial and challenging aspects of this disorder are the types of effective treatment, and the duration of medical therapy. No specific therapy has been proven to be effective for CVS in controlled trials. However, despite these controversies, medical therapy remains the only possible and promising tool to prevent, suppress or shorten the attacks[2,7]. Several empiric treatments have been shown to be effective in case series[18]. Treatment can be considered as abortive, supportive and prophylactic.

Agents that have been used empirically (with variable success) in children include: sumatriptan, erythromycin, carnitine, propranolol, cyproheptadine and tricyclic antidepressant. The aim of this prospective study was to evaluate the clinical presentation, response to prophylactic medical therapy, and outcome in children with CVS in southern Iran.

MATERIALS AND METHODS

Subjects

This prospective study included 181 consecutive children with a final diagnosis of CVS who were evaluated, managed and followed in our center between 1994 and 2005. All patients were evaluated by pediatric gastroenterologists and a neurologist (authors) and the diagnosis of CVS was based on typical clinical presentation (three or more episodes of intractable, self limited, non-bilious vomiting, separated by symptom-free intervals) and by exclusion of other possible causes with similar presentation (no identifiable organic cau
se). The initial assessment included a complete medical and family history, physical examination including evaluation of the patients' intellectual, motor, emotional and social development, and current level of functioning.

Methods

In addition to routine workup which was carried out at the time of the initial presentation, an upper gastrointestinal series (UGI) and small bowel follow through (SBFT) were performed in all patients. Sinus series and brain computed tomography (CT) were done in cases with suspicion of sinus or CNS problems. Endoscopy was carried out only in those patients with abnormal findings on UGI studies, and EEG in those with a history of seizure disorder. Patients were selected randomly for prophylactic treatment with either propranolol (1 mg/kg per day) or amitriptyline (1 mg/kg per day).

In non-responsive patients, the doses of drugs were increased to the maximum recommended level. If the symptoms did not improve, the medication was discontinued and was replaced with another drug. Patients were evaluated regularly in the pediatric GI clinic. The follow up period ranged from 6 mo to 12 years.

Table 1 Concomitant clinical symptoms in 181 children with CVS

Symptoms	*n* (%)
Abdominal pain	40 (22)
Abdominal pain + fever	33 (18)
Abdominal pain + fever + headache	45 (25)
Abdominal pain + headache	33 (18)
Abdominal pain + diarrhea	21 (12)
Fever	9 (5)

RESULTS

There were 88 boys and 93 girls. The mean age of onset of symptoms was 5 ± 3.3 years (range, neonatal period to 14), the mean age of diagnosis was 6.9 years (range, 1.5 to 14) and the mean time span between the onset of the first attack and diagnosis of CVS was 2 ± 1.8 years (range, 1/6 to 8). The mean duration of attacks was 4.25 d (range, few hours to 10 d) and the mean interval between attacks was 1.8 mo (range, 0.25 to 12). Eighty seven percent of patients had attacks of fairly uniform duration. Vomiting began at characteristic times specific for the individuals in 85% of cases, 70% patients were awakened during the night and/or had symptoms on arising in the morning and 15% patients had episodes beginning at other characteristic times of the day. Ninety percent of patients had attacks of fairly uniform duration and course. Most patients had concomitant symptoms in addition to vomiting (Table 1). Fever was usually of low grade and remitted spontaneously.

Abdominal pain was usually mild to moderate in intensity and was located in the periumbilical and/or epigastric area. About 40% of patients had history of identifiable specific conditions or events (i.e. parents' conflict, school examination, excitement, common cold) that seemed to trigger their episodes.

Forty one (25%) patients had a history of motion sickness and 8 (5%) had a history of seizures, which were controlled by anticonvulsant drugs. There was no temporal relation between the seizures and cyclic vomiting. About 55% of older children had history of recurrent headaches; in 20% the symptoms were typical of migraine. In 24% of cases, family history was positive for migraine. No patient had a positive family history of CVS. Barium studies and sinus series were normal in 97% and 95% patients respectively. Of the total of 181 patients, 164 (81 on propranolol and 83 on amitriptyline) had a regular follow-up. Of the 83 patients who received amitriptyline, 46 (56%) showed a good response, with decrease in the frequency and severity of attacks. A significant number of these cases had side effects of amitriptyline including: irritability, agitation, insomnia or lethargy.

Seventy four out of 81 (92%) patients who received propranolol had a good response ($P < 0.0001$), without any significant side effects. Patients who were non responsive to amitriptyline, were treated with propranolol, and most of them had a satisfactory response. A few patients who were non-responsive to both drugs, had repeated, severe attacks with frequent hospital admissions. This group was non-responsive to other medications as well including phenobarbital and cyproheptadine. There

was no explanation for the lack of response in this group.

The medication was discontinued in 52 (31%) patients. These subjects were > 10 years of age and were symptom free on prophylactic medication for at least 5 years. They were followed for one year or more, and only two cases had recurrence of symptoms.

DISCUSSION

The present report represents one of the largest studies of CVS in the literature. Although our findings do not provide further information on the etiology or pathogenesis of this disorders, it does supplement previous observations concerning the stereotypic nature of attacks, their precipitants, duration of illness, the time span between attacks and the time span between symptom onset and the final diagnosis[1,2]. The overall gender ratio in our series was equal. A similar gender distribution has been reported by Abu-Arafeh[9], although many have suggested a higher frequency in girls[10-12,19,20].

The mean age of onset of symptoms in our series was 5 ± 3.3 years, which is the same as that reported by Abu-Arafeh[9], but younger than that reported by Fleisher (6.75 years)[2] and older than that reported by Hoyt and Stickler (3.8 years)[11]. In present series, the first attack began during the neonatal period in 10 (5.5%) cases and at 14 years of age in 5 cases. In the series reported by Fleisher[2], the age range was 6 mo to 18 years. None of the patients reported in the previous series had disease onset in the neonatal period. Although CVS has been characterized as beginning in preschool and early school aged children, our data suggest that the onset in neonatal and pre-adolescent children is also possible. Adult onset cases have also been reported[21]. Therefore one cannot exclude the diagnosis of CVS on the basis of the age. In 85% of our cases the symptoms of CVS occurred from midnight to early morning. Further, 90% of the attacks had a uniform length, which is in agreement with previous studies[17].

Forty percent of our patients had a history of triggering events, which is approximately one-half of the incidence reported by Fleisher *et al*[2]. Twenty percent of our cases had history of motion sickness in early childhood, which is approximately 50% lower than that reported by Fleisher *et al*[2]. Fifty eight percent of our patients had history of recurrent headaches and of these 20% were migraine-like, which compares well with previous findings[2,4].

None of our patients had a family history of CVS, indicating that CVS is unlikely to be hereditary. Upper gastrointestinal series (UGI) and small bowel follow through (SBFT) were normal in 97% of our cases, suggesting that a thorough history and physical examination by an experienced physician can detect most of the typical cases of CVS without the need for invasive investigations. Therefore, it is recommended that routine barium studies are not necessary in typical cases of CVS as suggested by some authorities[16] and they should only be done in atypical cases, or in those patients unresponsive to medical treatment.

A significant number of our patients with a typical presentation suffered from multiple attacks spread over several months and even years with repeated hospital admissions before they were referred to our center. They were usually misdiagnosed and received treatment for other conditions such as food poisoning, metabolic disorders or acid peptic disease. The significant lag time between the onset of symptoms and final diagnosis of CVS in our series indicates that this disorder is usually not considered in the differential diagnosis of children with recurrent vomiting in this area. About 92% of our cases were responsive to propranolol without significant side-effects, while 56% were responsive to amitriptyline with considerable side-effects. The present data indicates that propranolol is much more effective and safe in comparison with amitriptyline, therefore propranolol is recommended as the first drug of choice for prophylaxis in children with CVS. It is concluded that: (1) the age of onset, sex distribution and clinical presentations of patients in southern Iran with CVS are similar to those in other regions of the world; (2) there may be a significant lag time between the onset of clinical symptoms and final diagnosis of CVS in our area; (3) in patients with a typical clinical presentation of CVS, invasive investigations are not necessary; and (4) propranolol is much more effective and has fewer side effects than amitriptyline for prophylactic therapy of children with CVS.

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RAPID COMMUNICATION

Knowledge, attitude and practice related to liver fluke infection in northeast Thailand

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Abstract

AIM: To investigate the knowledge, attitude and practice (KAP) in prevention and control of liver fluke infection in northeast Thailand.

METHODS: A descriptive KAP survey pertaining to liver fluke infection was carried out in June 2005 to October 2006 using structured questionnaires. Data were collected by questionnaires consisting of general parameters, knowledge, attitude, practice, and a history of participation in the prevention and control of liver fluke infection.

RESULTS: A total of 1077 persons who were interviewed and completed the questionnaires were enrolled in the study. The majority were females (69.5%) and many of them were 15-20 years of age (37.26%). The questionnaires revealed that information resources on liver fluke infection included local public health volunteers (31.37%), public health officers (18.72%), televisions (14.38%), local heads of sub-districts (12.31%), doctors and nurses (9.18%), newspaper (5.72), internets (5.37%), and others (12.95%). Fifty-five point eleven percent of the population had a good level of liver fluke knowledge concerning the mode of disease transmission and 79.72% of the population had a good level of prevention and control knowledge with regards to defecation and consumption. The attitude and practice in liver fluke prevention and control were also at a good level with a positive awareness, participation, and satisfaction of 72.1% and 60.83% of the persons studied. However, good health behavior was found in 39.26% and 41.42% of the persons studied who had unhygienic defecation and ate raw cyprinoid's fish. The result also showed that 41.25% of the persons studied previously joined prevention and control campaigns.

CONCLUSION: The persons studied have a high level of liver fluke knowledge and positive attitude. However, improvement is required regarding personal hygiene specifically with hygienic defecation and consumption of undercooked fish.

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Key words: Knowledge; Attitude; Practice; Liver fluke; Northeast Thailand

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INTRODUCTION

Liver fluke infection caused by *O. viverrini* remains a major public health problem in many parts of Southeast Asia including Thailand, Lao PDR, Vietnam and Cambodia[1]. Infection is acquired by ingestion of undercooked cyprinoid's fish harboring infective metacercariae (Figure 1)[2]. Liver fluke is endemic among human populations in northeast and north Thailand, where the most common raw fish "*Koi pla*" is frequently consumed[3-6]. In central Thailand where no indigenous human infection is ever encountered, cats and dogs are found to be naturally infected with human liver fluke (Figure 2). In the south, no infection is encountered. It has been extensively studied in Thailand where about 6 million people are infected with liver fluke accounting for 9.4% prevalence within the population in 2001[2,5-8]. The infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis[9]. Moreover, both experimental and epidemiological evidence strongly implicates that liver fluke infection is the etiology of cholangiocarcinoma, bile duct cancer development[10,11].

A community-level health education campaign has been conducted since late 1950s. Liver fluke control has been started as a small scale helminthiasis control program in some high risk areas. Presently, the program is operated in some provinces of the central and all provinces of the

northeast and north of Thailand. The main strategies for liver fluke control comprise three interrelated approaches, namely stool examination and treatment of positive cases with praziquantel for eliminating human host reservoir, health education for a promotion of cooked fish consumption to prevent infection, and improvement of hygienic defecation for the interruption of disease transmission. Between 1984 and 1987, the positive rate of liver fluke infection was 63.6%. In 1988, the positive rate went down to 35.6%. When the region wide control program was started in 1989, the annual positive rate decreased to 9.4% in 2001[8]. The overall prevalence indicates a considerable decrease of liver fluke in the region. However, the positive rates for several provinces remain high. Published studies provide biology, disease, diagnosis and treatment but the survey related to knowledge, attitude and practice (KAP) on liver fluke infection is weak and inconclusive. KAP on prevention and control of liver fluke infection are essential for decreasing the disease and planning of government. Also, no recent and update study has shown the data on KAP in northeast Thailand by questionnaires obtaining the general data, knowledge, attitude, practice, and a history of participation in the prevention and control of this parasite.

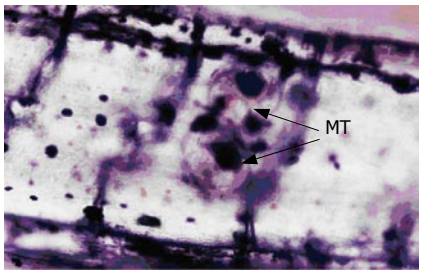

Figure 1 Metacercaria of *O. viverrini* in cavity of caudal fin ray of cyprinid's fish, *Cyclocheilichthys* spp (MT = metacercaria)[2].

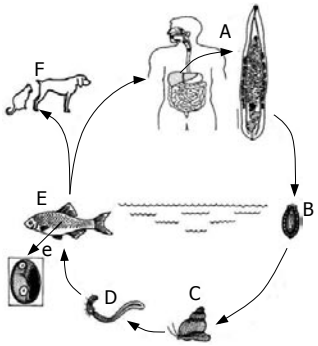

Figure 2 Life cycle of adult *O. viverrini* in bile duct (A), embryonated egg of *O. viverrini* (B), first intermediate host (Bithynia snail) (C), cercaria (D), second intermediate host (cyprinoid's fish) and metacercaria (E), reservoir host (dog and cat) (F)[2].

Figure 3 Map of Thailand showing location of Ubon Rachathani Province, northeast Thailand.

MATERIALS AND METHODS

Study area

The study was conducted in Ubon Ratchathani Province which is situated 630 km from Bangkok (central region) of Thailand. This province is located in the northeast broader line with Laos PDR (Figure 3). The geographic area has two rivers, namely Moon and Mae Kong where many people habit at the side of 2 main rivers. The infection rate of liver fluke in Ubon Rathcathani Province has been 19.5% in all age groups since 1997[12]. According to the Department of Communicable Disease Control, the prevalence of liver fluke infection is 12.8%[13].

Study design, study population and research instrument

A descriptive KAP survey pertaining to liver fluke was carried out in June 2005 to October 2006 using structured questionnaires administered by the research officers and trained research assistants. Persons enrolled in this study were from TaLad, KuMeung, NongKinPen, BungWai, NonPueng, KamKwang, and Srikai sub-District of WarinChamRap District, and HuaRue sub-District of Ubon Ratchathani District, Ubon Ratchathani Province in the northeast Thailand. Data were collected by Questionnaires. Before the study, permission and collaboration of the head of the public health centre and/or head of the sub-district were obtained. The questionnaire was tested before the study. The interviewers received an interview training of 1 d. The study was conducted at weekends to increase the possibility of meeting people at home. The staff comprised 3 interview teams each consisting of 5 interviewers from college of medicine and public health. Each team was responsible for a number of households in the sub-district. A supervising team visited the members of each interview team to check their performance and questionnaires were checked for inconsistencies. When correction was deemed necessary, the interview team visited the study participants on the same day again to gather the missing information. All inhabitants in a study site were asked to participate in the study. A total of 1077 persons were included. Written informed consent was obtained (those who could not write gave a fingerprint). Persons were asked about their KAP of liver fluke by a structured questionnaire. KAP of liver fluke were measured by asking questions related to the disease, mode of disease transmission, treatment, personal hygiene, and a history of participation in the prevention and control of Liver fluke. All questions related to KAP were multiple choices and open questions.

Statistical analysis

Data from structured interviews were analyzed using SPSS/PC version 5. Knowledge variables were summed up into a composite score and ranked as poor and good

Table 1 Characteristics of the 1077 persons older than 14 yr

Characteristic	*n*	%
Sex		
Male	328	30.5
Female	749	69.5 $P < 0.05$
Age (yr)		
15-20	401	37.26 $P < 0.05$
21-30	127	11.79
31-40	166	15.42
41-50	129	11.96
51-60	133	12.31
> 60	121	11.26
Occupation		
Farmer	470	43.67 $P < 0.05$
Trader	52	4.85
Housewife/unemployed	45	4.16
Employee	47	4.34
Student	414	38.47 $P < 0.05$
Other	49	4.51
Education		
Illiterate	47	4.36
Can read and write primary	564	52.37 $P < 0.05$
Middle and high	466	43.27

Table 2 Information resources on liver fluke infection

Information resources	*n*	%
Local head of sub-district	133	12.31
Local public health volunteer	338	31.37 $P < 0.05$
Public health officer	202	18.72
Doctor and Nurse	99	9.18
Television	155	14.38
Internet	58	5.37
Newspaper	61	5.72
Others	31	2.95
Total	1077	100

scores (lower or higher than the median score) with a range of 17 to 23. Attitudinal questions were structured in a 3 point Likert scale responses comprising agreement, disagreement and unawareness. The resulting score ranged from 1 to 3. The measure of internal consistency showed an acceptable level of reliability.

RESULTS

A total of 1077 persons who were interviewed participated in the study and completed the questionnaires. Females accounted for 69.5% and males 30.5%. The age of the majority persons was 15-20 years. Table 1 shows the characteristics of the persons older than 14 years in the eight sub-districts of the persons studied. Most of the persons were farmers (43.67%) and students (38.47%). Regarding the educational level, 95.64% completed primary school, middle and higher school education and could read and write. The questionnaires revealed that information resources on liver fluke infection included local public health volunteers (31.37%), public health officers (18.72%), televisions (14.38%), local heads of sub-districts (12.31%), doctors and nurses (9.18%), newspaper (5.72), internets (5.37%), and others (12.95%) (Table 2). In this survey, local public health volunteers were the main information resource. However, improvement was required regarding liver fluke knowledge especially with the disease transmission, prevention and control. Of the persons studied, 55.11% had a good level of liver fluke knowledge concerning the mode of disease transmission (Table 3), 79.72% had a good level of prevention and control knowledge with regards to defecation and consumption (data not show). Generally, the majority of the persons studied had a positive attitude towards liver fluke prevention and control. A good level of liver fluke prevention and control knowledge was found in 72.1% of the persons studied with a positive awareness, and satisfaction. Hygienic defecation was found in 60.83% of the persons studied, unhygienic defecation in 39.26% of the persons studied (Table 4). The risk food consumption was found in 27.38% and 14.04% of the persons studied who ate raw cyprinoid's fish 1 time/mo and ⩾ 2 times/mo, the intermediate hosts of liver fluke accounted for 27.38% and 14.04%, respectively. The questionnaire revealed that 20.12% of the persons studied were infected with liver fluke, *O. viverrini* (data not shown). The result also showed that 41.25% of the persons studied previously joined prevention and control campaigns.

Table 3 Knowledge and attitude level on liver fluke infection of the persons studied

Level	*n*	%
Knowledge		
Good	594	55.11 $P < 0.05$
Fair	306	28.42
Poor	177	16.47
Attitude level		
Good	777	72.1 $P < 0.05$
Fair	211	19.58
Poor	89	8.32

Table 4 Health behavior relating to liver fluke infection of the persons studied

Behavior	*n*	%
Health behavior		
Mostly hygienic defecation	655	60.83 $P < 0.05$
Occasionally unhygienic defecation	246	22.8
Frequently unhygienic defecation	176	16.46
Risk behavior: risk food consumption		
Low risk	631	58.58 $P < 0.05$
Moderate risk (1 time/mo)	295	27.38
High risk (> 1 time/mo)	151	14.04
Joined the prevention and control campaigns		
Yes	444	41.25
No	633	58.75

DISCUSSION

Eating raw or improperly cooked food including meat and fish is a source of infection with many parasitic zoonoses. The tradition of eating raw fish is becoming increasingly fashionable in many countries, principally with foods such as sushi, sashimi, koi-pla, kinilaw and ceviche. This has led to a dramatic rise in the incidence of fish borne zoonotic

parasitic infections in previously uninfected ethnic groups. Consumption of raw or improperly cooked or pickled freshwater fish results in about 70-100 million people infected with trematodes *Clonorchis sp. Opisthorchis spp. Echinostoma spp*. Control is through treatment with praziquantel, sanitation and education to discourage the consumption of raw fish. In Southeast Asia the prevalence is declining slowly due to effective and relatively cheap drugs and reduction in the use of night soil to fertilize fish ponds. A better sewage treatment system is required if the prevalence declines further[14]. A 10-year control program to alter raw fish eating habits in northeast Thailand has managed to reduce the regular consumption of raw fish, but occasional consumption of raw fish remains unchanged[12]. Opisthorchiasis is one of the diseases of public health importance in Thailand. It has been estimated that approximately 6 million people in Thailand harbor liver fluke[8]. Although the mortality rate is not high, the morbidity causes increased loss of man power (work days lost) and economic problems. The infection with liver fluke is common among the rural people of Northeast Thailand, which is associated with cholangiocarcinoma[10,11]. The majority of the people in Ubon Ratchathani Province have no clear-cut knowledge on transmission of liver fluke. Some (44.9%) still have incorrect knowledge about eating undercooked cyprinoid's fish. They also have incorrect knowledge about cooking raw fish with lemon and ant, considering that it can lead to infective stage of dead live fluke. These findings are consistent with the study of risk food consumption among northeasterner[13]. It might be one of the possible explanations for the failure to decrease liver fluke infection in the risk areas. Moreover, they also have incorrect knowledge about the fact that liver fluke infection is associated with cholangiocarcinoma. Those with misbelieve should be considered as the important target group that needs to promote their knowledge on transmission of liver fluke and related diseases so as to remove the potential barriers in control of liver fluke in the community. A positive attitude towards liver fluke prevention and control was found in 72.1% of the persons studied with a positive awareness of and participation in liver fluke disease prevention and control and participation in liver fluke disease prevention and control. However, 8.32% of the persons studied still had a poor attitude towards liver fluke prevention and control with awareness of and participation in liver fluke disease prevention and control, suggesting that personal hygiene specifically with the consumption of undercooked fish and liver fluke-associated with diseases should be improved. The percentage of people who have unhygienic defecation is 39.26%. Health history data obtained from questionnaires and interviews showed that 20.12% of the persons studied were infected with *O. viverrini*, which is consistent with reported data[8]. In the present study, 41.42% of the persons studied had consumption of undercooked cyprinoid's fish that may infect liver fluke. The result also showed that 58.75% of the persons did not join prevention and control campaigns, suggesting that it is necessary to increase the community awareness of the transmission of the disease in people who lack the knowledge about liver fluke. Local public health services with health education and mobile stool examination should be strengthened.

In conclusion, the population has a good level of liver fluke knowledge and positive attitude towards liver fluke prevention and control. However, improvement is required regarding personal hygiene specifically with hygienic defecation and consumption of undercooked fish.

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RAPID COMMUNICATION

Immunoscintigraphy of local recurrent rectal cancer with ^{99m}Tc-labeled anti-CEA monoclonal antibody CL58

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Abstract

AIM: To explore a specific diagnostic method for local recurrent rectal cancer.

METHODS: Immunoscintigraphy with ^{99m}Tc-labeled anti-CEA monoclonal antibody (MoAb) CL-58 was performed for patients suspected of having a postoperative local recurrent rectal cancer and the findings were compared with the results of conventional imaging and pathology.

RESULTS: A total of 36 patients with a suspected local recurrent rectal cancer underwent immunoscintigraphy with ^{99m}Tc-conjugated CL58. Local recurrence of rectal cancer was identified in 31 patients and established in 30 during operation, endoscopy and pathological examination. No local recurrence was found in 5 patients without specific accumulation of ^{99m}Tc during the follow-up. Immunoscintigraphy had a positive rate of 86.11%, a specificity of 83.33%, and a sensitivity of 100%.

CONCLUSION: Immunoscintigraphy has a highly specific and predictive value for detecting local recurrent rectal cancer, especially after abdominal perineal resection (APR).

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Key words: Immunoscintigraphy; Rectal cancer; Recurrence; Monoclonal antibody

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INTRODUCTION

Surgical intervention is the main therapeutic method for rectal cancer. However, local recurrence and metastasis are still two critical factors that determine the long-term survival and life quality of the patients. Early detection of any recurrent lesions can provide critical information for further therapy. Once recurrence or metastasis of rectal cancer is determined, early surgical intervention may help to gain long-term survival. Adjuvant chemotherapy, radiotherapy or other palliative modalities can improve the quality of life[1].

Based on the National Comprehensive Cancer Networks (NCCN) standard, serum CEA levels should be measured every three months in the first 2 postoperative years to monitor disease progression. For high-risk patients, CT scan and fibrocolonoscope should be performed every six months. However, these procedures often do not provide sufficient evidence for a final diagnosis of recurrent lesions. Although fibrocolonoscope can provide the pathological diagnosis, this method is suitable only for some patients undergoing low anterior resection (LAR). Ultrasonography or CT-guided needle biopsy is invasive with a higher risk. Moreover, the experience of different operators may lead to different outcomes. The advent of positive emission tomography (PET) can detect both local recurrent and distant metastatic lesions[2,3].

Monoclonal antibodies (MoAbs) against tumor-associated antigens have been utilized in targeted delivery of agents to tumor cells either for diagnostic imaging purposes or for therapeutic purposes[4,5]. Immunoscintigraphy is a targeted imaging technique that employs a radiolabeled MoAb specifically bound to a particular tumor antigen[6]. This approach produces a clear image of lesions expressing the specific tumor antigen, thus representing a highly specific method for detecting tumor recurrence.

This study was to assess whether immunoscintigraphy with ^{99m}Tc-labeled anti-CEA MoAb CL58 can provide useful information on the diagnosis and treatment of recurrent rectal cancer.

MATERIALS AND METHODS

Patients

The study group consisted of 36 consecutive patients (20 males, 16 females, mean age 58.8 + 3.30 years) with a suspected local recurrent rectal cancer treated at Peking University School of Oncology from September 2000 to December 2004. Patients were excluded from this study if they had bowel obstruction or extra-abdominal disease. Written informed consent was obtained from each patient. All the patients underwent total mesorectal excision (TME)

in accordance with the R0 criteria during the past 7-26 mo, of them 24 underwent low anterior resection (LAR) and 12 abdominal perineal resection (APR). Standard pathologic analysis of rectal specimens was performed. The rectal tumor was staged according to the American Joint Committee on Cancer (AJCC) Staging Manual, 6th edition. Postoperative staging of tumor, node and metastasis (TNM) showed that 2 cases were in stage Ⅰ, 5 in stage Ⅱ and 29 in stage Ⅲ. The distance from the anal margin in these patients ranged 4-15 cm. During the Follow-up (average 34 mo), 4 patients underwent a second operation, 28 adjuvant chemotherapy, and 14 pelvic radiotherapy. At the end of this study, 25 patients survived and 11 died. The average postoperative tumor-free survival time was 32 mo.

Clinical detection of local recurrence

Suspected local recurrence was defined as at least one lesion detected by radiology (CT, ultrasonography, endorectal ultrasound or PET) and/or fibrocolonoscopy. Patients after LAR were biopsied *via* fibrocolonoscopy. Others with suspected perineal recurrence after APR were biopsied under ultrasonography guidance if the tumor was reachable. For those cases post-APR with recurrent tumors invaded perineal skin, local cell smears or biopsies were used for pathological diagnosis. Of the 36 patients, 31 were positive and 5 were negative for recurrent tumors.

Anti-CEA antibody

CL58 is a murine anti-CEA monoclonal immunoglobulin (Ig)-G1. Hybridoma cell line was produced by cell fusion of spleen cells from human CEA-immunized Balb/c mice with SP2/0 myeloma cells. MoAb CL-58 hybridoma was grown in mouse ascites, CL-58 MoAb was purified on protein A affinity column (MAPS-100, Bio-Rad) and high performance hydroxylapatite (HPHT) column (Bio-Rad). Purity was more than 95% as confirmed by SDS-PAGE. The affinity constant of CL58 was 7.4×10^9 mol/L) as measured by competition ELISA. CL58 bound to colorectal cell line (CL-187, HT-29 and B-80) with a high affinity. Normal cells such as mixed lymphocytes, red blood cells, fibroblasts and bone marrow cells were proved negative for rectal cancer by ELISA. The specific CEA in colorectal and normal tissues recognized by CL-58 was confirmed by immunohistochemistry.

Radio labeling of CL58 McAb

^{99m}Tc-labeled CL58 was prepared by Schwartz method. Briefly, CL58 was reduced by 2-mercapthonal and purified on PD-10 column (Pharmacia, Sweden). One mg of the reduced CL58 was mixed with 1 mg glucoheptonate, followed by adding 5-10 μg of freshly prepared $SnCl_2$ (Sigma, American). Then about 0.5-1 mL of fresh e $Na^{99m}TcO4$ was added to the mixture with slight shake. The mixture was incubated at room temperature for 15 min before labeling efficacy was tested. When the labeling efficiency was less than 90%, the mixture was purified by running through a PD-10 column (Pharmacia, Sweden) and sterilized by filtration through a 0.22 μm filter (Millipore, USA). The agents produced by using the one- or two- step freeze drying method could be used to prepare antibodies for direct ^{99m}Tc labeling. Because the conjugation rate was greater than 90% and the level of free ^{99m}Tc was less than 2%, the produced ^{99m}Tc-labeled antibodies were not further purified for clinical application.

Immunoscintigraphy

Thirty-six patients with suspected recurrence of rectal cancer received immunoscintigraphy with ^{99m}Tc-labeled CL58. Single photon emission computed tomography (SPECT) was performed using an E.CAM™ dual-head emission imaging system (Siemens, Germany). The patients needed to have clear fecal residue before imaging. Head/neck-pelvis tomography was performed 4 and 22 h after intravenous injection of 1.11 GBq of ^{99m}Tc labeled CL58. To discriminate imaging artifacts in the intestinal tract, abdomen-pelvis tomography was conducted 28 h after CL58 injection. In addition, patients received abdominal CT, ultrasonography, and fibrocolonoscopy. Two- and three- dimensional immunoscintihtsphy images were analyzed by at least two experienced radiologists after completion of immunoscintigraphy. The areas of specific accumulation of ^{99m}Tc were regarded as malignant tumors. All images on X-ray films, abdominal CT and ultrasonography were compared except for the normal imaging sites in liver, spleen, kidney, bladder and major blood vessels.

Statistical analysis

Statistical evaluation was performed using the Student's *t* test and the positive predictive values were evaluated by Fisher's exact test using SPSS 11.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Imaging results

A total of 36 patients underwent immunoscintigraphy with ^{99m}Tc-labeled CL58. Tumor status and immunoscintigraphy data are summarized in Table 1. Of the 36 patients, 31 (86.11%) were positive for local tumor recurrence. Forty lesions were found in the pelvis: 20 in tissues adjacent to the bladder, 15 in presacral region, 2 in colon and 3 in peritoneal region (Table 2).Ten lesions were distant metastases: 1 in liver, 2 in sternum, 1 in retroperitoneal lymph nodes, 1 in free abdominal cavity, 4 in lung and 1 in tissues adjacent to the stomach (Table 3). Focal trace accumulation was found in bladder and lungs. Fourteen patients were found to have two lesions.

Tumor recurrence was pathologically confirmed in 30 of the 31 patients. Five patients with negative immunoscintigraphy had no pathological evidence of recurrence even though isolated masses were detected in the pelvis area. The overall positive rate was 86.11%, the specificity was 83.33%, and the sensitivity was 100%.

Immunoscintigraphy following APR

In this study, 12 patients underwent APR. Immuno-scintigraphy showed local recurrence in 11 patients and negative imaging in 1 patient. Of the 11 patients with a

Table 1 Surgical procedure, serum CEA level and immunoscintigraphy data

Patients	Gender	Operation LAR/APR*	RⅡ result	Serum CEA level
1	M	LAR	+	16.4
2	F	LAR	+	25.4
3	M	APR	+	25.4
4	M	LAR	+	16.4
5	M	LAR	+	13.3
6	M	LAR	+	2.1
7	F	APR	+	23.9
8	F	LAR	-	0.9
9	M	LAR	+	8.2
10	M	LAR	+	6.0
11	M	APR	+	52.8
12	F	APR	+	9.0
13	M	LAR	+	50.0
14	F	LAR	+	52.0
15	F	LAR	+	500.0
16	M	LAR	+	33.9
17	F	LAR	+	317.7
18	M	APR	+	51.8
19	M	APR	+	68.7
20	F	LAR	+	17.9
21	M	APR	+	14.5
22	F	APR	+	27.5
23	M	APR	+	34.2
24	M	APR	+	6.8
25	F	LAR	+	8.9
26	M	LAR	+	1.0
27	F	APR	+	7.5
28	F	LAR	+	6.1
29	M	LAR	+	13.7
30	F	LAR	-	1.3
31	M	LAR	+	24.9
32	F	LAR	-	7.7
33	F	APR	-	2.4
34	M	LAR	+	13.0
35	M	LAR	-	1.5
36	F	LAR	+	14.0

positive immunoscintigraphy finding, 7 were pathologically confirmed to have local recurrence by biopsy, 4 not confirmed by biopsy were eventually confirmed to have recurrence by pathology when the tumors invaded skin (Table 4).

Comparison of immunoscintigraphy with CT, ultrasonography and PET

To assess the reliability of immunoscintigraphy, we collected the pathological data from all cases which were compared with conventional diagnostic methods including CT, ultrasonography and fibrocolonoscope. Of the 31 patients with recurrences, radiolabled immunoscitigraphy (RⅡ), CT, and fibrocolonoscope identified lesions in 4 patients, accounting for 12.90%; RⅡ, CT and ultrasonography suggested recurrence in 20 patients, accounting for 64.52%; in the remaining 7 patients (22.58%) only ultrasonography and RⅡ suggested recurrence.

Immunoscintigraphy and serum CEA levels

Of the 31 patients with confirmed recurrence by positive CL-58 immunoscintigraphy, 29 had elevated serum CEA levels: 2-fold higher in 2 patients, 3-fold higher in 6 patients, and 4-fold higher in 19 patients.

DISCUSSION

TME can significantly reduce local recurrence of rectal cancer its recurrence rate[7]. It is estimated that approximately 8% of rectal cancer patients would experience a local recurrence even after TME. Most local recurrences of rectal cancer present only in the pelvic area, especially in stage T3 tumor with invaded serosa[8]. Diagnosis of local recurrence of rectal cancer in patients having undergone LAR is primarily based on pelvic CT and transrectal ultrasonography, partially on biopsy[9,10]. However, all these procedures are limited in the diagnosis of local recurrence of rectal cancer, especially following APR. Contrast-enhanced CT scan is difficult to discriminate small recurrent tumor nodules from postoperative scars[11]. Percutaneous punctures might injure adjacent organs. These methods are not sufficient to make a definitive diagnosis and it is difficult to treat it based only on conventional imaging and serum CEA levels[12]. About 40% of patients with rectal cancer, especially low rectal cancer, need to receive APR[13]. Because their rectum and anus are resected, the diagnosis of local recurrence of rectal cancer is difficult. Furthermore, a recent study revealed that the 5-year survival rate of patients with distant metastasis and local recurrence is 36.1% and 24% , respectively[14].

Immunoscintigraphy can obtain important information about tumors including their size and location. This technology is based on the principle that radioisotope-labeled anti-tumor antibodies can specifically bind to the corresponding tumor-specific antigens. It is characterized by a high specificity and sensitivity, and has been used in clinical practice[15]. Immunoscintigraphy with ^{99m}Tc-labeled anti-CEA antibodies appears to produce clear images and provide the precise location of recurrent rectal cancer, suggesting that it is an effective method for the identification of local recurrent rectal cancer, especially for the suspected local recurrent rectal cancer after APR. The sensitivity of immunoscintigraphy ranged 70%-100% for rectal cancer and the detection rate of abdominal and hepatic lesions is 86%. Our group has successfully used ^{99m}Tc labeled immuconjugates in more than 200 clinical cases. Based on previous preclinical experiments that assessed toxicity, body distribution, stability and availability of CL58 in animals[6]. we used ^{99m}Tc-labeled CL58 to detect malignancies in patients. In our study, of the 36 patients with suspected recurrent rectal cancer, 31 (86.1%) had positive immunoscintigraphy. Of the 54 recurrent lesions in 30 patients, 40 (74.07%) occurred in the pelvis.

Our data indicate that CL-58 immunoscintigraphy can detect small local recurrent lesions. Post-APR recurrent lesions which were too small to be detected by CT could be diagnosed by immunoscintigraphy in 7 patients. RⅡ can also be used to detect metastases of rectal cancer in the liver, lung, and peritoneum, and discriminate between recurrent malignancies and benign scar in the pelvic cavity. In addition, the cost of RⅡ is significantly lower than that of PET.

The detection rate of different methods for recurrent lesions at various locations is different. Ultrasonography is difficult to identify relatively small lesions and CT is

Table 2 Sites of local recurrence

Patients	Gender	Operation LAR/APR*	RⅡ (+/-)	CEA (+/-)	Parabladder	Presacral	Colon	Perineal
1	M	LAR	+	16.4	+	+	-	-
2	F	LAR	+	25.4	+	-	-	-
3	M	APR	+	25.4	-	+	-	-
4	M	LAR	+	16.4	+	+	-	-
5	M	LAR	+	13.3	+	-	-	-
6	M	LAR	+	2.1	+	+	-	-
7	F	APR	+	23.9	-	+	+	-
9	M	LAR	+	8.2	+	-	-	-
10	M	LAR	+	6.0	-	+	-	-
11	M	APR	+	52.8	+	-	-	-
12	F	APR	+	9.0	-	+	-	-
13	M	LAR	+	50.0	-	-	-	-
14	F	LAR	+	52.0	+	-	-	-
15	F	LAR	+	500.0	+	+	-	-
16	M	LAR	+	33.9	+	-	-	+
17	F	LAR	+	317.7	+	-	-	-
18	M	APR	+	51.8	-	-	-	-
19	M	APR	+	68.7	+	-	-	-
20	F	LAR	+	17.9	+	+	-	+
21	M	APR	+	14.5	+	-	-	-
22	F	APR	+	27.5	-	-	-	-
23	M	APR	+	34.2	-	+	-	-
24	M	APR	+	6.8	-	+	-	+
25	F	LAR	+	8.9	+	+	-	-
26	M	LAR	+	1.0	+	-	-	-
27	F	APR	+	36.2	-	-	-	-
28	F	LAR	+	6.1	+	-	+	-
29	M	LAR	+	13.7	+	+	-	-
31	M	LAR	+	24.9	+	+	-	-
34	M	LAR	+	13.0	-	-	-	-
36	F	LAR	+	14.0	+	+	-	-

Table 3 Distant metastases

Patients	Gender	Operation LAR/APR*	CEA (+/-)	Liver	Sternum	RPLN	FAC	Lung	Parastoma
1	M	LAR	16.4	-	-	-	-	-	-
2	F	LAR	25.4	-	-	-	-	+	-
3	M	APR	25.4	-	-	-	-	-	-
4	M	LAR	16.4	-	-	-	-	-	-
5	M	LAR	13.3	-	-	-	-	-	-
6	M	LAR	2.1	-	-	-	-	+	-
7	F	APR	23.9	-	-	-	-	-	-
9	M	LAR	8.2	-	-	-	-	-	-
10	M	LAR	6.0	-	-	-	-	-	-
11	M	APR	52.8	-	-	-	-	-	-
12	F	APR	9.0	-	-	-	-	-	-
13	M	LAR	50.0	-	+	-	-	-	-
14	F	LAR	52.0	-	-	-	-	-	-
15	F	LAR	500.0	-	-	-	-	+	-
16	M	LAR	33.9	-	-	-	-	-	-
17	F	LAR	317.7	-	-	+	-	-	-
18	M	APR	51.8	-	-	-	+	-	-
19	M	APR	68.7	-	-	-	-	-	-
20	F	LAR	17.9	-	-	-	-	-	-
21	M	APR	14.5	-	-	-	-	-	-
22	F	APR	27.5	-	-	-	-	-	+
23	M	APR	34.2	-	-	-	-	-	-
24	M	APR	6.8	-	-	-	-	-	-
25	F	LAR	8.9	-	-	-	-	-	-
26	M	LAR	1.0	-	-	-	-	-	-
27	F	APR	36.2	+	-	-	-	-	-
28	F	LAR	6.1	-	-	-	-	-	-
29	M	LAR	13.7	-	-	-	-	-	-
31	M	LAR	24.9	-	-	-	-	-	-
34	M	LAR	13.0	-	+	-	-	+	-
36	F	LAR	14.0	-	-	-	-	-	-

RPLN: Retroperitoneal lymph nodes; FAC: Free abdominal cavity.

Table 4 Immunoscintigraphy following APR

Patients	Gender	Operation LAR/APR*	Path (+/-)	CEA (+/-)	Parabladder	Presacral	Colon	Perineal	Liver	Sternum	RPLN	FAC	Lung	Parastoma
3	M	APR	Puncture	25.4	-	+	-	-	-	-	-	-	-	-
7	F	APR	LTS	23.9	-	+	+	-	-	-	-	-	-	-
11	M	APR	Puncture	52.8	+	-	-	-	-	-	-	-	-	-
12	F	APR	FTG	9.0	-	+	-	-	-	-	-	-	-	-
18	M	APR	Puncture	51.8	-	-	-	-	-	-	-	+	-	-
19	M	APR	Puncture	68.7	+	-	-	-	-	-	-	-	-	-
21	M	APR	Puncture	14.5	+	-	-	-	-	-	-	-	-	-
22	F	APR	Puncture	27.5	+	-	-	-	-	-	-	-	-	-
23	M	APR	LTS	34.2	-	+	-	-	-	-	-	-	-	-
24	M	APR	FTG	6.8	-	+	-	+	-	-	-	-	-	-
27	F	APR	Puncture	36.2	-	-	-	-	+	-	-	-	-	-

Puncture: Biopsy and pathological examination; LTS: Local tissue smear tumors invaded skin; FTG: Further tumor growth.

not able to discriminate between recurrent malignancies and benign postoperative scarring. Compared with these examination procedures, RⅡ has a higher sensitivity and specificity for both local recurrent lesions and metastases following surgery. Immunoscintigraphy in this study identified some suspected recurrent lesions not detected by CT, suggesting that RⅡ in combination with CT and ultrasonography may provide more accurate findings following tumor resection.

In our study, there was no false negative case, indicating that this method is highly specific. False positivity occurred only in the early period of this study mainly due to the vicinity of the bladder. Patients selected to undergo immunoscintigraphy were highly suspected of having recurrence. Since immunoscintigraphy is not routinely used after operation, there were few negative cases. This sampling bias limited the diagnostic specificity when it is routinely used after operation. We expect that ^{99m}Tc CL-58 immunoscintigraphy would provide more precise and specific data.

Serum CEA detection has become an important follow-up method after rectal cancer surgery[16]. CEA levels are increased in 75% of patients with rectal tumors[17]. We subjected 29 patients with suspected tumor recurrence to immunoscintigraphy because of elevated CEA serum levels. Immunoscintigraphy successfully identified local recurrence and metastasis in these patients. Moreover, it appeared to be more sensitive than blood CEA testing or other diagnostic modalities in detecting recurrent rectal cancer. Therefore, even when serum CEA levels are normal, immunoscintigraphy can be used as a routine screening method for early detection of recurrence and metastasis of rectal cancer.

Although immunoscintigraphy is highly specific, it cannot directly provide a pathological diagnosis. Furthermore, immunoscintigraphy has toxicity and side effects.

In conclusion, ^{99m}Tc CL-58 immunoscintigraphy after rectal cancer surgery has a high specificity and provides early diagnosis of local recurrence and distant metastasis of rectal cancer especially after APR. The eventual incorporation of this technique into the postoperative standard of care for rectal cancer patients may contribute to the early diagnosis of recurrent rectal cancer and prolong the survival time of such patients.

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RAPID COMMUNICATION

Screening for metronidazole-resistance associated gene fragments of *H pylori* by suppression subtractive hybridization

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Abstract

AIM: To screen for metronidazole (MTZ)-resistance associated gene fragments of *H pylori* by suppression subtractive hybridization (SSH).

METHODS: Five MTZ-resistant (tester, T) and 1 MTZ-susceptible (driver, D) clinical *H pylori* isolates were selected. Genomic DNAs were prepared and submitted to *Rsa* I digestion. Then two different adaptors were ligated respectively to the 5′-end of two aliquots of the tester DNA fragments and SSH was made between the tester and driver DNAs. The specific inserts of tester strains were screened and MTZ-resistance related gene fragments were identified by dot blotting.

RESULTS: Among the randomly selected 120 subtractive colonies, 37 DNA fragments had a different number of DNA copies (≥ 2 times) in resistant and susceptible strains and 17 of them had a significantly different number of DNA copies (≥ 3 times). Among the sequences obtained from the 17 DNA fragments, new sequences were found in 10 DNA fragments and duplicated sequences in 7 DNA fragments, representing respectively the sequences of depeptide ABC transporter periplasmic dipeptide-binding protein (dppA), permease protein (dppB), ribosomal protein S4 (rps4), ribonuclease Ⅲ (rnc), protease (pqqE), diaminopimelate epimerase (dapF), acetatekinase (ackA), *H pylori* plasmid pHP51 and *H pylori* gene 1334.

CONCLUSION: Gene fragments specific to MTZ-resistant *H pylori* strains can be screened by SSH and may be associated with MTZ-resistant *H pylori*.

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Key words: *H pylori*; Metronidazole; Drug resistance; Gene; Suppression subtractive hybridization

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INTRODUCTION

H pylori is a bacterial organism causing chronic gastritis and peptic ulcers. It also plays an important role in the pathogenesis of gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma. Metronidazole (MTZ) is an important component of many currently used *H pylori* eradication regimens. Antibiotic resistance of *H pylori* is known as an important cause of treatment failure of *H pylori*. The mechanism of MTZ resistance in *H pylori* treatment remains unclear, although some genes such as rdxA, frxA and fdxB have been found to be associated with MTZ resistance[1-5].

Suppression subtractive hybridization (SSH), one of the new methods for phenotypic cloning, has been developed for identifying genomic differences between the genomes of close relatives[6]. It is a powerful approach for screening genes associated with drug resistance. Using SSH, we have identified some DNA fragments which are specific to the resistant strains. The identified DNA fragments were sequenced and compared with GenBank database to search for some genes associated with MTZ-resistance in *H pylori*.

MATERIALS AND METHODS

Isolation and identification of clinical H pylori strains

Each of the clinical biopsy specimens was homogenized with a tissue grinder and then inoculated onto Columbia agar (bioMérieux) plates supplemented with 8.0% (V/V) sheep blood, 0.2% (W/V) cyclodextrin, 5 mg/L trimethoprim (Sigma), 10 mg/L vancomycin (Sigma), 2.5 mg/L amphotericin B (Sigma) and 2500 U/L

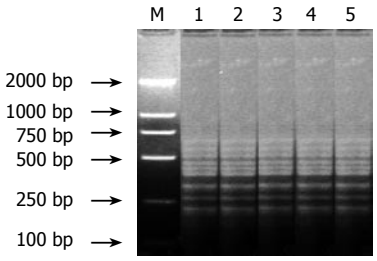

Figure 1 PCR-amplified different resistant isolates of *H pylori* subtraction. M: DNA marker; 1-5: different resistant isolates 60a, 45a, 51a, 28a, and 117a.

polymyxin B (Sigma). The plates were incubated at 37℃ under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) for 3-5 d. Isolates were identified as *H pylori* according to typical Gram stain morphology, biochemical tests positive for urease and oxidase.

Determination of minimal inhibition concentration

The minimal inhibition concentration (MIC) was determined by agar dilution method of the National Committee for Clinical Laboratory Standards (NCCLS). Agar dilution plates were prepared with two-fold serial dilution of MTZ, ranging from 0.25 to 128 mg/L. The inoculation concentration of *H pylori* was $1 \times 10^{6-7}$ CFU/5 μL. Results were read after 72 h incubation and the MIC was determined as the lowest concentration of MTZ in which no visible growth occurred. Strains with MIC value ⩾ 8 mg/L were classified as resistant[7]. NCTC11637 was used as a reference strain.

DNA extraction

Genomic DNA was extracted from *H pylori* strains according to UNIQ-10 genomics DNA isolation kit user manual provided by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd.

SSH

The subtractive DNA library was established according to Clontech PCR-selectTM bacterial genome subtraction kit (PT3170-1) user manual. Genomic DNA from five MTZ-resistant clinical *H pylori* strains was used as the tester respectively, and DNA from one MTZ-susceptible clinical strain was used as the driver. The sequences of adaptors and primers are as followings: adaptor 1: 5'-CTAATACG ACTCACTATAGGGCTCGAGCGGCCGCCCGGGCA GGT-3'; adaptor 2R: 5'-CTAATACGACTCACTATAGG GCAGCGTGGTCGCGGCCGAGGT -3'; P1: 5'-CTAAT ACGACTCACTATAGGGC-3'; NP1: 5'-TCGAGCGGC CGCCCGGGCAGGT-3'; NP2: 5'-AGCGTGGTCGCG GCCGAGGT-3'. The PCR products were analyzed by 2% agarose gel electrophoresis, purified by using the 3S PCR product purification kit (BBST).

T/A cloning and screening

The second PCR products were purified and cloned into the pUCm-T vector (BBST) following the protocols. The recombinant plasmids were transformed into *E. coli* DH5α, which was then cultured overnight on the selective agar plates. One hundred and twenty white colonies were randomly picked and cultured in Luria-Bertani medium containing ampicillin at 37℃ for 8 h. The plasmids were extracted and used as templates and the inserts were amplified under condition as in the second PCR for 25 cycles. The sizes of the inserts were identified by 2% agarose gel electrophoresis.

Dot blotting and DNA sequencing

Each insert PCR-purified products were dotted on the Hybond N+ membrane (BioRad) in duplicating forms and DNA fixation was carried out by baking the Hybond N+ membrane at 80℃ for two hours. In addition, 23S rRNA fragment of *H pylori* and pBR328 were dotted on the membrane as positive and negative control respectively. The *Rsa* I -digested genomic DNA fragments of four resistant and four susceptible *H pylori* strains (not tester and driver strains) were used as probes and dot blotting was preformed using the DIG DNA labeling and detection kit (Roche). Pre-hybridization and hybridization were carried out in the hybridization oven (HYBAID) at 50℃ for 2 and 20 h respectively. After the Hybond N+ membrane was stringently washed and blocked, the Anti-Dig-AP mixtures were added and the signals were detected by colorsubstrate solution (NBT/BCIP). The inserts that gave positive results (that is, obvious difference in gene copies between the DNA fragments of resistant and susceptible strains) were sequenced by BBST Company. The sequences were then submitted to gene homologous analysis based on GenBank database.

RESULTS

SSH

After subtractive hybridization of genomic DNA in resistant and susceptible isolates, PCR amplified products presented several similar and tight striples, indicating that most tester sequences formed with the driver were excluded, while tester-specific sequences were self-hybridized to form amplifiable fragments that were then enriched by PCR (Figure 1).

T/A cloning and screening

After SSH between the tester and driver DNA fragments, about 450 colonies grew on the ampicillin plates and one third of them were white in color. One hundred and twenty white colonies were randomly chosen and 101 positive colonies containing the target inserts were confirmed by nested primer PCR. The size of inserts ranged from 150 to 750 bp (the average was about 280 bp).

Dot blotting

From 101 positive subtractive colonies, 82 with their size longer than 180 bp were picked out for dot blotting with genomic DNA fragments of *Rsa* I digested resistant and susceptible strains. Thirty-seven DNA fragments with a different number of DNA-copies (⩾ 2 times) in resistant and susceptible strains were obtained and 17 of them differed significantly in the number of DNA-copies (⩾ 3 times) (Figure 2).

DNA sequencing and homologous analysis

Among the sequences obtained from the 17 specific

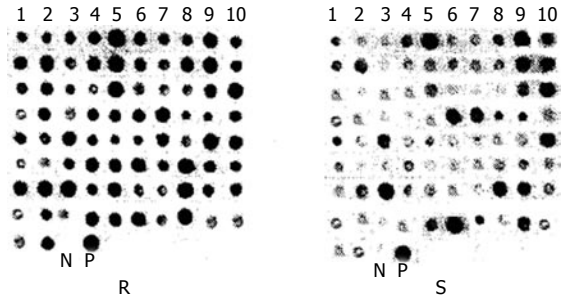

Figure 2 MTZ-resistant strain specific inserts screened by dot blotting using the RsaI digested resistant and susceptible strains as probes (R represents hybridized susceptible isolates with probe, S represents hybridized resistant isolates with probe; N: Negative control; P: Positive control).

DNA fragments, new sequences were found in 10 DNA fragments and duplicated sequences in DNA fragments, representing respectively the sequences of depeptide ABC transporter permease protein (dppB), periplasmic dipeptide-binding protein (dppA), ribosomal protein S4 (rps4), ribonuclease Ⅲ (rnc), protease (pqqE), diaminopimelate epimerase (dapF), acetatekinase (ackA), *H pylori* plasmid pHP51, *H pylori* gene 1334 and replication protein B (Table 1).

Table 1 Homologous analysis of some specific DNA sequences of MTZ-resistant strains

No.	GenBank accession No.	Homolog genes	DNA matches
R1	AE000548	Dipeptide ABC transporter periplasmic dipeptide-binding protein (dppA), S = 433, E = 0.0, I = 554/591	6142-6349
R2	AE000548	Depeptide ABC transporter, permease protein (dppB), S = 433, E = 0.0, I = 554/591	6349-6728
R3	AE000609	Protease (pqqE), S = 273, E = e-151, I = 309/321	9322-9642
R4	AE000579	Ribonuclease Ⅲ (rnc), S = 216, E = e-117, I = 265/280	10907-11185
R5	AY267368	PHP51, S = 115, E = 4e-57, I = 146/154	3018-3169
R6	AB078638	Replication protein B (REPB), S = 30, E = 2e-06, I = 55/62	1320-1381
R7	AE000633	Ribosomal protein S4 (rps4), S = 354, E = 0.0, I = 406/422	7294-7714
R8	AE000635	predicted coding region HP1334, S = 258, E = e-142, I = 457/523	1529-2050
R9	AE000570	Diaminopimelate epimerase (dapF), S = 215, E = e-116, I = 247/260	7955-8214
R10	AE000599	This region contains an authentic frame shift and is not the result of a sequencing artifact, acetate kinase (ackA), S = 99, E = 4e-47, I = 183/210	8555-8764

DISCUSSION

Genes that are present in certain isolates of a given bacterial species and absent or substantially different in others can be of great biological interest. Some may determine strain-specific traits such as drug resistance, pathogenicity, bacterial surface structure, or restriction-modification. SSH is a powerful technique that has been applied to many different fields, such as identification of PIs/genomic islands, mobile genetic elements, drug resistance associated genes and variations in gene expression, *etc*[8-12]. Here we used this method in *H pylori* to search MTZ-resistance associated gene fragments.

In this study, some gene fragments specific for MTZ-resistant *H pylori* strains were identified by SSH. Of them, 10 were identified by dot blotting, including depeptide ABC transporter permease protein (dppB), periplasmic dipeptide-binding protein (dppA), ribosomal protein S4(rps4), ribonuclease Ⅲ (rnc), protease(pqqE), diaminopimelate epimerase (dapF), acetatekinase(ackA), *H pylori* plasmid pHP51, *H pylori* gene 1334 and replication protein B. These gene fragments may be associated with MTZ-resistance of *H pylori*.

Dipeptide ATP-binding cassette (ABC) transporters dppA and dppB which are relative to transportation of bi-peptide and polypeptide, like the multidrug resistance protein (MDR), belong to bacterial periplasmic transport system and ABC transporters or traffic ATPase superfamily, take part in active efflux of intracelluar compounds, and function as a drug efflux pump[13]. It was reported that antibiotic efflux pumps exist in almost all bacteria and are one of the reasons for MDR. Our previous study showed that verapamil could reduce the MIC of MTZ-resistant *H pylori*, suggesting that drug efflux pump in the membrane of *H pylori* might be inhibited by verapamil[14]. In this study, the number of gene copies encoding DppA and DppB was obviously higher in the resistant *H pylori* isolates than in susceptible ones, indicating that DppA and DppB may play an important role in the active MTZ efflux and result in the resistance to MTZ.

Plasmid is a close-circular extra-chromosomal DNA form of bacteria *in vitro*. Antibiotic resistant plasmids widely exist in Gram-negative or positive bacteria. The resistant rate of *H pylori* to MTZ is very high and has an increasing trend. Researchers suspect that resistant plasmids exist in *H pylori*, but no relative evidence has been found. Our study showed that the number of gene copies in cryptic plasmid PHP051 was much higher in MTZ-resistant isolates, which may be due to its random insertion into the *H pylori* DNA, thus leading to some gene shift-code mutations and MTZ resistance.

In summary, the 10 new gene fragments identified in this study may be associated with *H pylori* resistance to MTZ. However, the relationship between these genes and their resistance to MTZ needs to be further investigated.

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RAPID COMMUNICATION

Contribution of mononuclear bone marrow cells to carbon tetrachloride-induced liver fibrosis in rats

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Abstract

AIM: To study the inhibitory effect of mononuclear bone marrow cell (BMC) transplantation on carbon tetrachloride (CCl_4) -induced liver fibrosis in rats.

METHODS: Rat liver fibrosis models were induced by CCl_4 and alcohol administration. After 8 wk, twenty rats were randomly allocated into treatment group (n = 10) and control group (n = 10). BMC were infused into the rats in treatment group *via* the portal vein, while heparinized saline was infused in control group. CCl_4 was hypodermically injected into the rats twice a week for 4 wk. At the end of wk 12, all rats were humanely sacrificed. Liver samples were taken and stained with HE or Masson trichrome. The general conditions, liver fibrosis (hydroxyproline and collagen fibre) and liver pathological grades in rats were evaluated.

RESULTS: The general conditions of the rats in treatment group improved markedly, but not in control group. Hydroxyproline was 504.6 ± 128.8 μg/g in treatment group, and 596.0 ± 341.8 μg/g in control group. The percentage of collagen fibre was 3.75% ± 0.98% in treatment group and 5.02% ± 0.44% in control group. There was a significant difference between the two groups (P < 0.05). Liver pathological grade decreased from grade Ⅳ to grade Ⅲ partially in treatment group (P < 0.05) with no obvious improvement in control group (P > 0.05). There was a significant difference between treatment group and control group (P < 0.05).

CONCLUSION: Transplantation of BMC can improve liver fibrosis due to chronic liver injury in rats.

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Key words: Bone marrow cell; Transplantation; Liver cirrhosis; Rat model

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INTRODUCTION

Liver fibrosis is caused by the excessive accumulation of extracellular matrix (ECM) proteins including collagen in most types of chronic liver disease[1]. Accumulation of ECM proteins distorts the hepatic architecture by forming fibrous scars. The subsequent development of nodules of regenerating hepatocytes defines cirrhosis. Cirrhosis leads to hepatocellular dysfunction and increases intrahepatic resistance to blood flow, resulting in hepatic insufficiency and portal hypertension[2], and often requires liver transplantation. However, donor livers are limited[3,4]. Cell transplantation has become the better choice of treatment for liver regeneration.

As a general rule, replication of existing hepatocytes is the quickest and most efficient way to generate hepatocytes for liver regeneration and repair. Nevertheless, there is evidence that the replicative activity of hepatocytes diminishes in advanced cirrhosis of humans and in chronic liver injury of mice, reaching a state of "replicative senescence", perhaps as a consequence of telomere shortening[5-7]. Recent reports have shown that bone marrow cells (BMC) are able to differentiate into non-hematopoietic stem cells[8-12]. The ability of BMC to differentiate into hepatocytes and intestinal cells has been shown by Y-chromosome detection in autopsy analysis of human female recipients of BMC from male donors[13,14].

Bone marrow is a complex tissue containing hematopoietic stem cells, mesenchymal stem cells, and other kinds of cell factors. Mesenchymal stem cells and cell factors are precursors of nonhematopoietic tissues.

The precursors of nonhematopoietic tissues can serve as a feeding layer that supports nonhematopoietic stem cell growth, self-renewing without differentiation, and become one phenotype, indicating that BMC with remarkable plasticity have become an attractive cell source in regenerative medicine.

However, contribution of BMC to the generation of hepatocytes in liver regeneration remains uncertain. In the present study, we employed a rat model of fibrosis induced by CCl_4 to evaluate the effect of BMC on fibrosis. Our results suggest that BMC treatment can improve fibrosis in rat models.

MATERIALS AND METHODS

Experimental protocol

Wistar rats weighing 200-300 g, purchased from Experimental Animal Center of Nanfang Medical University (Guangzhou, China), were housed in cages (5 in each cage) and subjected to 12-d/12-night cycle with free access to basic food. Rat hepatic cirrhosis model was induced by CCl_4 (Figure 1). On d 0, 3 mL/kg CCl_4 dissolved in paraffin oil (Guangzhou Chemical Factory, China) was injected subcutaneously into the rats (Guangzhou Chemical Factory, China) twice a week for 8 wk, with free access to alcohol gavage and high-fat diet. From the fourth week, a rat was killed weekly and liver fibrosis was determined by histopathology. At the end of wk 8, twenty rats were randomly divided into treatment group (n = 10) and control group (n = 10). BMC were infused into the rats in treatment group *via* the portal vein, while heparinized saline solution was infused in control group. CCl_4 was hypodermically injected into the rats twice a week for 4 wk. Then the trial ended, all rats were sacrificed to assess the extent of liver fibrosis at the end of experiment.

BMC preparation and transplantation

For BMC isolation, Fisher 344 rats (6-wk old) were killed by cervical dislocation and the limbs were removed. BMC were flushed with Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) from medullary cavities of tibias and femurs using a 25-G needle. According to the blood volume to be separated, 50 mL centrifuge tubes were filled with either 20 mL lymphocyte separation medium (D= 1077 g/L, at 20℃), then 25 mL heparinized bone marrow was diluted with an equal volume of PBS and poured over the lymphocyte separation solution. The separation process was performed by centrifugation at 1200 × g for 20 min. Mononuclear BMC concentrated in the interphase (white layer) between plasma and separation solution. They were subsequently extracted with a sterile Pasteur pipette and washed twice with heparinized saline before final resuspension in heparinized saline. The concentration of mononuclear cells was 6×10^9/L. After cirrhosis models induced by CCl_4 were confirmed, rats were placed in a dryer and anaesthetized with ether. Portal vein was exposed with abdominal midline and right transverse incision. Mononuclear BMC (0.5 mL, 3×10^6) and 0.5 mL heparinized saline were infused into liver respectively *via* portal vein in treatment group and control group.

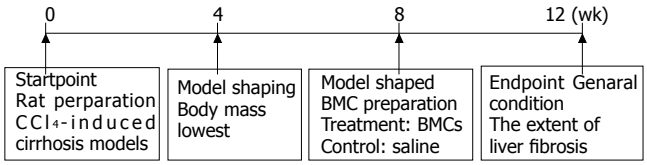

Figure 1 Fibrosis model protocol and BMC therapy for liver cirrhosis in rats.

This experiment was approved by the Committee of Animal Experiment Ethics at the Sun Yat-Sen University School of Medicine and was carried out under the guidelines for animal experiments at Sun Yat-Sen University School of Medicine.

Hydroxyproline (hyp) assay

Hyp was determined by the Kivirikko's method with modifications. Briefly, liver specimens were weighed, and 0.5 g freeze-dried sample was hydrolyzed in 6 mol/L HCl at 110℃ in an autoclave at a pressure force of 1.2 kg/cm^2 for 24 h, re-dissolved in water, and centrifuged to remove any impurities. Samples were incubated for 10 min in 0.05 mol/L chloramine-T (Fisher, Fair Lawn, NJ, USA) at room temperature, followed by 15-min incubation in Ehrlich's-perchloric acid solution at 65℃. Sample absorbency was measured at 560 nm and resulting values were compared to a standard hyp curve. Each sample was assayed in duplicate. The hyp was expressed as micrograms per gram of wet liver.

Collagen fibre percentage assay

Liver tissues were fixed in formalin and embedded in paraffin according to standard procedures (glass slide was cleaned with 950 ml/L ethanol, treated with APES solution and air dried). The liver tissue from the right lobes of liver was cut into 5-μm thick sections using microtome and applied to slides, deparafinized in xylenes and changed three times, each for 5 min. Hydrate sections were gradually passed through graded alcohol, washed twice in 100% ethanol, then twice in 95% ethanol for 10 min each, and finally washed in deodorized water for 1 min. Hematoxylin and eosin (HE) and masson trichrome (MT) staining was performed according to the standard procedure.

The liver fibrosis area with picro-sirius red staining was quantified under Zeiss Axiotron microscope equipped with a KY-F30B 3-CCD camera (JVC, Japan). Briefly, the red area was considered as the fibrotic area and was assessed by computer-aided image analysis with Meta-Morph software (Universal Imaging Corporation, Downingtown, PA) at a magnification of × 400. The semiquantitative data were expressed as percentage of positive staining. The average of scores taking from 5 randomly selected fields (4 corners and 1 center) per sample was used as the percent area of fibrosis.

Histopathological grading

Fibro-proliferation in liver sections was graded as 0: normal liver; 1: few collagen fibrils extending from the

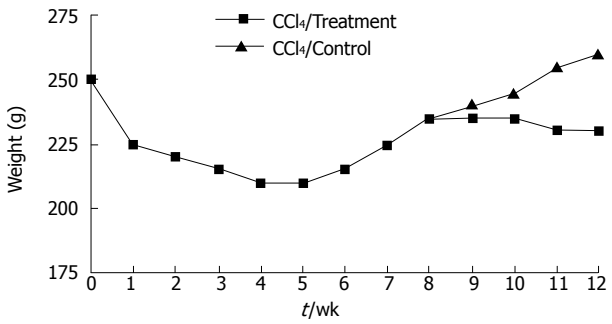

Figure 2 Average weekly body mass of rats.

central vein and portal tract; 2: obvious extension of collagen fibrils without encompassing the whole lobule; 3: collagen fibrils extending and encompassing the whole lobule; 4: collagen fibrils extending and separating lobule into pseudo-lobule mainly in square form; 5: formation of pseudo-lobule mainly in circle form. Two pathologists who had no knowledge about their sources and each other's assessment examined the stained slide independently.

Statistical analysis

Results are presented as mean ± SD. Differences between groups were analyzed by one-way ANOVA using SPSS 13.3. The grade of liver histopathology was examined using the χ^2 test.

RESULTS

General condition of the rats

In rat fibrosis model induced by CCl_4, the mean loss body mass was 40.67 g ± 10.88 g in the first 4 wk. The body mass increased by 25.93 g ± 8.67 g in the later 4 wk. The body mass of rats in treatment group was 26.57 g ± 8.58 g from wk 8 to the end of experiments, while body mass stopped increasing in rats of the control group (Figure 2).

In CCl_4-induced model, rats became lethargic, slower in reaction and diarrhea occurred in 40% of the rats. The fur of rats became gray, ulceration occurred in the injection area. Up to wk 8, cirrhosis model was pathologically confirmed with a mortality rate of 20% (6/30). After infusion of mononuclear bone marrow cells or heparinized saline, morphological and behavioral changes were observed in rats of the treatment group showing improvement in reaction, no diarrhea, fur scar healing and no ulceration. The rats in control group showed slow reaction, diarrhea, incurable fur ulceration, even new ulceration. Their eyes were pale and urine was yellow, and some animals had labored respiration. The mortality rate was 10% (1/10) in two groups. Some rats had a large amount of ascites at the time when they were killed, which might be a reason for their weight gain.

Liver histology

Two weeks after CCl_4 injection, histological examina-tion revealed fat denaturalization, centrilobular congestion and hemorrhagic necrosis, but no liver fibrosis (Figure 3A). Four weeks later, extensive fatty changes, centrilobular

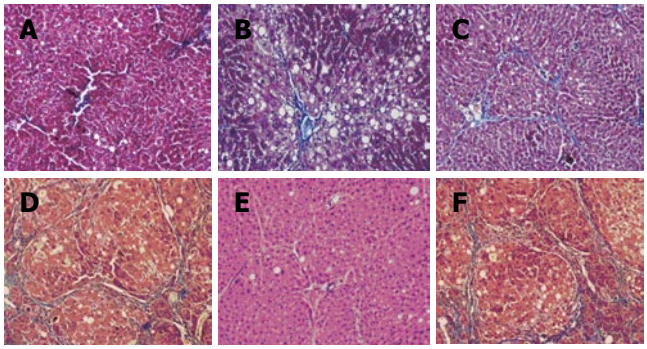

Figure 3 Histopathological changes of liver fibrosis 2 (**A**), 4 (**B**), 6 (**C**), 8 wk (**D**) after CCl_4 injection, 4 wk after BMC transplantation in treatment group (**E**) and 4 wk after saline transplantation in control group (**F**) (HE, × 100).

Table 1 Comparison between liver hyp and collagen fibre in treatment and control groups mean ± SD

Group	Hydroxyproline (μg/g)	Collagen fibre (%)
Treatment	504.6 ± 128.8[a]	3.75 ± 0.98[a]
Control	596.0 ± 341.8	5.02 ± 0.44

[a]$P < 0.05$ *vs* control group.

necrosis and intense neutrophilic infiltration were observed (Figure 3B). By 6 wk, liver fibrosis surrounding the central veins could be seen, with collagen fiber deposition, severe centrilobular necrosis and bridging necrosis, bile duct proliferation (Figure 3C). Massive fibrosis, extensive bridging, pseudolobules formed in the liver after exposure to CCl_4 for 8 wk (Figure 3D).

Surprisingly, 4 wk after BMC transplantation (12 wk after CCl_4 injection), the BMC-transplanted liver showed that the architecture of liver became better, with inflammation reduced, pseudolobules resolved, collagen accumulation and fatty degeneration alleviated, necrosis of hepatocytes replaced by the regenerated hepatocyres, and the thickened septal fibrosis became thinner or disappeared (Figure 3E). The BMC-transplanted liver showed improvement in liver fibrosis compared with the liver treated with CCl_4 alone (Figure 3F), although CCl_4 was injected throughout the experimental period.

BMC treatment decreased collagen deposition

BMC transplantation significantly reduced hydroxyproline in wet liver to 504.6 ± 128.8 μg/g liver in the treatment group compared to 596.0 ± 3418.0 μg/g liver in control group. Hyp was significantly reduced in treatment group compared with control group ($P < 0.05$, Table 1). Quantitative image analysis of liver fibrosis indicated that the percent area of liver fibrosis 4 wk after BMC transplantation was 3.75% ± 0.98 % in treatment group and 5.02% ± 0.44% in control group. Quantification of MT staining demonstrated that collagen was prominently decreased in rats of the treatment group compared to control group ($P < 0.05$, Table 1).

Moreover, the degree of fibrosis was decreased in treatment group compared to control group. Liver

Table 2 Liver pathological grading in two groups

Group	0	1	2	3	4	5	χ^2	*P*
Treatment								
Pre-treatment	0	0	0	0	0	10	10.55	0.005
Post-treatment	0	0	0	0	4	6		
Control								
Pre-treatment	0	0	0	0	0	10	-	1.0
Post-treatment	0	0	0	0	0	10		

pathological grades were improved from grade Ⅳ to grade Ⅲ in treatment group ($P < 0.05$) compared to control group ($P > 0.05$). There was a significant difference between treatment and control groups ($P < 0.05$, Table 2).

DISCUSSION

In this study, experimental fibrosis model was induced by CCl_4 and alcohol administration. The general conditions of rats improved after infusion of BM-derived mononuclear cells, suggesting that mononuclear bone marrow cells can improve liver fibrosis. CCl_4-induced liver fibrosis is a classical experimental fibrosis model. In this experiment, cirrhosis model was effectively induced by CCl_4 combined with alcohol gavage. CCl_4 could directly damage hepatocytes, thus leading to fatty degeneration and necrosis of hepatocytes. Alcohol is an enzyme inducer which damages mitochondria, increases oxygen consumption and enhances the toxicity of CCl_4 on hepatocytes, thus promoting formation of cirrhosis. During formation of cirrhosis, the body weight of rats dramatically reduced within the first 4 wk, then slowly increased within 4-8 wk. From wk 8, the body weight of rats slightly increased in treatment group with no obvious change in control group (Figure 1), showing that rats become adapted to toxicity. It was reported that this kind of model could undergo spontaneous resolution of fibrosis after CCl_4 challenging was stopped[15]. So it is necessary to establish a cirrhosis model to unceasingly inject CCl_4. We did not find recovery of liver fibrosis in this experiment.

BMC administration could repair injured liver by reducing inflammation, collagen deposition and remodeling[16,17]. In the current study, transplanted mononuclear bone marrow cells could degrade collagen fibers and improve liver fibrosis.

The classical paradigm that intrahepatic cell populations are replenished from resident oval cells has been revised to include extrahepatic stem cells[18]. Circulating bone marrow –derived cells contribute to the regeneration of a wide variety of organs. There is evidence that bone marrow-derived stem cells are able to enter parenchymal organs and participate in regenerative and structural modeling of transplanted tissues[8,10,11,19]. However, the mechanism by which extrahepatic stem cells contribute to the repair process (including regeneration) following injury remains controversial. The type of injury seems to play a major role in determining whether stem cells differentiate into hepatocytes (the process of stem cell "plasticity"), fuse with hepatocytes (the process of cell "fusion"), or change to form other cell lineages (the process of "trans-differentiation")[20,21]. Our previous study showed that BM-derived stem cells can inhibit the proliferation and activation of hepatic stellate cells by secreting some cytokines which might protect the liver against fibrosis[22].

In the current study, bone marrow-derived cells were infused without bone marrow ablation. It was reported that reconstitution of haematopoietic stem cells following ablation is necessary for their engraftment in the liver[23]. Additionally, significant engraftment of bone marrow-derived cells during liver injury is thought to require *in vivo* selection. However, Yamamoto *et al*[24,25] support the notion that bone marrow ablation is not required for stem cell engraftment in the liver. Mononuclear bone marrow cells include mesenchymal stem cells (MSC), hematopoietic stem cells (HSC), and many kinds of cell factors. These cells and cell factors may reduce fibrosis by interations between them.

Currently stem cells are mostly infused through peripheral vein in animal experiments of intrahepatic transplantation. Though this method is simple, the number of stem cells eventually reaching the liver is extremely limited, because of powerful macrophage phagocytosis in the lung. It severely impairs the efficacy of stem cell therapy. This problem may be solved by direct infusion of stem cells into the liver. In this experiment, the rats were anesthetized with ether and BMC were directly injected into the portal vein, which significantly improved liver fibrosis. However, Liechty *et al*[26] reported that BMC bear immune tolerance to allogenes even in different races. It may be related with the fact that BMC can inhibit the proliferation of T cells and induce T cell inaction[27-29]. Immune tolerance offers hope for BMC to be used in clinical practice.

The therapeutic potential of bone marrow-derived stem cells has led to increasing research interest in liver regeneration, especially in models of liver injury. Available data suggest that bone marrow-derived stem cells play a limited role in normal liver. The results of the current study demonstrate that stem cell administration can improve fibrosis and plays a significant role in liver injury and fibrogenesis.

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COMMENTS

Background

Liver fibrosis is due to the excessive accumulation of extracellular matrix (ECM) proteins including collagen that occurs in most types of chronic liver disease. Accumulation of ECM proteins distorts the hepatic architecture by forming fibrous scars, and subsequent development of nodules of regenerating hepatocytes defines cirrhosis. Cirrhosis leads to hepatocellular dysfunction and increases intrahepatic resistance to blood flow, which results in hepatic insufficiency and portal hypertension, and often requires liver transplantation. However, donor livers are limited. Cell transplantation has been used for liver regeneration.

Recent reports showed that bone marrow cells (BMC) are able to differentiate into non-hematopoietic cell lineages. The ability of BMC to differentiate into hepatocytes and intestinal cells has been shown by Y-chromosome detection in autopsy analysis of human female recipients of BMC from male donors. So, BMC

have an attractive cell source for regenerative medicine.

Research frontiers

It has recently been recognized that circulating bone marrow-derived cells contribute to the regeneration of a wide variety of organs. There is evidence that marrow-derived stem cells are able to enter parenchymal organs and participate in regenerative and structural modeling of transplanted tissues.

Bone marrow is a complex tissue containing hematopoietic stem cells, mesenchymal stem cells, and other kinds of cell factors. Mesenchymal stem cells and cell factors are precursors of nonhematopoietic tissues, indicating that BMC have become an attractive cell source in regenerative medicine. However, the contribution of BMC to the generation of hepatocytes in liver regeneration remains uncertain. In the present study, we employed a model of fibrosis induced by CCl_4 to evaluate the effect of BMC on fibrosis. Our results suggest that BMC treatment could improve fibrosis in rat models.

Innovations and breakthroughs

Bone marrow is a complex tissue containing hematopoietic stem cells, mesenchymal stem cells, and other many kinds of cell factors. It has been reported that infusion of hematopoietic stem cells or mesenchymal stem cells can improve liver fibrosis.

In this study, rats were anesthetized with ether, and portal vein was exposed by abdominal midline and right transverse incision. BMC were directly injected into portal vein. The results showed liver fibrosis was significantly improved.

Finally, our experiment used different genetic background rats between the donor and recipient. However, the results showed that BMC could bear immune tolerance in allogenes even between different races,suggesting that BMC can inhibit the proliferation of T cells and inactivate T cells. Immune tolerance offers hope for use of BMC in clinical practice.

Applications

The current study demonstrated that stem cell administration could improve fibrosis and BMC play a significant role in liver injury and fibrogenesis. Stem cell therapy will become the choice of treatment for liver disease in near future.

Terminology

Stem cell therapy: Stem cells have become an attractive cell source in regenerative medicine. Infusion of stem cells can repair or replace damaged cells.

Peer review

In this manuscript, the authors described the antifibrotic effect of mononuclear BMC infusion on carbon tetrachloride-induced liver fibrosis in rats. Intraportal infusion of mononuclear BMC improved the general conditions of rats and decreased hydroxyproline and the areas of collagen fiber in liver tissue. The results of this study are of certain importance.

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RAPID COMMUNICATION

A clinicopathological analysis in unsuspected gallbladder carcinoma: A report of 23 cases

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Abstract

AIM: To study the clinicopathological characteristics of unsuspected gallbladder carcinoma (UGC).

METHODS: We retrospectively studied 23 cases of UGC in Tongji Hospital, and compared their clinicopathological characteristics with 33 cases of preoperatively diagnosed gallbladder carcinoma (PDGC).

RESULTS: The proportion of UGC coexisting with cholecystolithiasis was significantly higher than that of PDGC (χ^2 = 13.53, P < 0.01). The infection rate of hepatitis B virus was 21.74% (5/23) in UGC and 30.30% (10/33) in PDGC. Nine (39.13%) of 23 patients with UGC and 8/33 (24.24) PDGC had contact with schistosome pestilent water. The rate of multiple pregnancies was 56.52% (13/23) in the patients with UGC and 42.42% (14/33) in PDGC. The primary location of the UGC was mostly in the neck and body of the gallbladder, and that of the PDGC was often in the body and bottom. The incidence of Nevin stage Ⅰ and Ⅱ UGC was significantly higher than that of PDGC (χ^2 = 4.44, P < 0.05 and χ^2 = 4.96, P < 0.05) while that of Nevin stage Ⅴ UGC was significantly lower than that of PDGC (χ^2 = 7.59, P < 0.01). According to the grading of carcinoma, the incidence of well-differentiated UGC was significantly higher than that of PDGC (χ^2 = 4.16, P < 0.05), and that of poorly-differentiated UGC was significantly lower than that of PDGC (χ^2 = 4.48, P < 0.05).

CONCLUSION: There are different characteristics between UGC and PDGC, such as in primary location, malignant degree and incidence of coexistence with cholecystolithiasis. Cholecystolithiasis, hepatitis B, schistosome and multiple pregnancies were high risk factors for gallbladder carcinoma.

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INTRODUCTION

The difficulty in early diagnosis of gallbladder carcinoma results from its poor specificity in clinical symptoms and ambiguous early symptoms, which seriously affects prognosis[1-6]. Some patients were found having gallbladder carcinoma only when they were diagnosed and treated because of other diseases[7-9]. Generally, gallbladder carcinoma is called unsuspected gallbladder carcinoma (UGC) when it was found incidentally during or after cholecystectomy because of gallbladder benign diseases[7,8,10,11]. We consider that UGC still includes gallbladder carcinoma found not only during or after cholecystectomy but also in the course of other diseases[12-17]. UGC found during cholecystectomy because of acute or chronic cholecystitis was reported in 1961[18], but more relative studies and close attention to UGC became available in the 1990s. To date, diagnosis and treatment of UGC still remain difficult[19]. This study aims at defining clinicopathological characteristics of UGC, which may provide some evidence on its diagnosis and treatment.

MATERIALS AND METHODS

Subjects

We retrospectively studied 23 cases of UGC visiting Tongji Hospital from 1990 to 2005, and compared the clinicopathological characteristics with that of 33 cases of PDGC, which included 15 diagnosed cases and 18 suspected cases.

Methods

All specimens were fixed in 4% formaldehyde solution, embedded in paraffin and cut into 4 μm thick sections. And all of them were diagnosed pathologically. Chi-square test and Fisher's exact probability test were used for statistical analysis.

Table 1 Analysis of past history of 23 UGC cases

Indications	UGC	PDGC
Age (yr)	59.57 ± 11.49	56.21 ± 11.62
Sex	M: 5, F: 18	M: 14, F: 19
Signs and symptoms		
Jaundice (*n*)	4	1
Stomachache (*n*)	20	24
Febrile (*n*)	1	1
Dyspepsia (*n*)	4	5
Body weight loss (*n*)	4	6
Abdominal mass (*n*)	0	3
Absence of symptoms (*n*)	1 (preoperative diagnosis is adenoma)	3
Multiple pregnancies (⩾ 3 times) (*n*)	13	14
Family malignancy history (*n*)	1, his father suffered with gastric cancer	2, their fathers respectively have lung cancer and hepatoma
Past history		
Gastritis (*n*)	4	3
Gastric ulcer (*n*)	3	2
Hepatitis B (*n*)	5	10
Contact with schistosome pestilent water (*n*)	9	8
Coexisted with cholecystolithiasis (*n*)	17	8[a]
Size of cholecystolithiasis (> 1 cm) (*n*)	7	5
Multiple (*n*)	17	6[b]
Complicated with choledoch carcinoma (*n*)	1	0
Biliary tract diseases found preoperatively		
Polypoid lesion of gallbladder wall (*n*)	3	7
Multiple (*n*)	2	6
Size (> 1 cm) (*n*)	2	3
Gallbladder wall thickening (*n*)	12	9
Gallbladder enlargement (*n*)	5	6
Atrophy of gallbladder (*n*)	2	1
Cholestasis (*n*)	2	0
Congenital capsular dilation of bile duct (*n*)	0	1
Coexist with extra-biliary-tract diseases		
Splenomegaly (*n*)	0	4
Kidney multiple cystis (*n*)	0	2
Hemangiomas of liver (*n*)	0	2
Heparadiposum (*n*)	1	5
Hepatic cirrhosis (*n*)	1	2
Esophageal hiatal hernia (*n*)	0	1
Lateral ventral hernia (*n*)	0	3

[a]$P < 0.01$ ($\chi^2 = 13.53$, *vs* UGC); [b]$P < 0.01$ ($\chi^2 = 17.39$, *vs* UGC).

RESULTS

Analysis of past history

The proportion of UGC coexisting with cholecystolithiasis was significantly higher than that of PDGC ($\chi^2 = 13.53$, $P < 0.01$). The infection rate of hepatitis B virus was 21.74% (5/23) in UGC and 30.30% (10/33) in PDGC. Nine (39.13%) of 23 patients with UGC and 8/33 (24.24) PDGC had contact with schistosome pestilent water. The rate of multiple pregnancies was 56.52% (13/23) in the patients with UGC and 42.42% (14/33) in PDGC. The primary location of the UGC was mostly in the neck and body of the gallbladder, and that of the PDGC was often in the body and bottom. The incidence of Nevin stage Ⅰ and Ⅱ of UGC was significantly higher than that of PDGC ($\chi^2 = 4.44$, $P < 0.05$ and $\chi^2 = 4.96$, $P < 0.05$) while that of Nevin stage Ⅴ UGC was significantly lower than that of PDGC ($\chi^2 = 7.59$, $P < 0.01$). According to the grading of carcinoma, the incidence of well-differentiated UGC was significantly higher than that of PDGC ($\chi^2 = 4.16$, $P < 0.05$), and that of poorly-differentiated UGC was significantly lower than that of PDGC ($\chi^2 = 4.48$, $P < 0.05$) (Table 1).

Condition of diagnosis and treatment

All cases of UGC in this study was found during or after open cholecystectomy, and no case was found during or after laparoscopic cholecystectomy. The ratios of UGC in open cholecystectomy and other cholecystectomies were 0.41% (23/5582) and 0.26% (23/8807), respectively. Preoperative misdiagnoses included cholecystolithiasis, adenoma, and hepatoma in order of frequency (Table 2).

Characteristics of pathology

The proportion of UGC with primary location in neck of gallbladder was significantly higher than that of the PDGC ($P = 0.020$) while the number of UGC with primary location in bottom of gallbladder was significantly lower than that of PDGC ($P = 0.023$). The number of UCG in the bottom and body of gallbladder was significantly lower than that of PDGC ($P = 0.047$). According to Nevin

Table 2 Diagnosis and treatment in 23 UGC cases

Indications	UGC	PDGC
Final diagnosis during operation	60.87% (14/23)	
Final diagnosis after operation	39.13% (9/23)	
Open cholecystectomy	0.41% (23/5582)	
Laparoscopic cholecystectomy	0% (0/3225)	
Imergency operation	13.04% (3/23)	
Main misdiagnosis causes (*n*)		
Hepatoma	2	
Cholecystolithiasis	14	
Adenoma	3	
Cholecystolithiasis coexisted with adenoma	4	
Special examination methods		
USG (*n*)	16	14
CT (*n*)	1	2
MRI (*n*)	0	1
USG + CT (*n*)	3	12
USG + MRI (*n*)	2	3
USG + CT + MRI (*n*)	1	1
Laboratory examinations		
Carcino-embryonic antigen (*n*)	1/2	0/10
Alpha-fetoprotein (*n*)	0/1	2/10
Alkali phosphatase (*n*)	0/0	2/4
γ-GT (*n*)	2/2	2/2
Lactate dehydrogenase (*n*)	0/0	1/1
5-nucleophosphatase (*n*)	0/0	1/1
Operations		
Cholecystectomy (*n*)	17	11
Cholecystectomy + regional lymph clearing (*n*)	2	4
Combined with partial hepatectomy (*n*)	3	10
Combined with partial excision of extrahepatic bile duct (*n*)	1	2
Combined with excision of outer metastasis (*n*)	0	5 (2 cases proved nontumorous metastasis postop)
Exploratory laparotomy (*n*)	0	1
Adjunctive therapy		
Postoperative radiotherapy (*n*)	0	3
Postoperative chemotherapy (*n*)	4	9
Treatment with TCM (*n*)	1	3
TACE with recrudescent tumor (*n*)	0	2

staging, the incidence of stage Ⅰ and Ⅱ was significantly higher in UGC than in PDGC (χ^2 = 4.44, P < 0.05 and χ^2 = 4.96, P < 0.05) while the incidence of stage Ⅴ was significantly lower in UGC than in PDGC (χ^2 = 7.59, P < 0.01). Based on the grading of carcinoma, the incidence of well-differentiated UGC was remarkably higher than that of PDGC (χ^2 = 4.16, P < 0.05), and the incidence of poorly-differentiated UGC was significantly lower than that of PDGC (χ^2 = 4.48, P < 0.05) (Table 3).

DISCUSSION

The proportion of UGC in gallbladder carcinoma ranged from 22% to 37.5%[20-22], and our result is 41.1% (23/56). The reported incidence of UGC found in open cholecystectomy were 1.7% in Germany[22] and 2.3% in Belgium[23], and it was 0.43% in China[11], and our result is 0.41% which is similar with domestic report.

Our results indicate that cholecystolithiasis play a more important role in the cancerization process of UGC than in PDGC. And hepatitis B, schistosome and multiple pregnancies may affect the cancerization process of gallbladder, which however, needs more evidences and studies in its mechanism. In diagnosis and treatment, our study indicates that there is no significant difference between UGC and PDGC in many aspects, such as age, sex, symptom, signs and complications. All cases of UGC in this study was found during or after open cholecystectomy, and no case was found during or after laparoscopic cholecystectomy. The ratio of UGC found in all cholecystectomies was 0.26% (23/8807). Preoperative misdiagnoses included cholecystolithiasis, adenoma, and hepatoma in order of frequency. These results indicate that more attentions should be paid to cholecystolithiasis, adenoma, and hepatoma in clinical diagnosis.

As for pathological characteristics in our study, the primary location of UGC was mostly in the neck and body of the gallbladder, and that of PDGC was often found in the body and bottom. According to Nevin staging and the grading of gallbladder carcinoma, the proportion of UGC was remarkably lower than PDGC when malignancy degree of carcinoma increased gradually. These results indicate that there are different characteristics between UGC and PDGC in primary location, Nevin staging and tumor grade.

Currently, there are more studies that have began to focus on UGC[24,25], but early diagnosis of gallbladder carcinoma is still difficult[26-30]. In order to improve the diagnosis of gallbladder carcinoma, epidemiological and clinicopathological studies should be conducted. Our

Table 3 Pathological characteristics of 23 UGC cases

Indications	UGC	PDGC
Primary location of gallbladder carcinoma (*n*)	(with record 16 cases)	(with record 21 cases)
Cystic duct	1	1
Neck of gallbladder	8	3[a]
Body of gallbladder	5	6
Bottom of gallbladder	0	6[b]
Boundary between neck and body of gallbladder	2	0
Body and bottom of gallbladder	0	5[c]
Nevin staging		
Ⅰ	6	2[d]
Ⅱ	5	1[e]
Ⅲ	7	8
Ⅳ	2	6
Ⅴ	3	16[f]
Cancerometastasis status (*n*)		
Gallbladder lymph node	1	0
Portal lymph node	0	1
Common bile duct lymph node	1	4
Latero-hepatic artery lymph nod	0	1
Nerve boundle in gallbladder wall	0	2
Extrahepatic bile duct	0	2
Liver	3	11
Diaphragmatic muscle	0	1
Peritoneum	0	1
Omentum	0	1
Pathohistological diagnosis		
Histological type (*n*)		
Adenocarcinoma	20	27
Adenoma canceration	2	2
Squamous carcinoma	1	2
Undifferentiated carcinoma	0	2
Grade (*n*)		
Well differentiated	14	11[g]
Moderately differentiated	3	4
Poorly differentiated	6	18[h]

[a]$P = 0.020$, *vs* UGC; [b]$P = 0.023$, *vs* UGC; [c]$P = 0.047$, *vs* UGC; [d]$P < 0.05$ ($\chi^2 = 4.44$, *vs* UGC); [e]$P < 0.05$ ($\chi^2 = 4.96$, *vs* UGC); [f]$P < 0.01$ ($\chi^2 = 7.59$, *vs* UGC); [g]$P < 0.05$ ($\chi^2 = 4.16$, *vs* UGC); [h]$P < 0.05$ ($\chi^2 = 4.48$, *vs* UGC).

study demonstrates that there are different characteristics between UGC and PDGC in some aspects, which may hopefully provide some beneficial evidence on early diagnosis of gallbladder carcinoma.

COMMENTS

Background

The difficulty in early diagnosis of gallbladder carcinoma results from its poor specificity in clinical symptoms and ambiguous early symptoms, which seriously affect prognosis. Some patients were found having gallbladder carcinoma only when they were diagnosed and treated because of other diseases. To date, diagnosis and treatment in unsuspected gallbladder carcinoma are still difficult. This study aims at clinicopathological characteristics of unsuspected gallbladder carcinoma, which may provide some evidence on its diagnosis and treatment.

Research frontiers

Unsuspected gallbladder carcinoma found during cholecystectomy because of acute or chronic cholecystitis was reported early in 1961, but more relative studies in and close attention to unsuspected gallbladder carcinoma became available after the 1990s. However, its epidemiology and clinicopathology are not well described.

Innovations and breakthroughs

There were different characteristics between unsuspected gallbladder carcinoma and preoperatively diagnosed gallbladder carcinoma, such as in primary location, malignant degree and incidence of coexistence with cholecystolithiasis. Cholecystolithiasis, hepatitis B, schistosome and multiple pregnancies were high risk factors of gallbladder carcinoma.

Applications

In order to improve the diagnosis of gallbladder carcinoma, epidemiology and clinicopathology need be studied. This study indicates that there are different characteristics between unsuspected gallbladder carcinoma and preoperatively diagnosed gallbladder carcinoma in some aspects, which hopefully could provide some beneficial evidence on early diagnosis of gallbladder carcinoma.

Terminology

Unsuspected gallbladder carcinoma (UGC): generally gallbladder carcinoma is called unsuspected gallbladder carcinoma (UGC) when it was found incidentally during or after cholecystectomy. We consider that unsuspected gallbladder carcinoma still includes gallbladder carcinoma found not only during or after cholecystectomy but also in the course of other diseases.

Peer review

The present study is interesting, well designed, and contained novel findings. The conclusions are well based and are of clinical value in some aspects.

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RAPID COMMUNICATION

Significance of blood analysis in hemophiliacs co-infected with human immunodeficiency virus and hepatitis viruses

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Abstract

AIM: To study the effect of hepatitis virus infection on cirrhosis and liver function markers in HIV-infected hemophiliacs.

METHODS: We have analyzed the immunological, liver function and cirrhosis markers in a cohort of hemophiliacs co-infected with human immunodeficiency virus (HIV) and hepatitis viruses.

RESULTS: There was no difference in immunological markers among co-infected patients and patients infected with HIV only and those co-infected with one or more hepatitis virus. Although liver function and cirrhosis markers remained within a normal range, there was a worsening trend in all patients co-infected with hepatitis virus C (HCV), which was further exacerbated in the presence of additional infection with hepatitis virus B (HBV).

CONCLUSION: Co-infection with HIV, HBV and HCV leads to worsening of hyaluronic acid and liver function markers. Increases in serum hyaluronic acid may be suggestive of a predisposition to liver diseases.

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Key words: Human immunodeficiency virus; Human immunodeficiency virus; Liver function markers; Liver cirrhosis markers

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INTRODUCTION

Co-infection with hepatitis virus B and C (HBV and HCV) is very common in individuals with human immunodeficiency virus (HIV) infection, presumably due to the shared transmission route of these viruses. The prevalence of HCV infection among HIV-infected individuals can be as high as 40% but varies substantially among risk groups[1]; cirrhosis is an increasingly prevalent cause of morbidity in such patients[2]. The prevalence of HCV infection among HIV-infected drug users (IDUs) is 50%-90%[1,3]. HCV co-infection increases the risk of cirrhosis by 10-20 fold in people infected with HIV[4]. This is especially apparent in the era of highly active antiretroviral therapy (HAART), possibly due to the improved survival rate. HAART itself may also influence liver diseases[4]. Co-infection with HBV and HIV is also common, and 70%-90% of HIV-infected individuals had past or active infection with HBV[5]. The prevalence of HIV within a cohort of IDUs in Yunnan, China was found to be 71.9%, 99.3% of them being co-infected with HCV[6]. In another Chinese study, HCV and HIV prevalence was 75% and 18.5% in IDUs, with 58% HCV patients being co-infected with HIV[7]. Treatment with contaminated blood products prior to 1990 resulted in a high prevalence of HIV, HBV and HCV in hemophiliacs; 85% of persons infected with HIV are co-infected with HCV[1,3]. In a multi-center hemophilia cohort study, 30% of HCV seropositive survivors had HIV and 4.6% were HBV carriers[8]. Co-infection data is not available for hemophiliacs in China.

Our data show that in a cohort of HIV-infected hemophiliacs, co-infection with HBV or HCV or both leads to worsening of a key cirrhosis marker, hyaluronic acid (HA). Taken together with recent literature[9], our data suggest that HA levels may be suggestive of progression to liver diseases in these patients.

MATERIALS AND METHODS

Patients

Laboratory data from the first visit of all hemophiliacs (previously diagnosed with HIV) reported to Shanghai Public Health Center between May and October 2004 were analyzed.

Table 1 Blood viral load, immune and biochemical markers in hemophiliac patients mean ± SEM

	Hemophiliac patients			
	HIV/HBV	HIV/HCV	HIV/HBV/HCV	HIV
No.	5	17	22	6
HIV viral load[1]	6.6×10^2 (< 50-10^5)	3.12×10^2 (< 50-10^5)	2.12×10^2 (< 50-10^5)	1.4×10^2 (< 50-10^4)
HBV viral load	Not detectable	Not applicable	Not detectable	Not applicable
HCV viral load	Not applicable	2.51×10^5 (< 10^3-10^7)	8.07×10^4 (10^3-10^7)	Not applicable
CD4[2] count	19.4 ± 3.4	21.56 ± 1.9	18.05 ± 1.7	25.17 ± 1.5
CD4/CD8	0.45 ± 0.13	0.46 ± 0.06	0.41 ± 0.06	0.63 ± 0.06
ALT (U/L)	18.2 ± 2	46 ± 8	47.6 ± 7.5	
AST (U/L)	24.2 ± 2	44.8 ± 9	53.8 ± 9	
TBIL (mol/L)	7.5 ± 1	10.4 ± 2	11.3 ± 1	
ALP (U/L)	188.2 ± 42	214 ± 16	207.3 ± 15	
γ-GT (U/L)	39.2 ± 17	103.5 ± 23	145.7 ± 25	
A/G	1.41 ± 0.1	1.4 ± 0.07	1.7 ± 0.4	
HA (ng/mL)	56.8 ± 12	73.8 ± 4.5	93 ± 15	
CG (μg/dL)	183 ± 15	230 ± 65	257 ± 15	
PC3 (μg/L)	108.3 ± 8	106.6 ± 3.2	107 ± 2.5	
LN (ng/mL)	121.2 ± 19	111.7 ± 4	114 ± 3	
IVC (ng/mL)	55.5 ± 22	46.5 ± 1.5	46 ± 1	

[1]Geometric mean viral loads and range of HIV RNA, HBV DNA and HCV RNA; [2]Represented as percent of the total T cell (CD3+) population.

All patients were infected with HIV-1, group M and subgroup B and were on HAART at the time of data collection as per the guidelines issued by China Center for Disease Control (The National AIDS Anti-viral Treatment Guidelines, stavudine (d4T), lamivudine (3TC), and efavirenz). Patients are started on antiretrovirals if their CD4 count is < 200/mm^3 or HIV-RNA is > 30 000 copies/mL.

All patients co-infected with HCV were on 180 mg PEGylated interferon weekly and ribavirin > 10.6 mg/kg daily. Patients are started on the therapy if their anti-HCV antibody is detected in the serum or HCV-RNA is > 1000 copies/mL, or if they have consistently abnormal aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values.

Patients with HBV co-infection were all on lamivudine therapy as part of HAART; and at the time of analysis, HBV DNA was below the limit of detection.

Patients infected with HIV only were compared with those co-infected with hepatitis viruses and patients co-infected with either HBV or HCV were compared with patients co-infected with both HBV and HCV.

Data was also collected from a control group of non-haemophiliac patients infected with either HIV or HCV or HBV. These control patients were not on any antiviral therapy at the time of data collection. Data from the control groups were compared with that from co-infected patients.

Laboratory analysis

HIV infection was diagnosed by a screening test on 50 L whole blood (immunogold silver staining kit, Dainablot Co. Ltd, Tokyo, Japan); confirmatory Western blot was performed at the Center of Disease Control, Shanghai. HBV and HCV infections were diagnosed by real time PCR on 200 L serum (PG Biotec, China, Bio-Rad iCycler). Anti-HBV and anti-HCV antibodies were estimated in 1 mL patient serum by microparticle enzyme immunoassay (ABBOT Diagnostics Division, USA) and ELISA (HENAN Sino-American Bio, China), respectively.

Cell markers were analyzed by FACS on a BD FACSCalibur using 50 μL whole blood and antibodies to CD3/CD8/CD45/CD4 (Becton and Dickinson Co. Ltd).

Cirrhosis markers (HA; cholylglycocholic acid, CG; N-terminal peptide of type Ⅲ collage, PC3; laminin, LN and collagen IV, IVC) were analyzed by radioimmunoassay (Tongji Unversity Shanghai Radioimmunological Technology Co. LTD). Liver function tests (ALT; AST; alkaline phophatase, ALP; glutamyl transferase, GT; ratio of albumin to globulin, A/G; total bilirubin, TBIL; pro-albumin, PA) were performed using the Roche multi-analyzer.

Statistical analysis

Unless otherwise stated, values are expressed as mean ± standard error (SEM). Comparisons were done using one-way ANOVA, with statistical software in Microsoft Excel. Statistical significance was accepted at $P < 0.05$.

RESULTS

Patients

The patient data are shown in Table 1. Of the 50 HIV-infected hemophiliacs, 22 (44%) were co-infected with HBV, 39 (78%) with HCV, and 17 (34%) were co-infected with both HBV and HCV. All patients were males, with age ranging from 14 to 68 years (mean = 38.08) at the time of data collection in 2004. No liver function data were available for those with HIV mono-infection ($n = 6$). Genotype data was available for 31 of the 39 patients co-infected with HCV; 16 were infected with genotype 1b, 10 with 2a and 5 with 2a/2b.

Viral load

HIV viral load in patient blood varied from undetectable (< 50 copies) to 100 000 copies/mL and was not different

Table 2 Blood viral load, immune and biochemical markers in non-hemophiliac patients mean ± SEM

	Non-hemophiliac patients		
	HBV	HCV	HIV
No.	17	14	20
HIV viral load[1]	Not applicable	Not applicable	1.4×10^2 (< 50-10^4)
HBV viral load	8.8×10^6 (2.6×10^3-10^8)	Not applicable	Not applicable
HCV viral load	Not applicable	9×10^5 (2.6×10^3-10^7)	Not applicable
CD4[2]	53.53 ± 4.15	41.43 ± 3.04	18.25 ± 1.83
CD4/CD8	1.93 ± 0.3	2.08 ± 0.26	0.63 ± 0.06
ALT (U/L)	266.6 ± 79.4	92.64 ± 22.6	34.35 ± 11.7
AST (U/L)	297.5 ± 108	77.5 ± 10	30.15 ± 3.82
TBIL (mol/L)	113.4 ± 36	31 ± 5.8	14.18 ± 1.42
ALP (U/L)	110 ± 7.9	118 ± 19.7	45.44 ± 0.63
γ-GT (U/L)	95.9 ± 17.8	87.6 ± 22	26.75 ± 5.52
HA (ng/mL)	323.2 ± 64.6	424.14 ± 78	83.9 ± 15.25
CG (μg/dL)	2678 ± 409	2050 ± 485	410 ± 61
PC3 (μg/L)	196 ± 21	236 ± 36	159.6 ± 28.4
LN (ng/mL)	122.6 ± 7.2	118 ± 11	106.65 ± 5.24
IVC (ng/mL)	100 ± 11	124 ± 15.6	74 ± 11.7

[1]Geometric mean viral loads and range of HIV RNA, HBV DNA and HCV RNA; [2]Represented as percent of the total T cell (CD3+) population.

significantly between mono- and co-infected patients, as well as among all three co-infected patient groups. HCV co-infected patients had moderate to high levels of HCV viral load, ranging from below 1000 to 10 million copies/mL (Table 1), there was no significant difference between HIV/HCV and HIV/HBV/HCV infected patients.

Patients infected with HCV and not on antiviral therapy (Table 2) had generally higher viral loads, ranging from more than 2600 to 10 million copies/mL but did not differ significantly from viral loads in co-infected patients.

Immunological markers

All three groups of co-infected patients had low CD4 counts; CD4 cells formed less than 22% of the total T cell population (Table 1). These levels were lower than, but not significantly different from the CD4 counts of HIV mono-infected hemophiliac patients. The mean ratio of CD4 to CD8 cells in peripheral blood was below 0.5 and did not differ between patient groups (Table 1).

Non-hemophiliac patients infected with HBV or HCV had higher CD4 levels, and CD4 cells formed more than 40% of the total T cell population. These levels were higher than those in non-hemophiliac HIV infected patients (18.25%). Similarly, the ratio of CD4 to CD8 cells was higher than 1.9 in HCV and HBV infected non-hemophiliac patients but only 0.63 in HIV infected non-hemophiliacs. When all patients were considered together, the CD4 to CD8 ratio differed significantly between HIV-infected and non-infected patients.

Liver function markers

Liver function data in hemophiliacs were available only for co-infected patients. Although all liver function markers analyzed were within normal range, there was a clear trend toward deterioration, i.e., patients co-infected with both

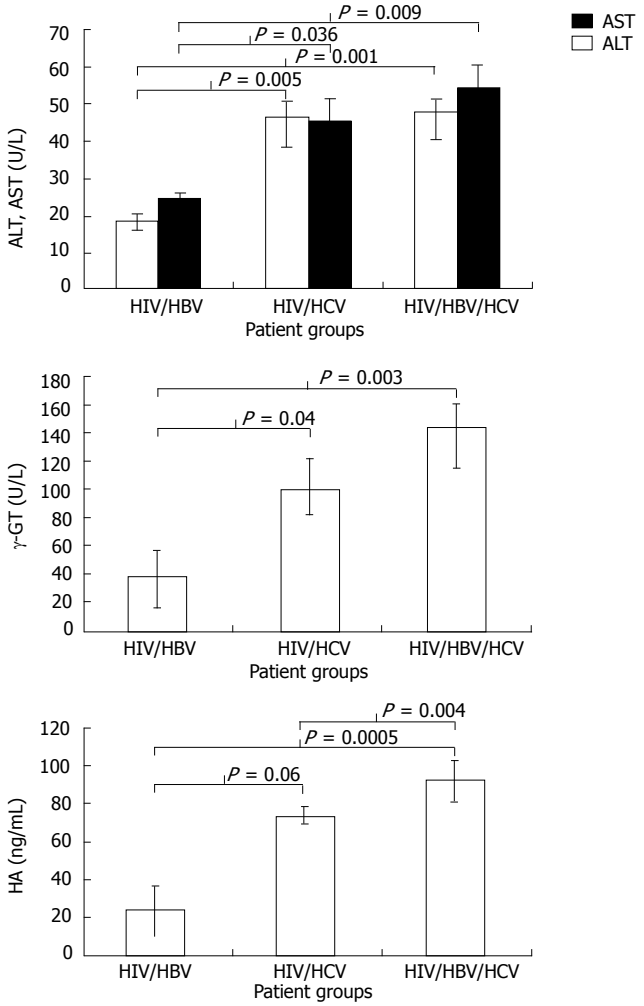

Figure 1 Liver disease markers in HIV patients co-infected with HBV, HCV or both.

HCV and HBV had poorer indicators than those with either HCV or HBV co-infection; liver function markers in patients with HCV co-infection were worse than in those with HBV co-infection. There was a significant difference in ALT between groups with and without HCV co-infection (HIV/HBV *vs* HIV/HBV/HCV, $P = 0.001$; HIV/HBV *vs* HIV/HCV, $P = 0.005$), but no significant difference between groups with HCV co-infection, although a trend toward further worsening of markers was observed in those with additional HBV co-infection. A similar progressive trend was also found in AST and γGT values (Figures 1A and B). There was no significant difference in ALP, TBIL and A/G among the three groups (Table 1).

Patients infected with HBV or HCV, prior to treatment with antivirals, had overall worsened liver function markers, but similar ALP and γGT levels to co-infected patients during treatment (Tables 1 and 2). All markers were higher in HBV-infected than HCV-infected patients. Liver function markers in non-hemophiliac HIV patients were within normal range and not different from those in hemophiliacs.

Cirrhosis markers

Among the five markers of cirrhosis measured and analyzed in this study, four were not different among

patient groups (Table 1). Serum HA was lower in patients with HIV/HBV infection than in those with HIV/HCV, with significant difference ($P = 0.06$), and was significantly lower than in patients with HIV/HBV/HCV infection ($P = 0.0005$, Figure 1C). Patients with HIV/HCV co-infection had significantly lower serum HA levels than those with HIV/HBV/HCV infection ($P = 0.00004$). The HA always remained within normal range, suggesting no fibrosis[10] except for one patient with HCV co-infection who had a very high serum HA level. This data were not included in the analysis.

Four of the five cirrhosis markers were considerably elevated in non-hemophiliac patients infected with HBV or HCV, and 2-10 fold that in hemophiliac patients (Tables 1 and 2). Only CG was increased in non-hemophiliac HIV infected patients compared with hemophiliacs.

DISCUSSION

Our study of a cohort of hemophiliacs infected with HIV show that co-infection with HCV leads to deterioration in liver function and cirrhosis markers, and HA is further worsened in the presence of additional HBV infection. Our data add to the accumulating literature, suggesting that serum HA levels may be used in initial test for progression to cirrhosis.

The prevalence of HCV co-infection within this group was similar to that previously reported in cohorts of IDUs and hemophiliacs[7,8]. Although the prevalence of HBV was lower than HCV (44%), it was higher than the previously reported prevalence of HBs antigenemia within a Chinese population and lower than any other markers of HBV infection[11]. HBV co-infection rates in our study may reflect the endemic nature of the disease rather than transmission *via* a common route with HIV and HCV. The high incidence (34%) of co-infection with all three viruses argues against this probability.

All patients were treated with lamivudine and HBV viral load was below the detection limit[12]. HCV viral load varied substantially and was below the detection limit in three patients with HCV co-infection only. The three patients also had undetectable HIV viral loads. There was no correlation in viral load or liver function markers between HCV and HIV. In contrast to previous studies[13], HCV infection did not appear to affect CD4 recovery as there was no difference in CD4 levels and CD4/CD8 ratios among the patient groups. Our data could not be used to evaluate the effect of HAART in liver diseases as all co-infected patients were on HAART throughout the study. HCV viral load did not differ significantly between patients on HAART or not, suggesting that HIV infection and HAART in these patients did not have any effect on HCV viral load.

Since the advent of HAART, liver disease has emerged as the major cause of morbidity and mortality in HIV patients co-infected with hepatitis viruses[14]. In our study, there was a trend towards deterioration of liver function associated with infection with HCV. Patients co-infected with HBV had the best results within the group. Patients co-infected with both HBV and HCV had the worst liver function indices, suggesting that HBV and HCV co-infection may have a synergistic effect on disease progression. Both AST and ALT values in HIV/HCV infected patients improved with ribavirin PEG-interferon therapy, reaching levels different from HIV/HBV infected patients after 3-5 mo (data not shown). The same markers in the serum of HIV/HBV/HCV infected patients did not respond to the therapy within a similar time frame.

Antiviral therapy was obviously effective in improving the liver function of HBV and HCV infected individuals as non-treated patients had significantly worse markers of liver disease.

HA levels have shown to correlate with fibrosis and may be used as diagnostic markers[10]. Higher serum HA levels were associated with HCV co-infection, and were further increased in the presence of additional HBV infection. Interestingly, serum HA increased in HIV/HBV/HCV co-infected patients at the time of the data collection from 129.8 ± 12 ng/mL to 210 ± 47 ng/mL, suggesting progression towards fibrosis while no such increase was observed in patients co-infected with HBV or HCV only. As none of the patients progressed to clinically evident cirrhosis during this study (based on normal liver ultrasound results), and liver biopsies were not performed, and we therefore, cannot directly correlate HA levels with cirrhosis. However, our data is in agreement with recent studies showing that some HIV patients co-infected with HCV and having normal liver function, have significant fibrosis as evidenced by liver biopsy[9,15]. Similar to the liver function tests, cirrhosis markers were significantly worse in those infected with either HBV or HCV but not treated with antiviral therapy, showing the efficacy of antiviral therapy in hepatitis patients.

To the best of our knowledge, this is the first study of the effect of hepatitis virus infection on cirrhosis and liver function markers in HIV-infected hemophiliacs from China, marking an important first step towards a better understanding of hepatitis virus co-infection in HIV patients. The cohort analyzed here represents a special group within China, they have free access to the best medical care through a special initiative of the government and may not reflect the situation in the general population of HIV infected persons.

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RAPID COMMUNICATION

Vagina vasorum dissection during D2 lymphadenectomy for gastric carcinoma

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Abstract

AIM: To explore the relationship between metastasis and vagina vasorum in the progress of gastric carcinoma and to find some facts and references for gastric surgeons.

METHODS: One hundred and seven specimens of left or right gastric arteries (55 left and 52 right) were gathered from 59 patients undergoing radical gastrectomy for gastric carcinoma. All the frozen specimens were cut into 3 μm-thick sections and stained with hematoxylin-eosin (HE) and immunohistochemical method separately. Cytokeratin (CK) and mesothelial cells (MC) were stained with immunohistochemical method. Cancer cells inside vagina vasorum were detected and the structure of artery wall was observed under microscope.

RESULTS: Metastatic cancer cells or tubercles were found inside vagina vasorum in some stage Ⅲ or Ⅳ specimens, but not in stage Ⅰ or Ⅱ specimens. Tumor cells in vagina vasorum were CK positive in 26 specimens of 14 tumors. Among them, stage Ⅲ was found in 4 specimens of 2 tumors, and stage Ⅳ in 22 specimens of 12 tumors. None of these specimens was positive for MC. The positive rate of CK increased with TNM staging. Compared with the lower part, tumors in the upper and middle parts of stomach were more likely to metastasize into vagina vasorum.

CONCLUSION: Vagina vasorum dissection should be performed during D2 lymphadenectomy for TNM stage Ⅲ or Ⅳ gastric carcinoma.

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Key words: Gastric carcinoma; Radical gastrectomy; Vagina vasorum; Lymphadenectomy; Cytokeratin

Peng JJ, He YL, Zhan WH, Xiao P, Cai SR, Zhang CH, Wu H. Vagina vasorum dissection during D2 lymphadenectomy for gastric carcinoma. *World J Gastroenterol* 2007; 13(12): 1867-1869

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INTRODUCTION

Whether vagina vasorum should be dissected is a hot problem. Extended lympathadenetomy (D2) is the standard radical gastrectomy for gastric carcinoma[1,2]. Now more and more gastric surgeons want to reach a consensus on this. However, vagina vasorum is a layer with tough dense connective tissue surrounding vessels. We want to know the function of vagina vasorum around gastric arteries during the progress of gastric carcinoma. We designed this study to elucidate the function of vagina vasorum around gastric arteries during the progress of gastric carcinoma and to find the relationship between vagina vasorum and metastasis of gastric cancer cells.

MATERIALS AND METHODS

Materials

One hundred and seven specimens of left or right gastric arteries (55 left and 52 right) were collected consecutively from 59 gastric cancer patients. All patients underwent radical gastrectomy for gastric carcinoma in the First Affiliated Hospital of Sun Yat-Sen University. These cases included TNM stages Ⅰ-Ⅳ gastric cancer. Thirty-three patients were male (55.93%) and twenty-six were formale (45.07%). Their average age was 55.2 ± 13.4 years. Pathologic grading was done after each radical gastrectomy for gastric cancer (Table 1).

Methods

All the 107 frozen specimens were cut into 3 μm–thick sections. The sections were divided into 3 groups (A-C). All the specimens were stained with hematoxylin-eosin (HE) to find metastatic cancer cells inside vagina vasorum of the left and right gastric arteries. Cytokeratin was used in immuno-histochemical method for its sensitivity to epithelial tumor. At the same time, we also used mesothelial cells (MC) to eliminate mesothelial cell cancer.

Table 1 Pathologic grading of specimens

TNM stage	Patients (*n*)	Gastric arteries		%
		GLA	GRA	
Ⅰ	6	5	5	10.2
Ⅱ	13	11	12	22.0
Ⅲ	17	16	14	28.9
Ⅳ	23	23	21	38.9

GLA: Gastric left artery; GRA: Gastric right artery.

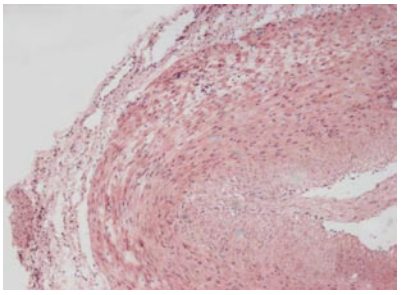

Figure 1 HE staining showing vagina vasorum and the structure of artery wall (× 200).

Table 2 CK positive rate in different stages of gastric cancer

TNM staging	CK		Total	CK positive rate (%)	*P*	*P*
	Positive	Negative				
Ⅰ	0	6	6	0		
Ⅱ	0	13	13	0		< 0.05
Ⅲ	2	15	17	11.8	< 0.05	
Ⅳ	12	11	23	52.2		
Total	14	45	59	23.7		

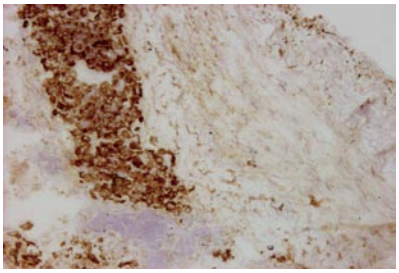

Figure 2 CK positive gastric cancer cells metastasized into vagina vasorum (IHC × 200).

Table 3 CK positive rate for lymph nodes

Lymph node	CK		Total	CK positive rate (%)	*P*
	Positive	Negative			
Positive	12	12	24	50.0	< 0.01
Negative	2	33	35	5.7	

Table 4 CK positive rate for different locations of stomach

Location	CK		Total	CK positive rate (%)	*P*
	Positive	Negative			
Upper	4	8	12	33.3	
Middle	4	9	13	30.8	
Lower	5	28	33	15.2	< 0.01
Total	13	45	58	22.4	

The sections were examined under light microscope.

Statistical analysis

All data were analyzed with SPSS11.0 for windows. The comparison of two sets of discrete differential counts was done with chi square test. $P < 0.05$ was considered statistically significant.

RESULTS

HE staining

Vagina vasorum structure and red blood cells in vessels could be found in 107 specimens with HE staining. Even metastatic cancer tubercles were found inside vagina vasorum (Figure 1).

Immunohistochemistry staining

Metastatic cancer cells or tubercles inside vagina vasorum were cytokeratin (CK) positive in 26 specimens of 14 tumors. Among them, stage Ⅲ was found in 4 specimens of 2 tumors, and stage Ⅳ in 22 specimens of 12 tumors (Figure 2). No cancer cell was found in stage Ⅰ or Ⅱ specimens of vagina vasorum. All the cancer cells were stained brown with CK. However, no malignant mesothelium was found, as none of them was MC positive.

With the progression of gastric cancer, cancer cells would move into the vagina vasorum around gastric arteries. In this study, we compared CK positive rates in different TNM stages (Table 2). Fifty-nine specimens met the criteria for the TNM staging in CK positive cases. The positive rate of CK was higher in stages Ⅲ and Ⅳ than in stages Ⅰ and Ⅱ ($P < 0.05$). Moreover, there was a significant difference between stages Ⅲ and Ⅳ ($P < 0.05$).

D2 lymphadenectomy is to dissect lymph nodes. Lymph nodes in the left gastric artery, common hepatic artery and celiac artery were dissected during extended lymphadenectomy. The dissected lymph nodes were counted. The criteria for positive and negative lymph nodes were metastatic cancer cells found in any lymph node and no cancer cells found in lymph nodes, respectively. The CK positive rate in positive lymph nodes was higher than that in negative lymph nodes (Table 3).

The stomach is anatomically divided into upper, middle, and lower thirds. Cases were divided into three groups according to the location of the tumors, except for one case of diffuse infiltrative gastric cancer (Table 4). Metastasis of vagina vasorum tumor in the upper and middle thirds was more common than that in the lower third.

DISCUSSION

Several factors can produce a good prognosis of gastric cancer. However, the only way to improve survival rate is to remove the carcinoma tissues thoroughly. During radical gastrectomy for gastric carcinoma, *en bloc* excision of tumor, dissection of lymph nodes and elimination of dissociative cancer cells in abdominal cavity are the

three goals[3]. The Japanese Gastric Cancer Association advised that D2 lymphadenectomy is the standard operation for gastric cancer[1,2]. This has been approved by more and more surgeons in the world. The comparison between D2 and less D2 lymphadenectomy has been reported[4-7]. The 5-year overall survival rate for patients treated with D2 lymphadenectomy is higher than that for those not treated with D2 lymphadenectomy[7-9]. It was reported that modified D2 lymphadenectomy sparing the spleen and pancreas could be performed safely and may prolong the survival of patients[10]. However, the postoperative mortality and morbidity are corresponding in two groups[5,11]. Whether the vagina vasorum of gastric artery should be removed during D2 lymphadenectomy remains controvisial. The vagina vasorum is a layer with tough dense connective tissue surrounding vessels containing a large number of capillaries, lymphangion and a few of neurofibrae[12]. It was reported that if the vagina vasorum was reserved, metastatic cancer cells inside vagina vasorum may be residual, although whether gastric cancer cells metastasize through vagina vasorum cannot be confirmed[3,13]. Rovertson *et al*[14] reported a prospective randomizaed trial comparing R1 with R3 gastrectomy in 1994, showing that skeletonization of the vessels in R3 gastrectomy for gastric cancer could lead to a high morbidity because of intra-abdominal sepsis. Dong *et al*[15] presumed that pseudoaneurysm of gastroduodenal artery following radical gastrectomy for gastric carcinoma is caused by the weakness in arterial wall due to the skeletonization resulting from lymphadenectomy.

In the earlier stage of gastric cancer, no cancer cells could be found inside vagina vasorum of gastric artery. However, tumor cells appeared in stage Ⅲ or Ⅳ vagina vasorum tumors, and even cluster of cancer cells could be found in vagina vasorum of artery in some more advanced cancer samples, suggesting that tumor cells have the tendency to metastasize into vagina vasorum during the progression of carcinoma. By comparing results in Table 2 and 3, metastasis of cancer cells inside vagina vasorum increased with the development of gastric carcinoma, especially in patients accompanying lymph node metastasis.

Tumors in the upper and middle thirds of stomach had a greater tendency to metastasize into vagina vasorum compared to gastric cancer in the lower third of stomach. Though we cannot explain the differences, the prognosis of tumors in the lower third of stomach is better than that in any other locations of stomach[16-18]. We presume that the prognosis of gastric cancer is associated with the metastasis of gastric cancer cells into the vagina vasorum.

In conclusion, vagina vasorum around gastric arteries should be dissected during D2 lymphadenectomy for gastric cancer. Metastatic gastric cancer cells should be removed thoroughly. Accurate pre-operative and operative pathologic staging is essential.

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RAPID COMMUNICATION

Significance of hepatitis B virus surface antigen, hepatitis C virus expression in hepatocellular carcinoma and pericarcinomatous tissues

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Abstract

AIM: To investigate the correlation between hepatitis B virus surface antigen (HBsAg), hepatitis C virus (HCV) expression in hepatocellular carcinoma (HCC), the HAI score of the noncancerous region of the liver and the serum Alpha fetoprotein (AFP) level.

METHODS: The patterns of HBsAg and HCV in 100 cases of HCC and their surrounding liver tissues were studied on paraffin-embedded sections with immunohistochemistry, the histological status was determined by one pathologist and one surgeon simultaneously using the hepatitis activity index (HAI) score, and AFP was detected by radioimmunity. The study included 100 consecutive patients who underwent curative resection for HCC. Based on HBsAg and HCV expression, the patients were classified into 4 groups: patients positive for HBsAg (HBsAg group), patients positive for HCV (HCV group), patients negative for both HCV and HBsAg (NBNC group) and patients positive for both HBsAg and HCV (BC group).

RESULTS: The BC group had significantly higher HAI scores than the other three groups. (BC > HCV > HBsAg > NBNC). HBV and HCV virus infection was positively correlated with HAI (r_s = 0.39, P = 0.0001). The positive rate of AFP (85.7%) and the value of AFP (541.2 ng/mL) in the group with HBV and HCV co-infection were the highest among the four groups. The positive rate (53.3%) of AFP and the value of AFP (53.3 ng/mL) in the group with none-infection of HBV and HCV were the lowest. HBV and HCV virus infection was positively correlated with AFP(r_s = 0.38, P = 0.0001).

CONCLUSION: The AFP increase in patients with liver cancer was positively correlated with the infection of HBV and HCV. The serum AFP elevation by the infection of HBV and HCV is one of mechanisms which lead to hepatocarcinogenesis, and the antivirus intervening treatment of hepatitis is significant for the prognosis of liver cancer. From our Spearman's rank correlation analysis, we can conclude that the severity of virally induced inflammation is correlated with HBsAg and HCV expression in HCC tissues and noncancerous tissues. Prior co-infection of HBV in HCV patients may be an adverse risk factor for intrahepatic inflammation.

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Key words: Hepatitis B virus surface antigen; Hepatitis C virus antigen; Histological activity index; Immunohistochemistry; Hepatocellular carcinoma; Alpha-fetoprotein.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide[1,2]. Although *H pylori* can be detectable in the HCC tissues[3], HBV has been reported to be the prime cause for the development of HCC with regard to the major microorganism etiologies of HCC. The incidence of HCC is clearly linked to the rate of chronic HBV infection. A large percentage of HCC patients show serological or histological markers of past contact with hepatitis B virus (HBV)[4]. Likewise, hepatitis B virus (HCV) infection predisposes to HCC[5].

Most patients with HCC in China have hepatitis virus-induced chronic liver disease (HBV or HCV). The infection rate of HBV has reached 10% in China, and HCV has reached 3.2%. This causes loss of hepatic reserve, and subsequently limits the extent of resection for HCC patients. Poor hepatic reserve can also be a major obstacle to multidisciplinary treatments for post-hepatectomy recurrences. HBsAg and HCV expression can really reflect HBV and HCV infection in HCC. In order to discuss the relationship between the patients'

viral histological status and the degree of virally induced inflammation, we studied the clinical data of HCC and examined HBsAg and HCV expression in HCC tissues and noncancerous tissues.

MATERIALS AND METHODS

Patients

All patients participating in this study gave informed consent before surgery. A total of 100 patients (66 males and 34 females, mean age 53.6 ± 24.1 years, range 22-78 years) who underwent hepatectomy for HCC from January 2000 to September 2005 were included in this study. The patients, who had undergone hepatectomy and hepatic arterial chemoembolization (TACE) or who had alcoholic liver cirrhosis, were excluded. HCC tissues were obtained from all patients. HCC tissues and surrounding liver tissues were examined for HBsAg and HCV. We also investigated the HBsAg and HCV expression in normal liver tissues without HCC cells or HBV and HCV, which was surgically obtained from the pathological specimens of patients undergoing surgical resection for other diseases or at autopsy.

The pathologic diagnosis and classification of variables were based on the criteria recommended in the General Rules for Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of China 2001). Clinicopathological parameters analyzed included sex, age, liver pathology (HAI score) (Table 1) and Alpha fetoprotein (AFP) (Table 2). Eighty-seven patients were positive for HBsAg (HBsAg group), 27 were positive for HCV (HCV group), 6 were negative for both HCV and HBsAg (NBNC group) and 21 were positive for both HBsAg and HCV (BC group).

HAI score

Based on the microscopic findings of the non-cancerous region of the surgical specimen by hematoxylin and eosin staining, the histological status was determined by one pathologist and one surgeon simultaneously using the hepatitis activity index (HAI) score. The inflammatory activity of coexisting hepatitis was graded according to the histological activity index (HAI score) of Knodell *et al*[6] as follows (Table 3). The scores for three features (degree of hepatocellular periportal necrosis and bridging, degree of introlobular degeneration and focal hepatocellular necrosis, and degree of portal inflammation) were calculated for each individual. The HAI score in each group was expressed as the average of the scores in three visual fields. The degree of histopathological derangement was comparable in the HBsAg and HCV groups; however, the HAI score in the BC group was significantly higher than that in the other three groups (Table 1).

All primary antibodies (polyclonal antibodies) to HBsAg, HCV and secondary antibodies were purchased from Zhongshan Biotechnology Limited Company (Beijing, China).

Immunohistochemistry

To obtain more accurate dada, we selected the tissue blocks containing HCC and surrounding liver tissues. Each specimen was cut into 4-μm thick sections. Tissue wax sections were unfolded on glass sheet. Immunohistochemistry (strepolin-biotin-peroxidase method) was used to detect the expression of HBsAg and HCV. Briefly, paraffin-embedded tissue sections were dewaxed, treated with 3% H_2O_2 at 37℃, washed with PBS, incubated with HBsAg and HCV antibodies separately, washed with PBS for 15 min, incubated again with strepolin-biotin-peroxidase at 37℃. Finally, the sections were washed with PBS for 15 min, visualized

Table 1 Correlation between HAI scores and HBsAg and HCV expressions in HCC and noncancerous tissues

Groups	*n*	Rate (%)	HAI score in noncancerous tissues
NBNC	6	6	6.67 ± 2.58
HBsAg	66	66	9.80 ± 2.97
HCV	7	7	10.71 ± 4.82[a,c]
BC	21	21	12.62 ± 3.88[b]

[a]$P > 0.05$ *vs* the other three groups; [c]$P < 0.05$ *vs* NBNC group *vs* HBsAg group; [b]$P < 0.01$ *vs* NBNC group and HBsAg group. Spearman correlation analysis between HAI scores and expressions of HBsAg and HCV in HCC and noncancerous tissues, $r_s = 0.393$, $t = 4.23$, $P = 0.0001$.

Table 2 Correlation between AFP and expressions of HBsAg and HCV in HCC and noncancerous tissues

Groups	*n*	AFP negative	AFP positive	Positive rates of AFP (%)	Mean of AFP (ng/mL)	Average sum of ranks
NBNC	6	4	2	33.3	53.3	14.3
HBsAg	66	22	44	66.7	352.6	48.7
HCV	7	3	4	57.1	348.2	50.1
BC	21	3	18	85.7	541.2	66.7

AFP negative means AFP value ≤ 20 ng/mL and AFP positive means AFP value ≥ 20 ng/ mL. Rank-sum test, H = 16.11, $P = 0.001$; coparation between two groups, $P < 0.001$. Spearman correlation analysis, $r_s = 0.38$, $t = 4.06$, $P = 0.0001$. chi square test, Fisher probabilities $P = 0.0415$.

Table 3 Knodell scoring

Periportal Bridging Necrosis	Intralobular degeneration and focal necrosis	Portal inflammation	Fibrosis
0: None	0: None	0: None	0: None
1: Mild	1: Mild (< 1/3 lobules)	1: Mild (< 1/3 lobules)	1: Fibrous portal expansion
3: Moderate (< 50% circumference)	3: Moderate (< 2/3 lobules)	3: Moderate (< 2/3 lobules)	3: Bridging fibrosis
4: Marked	4: Marked (> 2/3 lobules)	4: Marked ,dense (> 2/3 lobules)	4: Cirrhosis
5: Moderate + bridging			
6: Marked + bridging			
10: Multiple lobular necrosis			

HAI score is the combined score of necrosis, inflammation, and fibrosis.

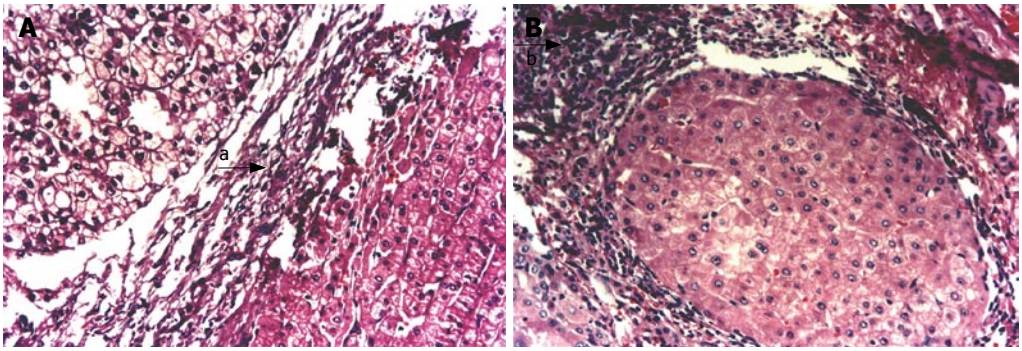

Figure 1 **A**: Fibrous connective tissues increased in surrounding HCC tissues, hepatic sclerotic tissue can be seen (arrow a); **B**: Inflammatory cells infiltrated surrounding noncancerous tissues (arrow b) (HE, × 200).

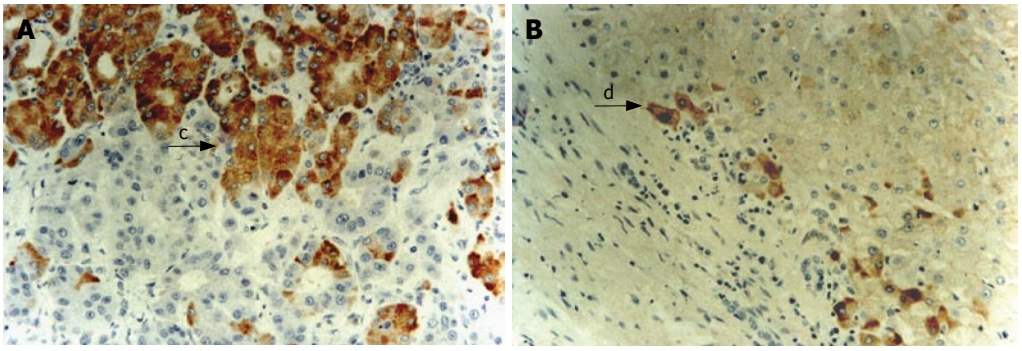

Figure 2 **A**: Expressions of HBsAg in HCC tissues (arrow c); **B**: Expressions of HCV in noncancerous tissues (arrow d) (Immunohistochemistry, SP, × 200).

with DAB reagent and counterstained with hematoxylin. Negative and positive controls were used simultaneously to ensure specificity and reliability of the staining process. The negative controls were incubated with PBS instead of primary antibody and a positive section supplied by the manufacturer of the staining kit was taken as positive control. Sections were observed under microscope after being mounted.

Criteria for data analysis

Immunostaining of HBsAg and HCV showed brown-yellowish granules either in the cytoplasma or the nucleus with a positive expression. Results estimation: the specimens with no positive cells or positive cells < 10% were negative (-); with light brownish yellow cells or positive cells > 10% were positive (+).

Statistical analysis

All statistical analyses were performed using the Microsoft SPSS 12.0 software. A P value less than 0.05 was considered to indicate significance. Quantitative data were expressed as mean ± SD. Chi-square test was used for comparison between groups. Rank-sum test and Spearman's rank correlation were used in the four groups.

RESULTS

Expressions of HBsAg and HCV in HCC and noncancerous tissues

Expressions of HBsAg and HCV in HCC and noncancerous tissues (Figures 1 and 2). Different expressions of HBsAg and HCV were observed in HCC and noncancerous tissues. We presumed HBsAg positive when HBsAg was detected either in HCC or noncancerous tissues. Of the 100 biopsies, HBsAg was detected in the hepatocytes in 87 (87%), HCV 28 (28%), and HBsAg and HCV 21 (21%), respectively.

Location and distribution of HBsAg and HCV antigen in tissues

Three patterns of HBsAg staining-cytoplasmic, submembranous and membranous, have been described in patients with chronic HBV infection. In our study, all patients with high replication (HBeAg-positive) had discrete cytoplasmic HBsAg expression. A mixed pattern of staining was seen in the other two groups. There was no difference in membranous HBsAg expression and histological activity in these groups. There was a significant difference in the positive expressions of HBsAg between HCC and noncancerous tissues. (23% *vs* 79%, χ^2 = 62.75, P < 0.001).

HCV Ag could be seen in the nuclei and/or cytoplasms of cancer cells and/or pericancerous hepatocytes of HCC. In the 28 positive cases of HCC, HCV Ag was more often seen in nucleus than in cytoplasm (20 cases in nuclei, only 8 in cytoplasms). The HCV Ag positive cells were scattered singly or gathered in small groups in some parts. The detecting rate of HCV Ag in cancer tissues was 15% (15/100), whereas in the pericancerous tissues it was as high as 23% (23/100). There was no significant difference in the positive expressions of HCV between HCC and noncancerous tissues (15% *vs* 23%, χ^2 = 2.08, P = 0.15).

HAI score

The score of HAI in the group with co-infection of HBV and HCV is the highest among the four groups (12.62 ± 3.88) while that in the group without infection of HBV and HCV is the lowest (6.67 ± 2.58). HBV and HCV

infection was positively correlated with HAI (r_s = 0.39, P = 0.0001) (Table 1).

DISCUSSION

HCC is one of the most common malignant tumors, representing more than 5% of all cancers. The estimated annual number of cases exceeds 500 000[7], with a mean annual incidence of around 3%-4%. In terms of relative frequencies, HCC ranks as the fifth most common cancer in the world (the fifth among men and eighth among women) and the second among cancers of the digestive tract after stomach cancer[8]. Chronic hepatitis B and C, mostly in the cirrhotic stage, are responsible for the majority of the hepatocellular carcinomas worldwide. In cases with persistent HBV infection, HBV is one of the most important risk factors for HCC. It has been noted that the probability of acquiring HCC increases with severity of liver disease. The annual risk of HCC is 0.5% for asymptomatic HBsAg carriers and 0.8% for patients with chronic hepatitis B[9]. Patients with HBV-cirrhosis have a 1000 times higher risk of developing HCC compared to a HBsAg negative control group. The incidence of HCC in compensated cirrhosis due to HBV from Asia was 2.7%. In Japan, the mean interval between the time of initial infection with HBV and the occurrence of HCC is 50 years. As most people are infected at birth, HBV related liver cirrhosis usually develops in patients in their 40's and HCC in their 50's[10].

Likely, HCV is associated with HCC, and HCV infection enhances the development of liver diseases. HCV affects the initiative period of HCC and induces the malignant phenotypic alteration of hepatocytes[11].

Co-infection of HBV and HCV seems to result in more severe liver diseases than either infection alone[12]. The risk of developing HCC in subjects with both infections has been investigated in a meta-analysis of 32 epidemiological studies between 1993 and 1997[13]. The odds ratio for development of HCC in HBsAg positive and anti-HCV/HCV RNA negative subjects was 20.4; in HBsAg negative and anti-HCV/HCV RNA positive subjects 23.6; and subjects positive for both markers 135. These data suggest a more than additive but less than multiplicative effect of HBV and HCV co-infection on the relative risk for HCC. The viruses may act through common as well as different pathways in the carcinogenic process.

AFP is a fetal specific glycoprotein produced primarily by the fetal liver. Normally, its serum concentration falls rapidly after birth and its synthesis in adult life is repressed. However, greater than 70% of HCC patients have a high serum concentration of AFP because of the tumor excretion. Forty years after its discovery, serum AFP remains a most useful tumor marker in screening HCC patients. The serum concentration of 20 ng/mL is the most commonly used cut-off value to differentiate HCC patients from healthy adults in clinical researches. Alpha-fetoprotein expression is a potential prognostic marker in hepatocellular carcinoma. In our study, there was direct correlation between HBsAg, HCV expression in HCC tissues, noncancerous tissues and serum AFP. The level of AFP in BC group is the highest among the four groups. We conclude that HBV/HCV infection is related to the increased level of AFP. That is to say, the increased level of serum AFP in HBV/HCV infection is associated with HCC. AFP, as a hepatoma-promoting factor, has become a new target of anti-hepatoma agents. Wang XW *et al*[14] suggested that it is a new approach for the treatment of tumors to inhibit or block oncogene expression. The effect of AFP on the growth of tumor has been confirmed. The functional mechanism of AFP on the growth of tumors may be attributed, at least in part, to receptor mediated cAMP pathway and/or calcium signaling resulting in overexpression of certain genes. The increase of AFP in the patients with liver cancer was positively correlated with the infection of HBV and HCV. The AFP increase by the infection of HBV and HCV viruses was one of mechanisms which lead to hepatocarcinogenesis, and the antivirus intervening treatment of hepatitis is significant for the prognosis of liver cancer. We therefore, can prevent HCC through decreasing the level of AFP by anti-virus therapy.

HAI score describes the histological status of accompanying chronic hepatitis and was established by pathologists. A reprospective trial has been performed that the patients with moderate hepatitis (HAI score of 6–9) had the highest rate of intrahepatic metastatic recurrence among the various HAI groups[15]. In our study, the BC group had significantly higher HAI scores than the other three groups. (BC > HCV > HBsAg > NBNC). From our Spearman's rank correlation analysis, we can conclude that the severity of virally induced inflammation was well correlated with HBsAg and HCV expression in HCC tissues and noncancerous tissues. Prior co-infection of HBV in HCV patients may be an adverse risk factor for intrahepatic inflammation. As for the tissues of liver cancer with virus infection background, the HAI is obviously higher than that without virus infection. HBV and HCV virus infection was correlated with HAI significantly, perennial viremia will aggravate pathological changes of liver tissue.

Based on our experiments, therefore, HCC might be prevented by early antiviral therapy. In patients with chronic hepatitis B or C, antiviral therapy in a noncirrhotic stage is protective for HCC development in responders, probably by prevention of cirrhosis development.

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CASE REPORT

Diphyllobothrium latum: A case of an incidental finding

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Abstract

Colorectal cancer screening can be performed by fecal occult blood testing, sigmoidoscopy, double contrast barium enema or colonoscopy. Colonoscopy has the significant advantage that polyps can be removed during the same procedure. Occasionally, colonoscopy can also reveal unexpected findings, including parasitic infections, even in asymptomatic patients. Tapeworms or cestodes are hermaphroditic parasites, which can live for considerable periods of time in the human gastrointestinal tract. Fish tapeworm (Diphyllobothrium species) are endemic in various parts of the world, the commonest species being Diphyllobothrium latum. Humans are the main definitive host for *D. Latum* and the majority of individuals harbouring the parasite are asymptomatic, while 40% of infected individuals may have low vitamin B12 levels. We describe a case of *D. latum* infection found on routine colonoscopic screening for colorectal cancer in an asymptomatic patient, which was successfully treated with praziquantel. The infection likely arose following raw fish (sushi) consumption.

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Key words: *Diphyllobothrium latum*; Colonoscopy; Colorectal cancer screening

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INTRODUCTION

Tapeworms or cestodes are hermaphroditic parasites, which can live for considerable periods of time in the human gastrointestinal tract. Fish tapeworm (Diphyllobothrium species) are endemic in various parts of the world, the commonest species being Diphyllobothrium latum.

Colorectal cancer screening can be performed by fecal occult blood testing, sigmoidoscopy, double contrast barium enema or colonoscopy. Colonoscopy has the significant advantage that polyps can be removed during the same procedure. Occasionally, colonoscopy can also reveal unexpected findings, including parasitic infection, even in asymptomatic patients.

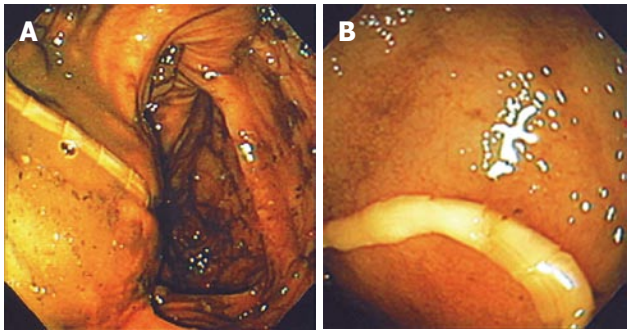

Figure 1 Colonoscopic images revealing a segmented worm in the ascending colon (**A**), passing through the ileo-caecal valve into the small intestine (**B**).

CASE REPORT

A 70-year-old woman, with a family history of colorectal carcinoma, was referred for evaluation of minimal bright red bleeding per rectum. Laboratory data, including complete blood count, serum ferritin, B12 and folate were within normal limits. Colonoscopy revealed first-degree hemorrhoids, with an unusual finding in the ascending colon and terminal ileum (Figure 1A and B).

DISCUSSION

The colonoscopic images revealed a segmented worm, 2 mm wide, in the ascending colon (Figure 1A), passing through the ileo-caecal valve into the small intestine (Figure 1B). Microscopy of a retrieved portion of the worm showed segments and eggs (Figure 2) characteristic of *Diphyllobothrium latum*; these typically measure 40 by 60 m and display a small knob or 'abopercular' on the opposite end of a lid-like opening or 'operculum', which may be inconspicuous. The patient was treated with a single dose of praziquantel. No eggs were seen in stool samples checked one and three months later.

Humans are the main definitive host for *D. Latum* (broadfish tapeworm), which can measure up to 12 m.

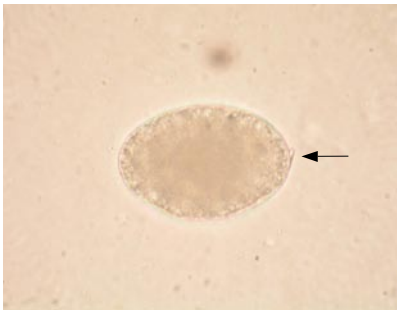

Figure 2 Microscopic image of *Diphyllobothrium latum* egg, displaying the abopercular (arrow) on the opposite end of the operculum.

Most individuals harboring the parasite are asymptomatic, and 40% may have low B12 levels but only 2% develop anaemia[1]. Freshwater fish act as intermediate hosts and patients consuming raw fish may risk infection, which is the likely source in our patient.

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CASE REPORT

Localized amyloidosis of the stomach: A case report

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Abstract

We report an unusual case of primary amyloidosis of the stomach in a patient complaining of dyspeptic symptoms. The diagnosis was confirmed histologically and other gastrointestinal site or systemic involvement was ruled out. Uncharacteristic dyspeptic symptoms may hide this rare metabolic disease.

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Key words: Amyloidosis; Gastrointestinal tract; Dyspepsia

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INTRODUCTION

The term amyloidosis refers to a group of disorders characterized by the extracellular accumulation of insoluble, fibrillar proteins in various organs and tissues. Amyloidosis commonly shows a systemic involvement. Localised deposition of amyloid is a rather uncommon form, and amyloidal deposit confined to the stomach is extremely rare. The clinical manifestations of gastric amyloidosis are often uncharacteristic and subclinical[1].

CASE REPORT

A 55-year old Caucasian male was referred to our open-access Digestive Endoscopy Unit by his family physician due to epigastric pain, heartburn and mild weight loss (< 3 kg). Physical examination showed reduced lower thoracic basis sound, more evident on right side, no wheezes, crackles, or rhonchi. Blood tests and urinalysis were normal. Chest X-rays were normal. Upper gastrointestinal (GI) endoscopy showed two white-yellowish 3-cm circular areas with fine granular appearance on the distal portion of the gastric body and angulus (Figure 1). Histologic examination of biopsy specimens taken at the edges of the lesions showed deposition of fibrillar eosinophilic substance, infiltration of lymphocytes and polyclonal mature plasma cells in the lamina propria, compatible with a pattern of gastric amyloidosis (Figure 2). Endoscopic examination and biopsy samplings of the esophagus, duodenum and rectum excluded other gastrointestinal localizations. Endoscopic ultrasound showed mild thickening of the first two layers of the gastric wall with no pathological perigastric or coeliac lymph nodes. The patient was then evaluated in the Centre for the Study of Systemic Amyloidosis of the University of Pavia, where examination of serum, liver, kidney and heart ruled out any multiorgan involvement.

Figure 1 Upper GI endoscopy showing a white-yellowish 3-cm circular area with fine granular appearance at the gastric body (above the angulus).

DISCUSSION

Amyloidosis is a disorder characterized by extracellular deposition of amyloid in various tissues and organs. Common gastrointestinal manifestations include gastroparesis, constipation, malabsorption, intestinal pseudo-obstruction, and bleeding[2,3]. The absence of systemic symptoms may make diagnosis difficult. Endoscopic findings such fine granular appearance and polypoid protrusions are common and may reflect amyloid deposition in the mucosa or submucosa of the alimentary tract[4].

Localized gastric amyloidosis is an uncommon form

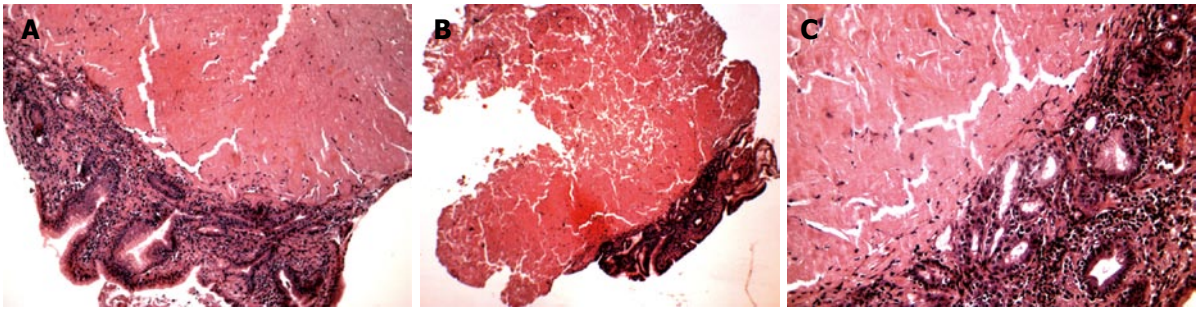

Figure 2 Photomicrograph of the endoscopic biopsy specimens showing deposition of fibrillar eosinophilic substance (**A**) (HE, x 10), positive Congo red staining (**B**) (Congo red, x 10), and high power image showing infiltration of the lamina propria with lymphocytes and polyclonal plasma cells (**C**) (HE, x 25).

of the disease, sometimes associated with gastric malignancies, such as carcinoma and stromal tumours[5,6]. The patient reported herein complained of dyspeptic symptoms, and had isolated gastric involvement without any other gastrointestinal or systemic localizations. Proton pump inhibitors (pantoprazole 40 mg daily per os) were administered as intermittent "on demand" therapy for his dyspeptic complaints, associated with a prokinetic drug (domperidone twice daily before meals). No further treatment was deemed necessary, since the patient was symptoms-free on a clinical follow-up of about 10 mo. Periodic controls have been scheduled to follow the evolution of disease and early recognition of multiorgan involvement.

Although gastric amyloidosis is rare, it should be considered among the possible diagnoses in dyspeptic patients and clinical follow-up in specialized centres is warranted in the management of these cases.

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CASE REPORT

Unusual causes of intrahepatic cholestatic liver disease

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Abstract

We report five cases with unusual causes of intrahepatic cholestasis, including consumption of *Teucrium polium* (family Lamiaceae) in the form of tea, Stauffer's syndrome, treatment with tamoxifen citrate for breast cancer, infection with *Coxiella Burnetii* (acute Q fever), and infection with *Brucella melitensis* (acute brucellosis).

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Key words: Brucella melitensis; *Coxiella Burnetii*; Cholestasis; Hepatitis; Stauffer's syndrome; Tamoxifen; *Teucrium polium*

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INTRODUCTION

The term "cholestasis" indicates stoppage or *stasis* of bile. Cholestasis is not a disease but a symptom of many diseases. It is defined as a pathologic state of reduced bile formation or flow. This definition applies more to the experimental situation, where the rates of bile formation and flow can be measured, than to human cholestasis, where neither can be assessed. Therefore, the clinical definition of cholestasis is any condition in which substances normally excreted into bile are retained. The mechanisms of cholestasis can be broadly classified into intrahepatic, where an impairment of bile formation occurs, and extrahepatic, where impedance to bile flow occurs after it is formed[1]. Intrahepatic cholestasis occurs in certain instances of viral, alcoholic, drug-induced, and chronic liver diseases, such as genetic defects[2] (Table 1). Gallstones, bile duct strictures, and tumours are the most frequent causes of extrahepatic (mechanical) bile duct obstruction[2] (Table 1). Cholestatic liver disease is characterised by a predominant elevation of serum alkaline phosphatase and bilirubin, although serum bilirubin may be normal until a late stage of the disease. In this paper we discuss five patients with unusual causes of intrahepatic cholestatic liver disease.

CASES REPORT

We retrospectively studied the case notes of 100 patients with intrahepatic cholestatic liver disease who were hospitalised in two divisions of internal medicine for investigation in 2002-2004. Five unusual causes of intrahepatic cholestasis were revealed in our patients. The causes of intrahepatic cholestasis were defined as unusual with a prevalence < 10%. The medical histories of five representative patients with these causes of cholestasis are reported below:

Case 1

A 67-year old man was admitted to hospital for a 5-d history of painless jaundice, pruritus, dark urine and light stools. His medical history included fatty liver, associated with hyperlipidemia and the consumption of ethanol, which was confirmed by an abdominal ultrasound scan and liver biopsy performed in the previous year. Consumption of *Teucrium polium* (family Lamiaceae) in the form of tea for the treatment of hyperlipidemia during the previous 6 mo (increasing to 2 litres per day in the preceding month) was also mentioned. Physical examination revealed yellow pigmentation of the sclera and skin. Laboratory tests showed the following values: haematocrit (Ht) 46%, white blood cell (WBC) count 7000 cells/mm^3 (neutrophils 53%, lymphocytes 29.3%, monocytes 10.2%, eosinophils 6.6%), MCV106 fL (normal range: 80-99 fL), platelet (PLT) count 133 000/mm^3 (normal range: 150 000-450 000/mm^3), international normalized ratio (INR) 2.9 (normal value: < 1.7), alanine aminotransferase (ALT) 1272 U/L (normal range: 5-40 U/L), aspartate aminotransferase (AST) 1739 U/L (normal range: 5-40 U/L), γ-glutamyl transpeptidase (GGT) 302 U/L (normal range: 10-75 U/L), alkaline phosphatase (ALP) 190 U/L (normal range: 35-125 U/L), total bilirubin (Bil) 11.37 mg/dL (normal range: 0.1-1.3 mg/dL), direct-bilirubin (d-Bil) 7.97 mg/dL, albumin (Alb) 3.3 gr/dL (normal range: 3.5-5.0 g/dL), lactate dehydrogenase (LDH) 380 U/L (normal range: 80-230 U/L), bilirubin and urobilinogen in the urine. Serological and immunologic tests, such as tests for hepatotropic

Table 1 Causes of cholestasis[2]

Intrahepatic cholestasis	Extrahepatic biliary tract diseases
Primary biliary cirrhosis (PBC)	Choledocholithiasis
Primary sclerosing cholangitis (PSC)	Bile duct tumours (benign and malignant)
Drugs and toxins	Ampullary tumours (benign and malignant)
Sepsis	Pancreatic carcinoma
Malignancy	Mirizzi's syndrome
Granulomatous liver disease (infections, chemicals, drugs, miscellaneous)	AIDS cholangiopathy
Intrahepatic cholestasis of pregnancy	Parasites
Hepatitis (viral and alcoholic)	PSC (primary sclerosing cholangitis) (see left)
Genetic disorders	
Total parenteral nutrition associated cholestasis	
Graft versus host disease	
Post liver transplant cholestasis	

viruses were negative. Histopathology of a liver biopsy revealed cholestatic hepatitis compatible with drug-induced hepatitis. The management of the patient was successful mainly consisted of careful parenteral administration of suitable fluids and vitamin K, per os administration ursodeoxycholic acid and a frequent assessment of the liver function tests. Liver dysfunction was restored one month after his admission.

Final diagnosis: Acute cholestatic hepatitis caused by *Teucrium polium* L.

Case 2

A 50-year old man [farmer, smoker (40 packs/year)] was admitted to hospital because of a 5-d history of high-grade intermittent fever (up to 38.5℃) associated with mild left abdominal pain, fatigue, and anorexia. Weight loss (approximately 12 kg) during the previous two months was also mentioned. His medical history included Hashimoto thyroiditis and vitiligo. Physical examination revealed a palpable abdominal mass on the left kidney that was confirmed by ultrasound and CT scans of the abdomen. Laboratory tests showed the following values: Ht 31%, WBC 9000 cells/mm^3 (neutrophils 53%, lymphocytes 27%, monocytes 13%, eosinophils 7%), PLT 470 000/mm^3, erythrocyte sedimentation rate (ESR) 140 mm/h, C-reactive protein (CRP) 8 mg/dL (normal range: 0.08-0.8 mg/dL), ALT 260 U/L, AST 227 U/L, GGT 140 U/L, ALP 187 U/L, Bil 2.1 mg/dL, d-Bil 1.8 mg/dL, Alb 3.2 gr/dL, LDH 370 U/L, INR 2.9, microscopic haematuria in urinalysis. Serological and immunologic tests were negative. A left radical nephrectomy was performed. Pathologic examination revealed stage Ⅱ renal cell carcinoma (RCC). Six weeks after the nephrectomy, the liver function tests, the ESR and CRP level were normal. There was no recurrence or metastasis throughout the 3-year follow-up period.

Final diagnosis: Non-metastatic cholestatic paraneoplastic syndrome associated with RCC stage Ⅱ (Stauffer's syndrome).

Case 3

A 60-year old woman was admitted to hospital for the check of elevated liver function test levels in blood examinations. Her medical history included fatty liver confirmed by an abdominal ultrasound scan and liver biopsy performed in the previous year (steatohepatitis). She had a previous history of modified mastectomy with axillary lymphadenectomy for a retroareolar, canalicular breast carcinoma 6 months ago. She received adjuvant radiation and started tamoxifen citrate (20 mg daily) two months before admission. Physical examination revealed hepatomegaly that was confirmed by an abdominal ultrasound and CT scan. No hepatic metastasis or main bile duct obstruction was detected. Laboratory tests showed the following values: Ht 43%, WBC 7600 cells/mm^3 (neutrophils 60%, lymphocytes 28%, monocytes 8%, eosinophils 4%), MCV 102 fl, PLT 142 000/mm^3, INR 1.9, ALT 377 U/L, AST 284 U/L, GGT 342 U/L, ALP 290 U/L, Bil 1.5 mg/dL, d-Bil 1.2 mg/dL, Alb 3.2 gr/dL, LDH 320 U/L. Serological and immunologic tests, tumour markers, such as tests for hepatotropic viruses were also negative. Histopathology of a liver biopsy revealed cholestatic hepatitis compatible with drug-induced hepatitis. Tamoxifen was withdrawn and methylprednisolone (1 g daily) was administered for 3 d. Thereafter, 0.5 mg/kg prednisone was indicated for 7 d and subsequently decreased. Five weeks after the discontinuation of tamoxifen citrate, serum ALP and GGT levels were normalized, while serum ALT and AST concentrations were approximately a little above the normal limits. Currently she is receiving anastrazole (aromatase inhibitor).

Final diagnosis: Acute cholestatic hepatitis caused by Tamoxifen citrate.

Case 4

A 39-year old man presented with a one-month history of painless jaundice and after a 5-day period of dark urine without fever. His personal and family medical history was unremarkable. Physical examination revealed yellow pigmentation of the sclera and skin. Laboratory tests showed the following values: Ht 47%, WBC 6700 cells/mm^3, PLT 215 000/mm^3, ALT 762 U/L, AST 735 U/L, GGT 167 U/L, ALP 417 U/L, Bil 3.19 mg/dL, d-Bil 2.15 mg/dL, LDH 390 U/L, urobilinogen in the urine. Immunologic tests, such as tests for hepatotropic viruses were negative. A four-fold increase was found by comparing the titers of IgG antibodies to phase Ⅱ antigen of *Coxiella Burnetii* in two consecutive assays (indirect immunofluorescence antibody technique, IFAT). Histopathology of a liver biopsy revealed acute hepatitis. The patient was treated successfully with doxycycline (100 mg per os twice daily) for 2 wk. Liver dysfunction was restored two months after his admission.

Final diagnosis: Acute cholestatic hepatitis caused by *Coxiella Burnetii* (acute Q fever infection).

Case 5

A 47-year old man shepherd was admitted to hospital, because of a 2-wk period of intermittent low-grade fever, fatigue, malodorous sweating and headache. His personal

and family medical history was unremarkable. A record of the consumption of unpasteurised dairy products during the previous months was reported. Physical examination revealed hepatomegaly that was confirmed by an abdominal ultrasound scan. Laboratory tests showed the following values: Ht 36, WBC 7900 cells/mm^3 (neutrophils 60%, lymphocytes 30%, monocytes 7%, eosinophils 3%), PLT 123 000/mm^3, ESR 53 mm/h, CRP 4.43 mg/dL, ALT 672 U/L, AST 769 U/L, GGT 432 U/L, ALP 342 U/L, Bil 2.3 mg/dL, d-Bil 2 mg/dL, Alb 3.3 gr/dL, LDH 380 U/L. Brucella agglutinin titter was positive at 1/320 and growth of *Brucella melitensis* in blood culture after 5 d. After a putative diagnosis of brucellosis, the patient was administered doxycycline (100 mg per os twice daily) for six weeks plus 1 g streptomycin im for the first 21 d, beginning on the third day of hospitalisation, while waiting the serological and cultural confirmation. Liver dysfunction was restored five weeks after his admission.

Final diagnosis: Acute cholestatic hepatitis caused by *Brucella melitensis*.

DISCUSSION

Case 1

The final diagnosis was based on the excessive consumption of *Teucrium polium* tea during the month before admission, and the exclusion of other cholestatic syndromes. The histopathological change in the findings of the liver biopsy, taken before and after the consumption of the excessive amount of tea was also conclusive evidence of the cause of cholestatic hepatitis. Although the mechanism of *Teucrium polium* hepatotoxicity is unclear, teucrin A and several neoclerodane diterpenoids, present in the aerial parts of the plant, have been reported as the probable hepatotoxic precursors of this herb[3]. In some instances, liver injury has been associated with the presence of autoantibodies in the serum[4,5]. It has also been reported that some flavonoids have antihyperlipidemic properties, while some terpenoids could inhibit lipid peroxidation[3]. In 1995 Mattei *et al*[6] reported massive hepatocyte necrosis predominantly in the centrilobular areas of the liver in a patient with acute liver failure after the consumption of *Teucrium polium*.

Case 2

Stauffer's syndrome is a non-metastatic cholestatic paraneoplastic syndrome usually associated with RCC, with an incidence of 3%-6% among these patients[7,8]. The syndrome is also associated with other malignancies, such as bronchial adenocarcinoma, leiomyosarcoma and prostate adenocarcinoma[9-11]. Stauffer's syndrome may result from tumour production of cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and possibly interleukin-6 (IL-6)[12,13]. Nephrectomy may result in the amelioration of hepatic dysfunction, but persistent or recurrent elevations may herald local recurrence or metastatic disease[14]. It is remarkable that RCC was an unexpected finding diagnosed during the investigation of systemic symptoms and hepatic dysfunction in our patient. The hepatic dysfunction was returned to normal after nephrectomy.

Case 3

Tamoxifen is a synthetic antioestrogen with both agonist and antagonist properties and plays an important role in prevention and treatment of breast cancer[15]. It is believed to act primarily through binding to oestrogen receptors in breast cancer cells, acting as a competitive inhibitor of oestrogen. Several forms of drug-induced liver disease, such as clolestasis, benign liver tumours, hepatocellular carcinoma, acute hepatitis, submassive hepatic necrosis, steatosis, and steatohepatitis, have been attributed to tamoxifen[15,16]. Although tamoxifen is a known cause of cholestasis, very little attention has been focused on its use in patients with pre-existing liver dysfunction and the possible need for dose adjustment[17]. The pre-existing steatohepatitis might contribute to the early development of tamoxifen-induced cholestasis (2 mo after tamoxifen administration) in our patient.

Case 4

The final diagnosis was based on both serological and clinical criteria. *Coxiella Burnetii* has a worldwide distribution and reports from various countries show a variety of clinical and epidemiological features of Q fever from one area of the world to another[18]. Hepatic involvement is frequent in Greece as well as in France where it accounts for approximately 40%-50% of acute cases[19,20]. Usually hepatic involvement is asymptomatic with a mild increase (2-3 times the normal level) of ALP, AST, and ALT[21]. Jaundice is a rare finding in Q fever hepatitis while hepatomegaly is quite common[20]. The three clinical presentations of Q fever hepatitis are infectious viral-like hepatitis, fever of unknown origin with granulomatous hepatitis, and incidental finding of elevated transaminases in a patient with pneumonia. In patients with granulomatous hepatitis liver biopsy shows a typical doughnut granuloma with dense fibrin ring surrounded by a central lipid vacuole, that is highly suggestive of the disease caused by *Coxiella burnetii*[21,22]. Extensive destruction of liver tissue leading to hepatic coma and death have occasionally been reported[23,24].

Case 5

Brucellosis, a world-wide zoonotic disease, is a systemic infection caused by facultative intracellular bacteria of the genus *Brucella* that can involve many organs and tissues[25]. Brucellosis is a major public health concern in Greece, especially in areas where animal breeding is an important economic resource. *Brucella melitensis* is the most pathogenic and invasive species of *Brucella* and occurs more frequently in general human population than in other known species. Hepatic tenderness and mild elevation of transaminases and ALP may be found in acute disease[26]. Asymptomatic, mild cholestatic hepatitis accounted for approximately 16% of acute cases of brucellosis in a small study from Greece[27].

CONCLUSION

In conclusion, use of herbal remedies, as *Teucrium polium* must be considered a possible aetiology in the setting of clinical and/or biochemical manifestations of liver injury, since alternative medicine and herbal treatments

have growing appeal to many societies. Malignant diseases including hypernephroma, treatment with tamoxifen citrate, and infections with *Coxiella Burnetii* or *Brucella melitensis* are also unusual causes of cholestatic hepatitis. Clinicians should consider these causes during diagnostic workup of intrahepatic cholestatic liver disease.

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Relationship between the pathogenesis of sarcoidosis and hepatitis C

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TO THE EDITOR

I read the article by Kim *et al*[1]with great interest. This is a rare case of systemic sarcoidosis in a patient with chronic hepatitis C without treatment for neither sarcoidosis nor hepatitis C. In this case, spontaneous resolution of sarcoidosis was noted while hepatitis activities were aggravating[1]. The authors suggest that this relationship mentioned above was not a fortuitous coincidence and these two diseases share a common pathogenesis due to the fact that interferon-induced immune activation is an important mechanism of viral replication suppression in chronic hepatitis C, hepatitis activities may be suppressed when chronic hepatitis C is combined with active sarcoidosis[1].

Before a conclusion like this, we consider that some points must be further clarified. Firstly, if there had been no spontaneous regression of sarcoidosis in the related case, one might have easily suggested that hepatic granulomas and cutaneous lesions were not associated with sarcoidosis in a patient who had no lung involvement confirmed by transbronchial biopsy. Hepatic granulomas have been described in up to 9.5% of liver biopsies from HCV-infected patients[2]. Additionally, in another series with hepatitis C, the prevelance of hepatic granulomas was found to be 1.3% before the initition of interferon therapy[3]. Furthermore, a study investigating the dermatopathologic manifestations of hepatitis C virus infection showed that palisading granulomatous inflammation may also present[4]. By contrast, a prospective controlled study recently reported that anti-HCV antibodies were not found in any patients of sarcoidosis[5].

In another aspect, the main controversy about sarcoidosis is its unkown pathogenesis. T-helper-1 (Th1) mediated cytokines, especially IFN-γ have a crucial role in the pathogenesis of the disease but IL-1, TNF-α and IL-2 are also contributory cytokines in inducing and maintaining of granuloma formation. This exaggerated Th-1 immune response is thought to be secondary to stimulation by either exogenous antigens (infectious or environmental) or autoantigens[6]. Although the potential role of IFN-α in the immunopathogenesis of sarcoidosis is not clear, the observations of coincidence of manifestation of sarcoidosis with IFN-α therapy for hepatitis C may support the concept of the cytokine network in pathogenesis of sarcoidosis[7,8]. Reported cases with spontaneous resolving sarcoidois after cessation of IFN-α treatment for hepatitis C may also support this hypothesis[9]. But it is important to remember that a considerable amount of IFN-α is being used in treatment of hepatitis C which may probably modulate an immune response resulting in an expression of a Th-1 mediated granulomatous reaction. Otherwise it is not known whether the amount of IFN-γ releasing during the natural course of sarcoidosis is sufficient to supress viral replication in hepatitis C. As known well in the early phase of hepatitis C, HCV continues to replicate in spite of high level IFN-α expression in the liver[10]. Additionally, plasma cytoid dentritic cells (PDC) predominantly release a considerable amount of IFN-α simultaneously[10]. On the other hand, not only non-cytolytic mechanism (IFN-γ) but also cytolytic mechanisms (Fas ligand, perforin, granzyme, TNF-α) mediated by cytotoxic T lymphocytes play an important role in clearance of HCV from the liver in chronic hepatitis C[10].

Moreover, in this case the duration of sarcoidosis was shorter (20 mo) than that of hepatitis C (20 years). In this regard, the absence of convincing evidence for the interaction of these two diseases has led us to consider a fortutious coincidence.

Certainly, this is a single case and it is obvious that genetic polymorphisms may contribute to individual difference=s in the regulation of cytokine release in sarcoidosis.

Further large and prospective clinical and basic studies are necessary to clarify the relationship between chronic hepatitis C and sarcoidosis.

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Short mucin 1 alleles are associated with low virulent *H pylori* strains infection

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TO THE EDITOR

We read with interest the article published by Nguyen *et al*[1] in the World Journal of Gastroenterology, showing an association between short MUC6 alleles and *H pylori* infection. These results, together with previous observations by Vinall *et al*[2], reinforce the role of mucin genes (MUC6 and MUC1) VNTR polymorphisms for the variability in individual susceptibility to *H pylori* infection that cannot be explained by differences in environmental factors.

We conducted a similar analysis based on a previously described[3] survey of gastric pathology in shipyard workers at Viana do Castelo, north of Portugal. Briefly, volunteer workers underwent an upper digestive endoscopy, completed symptoms and lifestyle questionnaires, and had a blood sample drawn. *H pylori* infection status was determined by serology and PCR, and typing of *cagA*, *vacA s* and *vacA m* was performed directly in gastric biopsy specimens by multiplex PCR and reverse hybridization[4]. The MUC1 gene polymorphisms were evaluated by Southern blot analysis[5].

Information on MUC1 genotypes and *H pylori* infection status was available for 216 subjects (median age: 43 years; 91% males). For analysis, each participant was considered infected if positive by either serology or PCR (n = 172, 79.6%), and classified according to MUC1 genotype [long-long (LL), long-short (LS), short-short (SS)]. Data on *H pylori* genotypes were available for 161 participants. The association between MUC1 genotypes and *H pylori* infection was quantified through the calculation of odds ratios (OR) and the respective 95% confidence intervals (CI) by unconditional logistic regression using STATA®, version 9.2.

Short MUC1 alleles were associated with *H pylori* infection (SS *vs* LL: OR = 2.7, 95%CI: 1.0-7.1; LS *vs* LL: OR = 1.6, 95% CI: 0.7-3.7). The risk of infection (SS *vs* LL) was increased nearly three-fold for low-virulent strains (*cagA*$^-$, *vacA s2* or *vacA m2*) and less than two-fold when considering high-virulent *H pylori* strains (Table 1).

The present study, performed in a population at high risk for gastric cancer, confirms earlier findings on the effect of mucin genes VNTR variability on *H pylori* infection, and adds to previous investigations the evidence of a stronger association between the short mucin alleles and low-virulent strains. However, an increased risk of infection also with high virulence strains cannot be excluded and may be shown in investigations with larger sample sizes, powered enough to disclose an association of this magnitude.

Our results are compatible with previous findings of an increased risk for both intestinal metaplasia[5] and gastric carcinoma[6] in individuals with short MUC1 mucin alleles, but additional studies should explore the combined effect of *H pylori* genotypes and mucin genes polymorphism on the infection outcome.

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Table 1 Association between MUC1 polymorphisms and *H pylori* infection according to bacterial genotype

		H pylori positive											
		cagA genotypes				*vacA s* genotypes				*vacA m* genotypes			
	H pylori negative	*cagA*⁻	OR	*cagA*⁺	OR	s_2	OR	s_1 or s_1/s_2	OR	m_2	OR	m_1 or m_1/m_2	OR
	n (%)	*n* (%)	(95% CI)	*n* (%)	(95% CI)		(95% CI)	*n* (%)	(95% CI)	*n* (%)	(95% CI)	*n* (%)	(95% CI)
MUC1 genotypes													
LL	10	8	1	21	1	5	1	24	1	8	1	21	1
	(25.6)	(20.5)	[reference]	(53.8)	[reference]	(12.8)	[reference]	(61.6)	[reference]	(20.5)	[reference]	(53.9)	[reference]
LS	25	30	1.5	47	0.9	24	1.9	53	0.9	34	1.7	43	0.8
	(24.5)	(29.4)	(0.5-4.4)	(46.1)	(0.4-2.2)	(23.5)	(0.6-6.4)	(52.0)	(0.4-2.1)	(33.3)	(0.6-4.9)	(42.2)	(0.3-2.0)
SS	9	22	3.1	33	1.8	16	3.6	39	1.8	23	3.2	32	1.7
	(14.1)	(34.4)	(0.9-10.2)	(51.6)	(0.6-5.0)	(25.0)	(0.9-13.7)	(61.0)	(0.6-5.1)	(35.9)	(1.0-10.7)	(50.0)	(0.6-4.9)

LL: long-long; LS: long-short; SS: short-short; OR: odds ratio CI: confidence interval.

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LETTERS TO THE EDITOR

A different approach for sterilization of liver hydatid cysts

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TO THE EDITOR

Many different methods have been used in surgical treatment of liver hydatid cysts[1]. However, two methods have been frequently used recently: one is used to radically remove the cyst and the other is used to shrink the cyst cavity after treated with scolocidal agents. One of the most important principles for the treatment of hydatid cyst is to avoid the contact of fluid in the cavity with the adjacent tissues during cyst cavity contacts with scolek killers at certain time.

If the cysts are easily accessible, sterilization of the cavity is not a problem. However, it is difficult to give a desirable amount of scolek killer agents to the cavity in some cases because the cyst is located in posterior lung segments. For this reason, we present an easy but effective method.

The cyst can be accessed by performing a suitable incision. The surrounding of the cyst is isolated with cloth absorbing saline solution. The cyst ingredients are evacuated with a trochar aspirator. When there is no bile in the content, a thick foley catheter is placed into the cyst and its balloon is blown (the balloon should be blown to the level not to be taken out when pulled tightly from the orifice) (Figure 1A and B). The cavity is filled with scolek killers and clamped. A balooned catheter is fixed tightly through a retractor. After a certain period of time, the solution given to the cyst through the balloon catheter kept in tension is taken out and the cyst becomes smaller and smaller.

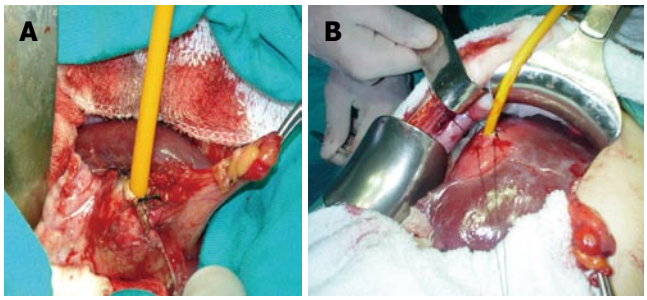

Figure 1 A foley cather inserted into the liver hydatid cyst (**A**) and its inactivation period (**B**) during operation.

Two different techniques have been used in the treatment of hydatid cyst: one is used radically remove the cyst and the other is used to evacuate the content in the cyst and to make the cyst cavity smaller. The second technique is used more often than the first, and the biggest disadvantage of these two techniques is that there is a risk of developing peritonea disease if the fluid leaks into the abdomen during operation. Many different techniques have been used to avoid this[1-3]. Here the most important thing is to prevent fluid leaking into the abdomen and keeping the scolosid killer agents in the cyst for a certain period of time in order to kill the live scoleks. It is easy to do this with the lung cysts on the frontal surface of the lungs. However, it is quite difficult to perform this for the cysts on diaphragmatic surface. In our clinic, the technique has been used in 45 patients between 2002 and 2005. No recurrence of liver hydratid crysts was found in any patients, suggesting that this technique which provides adequate contact with scoleks in the cyst and prevents liquid leaking into peritonea can be used in the treatment of lung cyst disease.

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symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk/

NEXT 6 MONTHS

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
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easl2007@easl.ch
www.easl.ch/liver-meeting/

Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
Washington-DC
tkoral@asge.org

Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
12-15 June 2007
Lisbon
fca@netvisao.pt

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15-16 June 2007
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Meeting ILTS 13th Annual International Congress
20-23 June 2007
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www.ilts.org

Meeting 9th World Congress on Gastrointestinal Cancer
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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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]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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

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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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