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EDITORIAL

Current treatment options and response rates in children with chronic hepatitis C

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Abstract

Vertical transmission has become the most common mode of transmission of hepatitis C virus (HCV) in children. The rate of perinatal transmission from an HCV-infected mother to her child ranges from 2% to 5% and the prevalence of HCV in children in developed countries ranges between 0.1% and 0.4%. Spontaneous viral clearance seems to be dependent on the genotype and has been reported between 2.4%-25%. For chronically infected patients, treatment with recombinant polyethylene glycol (PEG)-interferon α-2b and daily ribavirin has now been approved as standard treatment for children 2-17 years of age. In five large prospective studies, a total of 318 children and adolescents aged 3-17 years were treated either with subcutaneous PEG-interferon α-2b at a dose of 1-1.5 μg/kg or 60 μg/m² once a week in combination with oral ribavirin (15 mg/kg per day) or PEG-interferon α-2a with ribavirin. Subjects with genotype 1 and 4 received the medication for 48 wk and individuals with genotype 2 and 3 mainly for 24 wk. Overall sustained viral response (SVR) was achieved in 193/318 (60.7%) of treated patients. Stratified for genotype; 120/234 (51%) with genotype 1, 68/73 (93%) with genotype 2/3, and 6/11 (55%) with genotype 4 showed SVR. Relapse rate was between 7.7% and 17%. Overall, treatment was well tolerated; however, notable side effects were present in approximately 20%. According to recent experiences in the treatment of chronic hepatitis C in children and adolescents, a combination of PEG-interferon α with ribavirin has been found to be well tolerated and highly efficacious, particularly in individuals with genotype 2/3. Thus, this treatment can be recommended as standard of care until more effective treatment options will become available for genotype 1 patients.

Key words: Chronic hepatitis C; Treatment; Children; Polyethylene glycol-interferon and ribavirin; Response rate

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INTRODUCTION

Combination therapy of polyethylene glycol (PEG)-interferon α-2a or α-2b with ribavirin is standard of care for adults with chronic hepatitis C. Clear benefits in terms of sustained viral response (SVR) and side effect profile have been documented with PEG-interferon α compared with recombinant interferon α with and without ribavirin. An additional advantage of the pegylated form of interferon is the extended serum half-life, which allows a once-weekly administration regimen. Until recently, only recombinant interferon α-2b in combination with ribavirin had been approved by the Food and Drug Administration

(FDA) and European Medicines Agency (EMA) for use in children and adolescents. Since December 2008 and September 2009, respectively, the FDA and EMA approved PEG-interferon α-2b in combination with ribavirin in the United States and Europe for children aged 3 years and older[1]. Although most experts believe treatment is beneficial, due to several factors associated with treating young patients with chronic hepatitis C, this topic remains controversial[2-4]. However, there is no doubt that chronic hepatitis C remains an epidemiologically important health care issue in children and adolescents. Associated costs in the United States are estimated between $17 and $40 million annually[5]. Effective treatment of chronic hepatitis C virus (HCV) at an early age would help to prevent the long-term sequelae of chronic infection, improve the prognosis of patients, and reduce health care expenditure.

The prevalence of HCV in children in developed countries ranges between 0.1% and 0.4% but may even exceed 10% in some regions of Saudi Arabia and Africa[6-8]. The rate of perinatal HCV transmission from an infected mother to her child ranges from 2% to 5%. Clinically most relevant are genotypes 1, 2 and 3; considerably less spread is genotype 4[2]. It is estimated that there are 1 million individuals aged less than 18 years infected with chronic hepatitis C worldwide[9].

Since the early 1990s, transmission of HCV infection has occurred predominantly by parenteral transfusion of blood products or by non-use of disposable syringes. However, transfusion-associated hepatitis C has now become extremely rare in countries with adequate hygienic facilities. Subjects who were particularly at risk such as premature infants, hemophiliacs, patients with thalassemia, and children with malignant diseases or organ transplantations have now reached adulthood and vertical transmission from HCV-infected mothers to their offspring has become the most common cause of chronic hepatitis C in children. Importantly, in the case of vertical infection, the chronicity rate is very high[10].

Children chronically infected with HCV may be at risk for social disintegration and impaired quality of life. A possible psychological burden may be present and some physical impairment has been described. To date, only two rather small studies have been published reporting significantly lower physical and psychosocial scores and worse cognitive functioning compared with non-infected controls[11,12].

NATURAL COURSE

Spontaneous viral clearance in vertically infected children seems to be dependent on genotype and was found to range from 2.4%-25%[13,14]. It may be higher in parenterally infected individuals and was reported to reach 35%-45% by adolescence[15,16]. Children infected with genotype 3 have a higher spontaneous clearance rate than those infected with genotype 1. Beyond the age of 4 years, spontaneous viral clearance seems to become rather unlikely[13]. Patients who do not clear the virus within the first years of life will develop chronic hepatitis C. Overall, the cumulative probability of progression to chronicity is approximately 80%[17,18]. Most children are clinically asymptomatic or show only mild unspecific symptoms. In roughly 10% of patients, hepatomegaly may be present[17]. During the chronic course, alanine aminotransferase (ALT) levels may be normal or intermittently elevated. Only few patients show persistent markedly elevated ALT levels. Inflammatory activity in liver tissue is usually mild and the risk of severe complications is low. However, despite the favourable prognosis during the first and second decade of life, approximately 4%-6% of children will develop evidence of advanced liver fibrosis or cirrhosis[19,20]. A recently published study in pediatric patients with chronic hepatitis C cured of malignancy reported liver cirrhosis in 5% after three decades of observation[21]. Progression of fibrosis depends on age and additional risk factors such as obesity and alcohol consumption. Thus, progression usually starts beyond the second life decade and there is evidence that it seems to proceed more rapidly in patients with genotype 3[22]. Large liver transplantation units have reported on children who needed liver transplantation due to progressive HCV infection[23].

TREATMENT OPTIONS

Many years ago, treatment started in adults with the use of interferons, yielding SVR rates in the 10%-15% range. According to the use of different treatment regimens and small numbers of treated children, it was difficult to compare the response rates in children to those in adults. Overall, SVR seemed to be better in children. Nineteen studies using recombinant α-interferon were published between 1992 and 2003[24]. A meta-analysis of trials with interferon-α monotherapy revealed a wide range (0%-76%, mean 27%) of SVR. Subjects infected with genotype 2 and 3 clearly responded better than patients harbouring genotype 1. Based on an increasing number of randomized controlled trials in adults, ribavirin was added to interferon-α in treatment trials for children. Between 2000 and 2005, six studies were published all demonstrating an SVR from 27% to 64%[25]. The stratification according to genotypes showed a very good response (> 80%) in patients with genotype 2 and 3 and an SVR of approximately 36%-53% in those with genotype 1. Results of an extensive trial in children published by Gonzalez-Peralta led to the approval of recombinant interferon α-2b in combination with ribavirin[26].

However, when PEG-interferon in combination with ribavirin became the standard of care for adults with chronic hepatitis C, trials in children promptly started. Some advantages were present such as a reduced injection frequency to once per week, better SVR, and better interferon tolerance. Interestingly, the sole controlled randomized trial, comparing a pegylated interferon α (PEG-interferon α-2a) with and without additional ribavirin, was only published in 2011. It clearly demonstrated that in the pediatric age group, the addition of ribavirin was necessary to obtain significantly better treatment re-

Table 1 Sustained viral response in five representative prospective trials using polyethylene glycol-interferon alpha-2b and polyethylene glycol-interferon alpha-2a in combination with ribavirin and stratified for different clinical and laboratory parameters and genotypes, published between 2005 and 2011

	Wirth 2005[1]	Jara 2008[1]	Wirth 2010[1]	Total PEG-interferon a-2b trials	Schwarz 2011[2]	Sokal 2010[2]	Total all trials
Dosage	1.5 μg/kg per week	1.0 μg/kg per week	60 μg/m² per week		180 μg/1.73 m² per week	100 μg/m² per week	
Total (%)	36/61 (59)	15/30 (50)	70/107 (65.4)	121/198 (61.1)	29/55 (53)	43/65 (66.1)	193/318 (60.7)
Genotype (%)							
1	22/46 (48)	12/26 (46)	38/72 (53)	72/144 (50)	21/45 (47)	27/47 (59)	120/236 (51)
2/3	13/13 (100)	3/3 (100)	28/30 (93)	44/46 (96)	8/10 (80)	16/17 (94)	68/73 (93)
4	1/2	0/1	4/5 (80)	5/8 (62)		Included in G1	
ALT-levels (%)							
Elevated	12/25 (48)		27/44 (61)			19/33 (58)	58/102 (57)
Normal	24/36 (67)		42/63 (67)			24/30 (80)	90/129 (70)
Mode of infection (%)							
Parenteral	19/27 (70)	7/9 (78)	5/5 (100)	31/41(76)			
Genotype 1	13/21 (62)		1/1				
Vertical	12/25 (48)	8/21 (38)	46/75 (61)	66/121 (55)			
Genotype 1	7/20 (35)		26/52 (50)	33/72 (46)			
Break through	9.8%				6/41 (15)		
Relapse	7.7%		8%		6/35 (17)		

ALT: Alanine aminotransferase; PEG: Polyethylene glycol. [1]PEG-interferon α-2b; [2]PEG-interferon α-2a.

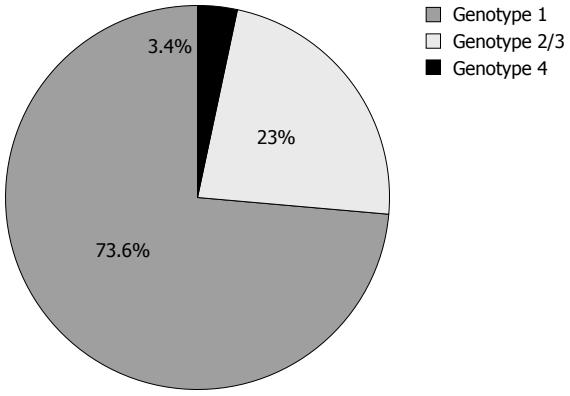

Figure 1 Distribution of genotypes in the five representative prospective trials using polyethylene glycol-interferon α-2b and polyethylene glycol-interferon α-2a in combination with ribavirin, published between 2005 and 2011.

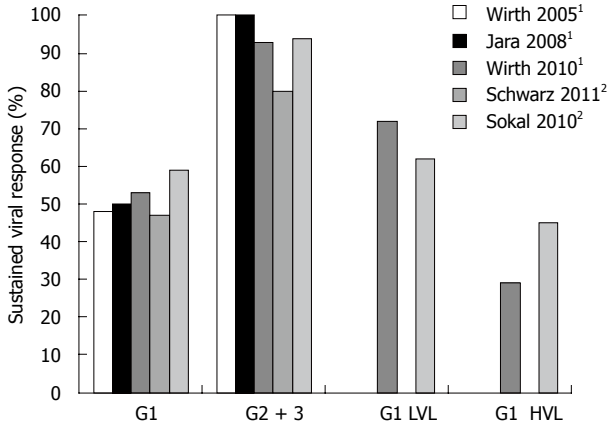

Figure 2 Sustained viral response in five large prospective trials with polyethylene glycol-interferon α-2b[1]/α-2a[2] and ribavirin stratified for genotype and viral load[27,28,30-32]. G: Genotype; HVL: High viral load, > 600 000 U/mL, (Wirth *et al*[32]), > 500 000 U/mL (Sokal *et al*[30]); LVL: Low viral load, < 600 000 U/mL (Wirth *et al*[32]), < 500 000 U/mL (Sokal *et al*[30]).

sults[27]. Specifically, in genotype 1 patients, SVR rate was 17% with PEG-interferon monotherapy compared with 47% in individuals with combination treatment. The difference was also striking in subjects infected with genotype 2 and 3 (36% *vs* 80%).

Up to now, results of seven trials using PEG-interferon α in combination with ribavirin have been reported[27-32]. SVR rates in patients with genotype 1 from 5 trials with more than 30 patients ranged from 44% to 59%. Achieving SVR in children with genotype 2 and 3 was very successful and yielded rates of more than 90%. The relapse rate was between 7.7% and 17%. Four trials used PEG-interferon α-2b and two used PEG-interferon α-2a in combination with ribavirin. An additional report presented the retrospective data in 33 treated Japanese children and young adults[33]. SVR rate in these patients was approximately 82%. Unfortunately, no information regarding genotypes was provided. Table 1 and Figure 1 summarize the characteristics of the five prospective studies. Peg-interferon α-2b and ribavirin were approved for patients aged 3 to 17 years of age by the FDA in December 2008 and the EMA in September 2009.

Baseline viral load

Two studies stratified the results in genotype 1 patients according to the viral load before treatment. In the first study, the cut-off level was 600 000 IU/mL: 32% of children with genotype 1 and high viral load (> 600 000 IU/mL) and 73% with low viral load (< 600 000 IU/mL) achieved SVR[32]. In the second trial, the cut-off value was 500 000 IU/mL: 45% of children with genotype 1 and > 500 000 IU/mL and 62% with < 500 000 IU/mL achieved SVR[30].

Figure 2 summarizes the SVR in relevant pediatric trials using PEG-interferon in combination with ribavirin.

Table 2 Most frequent adverse events during polyethylene glycol-interferon treatment in combination with ribavirin and its appraisal of clinical significance

Interferon α-treatment:
Leukopenia, thrombocytopenia: Frequent, not really significant; if necessary dose reduction
Flu-like symptoms: In all treated patients, not significant
Alopecia: Not significant
Autoimmune thyroiditis: At least 15 %, significant, mostly reversible
Acute psychosis, depression: Very seldom before puberty (< 1 %), rare in adolescents, significant in cases with manifestation; should be under investigation in future trials
Growth delay: Clinically not significant, catch-up growth, but under investigation with relative high priority
Anorexia, weight loss: Mostly not significant with exceptions, normalisation after therapy stop
Ribavirin:
Anemia: Mostly clinically not significant with exceptions, reversible

Most side effects' intensity is decreasing after some weeks of treatment.

Baseline aminotransferases

It is remarkable that the level of aminotransferases or histological findings by liver biopsy do not significantly correlate with SVR. However, interestingly, there was a trend towards a slightly better SVR in patients with normal aminotransferases.

Mode of infection

There is no significant correlation between SVR and the mode of infection. Nevertheless, it seems that individuals with parenteral infection may have a slightly higher probability to obtain SVR. However, the overall response rate in vertically infected subjects was 55% and in genotype 1 patients 46%, which is comparable to the SVR in adults who are mainly parenterally infected (Table 1).

Standard of care

According to approval, in principle, treatment with interferon α-2b and ribavirin administering injections thrice per week can be performed. However, the majority of experts will prefer once weekly dosing using PEG-interferon. To date in America and Europe, only PEG-interferon α-2b (60 μg/m² per week) in combination with ribavirin (15 mg/kg per day) is approved by the FDA and EMA[1]. Patients with genotypes 1 and 4 should be treated for 48 wk, with treatment discontinued at 4-6 mo if there has been no viral response. Patients with genotypes 2 and 3 should be treated for 24 wk irrespective of pre-treatment viral load. In routine clinical practice, there is no need to perform liver biopsy before initiating treatment. In addition, pre-treatment levels of aminotransferases and mode of infection are not predictive for SVR. A five-year follow-up study of children with SVR treated with interferon α and ribavirin showed permanent viral elimination in 98% (Kelly D, personal communication).

Re-treatment

Response rates in patients retreated with a standard of care protocol are dependent on the primary treatment regimen. Individuals with previous interferon α monotherapy or recombinant α-interferon in combination with ribavirin may achieve a higher response rate. There are no studies specifically addressing re-treatment except for the trial by Gerner *et al*[34], which has been performed with a natural interferon α in combination with ribavirin. Previously published reports have only included small numbers of children with failed response, demonstrating a re-treatment response rate of 40%-50% in those with previous interferon α monotherapy. Gerner *et al*[34] reported SVR in only 2/18 patients. Thus, re-treatment, particularly in individuals who have been primarily treated with PEG-interferon and ribavirin, remains prognostically difficult and cannot be recommended until new combination treatment options including directly acting antivirals such as protease inhibitors become available.

ADVERSE EVENTS

The majority of treated children and adolescents will tolerate PEG-interferon and ribavirin well. Nevertheless, almost all patients will experience at least one side effect. The clinical significance of adverse events is summarized in Table 2. Most adverse events are mild to moderate, such as flu-like symptoms including fever, anorexia, fatigue, dry skin and moderate hair loss. In some patients, dose reduction of PEG-interferon may be necessary due to decreased white blood cell counts. Severe anemia is very rare; hence, the need for dose reduction of ribavirin is extremely infrequent. The rates of discontinuation of treatment due to adverse events were low in all trials published. Severe psychiatric side effects were rare in prepubertal individuals, but may be of significance in affected individuals. Appearance of thyroid autoantibodies and thyroid dysfunction during long-term treatment (> 24 wk) has to be considered and carefully monitored. Up to 20% of treated patients, particularly with genotype 1, may have abnormal thyroid stimulating hormone levels or other signs of thyroid dysfunction[31,35]. Another notable side effect is transient growth impairment. Inhibited growth can be observed in 50%-70% with decrease of growth velocity below the 3rd percentile. Shortly after the end of treatment, catch-up growth usually starts with an increased growth velocity followed by achievement of previous growth velocity levels, which can be observed during the follow-up period. Nevertheless, if possible, treatment during pubertal growth spurt should be avoided[36]. In addition, weight loss is very common during the treatment phase; however, most patients experience compensatory weight gain after treatment ends[32]. Regarding quality of life, and behavioral, emotional and cognitive outcomes during and after treatment, no significant impairment has been detected in the PEDS-C trial[37]. More follow-up studies are in progress to evaluate long-term sequelae.

NEW DEVELOPMENTS

There is no doubt that treatment response in patients with genotype 1 is not entirely satisfactory and improved treat-

Table 3 Indication for hepatitis C virus treatment in children-pros and cons

In favour of treatment	Deferral might be considered
High response rate, sustained viral response means cure of the disease	Before 3-4 years of age because of possible spontaneous viral elimination
Prevention of disease progression and social burden	Psychiatric disorder
Better tolerability and less side effects in younger patients (particularly before puberty)	Low response rate in subjects with genotype 1 and high viral load
More favourable factors for response in children (e.g., low viral load)	Pubertal growth spurt
Parents facilitate compliance	More effective treatments in future in genotype 1 non-responders

ment regimens are desirable. A number of directly acting antiviral agents, designed to target viral encoded proteins essential to the HCV life cycle, are currently under development. Phase Ⅲ trials in adults have been completed for two protease inhibitors (telaprevir and boceprevir) and have shown a significantly increased viral elimination rate in combination with PEG-interferon and ribavirin[38-40]. Brand new data indicate that in a considerable number of patients with rapid response, exposure to PEG-interferon and ribavirin may be shortened and response-guided therapy will become the treatment of choice[41]. Approval of telaprevir and boceprevir has been sanctioned by the FDA and EMA in 2011, and pediatric trials will follow in the near future. In adults, genotype 1 non-responders have also demonstrated SVR rates ranging between 59% and 66%, depending on the duration of boceprevir treatment, compared to 20% with standard of care[42]. Given that efficacy data could be extrapolated from adults to children, an approved triple therapy regimen should be expected for non-responders. Nevertheless, they should definitely be included in future pediatric trials[36].

CONCLUSION

In children and adolescents, PEG-interferon treatment in combination with ribavirin for 48 wk produces a sustained viral response rate in approximately 50% of adequately treated individuals. Thus, this option can be offered to all patients irrespective of the level of aminotransferases or mode of infection. There is evidence that subjects with low viral load may respond better than patients with high viral load. In patients infected with genotype 2 or 3, a 90% or even better SVR rate can be achieved. Thus, treatment for 24 wk should be administered in all patients with genotype 2 and 3. According to the approval of the drugs, treatment start is possible beyond three years of age. However, because spontaneous viral elimination may occur within the first 4-5 years of life in vertically infected individuals, watchful waiting for up to five years of age is a justified alternative to an early treatment start. Additionally, different individual and family variables may influence the appropriate time to initiate therapy. An experienced pediatric gastroenterologist should supervise the management of treating these patients. Mid-childhood age before pubertal growth spurt is preferable. Table 3 summarizes pros and cons to indicate or possibly to defer treatment. Adverse events are usually well tolerated, but severe side effects may occur in a small number of patients making dose adjustment necessary. Overall, the encouraging results, particularly in patients with relatively low viral load and/or favourable genotypes and in line with an appropriate consideration of early stopping rules, endorse application of treatment in eligible patients. Re-treatment in non-responding genotype 1 patients should be deferred until a combination of standard care with direct acting antivirals has become available.

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TOPIC HIGHLIGHT

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Role of genetics in the diagnosis and prognosis of Crohn's disease

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Abstract

Considering epidemiological, genetic and immunological data, we can conclude that the inflammatory bowel diseases are heterogeneous disorders of multifactorial etiology in which hereditability and environment interact to produce the disease. It is probable that patients have a genetic predisposition for the development of the disease coupled with disturbances in immunoregulation. Several genes have been so far related to the diagnosis of Crohn's disease. Those genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the most strong and replicated associations with Crohn's disease have been done with *NOD2*, *IL23R* and *ATG16L1* genes. Many genes have so far been implicated in prognosis of Crohn's disease and many attempts have been made to classify genetic profiles in Crohn's disease. *CARD15* seems not only a susceptibility gene, but also a disease-modifier gene for Crohn's disease. Enriching our understanding on Crohn's disease genetics is important but when combining genetic data with functional data the outcome could be of major importance to clinicians.

Key words: Crohn's; Genetics; Polymorphism; Diagnosis; Prognosis; Genome wide scan; Genetic consortium

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EVOLVING ROLE OF GENETICS IN CROHN'S DISEASE

Despite decades of research the etiology of inflammatory bowel diseases (IBD) remains largely unexplained, but considering together epidemiological, genetic and immunological data, we can conclude that IBD are heterogeneous disorders of multifactorial etiology in which hereditability (genetic) and environment (microbial, behavior) interact to produce the immunological background of the disease. It is probable that patients have a genetic predisposition for the development of the disease coupled with disturbances in immunoregulation. The disease can then be triggered by any of a number of different unknown environmental factors and sustained by an abnormal immune response to these factors. Rather, the intensive interaction between intestinal epithelial cells and immune competent cells is critical to maintain and perpetuate the chronic inflammatory process characteristic

for IBD[1].

Early epidemiologic evidence for the role of genetic factors in the pathogenesis of Crohn's disease (CD) came from studies demonstrating higher rates of CD among individuals of Caucasian and Jewish ethnicity, familial aggregation of CD and higher concordance rates of both twins developing CD in monozygotic compared with dizygotic twins. The search for specific CD susceptibility genes, however, has been difficult due to complex genetics, including factors such as the lack of simple Mendelian inheritance patterns, involvement of several genes, and the influence of environmental factors and intestinal microflora on disease development. More than 30 distinct genomic loci encode genes involved in a number of homeostatic mechanisms and have been suggested to be involved in CD etiopathogenesis and prognosis[2].

Until very recently, two main approaches could be undertaken to identify genes in complex diseases: the positional cloning approach, based on linkage analysis, and the candidate gene approach, based on association studies. Linkage analysis studies the co-segregation of the disease with a marker within families. The candidate gene approach uses case-control cohorts or trios of affected offspring with both parents. Here, a specific gene with known or potential interest for the disease is studied. The allelic frequencies (in the case of case-control study) or the transmission of a single nucleotide polymorphism (SNP) towards affected offspring (in the case of trios) are analyzed, and differences between patients and controls, or distortion of transmission towards affected children, will point towards implication of the gene in the pathogenesis of the disease under investigation.

Despite the large numbers of genome-wide association studies (GWAS) established to date, most diseases have only managed to explain some additional percentage of the hereditability estimates. In an attempt to explain some of this missing hereditability, researchers have adopted several complementary strategies. Larger cohorts of cases are being collected, through either further patient recruitment or collaborations. The meta-analysis data generated to date has demonstrated how increasing the cohort sample size generates additional statistical power to detect smaller and smaller odds ratios[3]. Advances in technology and particularly bioinformatics have now made it possible to perform GWAS using common copy number variation probes. Many groups are looking to high-throughput sequencing technology, with the aim of sequencing candidate gene regions identified by GWAS, to hopefully identify either the causal or rare variants[4,5]. Several GWAS have been published in the last decade and have identified many genes associated with Crohn's disease (Table 1). Among these there are recognition-related genes such as *NOD1* and *TLRs*, other susceptibility genes including *DLG5*, *OCTN* and *HLA* and the newest susceptibility genes in CD resulting from GWAS: *IL23R* gene, *ATG16L1* gene and *IRGM* gene[6].

Table 1 Genetic polymorphisms related to Crohn's disease

Genes and the diagnosis of Crohn's disease
Genes related to innate pattern recognition receptors
NOD2/CARD15
OCTN
TLR
Genes related to epithelial barrier homeostasis
IBD5
DLG5
Genes related to molecular mimicry and autophagy
ATG16L1
IRGM
LRRK2
Genes related to lymphocyte differentiation
IL23R
STAT3
Genes related to secondary immune response and apoptosis
MHC
HLA
Genes and the prognosis of Crohn's disease
Genes related to age of Crohn's disease onset
TNFRSF6B, CXCL9, IL23R, NOD2 , ATG16L1, CNR1, IL-10, MDR1, DLG5, IRGM
Genes related to Crohn's disease behaviour
Stenotic/structuring behaviour: *NOD2, TLR4, IL-12B, CX3CR1, IL-10, IL-6*
Penetrating/fistulizing behaviour: *NOD2, IRGM, TNF, HLADRB1, CDKAL1*
Inflammatory behaviour: *HLA*
Granulomatous disease: *TLR4/CARD15*
Genes related to Crohn's disease location
Upper gastrointestinal: *NOD2, MIF*
Ileal: *IL-10, CRP, NOD2, ZNF365, STAT3*
Ileocolonic: *ATG16L1, TCF-4 (TCF7L2)*
Colonic: *HLA, TLR4, TLR1, -2, -6*
Genes related to Crohn's disease activity
HSP70-2, NOD2, PAI-1, CNR1
Genes related to surgery
NOD2 , HLA-G
Genes related to dysplasia and cancer
FHIT
Genes related to extraintestinal manifestations
*CARD15, FcRL3, HLADRB*103, HLAB*27 HLA-B*44, HLA-B*35, TNFa-308A, TNF-1031C, STAT3*
Pharmacogenetics in Crohn's disease
CARD15, NAT, TPMT, MDR1, MIF, DLG5, TNF, LTA

ROLE OF GENES IN THE DIAGNOSIS OF CROHN'S DISEASE

Several genes have been related to the diagnosis of Crohn's disease so far. Those genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the strongest and replicated associations with CD have been done with *NOD2*, *IL23R* and *ATG16L1* genes.

Genes related to innate pattern recognition receptors

***NOD2/CARD15* gene:** *NOD2*/Caspase Recruitment Domain Family member 15 (*CARD15*) acts as a pattern recognition receptor (PRR); this locus has been characterized as the IBD1 locus on 16q12-13[7].

Fine mapping of the IBD1 locus identified the underlying gene on chromosome 16 as the *CARD15* (previous *NOD2)* gene. *CARD15* represents homology with the R genes in plants, genes that confer resistance to infection[8]. Thirty nonconservative polymorphisms have been identified within the gene, which are associated with CD, but only three are common (Arg702Trp, Gly908Arg and Leuc1007insC). The three common variants account for approximately 82% of the mutated alleles. CARD15 is associated with CD only and not with UC. CARD15 codes for a protein expressed in monocytes, macrophages, dentritic cells, epithelial cells and Paneth cells. CARD15 is involved in the recognition of bacterial peptidoglycan-derived muramyl dipeptide through the leucine-rich repeat (LRR) region. Of importance, the frameshift mutation 1007fsinsC that leads to a truncated protein lacking the 33 distal amino acids was associated with impaired activation of the transcription factor NF-κB after stimulation.

It has been shown that Paneth cells play an important role in innate host defense via their ability to secrete antimicrobial peptides and proteins. Although NODs are expressed at low levels in absorptive and secretory intestinal epithelial cells, Paneth cells in the small intestine have been recognized as the predominant site of expression of NOD2 in the epithelium. Furthermore, NOD2 mutations have been associated with decreased expression of antimicrobial peptides, the α-defensins, by Paneth cells. In addition, a distinct gene polymorphism resulting in low β-defensin 2-gene copy number has been associated with a predisposition to colonic Crohn's disease. In addition, NOD2 plays important roles in the promotion of antibacterial T-helper-17 (Th-17) cells in the IL-23-IL-1-IL-17 axis.

CARD15 variants are found in 35% to 45% of white CD patients, with the exception of Scandinavian, Irish and Scottish patients[9,10], in whom the prevalence is much lower. Genotype relative risks of 3 (simple mutation) and 10-44 (double mutations) have been reported in European Caucasians[9,10]. However, CARD15 mutation is not frequent or even absent in African-American populations, in Indians, Chinese and Japanese[11-13]. Other CARD related genetic loci that have been associated with CD diagnosis are the CARD4 (NOD1), CARD8 and CARD9 loci[14,15].

Organic cation transporter genes: Organic cation transporters (*OCTNs, 5q31-33*) are membrane transporters for drugs and positively charged endogenous metabolites. The novel OCTN subfamily may also transport carnitine, which is essential for metabolism of lipids and is involved in transport of light chain fatty acids into mitochondria for b-oxidation. The first study reported on two functional mutations in the carnitine/OCTN cluster on 5q31 (the IBD5 locus) that were associated with Crohn's disease. As membrane transporters of organic cations, OCTNs are therefore important in the maintenance of intracellular homeostasis. In humans OCTN1 and OCTN2 map to IBD5 on 5q31. An OCTN3 has recently been described in humans[16].

Toll-like receptor genes: Host response to microbial pathogens includes self-defense mechanisms such as defensins, PRRs, pathogen-associated molecular patterns and toll-like receptors (TLRs). TLRs recognize conserved motifs on pathogens that are not found in higher eukaryotes and initiate an "innate" (rapid and non-specific) immune response[17]. Subsequently, specific receptors recognizing chemo-attractant molecules mobilize phagocytic leukocytes and induce their migration to inflammatory sites. There, leukocytes encounter the invading microorganisms and ingest them through the activation of phagocytic receptors that mediate the uptake process. Innate immune responses are linked to the generation of corresponding adaptive immune responses and studies of genetically engineered or cellularly manipulated animal models have generated a great deal of new information[18].

Leucocyte-epithelial interactions are of special interest as exposure of epithelial TLRs to microbial ligands has been shown to result in transcriptional upregulation of inflammatory mediators whereas ligation of leucocyte TLRs modulate specific antimicrobial responses[19]. It has been shown that Paneth cells play an important role in innate host defense *via* their ability to secrete antimicrobial peptides and proteins. In addition, it has been shown that NOD2 mutations lead to loss of negative regulatory effects on TLR signaling while activation of the CARD domain results in activation of NF-κB[20].

TLRs are the most important receptors of the innate immune system. They are expressed by immune cells and by intestinal epithelial cells in IBD patients. In humans, at least 10 different TLRs are described and each recognizes a specific pathogen-associated molecular pattern. A transmission disequilibrium test on Belgian IBD trios with CD demonstrated preferential transmission of the TLR4 Asp299Gly polymorphism from heterozygous parents to affected children[21]. TLR9 modulates CD susceptibility and there is interaction between other polymorphisms such as NOD2, IL23R and DLG5[22,23].

Genes related to epithelial barrier homeostasis

The gastrointestinal tract uses a system of tolerance and controlled inflammation to limit the response to dietary or bacteria-derived antigens in the gut[24]. When this complex system breaks down, either by a chemical or pathogenic insult in a genetically predisposed individual the resulting immune response may lead to IBD[25]. Genes or loci involved in the maintenance of epithelial barrier integrity and associated with Crohn's disease are the *IBD5* and the Discs Large Homolog 5 (*DLG5*)[26].

The *DLG5* gene is a 180-kb protein containing 1900 amino acids. DLG5 protein harbours a CARD domain, is a further CD susceptibility gene of the CARD family and contributes to CARD-mediated mechanisms of host defense. In fact, the *DLG5* gene associated protein is a member of Membrane Associated Guanylate Kinase family of scaffolding proteins. Scaffolding proteins

organize protein complexes at cellular junctions to integrate the tethering of adhesion molecules, receptors and intracellular signaling enzymes. Of interest is a population variation in DLG5 variants. For example, DLG5 R30Q variant was not confirmed in other European studies[27,28]. Other genes of potential importance in the same panel are the *PTGER4, ITLN1, DMBT1, BPI* and *XBP1* genes[29].

Genes related to molecular mimicry and autophagy

The innate immune system is the first line of defense against infection. Of interest, virulence factors from bacteria and viruses have been identified that manipulate host innate immune signaling pathways through molecular mimicry. These microbial proteins contain signaling domains that bear sequence and structural similarity to their host targets, and thereby potentially sabotage host immunity by hijacking crucial signaling pathways and uncouple receptor activation from effector induction. Several protein families have evolved to function as receptors or sensors of pathogen invasion. There are two types of signaling domains for the above receptors: the TIR domain for the TLRs and the Pyrin domain or CARD for the NOD-like receptors (NLRs) and retinoic acid-inducible gene 1-like receptors or helicases (RLRs or RLHs).

Molecular mimicry has been invoked as one of the mechanisms responsible for the activation of autoreactive cells by microbial peptides that have structural similarities to self peptides but there is also evidence that antigenically unrelated infections or specific inflammatory signals can result in autoaggressiveness and induction of organ-specific autoimmunity including the gut. The extent and severity of this loss of tolerance is still being defined, as it has demonstrated that loss of tolerance in IBD patients is not exclusive for bacterial antigens and occurs also to orally administered soluble proteins[30]. This subversion of innate immune signaling through molecular mimicry is closely related to the phenomenon of autophagy. Autophagy is the tightly orchestrated cellular 'housekeeping' process responsible for the degradation of damaged and dysfunctional cellular organelles and protein aggregates and is well recognized as playing an important role in maintaining cellular homeostasis under physiological and pathophysiological conditions. Regulated degradation and turnover of subcellular components is essential for normal cellular function, growth, and development. The major catabolic pathway responsible for the disposal of obsolete or damaged organelles and protein aggregates is autophagy (i.e., "self-digestion"). During this process organelles and proteins are encircled in a double-membrane vesicle (the autophagosome), delivered to lysosomes, and the substrates for ATP generation that can be recycled to synthesize new proteins, high-energy phosphates, and other cellular components. Autophagy has evolved as a conserved mechanism for cell survival under conditions of starvation and stress. In addition to (macro)autophagy, characterized by the sequestration of organelles and proteins within an autophagosome, there are two additional subtypes of self-digestion, microautophagy which is protrusion of the lysosomal membrane per se around a region of cytoplasm and chaperone-mediated autophagy in which degradation is restricted only to those proteins with a consensus peptide sequence recognized by specific chaperone complexes[31]. Autophagy is now considered to be important for host defense against intracellular microorganisms. The associations of these autophagy-associated genes with Crohn's disease strongly support the hypothesis that abnormal innate immune responses to intracellular pathogens contribute to the pathogenesis of Crohn's disease. In fact, the pathological characteristics of human Crohn's disease represent "granuloma" formation. The mechanisms of granuloma formation remain unclear. Recent studies have demonstrated functional roles for IL-23 in the differentiation and promotion of Th-17 cells. Autophagy genes that have been related to CD diagnosis are the *ATG16L1*[32,33], *IRGM* and the *LRRK2* gene[34]. Unraveling the mechanisms of such molecular mimicry is crucial to our understanding and clinical intervention of infectious diseases and inflammatory disorders of unkown aetiopathogenesis including Crohn's disease.

Genes related to lymphocyte differentiation

***IL23R* gene:** Dysregulated cytokine production by mucosal lymphocytes and macrophages has been implicated in the pathogenesis of CD. In fact, an exclusive increase of $CD4^+$ T cells in inflammatory bowel disease and their recruitment as intraepithelial lymphocytes has been demonstrated[35]. $CD4^+$ T cells secreting interleukin-17 (T helper type 17) cells have emerged as a key effector population driving colitis in animal models previously associated with exaggerated T helper type 1 responses.

Of the genes involved in the differentiation of Th-17 lymphocytes the *IL23R* gene has been proved of great importance and has been related to Crohn's disease[36,37].

The IL23R, consisting of an IL-12β1 and an IL23R chain, is highly expressed on memory T cells. IL23 is a novel cytokine formed *via* the binding of IL12p40 to a p19 protein. After binding to the IL23 receptor, IL23 preferentially activates memory T cells. IL23 does exhibit some similar biological activities to IL-12; however, IL-12 is more involved in the differentiation of naïve T-cells into Th1 lymphocytes and subsequent interferon-gamma production. IL23, on the other hand, mediates proinflammatory activities in part by the production of IL17 through activation of Th17 lymphocytes[38].

Signal transducer and activator of transcription 3 gene: Signal transducer and activator of transcription 3 (STAT3) play an important role in various autoimmune disorders including IBD[39,40]. STAT3 was initially identified as an acute phase response factor, an inducible DNA binding protein that binds to the IL-6 responsive element within the promoters of hepatic acute phase protein genes and is involved in IL-6 dependent T-cell proliferation through prevention of apoptosis. Subsequent studies indicate that STAT3 becomes activated in response to

a wide variety of cytokines and growth factors. Recent studies have revealed that STAT3 activation plays distinctly different roles between innate immune responses and acquired immune responses in colitis. STAT-3 mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing the survival of pathogenic T-cells. In contrast, STAT3-mediated activation of innate responses contributes to the suppression of colitis. Emerging data indicate that STAT3 is one of the crucial targets for the treatment of IBD. However, as the receptors of these cytokines and growth factors are present in both innate and acquired cells, activation of STAT3 is likely to occur in both cell types. Therefore as the function of STAT3 is a double-edged sword, careful attention should be directed toward the cell population that is being targeted when one contemplates STAT3 inhibition or activation in human IBD[41]. Within the same panel, other than *STAT3* genes, and with probable importance are the *TNFSF15, JAK2, CCR6* and *ICOSLG* genes[42-44].

Genes related to secondary immune response, apoptosis and other pathways

Chemokines play a central role in the pathogenesis of IBD as they are able to trigger multiple inflammatory actions including leukocyte activation and chemoattraction, granule exocytosis, production of metalloproteinases for matrix degradation and upregulation of the oxidative burst[45]. Therefore, further support is given for genes that relates to secondary immune response, apoptosis and other pathways. For example, in the IBD4 locus 4 several interesting candidate genes, which may be relevant in the pathogenesis of CD, lie within this region (e.g., genes regulating apoptosis, signal transduction proteins, chemokine receptors, T cell receptor, metalloproteinases).

Gene expression profiles from colon lamina propria fibroblasts have demonstrated several functional changes in some proteins coded from the corresponding genes: collagen types Ⅰ, Ⅳ, XIV, matrix metalloproteinase 1, cathepsin K, stroma cell-derived factor-1, chitinase3-like-1 and many others[46]. The major histocompatability complex (MHC) has been extensively investigated. Human leucocyte antigen (HLA) class Ⅱ molecules present partially digested antigen to the T-cell receptor and play a central role in the immune response. In CD MHC and HLA studies have yielded conflicting and heterogenous results. HLADR1 has been implicated with CD[47].

Many other genes, loci and chromosomes involved in CD have also been advocated in several studies that however still require wide replication and association with clinical practice. These include *CNR1, MCP-1*[48], *PTPN2* (protein tyrosine phosphatase)[49], *PTPN22, NKX-3, IL-18 RAP /IL-18R1, IL12/IL23* pathway[50], *PTGER4, MST1/BSN/MST1R*[50,51,52], *IL-2/IL-21*[53], *TYK2, JUN, NAT2*[54], *IL-10, NELL1, NKX2-3*[55], *Cyclin Y, Hect domain, 1q24, 10q21, 5p13, RCC1-like domain, ICOSLG, CDKAL1*[56], *13q13.3, 1p35.2, 3p29, 5p13.1*[57,58], *X chromosome*[59], *NLRP3*[60], *Vitamin D receptor polymorphisms*[61] and many others as well.

Genes in family and ethnic group studies

Linkage studies performed in complex genetic disorders such as CD frequently use model-free analytic methods, which are non-parametric analyses that do not assume Mendelian recessive or dominant models of inheritance.

The strongest risk factor for IBD is having a relative with the same disease. First-degree relatives of patients with CD have a 12-to-15 times greater risk of developing CD than do people of comparable age in the general population[61]. Familial clustering can also result from exposure to common environmental risk factors. Twin studies are very useful to determine the degree of genetic versus nongenetic etiologies for a trait. Today, there is no evidence of a separate entity of familial IBD[62,63]. Based on the current literature, phenotypic differences between familial and sporadic cases of IBD are weak. Available data are to be accepted with caution, however, as they are mostly retrospective and may be biased. CARD15 explains around 20% of the genetic predisposition to Crohn's disease[64]. The relative risk of developing CD in the presence of one mutation is 2-4, but increases dramatically in the case of two mutations (compound heterozygous or homozygous).

Although NOD2 provides no clear familial predisposition, unaffected relatives carry an increased rate of CARD15 variants (37.1%) compared to controls, and it would be interesting to see if they will eventually develop symptoms[65-67]. In addition, maternal transmission of CARD15 variants seems protective with a lower ratio of affected/unaffected children when compared to fathers[68,69]. In the light of the foregoing data, it seems that genetic counselling should be done with caution. In addition, families should not receive genetic counseling/information about age at onset and disease severity. Ethnic group studies and ethnic variation were first demonstrated in the Jewish population, and those studies are of major importance in this context[70].

ROLE OF GENES IN PROGNOSIS OF CROHN'S DISEASE

This is a major issue that greatly concerns patients. Many genes have so far been implicated in the prognosis of CD and numerous attempts have been made to classify the genetic profiles in CD. Of interest, CARD15 seems not only a susceptibility gene, but also a disease-modifier gene for CD. Of the many studies published on the clinical relevance of CARD15 mutations, there are several providing data on disease location, and the majority of them support a significant association of CARD15 mutations with ileal disease site, while some demonstrate a connection with the absence of colonic location. Some studies also provide data supporting the relevance to CARD15 variants with stricturing disease behavior, and also penetrating behaviour. Other pertinent studies revealed an association with early onset of the disease. These investigations also support the thesis that pediatric Crohn's is like a "more genetic disease" consistent with other polygenic disease

models. Other reports provide data on an increased risk or need of surgery related to CD[71].

Differences among studies are difficult to explain, and we could argue about the low number of patients in some of the studies, the disease variability among Caucasians and finally differences regarding disease assessment and interobserver agreement. Whether the described relationship between the CARD15 variants and both stenosing phenotype and increased need for surgery in CD patients is a true association or only reflects the high proportion of ileal CD developing bowel stenosis and, therefore, requiring surgery, is still a matter of controversy.

Genes related to age of Crohn's disease onset

With respect to age of CD onset and more specially to childhood or early-onset Crohn's disease, many genes/loci have been implicated: *TNFRSF6B, CXCL9*[72], *IL23R*[73,74], *NOD2*[75], *ATG16L1 rs2241880*[76], *CNR1*[77], *IL-10*[78], *MDR1*[79]. Of interest DLG5 seems protective for female children[80] while there are also studies not supporting the relation of genes and early onset of CD[81] or supporting the relation of IL-10 and IRGM with adult onset[82].

Genes related to crohn's disease behaviour

Genes related to stenotic/structuring behaviour in CD are: *NOD2/CARD15*[83], *TLR4*[84], *IL-12B*[85], and *CX-3CR1*[86,87]. Of importance NOD2/CARD15 has been also related to acute intestinal obstruction[88]. IL-10 and IL-6 are also potentially related to stenotic/structuring behaviour in CD while genetic variants of several metalloproteinases and their inhibitors would be excellent candidate genes, since these molecules are considered to play a key role in the abnormal fibrogenesis that underlies the development of bowel stenosis in CD patients. Genes related to penetrating/fistulizing behaviour in CD are as follows: *NOD2, IRGM, TNF*[89], *HLADRB1*[90]; the C-allele in CDKAL1 rs6908425 SNP is associated with NOD2 (-) perianal fistula, whereas OCTN and the near *IL-12B* gene rs12704036 T-allele have a relationship with non perianal fistula[91]. Inflammatory CD behaviour has been related to HLA variation[92] while granulomatous disease has been related with TLR4/CARD15 variants[93].

Genes related to Crohn's disease location

Upper gastrointestinal Crohn's disease has been related to NOD2[94] and MIF variants[95]. Ileal CD has been related to the following genes: *IL-10*[96], *CRP* gene[97], *NOD2, ZNF365* and *STAT3*[98]. Genes/loci associated with ileocolonic CD are *3p21*, *ATG16L1*[98] and *TCF-4* (*TCF7L2*)[99]. No role for phenotype in IL23R gene has been demonstrated[100] while a detailed genotype-phenotype analysis revealed weak associations of the IL23R rs10024819 variant with ileal involvement and stenoses in carriers of the TT genotype. Finally, the HLADRB1*0701 has been associated with ileal CD, but only in patients that have no CARD15 variants[101]. Colonic CD has been related to the following genes: the HLA region was associated with inflammatory colonic phenotype and TLR4[102], TLR1, -2, -6[103]. *TNF* gene showed a negative association with stricturing behaviour or colonic location[104]. For IBD5 and OCTN1 and 2, results have not been consistent but associations with perianal and ileal disease have been reported.

Genes related to Crohn's disease activity

Genes implicated in disease activity are the following: *HSP70-2* heat shock protein gene[105], *NOD2*[106], *PAI-1* (type 1 plasminogen activator inhibitor[107]), while the combination of NOD2 and PAI-1 predicted complicated disease behavior[108]. Of importance, NOD2 predicted lower weight in children[109], and CNR1 low BMI[110].

Genes related to surgery

NOD2 gene has been related to early pediatric surgery[111], stenosis and need for surgery[112], previous surgeries[113], increased number of surgeries[107] and surgical costs[114]. NOD2 has no relation to the risk of re-operation[115]. Finally, HLA-G has been associated with higher risk for ileocolonic resection[116].

Genes related to dysplasia and cancer

The *FHIT* gene (fragile histidine triad gene) located at 3p14.2 has been identified as a candidate tumor-suppressor gene. The gene spans the t (3; 8) translocation breakpoint of familial renal-cell carcinoma and contains the FRA3B fragile site. It encodes the human diadenosine triphosphate hydrolase, which in vitro cleaves the diadenosine substrate into ADP and AMP. It has been suggested that *FHIT* gene plays a role in the pathogenesis of IBD and the development and progression of a subgroup of IBD-related carcinomas at an early phase[117-119].

Genes related to extraintestinal manifestations and concommitant diseases

Extraintestinal manifestations are common in CD. Genes related to CD extraintestinal manifestations have been reported, as follows. Peripheral arthritis was related with FcRL3[120], HLADRB*103, HLAB*27 HLA-B*44, HLA-B*35, TNFalpha-308A[121]. CARD15 has been related to spondyloarthropathy[122] and uveitis[123] but not with sacroileitis[124]. TNF-1031C was associated with erythema nodosum while certain HLA alleles (HLA-B27, HLA-B35, HLA-B44) were connected with different disease behaviour and extraintestinal manifestations such as arthropathy, eye and skin manifestations. Genes/loci related to other chronic diseases concomitant to CD are 10p12.2 (sarcoidosis and CD)[125], STAT3 (multiple sclerosis and CD)[126], and a parallel genetic fingerprint between leprosy and CD[127].

Pharmacogenetics in Crohn's disease

Pharmacogenetics is of major importance in CD therapeutics and prognosis. Genes have been implicated in influencing the efficacy and side effects of drugs and reflect a complex interplay regarding absorption, elimina-

tion and transport. Future studies need to be large and prospective with uniformly phenotyped patients and correlating genetic associations with functional data. In addition hypotheses such as whether observations about drug response in IBD lead us to IBD etiology or whether the genes that control the drug response are related to genes that control the disease still remain unanswered. Pharmacogenetic studies to date have found no association between CARD15 variants and prediction of response to various IBD therapies. In addition, responses to azathioprine, steroids and infliximab are not related to NOD2[128]. Of note, NOD2 was related to antibiotic failure[129].For mesalazine, variability in drug acetylation was demonstrated many years ago with patients divided in slow and rapid acetylators, because of polymorphisms in the N-acetyltransferase (*NAT*) genes. Two isoenzymes NAT1 and NAT2 have been identified in humans and more than 50% of Caucasians are NAT2 slow acetylators. Mesalazine is acetylated in the liver by NAT1 into N-acetyl-5 aminosalicylates and excreted in the urine[47].

The clinical usefulness of pharmacogenetics in CD is limited to AZA and TPMT at this moment. The human TPMT gene, consisting of 10 exons, is located on chromosome 6p22.3. The hereditary nature of the TPMT deficiency in humans was initially identified in a study of TPMT activity in red blood cells (RBC). This and subsequent studies determined the distribution of TPMT activity in RBC to be trimodal; 90% of persons have high activity, 10% have intermediate activity and 0.3% have low or no detectable enzyme activity. To date, numerous mutant TPMT alleles have been identified, including the three most frequent alleles (TPMT*2, TPMT*3A and TPMT*3C), which account for 80%-95% of intermediate or low TPMT enzyme activity cases. The prevalence of the most frequent SNPs in the TPMT gene has been reported to vary worldwide. However, it is of interest that studies on the prevalence of TPMT SNPs in large IBD cohorts are lacking. Although AZA is an effective drug for maintenance of remission in IBD, it is associated with side effects. Clinically sound pharmacogenetic studies over the last two decades have shown that polymorphisms in the *TPMT* gene locus play a significant role in the occurrence of various side effects of thiopurine drugs including life-threatening bone marrow toxicity (BMT), a serious dose-related toxicity[130-134].

The G2677T variant in the *MDR1* gene predicted gastrointestinal and unspecified intolerance to azathioprine and methotrexate in IBD patients. These findings suggest a role for MDR1/P-gp in the mechanism of action of azathioprine and methotrexate[135,136].

Twin studies have linked polymorhisms of the vitamin D receptor (*VDR*) gene with bone mineral density in healthy women and in addition VDR is an important regulator of calcium metabolism and bone cell function and influences calcium absorption from the intestine. VDR polymorphisms have also been implicated in susceptibility to CD[137].

The HLADR region has been associated with failure to budesonide[138] while DLG5R30Q predicted response to steroids[139]. Other genes such as MIF (macrophage migration inhibition)[140] and MDR have been also related to steroid therapy[136]. In addition, 1082 AA IL-10 genotype was associated with steroid dependency, whereas the allele 113A of the *DLG5* gene conferred resistance to steroids.

Regarding response to infliximab the data for TNF gene are conflicting. Specifically, there are conflicting data regarding the role of FcGR3A, which has been supported by some authors[141,142], but was not confirmed in patients of the ACCENT I study. Response to infliximab is not related to *TNFa-308*[143] or *TNFR1* and *TNFR2*[144] or *NOD*[145] or *CRP* gene[136]. The association between the Fas ligand-843 TT genotype and lack of response to infliximab seemed to be the most relevant observation[136]. The relationship of infliximab response and lymphotoxin alpha gene (*LTA*) is also conflicting[144].

WHAT LIES AHEAD

Gene-to-gene crosstalk and epistasis

With new methodologies like genome wide association studies, microarrays, and fine SNP analysis becoming available during the last decade, our investigative armamentarium has been considerably enriched. As many studies with complex statistics arise, we understand increasingly the real crosstalk present among genes and the need of a genetic panel for disease diagnosis and prognosis. It is now evident that gene-to-gene interaction and epistasis modulate disease activity and susceptibility[146]. Some data have come to light. A genome-wide scan in a Flemish population of IBD affected families supports the existence of *IBD4* on 14q11, and has shown additional evidence for the existence of other susceptibility loci (1p, 4q and 10p). This study has further demonstrated that epistasis and gene to gene interactions (CARD15-TLR4) are also present in IBD and that population heterogeneity is not to be underestimated[147]. Crosstalk has been demonstrated for TLR9 with NOD2, IL23R and DLG5, and epistasis has been shown between IL23R and DLG5. Also potential epistasis between IL23R variants and the three other previously described CD susceptibility genes CARD15, SLC22A4 and SLC22A5 (OCTN 1 and 2) has been shown[116].

Genetic consortium studies and genome wide scans

Over the past few years, a combination of progress in high throughput genotyping technology and growing knowledge about the human genome through the International HapMap project and the Human Genome Project have enabled genome-wide association studies (GWAS) for several complex diseases. To understand the approach to conducting GWAS in this setting it is important to expound on the concept of linkage disequilibrium, which refers to the nonrandom association of alleles at nearby loci. Specifically, linkage disequilibrium refers to adjacent alleles assorting together nonindepen-

Table 2 Predicted future developments in the genetics of Crohn's disease

What lies ahead in the genetics of Crohn's disease
Gene-to-gene crosstalk and epistasis
Genome wide association studies
Microarrays
Fine single nucleotide polymorphism analysis
Genetic consortium studies and genome wide scans
Genome-wide association studies
Genetic consortium studies
Future perspectives
Functional studies to understand the mechanisms
Combining genetic data with functional data
Combination of a panel of clinical, biochemical, serological and genetic factors
Functional consequences of polymorphisms
Molecular and cellular mechanisms leading to Crohn's disease
Predict disease outcomes
Redesigning the methods of treatment

dently from generation to generation because they are tightly linked and thus less likely to become separated by recombination. Genetic consortium studies are of major importance and homogeneity in methodology issues is of paramount value[148-151]. Appropriate study design[152], power analysis[153] and overall data analysis and meta-analysis[154] are mandatory. Accurate estimation of sample sizes required in a genetic association study is essential before commencing genotyping, to ensure that the study is sufficiently powered to detect the subtle genetic effects that contribute to most complex diseases. The extensive genetic variation and complex linkage disequilibrium across even a small genomic region will give rise to several alternative scenarios. Genetic variation across a region studied should be carefully evaluated and consideration should be given to possible linkage disequilibrium and allelic heterogeneity when evaluating power of an association study. As larger datasets are studied and combined, as genotyping platforms provide even greater depth of coverage of the genome and as modest hits are followed up in large independent panels so that the vast majority of true signals should be identified. These robust genetic data will truly provide a solid platform for functional studies to understand the mechanisms by which these genetic variants predispose to CD. Finally studies at post-transcriptional level become more and more urgent[155]. Enriching our understanding of CD genetics is important but when combining genetic data with functional data the outcome could be of major importance. In fact, improved understanding of immune mechanisms, on which manifold genetic and environmental traits might converge, and which ultimately mediate all phenomena in inflammatory bowel disease, holds promise (Table 2).

CONCLUSION

The recent advances in the understanding of CD genetics have been tremendous[156]. Starting with the susceptibility area, whole genome linkage and association scans have already led to the identification of a number of susceptibility genes (*NOD2/CARD15, DLG5, OCTN1* and *2, NOD1, IL23R, PTGER4, ATG16L1* and *IRGM*) of which the *NOD2/CARD15* gene is the most replicated and understood at present. Although it is clear that genetic research in IBD has advanced our understanding of the clinical heterogeneity of the disease, new efforts are required and point towards the complex combination of a panel of clinical, biochemical, serological and genetic factors, in order to achieve the optimal prediction of both clinical behaviour and response to therapy.

Genome-wide association studies have allowed an unprecedented rapid unraveling of the genetic basis of IBD; however there will be much more follow-up work needed in this field. First, ongoing work including meta-analysis of the Crohn's disease genome wide association studies will probably reveal additional Crohn's disease susceptibility genes. It will then be essential to investigate the functional consequences of polymorphisms in these genes so the molecular and cellular mechanisms leading to CD can be better characterized. Finally, genotype-phenotype correlation studies should help clinicians predict disease outcomes with more accuracy, including the risk for complications, need for surgery, and response to therapy, and finally lead to redesigning the methods of treatment of CD patients.

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REVIEW

Immune mechanisms of Concanavalin A model of autoimmune hepatitis

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Abstract

As a chronic inflammatory disease of the liver, the pathogenic mechanisms of autoimmune hepatitis (AIH) have not yet been elucidated, with prognosis and diagnosis remaining unsatisfied. Currently the only viable treatments of AIH are immunosuppressant application and liver transplantation. It is considered that lack of good animal AIH models is the main reason for the shortage of a simple and efficient cure. The Concanavalin A (Con A) model is a typical and well established model for investigating T-cell and macrophage dependent liver injury in mice, which closely mimics the pathogenesis mechanisms and pathological changes of patients, and is regarded as the best experimental model for AIH research so far. In this paper we elucidated the pathogenic mechanisms of AIH and the evolution of relative animal models. We go on to further focus on Con A-induced liver injury from the point of immunological mechanisms and the change of cytokine levels. Finally, we manifested the clinical significance of the AIH animal models and the challenges they would meet during their future development.

Key words: Autoimmune hepatitis; Animal models; Concanavalin A

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory disease of the liver, characterized by a loss of self-tolerance leading to the appearance of autoantibodies, pathological changes and dysfunctions (the detailed pathogenic mechanisms of which still remain vague). According to different antibodies profiles, AIH is classified into three categories: AIH type 1 is characterized by the presence of antibodies to nuclear antigens (ANA) and/or anti-smooth muscle antigen (SMA) antibodies; AIH type 2 is characterized by anti-liver kidney microsomal (LKM)-1 and low level of LKM-3 antibodies (with or without ANA or SMA antibodies); AIH type 3 is characterized by autoantibodies against soluble liver

antigen/liver pancreas (with or without ANA or SMA antibodies)[1].

Around the world, the incidence of AIH is 0.1-1.9 cases out of 100 000 persons per year, which is not very high[2]. However, the prevalence of autoimmune hepatitis in Europe is in the range of 11.6-16.9 cases per 100 000 persons[2], and in the United States, the proportion of hepatitis among patients with liver cancer is about 11%[3]. Incidence is also different between men and women. It was reported that women are more vulnerable to AIH[2,4,5].

Unfortunately, we do not have any better choice of medicines other than immunosuppressants, which can be classified into four generations[6,7]. In the 1950s, the first generation immunosuppressants were limited to azathioprine and steroids, which were enriched by polyclonal anti-lymphocyte and anti-thymocyte globulins in the 1960s[6]. For this generation, 70%-80% patients might relapse after withdrawal of treatment[8]. More seriously, they have many side effects[9]. Corticosteroids, Tacrolimus and Cyclosporine are typical of the second generation[6]. In the early 1990s a broad range of third-generation immunosuppressants emerged[6], most of which are monoclonal anti-lymphocyte and anti-thymocyte globulins followed by the fourth generation, such as the IL-2 monoclonal antibody with its highly specific sites of action[7,10]. The second and the third generation immunosuppressants are in most cases successfully used for treatment of AIH[11,12]. But long term applications of these immunosuppressive drugs carries serious risks[13] and sustained remission[9], even at low doses. Non-system steroids may be the best candidates[14]. Patients with liver failure or fulminant presentation who fail to improve under immunosuppressive therapy should be considered as candidates for liver transplantation. Without treatment, nearly 50% of patients with severe autoimmune hepatitis die in approximately 5 years[15]. Taking this into consideration, it is significantly important to develop new specific drugs. Animal models are the basis of drug discovery and development. Up to the time of writing, there are still no universal animal models of AIH which can be used as pathogenic models as well as therapeutic ones.

As the most important AIH research model, the Con A animal model plays a key role in AIH drug development. In this article we attempt to review the evolution of the Con A animal model of AIH, to sum up the mechanisms of Con A-induced liver injury, and to illustrate its statue in AIH drug development. Furthermore, the future challenges of the animal model are also discussed.

EVOLUTION OF AIH MODEL

AIH models have evolved from crude liver homogenates and adjuvants to the genetic engineering level, which can be classified into five phases[16]. The first phase was in 1972 when Buschenfelde *et al*[17] induced chronic active hepatitis in rabbits immunized with human liver proteins combined with complete Freund's adjuvant. This work built a solid foundation for AIH models. The second phase began in 1983, when Mihas *et al*[18] established transient hepatitis in mice by immunization with syngeneic liver proteins together with the polysaccharide of Klebsiella pneumoniae. In the third phase, taking place from 1987 to 1990, many scientists used inbred or neonatal thymectomy mice to establish the T-cell reactive AIH model. They induced transient hepatitis by immunizing C57BL/6 mice with the supernatant of liver syngeneic liver homogenates with complete Freund's adjuvant and used adoptive transfer technology to study the roles of T-cell, which allowed studies of the pathogenesis of AIH[19]. The fourth phase, from 1992 to 2003, had endotoxin and plant lectin-induced hepatitis models receive extensive attention. Three types of inducers were wildly used during this period: Con A[20], D-galactosamine (GalN) with low dosage of lipopolysaccharides (LPS)[21], and high dosage of LPS[22]. In the fifth phase, from 2002 to 2008, the application of genetic engineering technology accelerated the development of AIH model[23]. From one aspect, gene knockout and transgenic animals facilitated the study of the functions of certain genes[24]. From the other, production of designated antibodies using genetic engineering methods made it possible for scientists to get specific types of autoantibodies[25], and also made it possible for the Con A models to mimic a specific subtype of AIH. Significantly, the production of designated autoantibodies is based on known antigens. Scientists have now clarified the antigens to the following autoantibodies: the antigen to LKM-1 is cytochrome P450 2D6[26,27], the antigen to LKM-2 is cytochrome P450 2C9[1], the antigens to Liver Microsomal are cytochrome P450 1A2 and cytochrome P450 2A6[1]. The animal models of type 2 AIH[28] have been reported, but obviously type I animal models have more clinical significance than type Ⅱ[2]. As is widely known, it is difficult to find the antigen of autoantibodies, which is the limitation of the gene engineering AIH model. The features and parameters of the three models are listed in Table 1[29].

From the information in Table 1, it is obvious that the Con A-induced hepatitis model possesses more advantages than the other two. Firstly, the Con A model includes only one inducer, making it easier to be established compared with the GalN/LPS model. Secondly, there is no significant change of the level of transaminase, which is considered a valid index of the severity of liver injury, in the LPS model, while such change is remarkable in the Con A model. Thirdly, in the Con A model, the serum level of many cytokines relevant to inflammation change dramatically, which is favorable for the study of the pathogenic mechanisms of AIH[29]. Furthermore, besides AIH, Con A animal models with different parameters are adaptable to many clinical diseases, such as fulminant hepatitis[30], virus hepatitis[31], hepatotoxin[32,33] and alcoholic liver diseases[34]. In summary,

Table 1 The features of the autoimmune hepatitis model induced by endotoxins and plant lectins

	Con A[29]	GalN/LPS[29]	LPS[29]
Animal	BALB/c-mice (6-8 wk)	BALB/c-mice (6-8 wk)	BALB/c-mice (6-8 wk)
Inducer	Con A	GalN/LPS	LPS
Dosage	20 mg/kg	LPS: 5 μg/kg GalN: 700 mg/kg	10 mg/kg
Application method	Tail vein	Subcutaneous	Subcutaneous
Transaminase level (max)	8 h	8 h	No significant change

Con A: Concanavalin A; GalN: D-galactosamine; LPS: Lipopolysaccharides.

Con A AIH model is easy, convenient, inexpensive and repeatable, as well as a T-cell activated model and could greatly facilitate the study of the mechanisms of AIH-induced liver injury.

IMMUNOLOGICAL MECHANISMS OF CON A-INDUCED LIVER INJURY

Con A is one kind of lectin, which is purified from *Canavalia brasiliensis*[35]. Tiegs *et al*[20] injected Con A, Succinyl Con A with no agglutination activity, and *Vicia faba* lectin with strong agglutination activity to nuclear magnetic resonance imaging mice *via* tail vein, respectively. The results showed that only Con A could induce liver injury, which indicated that the *in vitro* agglutination activity of this lectin does not correlate with its hepatotoxic potential *in vivo*. They also studied the correlation between the hepatotoxic potential of Con A and its sugar-binding site[20]. Con A has specific sugar-binding sites, whose ligands are α-D-mannoside, methyl α-D-mannopyranoside, α-D-glucose, and methyl-α-D-glucose[36]. They co-administrated Con A with α-D-mannoside or methyl α-D-mannopyranoside to mice, which prevented the induction of hepatic injury by the lectin[20]. This suggested that free sugar-binding sites are indispensible for the induction of liver injury by lectin. Sato *et al*[37] also confirmed that Con A/glycogen multilayer films can be decomposed by exposing them to sugar solutions (D-glucose, D-mannose, methyl-alpha-D-glucose and methyl-alpha-D-mannose), as a result of the displacement of sugar residues of glycogen from the binding sites of Con A by the free sugar added in the solution. This suggested that sugar-binding sites are prerequisites of activated Con A. But among Con A, Succinyl Con A and *Vicia faba* lectin, which have the same sugar-binding site, only Con A can lead to high level of transaminase[20]. These two results indicated that the hepatotoxic potential of Con A is not determined by its agglutination activity or sugar-binding site. Other mechanisms may exist.

The mechanisms of the Con A model have interested many scientists. Previews papers describe that the aminotransferase of mice in thymus[38] and CD4[39] neutralized groups decreased significantly compared with the control group, while the CD8 neutralized group show no significant change. What is more, after injection of Con A, the blood level of interleukin 2 (IL-2), IL-4 and interferon gamma (IFN-γ) all increased dramatically[40]. This suggested that the $CD4^+$ T helper (Th) cell was involved in the liver injury[40]. It is reported that $CD4^+$-positive Th cells recognize the Con A-modified major histocompatibility complex (MHC) structures of macrophages and become activated, followed by an inflammation reaction and the release of IL-1 and IL-2 to the blood[41]. In the experiment of CD8 neutralization, there was a minor decrease of the transaminase level, which suggested that the target cell lysis by cytotoxic $CD8^+$ T lymphocyte (CTL) also contributes to liver injury, but not as the major factor. In conclusion, the main mechanism of the Con A model is that Th cell activation increases the relevant cytokine level, which leads to liver injury. Meanwhile, the CTL mediated target cell lysis may be the secondary mechanism.

In the liver, lymphocytes, sinusoid endothelial cells (SECs), Kupffer cells (KCs) and stellate cells are all involved in the immune response[42]. Lymphocytes can be classified into two groups, exogenous and endogenous[43]. Exogenous lymphocytes originate from the thymus[44], bone marrow[44,45], intestinal tract[46], spleen[47] and lymph gland[48], and enter the liver through circulation. Endogenous lymphocytes are enriched in the portal area of the liver, which count for 25% of non-parenchyma cells in the liver[49]. The endogenous lymphocytes are mainly T cells, while B cells only count for 5% of them. This is why lymphocyte infiltration is mainly focused in the portal area[50].

For a long time, there have been debates about whether KCs or SECs plays a major role in immunological liver injury[51-53]. Knolle *et al*[52] established the spontaneous and LPS activated cell model, and found that SECs and KCs both secreted IL-1 and IL-6, which suggested that SECs are also key cells in liver injury. It has been found that fifteen minutes after intravenous injection of Con A, Con A binds to SECs first; 4 h later, Con A begins to bind to the KCs[52]. Using Scanning Electron micrograph, it is clearly seen that 4 h after intravenous injection of Con A, blood cell endothelium attaches to the SECs first[52]. Then lymphocytes or neutrophils are trafficked into the hepatocytes, leading to inflammation[52]. We can conclude that SECs and KCs are both important, but they play their roles in the different phases. After injection, Con A binds to the mannose gland in the SECs surface first, leading to the breakdown of the SECs membrane, bleb formation and cytoplasm disappearance[30]. SECs detachment facilitates the binding of Con A to the KCs. $CD4^+$ Th cells recognize the MHC class Ⅱ and T cell receptor of KCs modified by Con A and are then activated[30]. Such liver injury is mainly mediated by T helper cells, including Th1 and Th2 cells. Figure 1 depicted the mechanisms of T cell activated liver injury.

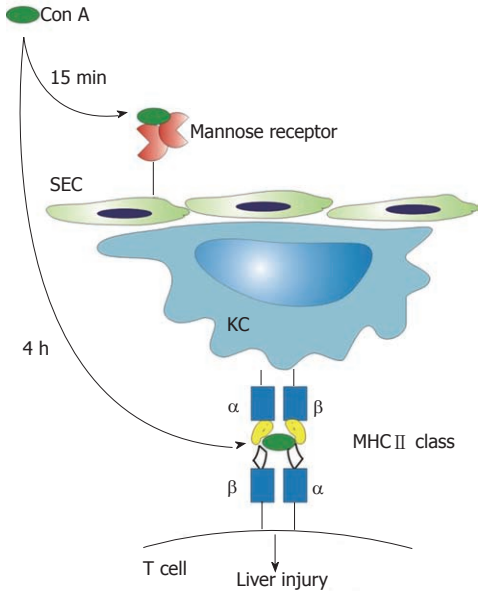

Figure 1 Mechanisms of Concanavalin A induced T cell activated liver injury. Con A: Concanavalin A; KC: Kupffer cell; SEC: Sinusoid endothelial cell; MHC: Major histocompatibility complex.

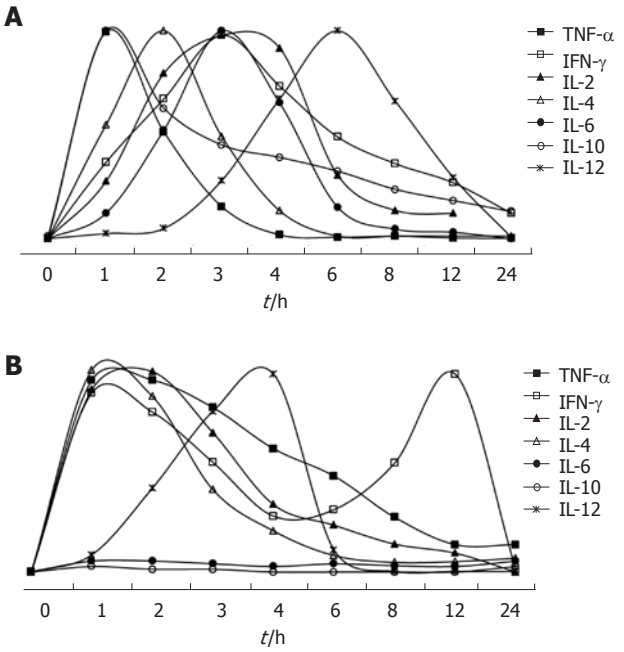

Figure 2 Different cytokines levels within 24 h. A: Plasma level; B: Liver level. TNF: Tumor necrosis factor; IFN: Interferon; IL: Interleukin.

CHANGES IN THE EXPRESSION LEVELS OF RELEVANT CYTOKINES

Some major cytokines involved in the Con A-induced liver injury are IFN-γ[54-55], IL-2[55], IL-4[56], IL-6[56] and tumor necrosis factor α (TNF-α)[56], of which TNF-α and IFN-γ are the major ones.

Figure 2 shows the time when different cytokines reach their peak level in the plasma and liver. In the plasma, TNF-α and IL-10[29,57] first reach their peak level after 1 h, followed by IL-4 after 2 h. IFN-γ, IL-2 and IL-6[29,57] reach their peak after 3 h, followed by IL-12. However, in the liver, TNF-α, IFN-γ, IL-4[29,57] reach their peak level in 1 h, followed by IL-2 and IL-12. There is no significant change for IL-6[57] and IL-10[29,57] in the liver. Especially, the level of IL-10[29] is very low in the liver compared with that in the plasma, which suggested that IL-10 might originate from other tissues, such as the spleen. But one previous paper reported that IL-10 expression in the liver is higher than that in the spleen[57]. As yet, where IL-10 originates remains unanswered.

Comparing the acute and chronic animal models, the expression profiles of IL-10 are quite different. For example, in the acute model induced by Con A, TNF-α, IFN-γ and IL-12 levels increased to 2.11, 1.92 and 8.30 times of their normal level, respectively, after neutralization of IL-10. Reversely, administration of recombinant IL-10 prior to injection of Con A decreased by 47%, 47% and 80% of TNF-α, IFN-γ and IL-12 expression levels respectively. IL-10 is considered to be an anti-inflammatory cytokine in a murine model of Con A[58]. Kato *et al*[59] described that the IL-10 level is increased at 12 h after the Con A injection. After neutralizing antibodies to IL-10, it was intraperitoneally injected into animals of the same model at 6 h before Con A treatment, with serum alanine aminotransferase level being significantly higher than in the control group. Histological studies showed spotty necrosis in the group treated with anti-IL-10 antibodies. These results suggest that IL-10 has an inhibitory effect on liver injury in a murine model of Con A-induced experimental liver injury mediated by cellular immunity[58]. These studies suggested that both endogenous and exogenous IL-10 can protect the liver from acute injury[59].

However, there is evidence indicating that IL-10 could accelerate liver injury in the chronic model[60]. When Con A was administrated intravenously to BALB/c mice once a week, the IL-10 expression level in plasma increased to 7 times higher 20 wk later. Accordingly, in this model, inflammatory infiltration also lasted for 20 wk and activated stellate cells also dramatically increased[60]. All these results suggested that IL-10 aggravated liver injury in the chronic Con A model.

Paradoxically, IL-10 does not play the same role in all chronic models. For example, in the CCl_4 chronic model, IL-10 slows down the process of fibrosis[61]. This may be due to the fact that the mechanisms of liver injury in these two models are different, and the latter does not involve T cell activation. In the acute Con A model, IL-10 may inhibit macrophages and Th1 cells from releasing inflammatory cytokines, which explains why it plays an anti-inflammation role in the acute model[58]. Though IL-10 can inhibit the secretion of anti-inflammation cytokines, secretion of IFN-γ is also inhibited[62]. Some previous studies reported that, to some extent, IFN-γ may relieve liver fibrosis. Therefore, a long duration of IFN-γ deficiency may aggravate fibrosis. As for the CCl_4 model, liver injury is mediated only by free

Table 2 New drugs developed based on the Concanavalin A model

	Classification	Target	Pathway
Hu 23C3[63]	Monoclonal antibody	Human osteopontin	NF-κB
Anti-his H1[64]	Polyclonal antibody	Histone H1	NF-κB
ApoA Ⅱ[65]	High density lipoprotein	Leukocytes and T cells	-
CpG ODN[66]	Oligodeoxynucleotides	DNA binding ability of NF-κB	NF-κB

CpG ODN: CpG-containing oligodeoxynucleotides; ApoA: Apolipoprotein A; Anti-his H1: Antibody against histone H1; NF-κB: Nuclear factor kappa B.

radicals, which is not relevant to the activation of the immune response and the release of inflammation cytokines. In conclusion, the expression profiles in different models, even with the same inducer, are not the same. The various mechanisms, cell types and micro-environments should be taken into consideration in experimental design and execution.

CON A MODEL AND NEW DRUG DEVELOPMENT

In recent years, based on the Con A animal model, many new therapeutic antibodies or proteins have been developed to attenuate liver injury in experimental models (Table 2)[63-66].

Fan *et al*[63] humanized a murine monoclonal antibody 23C3 against human osteopontin by a complementary-determining region grafting method based on computer-assisted molecular modeling, denoted as Hu23C3. They demonstrated that Hu23C3 could have the potential for attenuating Con A-induced liver injury through the nuclear factor kappa B (NF-κB) pathway.

Nakano *et al*[64] intraperitoneally injected a polyclonal antibody against histone H1 immediately after Con A injection; they found that injection of anti-histone H1 antibodies could reduce Con A-induced liver damage, also *via* the NF-κB pathway.

It is reported that Con A-induced hepatitis was attenuated by the administration of apolipoprotein A-Ⅱ, which is the second major apolipoprotein of high-density lipoprotein[65]; this inhibited leukocytes infiltration and the expression of T-cell related cytokines and chemokines.

The survival rate of mice was markedly enhanced by the administration of CpG-containing oligodeoxynucleotides (CpG ODN)[66]. This is because CpG ODN pretreatment inhibits the DNA binding ability of NF-κB, leading to the decrease of systemic/liver levels of TNF-α and IFN-γ. These results suggest that CpG ODN pretreatment protects the mice from Con A-induced liver injury, also *via* NF-κB pathway.

CONCLUSION

In this article we reviewed the evolution of the AIH model and emphasized the importance of the Con A AIH model. Based on the previous papers, we summarized the mechanisms of Con A-induced liver injury, its pathogenic changes and cytokines expression levels. The Con A animal model, which is a typical T cell dependent model, can mimic the mechanisms of clinical AIH diseases. Therefore, we think that it is a good and convenient model for studying the mechanisms of AIH and developing new therapeutic drugs.

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ORIGINAL ARTICLE

Suppression of esophageal cancer cell growth using curcumin, (-)-epigallocatechin-3-gallate and lovastatin

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Abstract

AIM: To determine the effects of curcumin, (-)-epigallocatechin-3-gallate (EGCG), lovastatin, and their combinations on inhibition of esophageal cancer.

METHODS: Esophageal cancer TE-8 and SKGT-4 cell lines were subjected to cell viability methyl thiazolyl tetrazolium and tumor cell invasion assays *in vitro* and tumor formation and growth in nude mouse xenografts with or without curcumin, EGCG and lovastatin treatment. Gene expression was detected using immunohistochemistry and Western blotting in tumor cell lines, tumor xenografts and human esophageal cancer tissues, respectively.

RESULTS: These drugs individually or in combinations significantly reduced the viability and invasion capacity of esophageal cancer cells *in vitro*. Molecularly, these three agents reduced the expression of phosphorylated extracellular-signal-regulated kinases (Erk1/2), c-Jun and cyclooxygenase-2 (COX-2), but activated caspase 3 in esophageal cancer cells. The nude mouse xenograft assay showed that EGCG and the combinations of curcumin, EGCG and lovastatin suppressed esophageal cancer cell growth and reduced the expression of Ki67, phosphorylated Erk1/2 and COX-2. The expression of phosphorylated Erk1/2 and COX-2 in esophageal cancer tissue specimens was also analyzed using immunohistochemistry. The data demonstrated that 77 of 156 (49.4%) tumors expressed phosphorylated Erk1/2 and that 121 of 156 (77.6%) esophageal cancers expressed COX-2 protein. In particular, phosphorylated Erk1/2 was expressed in 23 of 50 (46%) cases of esophageal squamous cell carcinoma (SCC) and in 54 of 106 (50.9%) cases of adenocarcinoma, while COX-2 was expressed in 39 of 50 (78%) esophageal SCC and in 82 of 106 (77.4%) esophageal adenocarcinoma.

CONCLUSION: The combinations of curcumin, EGCG and lovastatin were able to suppress esophageal cancer cell growth *in vitro* and in nude mouse xenografts, these drugs also inhibited phosphorylated Erk1/2, c-Jun and COX-2 expression.

Key words: Chemoprevention; Curcumin; Cyclooxygenase-2; (-)-epigallocatechin-3-gallate; Esophageal cancer; Statin

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INTRODUCTION

Esophageal cancer is one of the least studied and deadliest cancers. Tobacco smoke is the most firmly established risk factor for esophageal cancer and has been associated with the development of esophageal squamous cell carcinoma and adenocarcinoma; gastroesophageal reflux with bile acid is another important cause of adenocarcinoma[1-3]. Molecularly, the expression of retinoic acid receptor-β2 (RAR-β2) is frequently lost in premalignant and malignant esophageal tissues, and benzo[a]pyrene, a carcinogen present in tobacco and environmental pollution, and bile acid, a tumor promoter for gastrointestinal cancer, may be responsible for its loss. Restoration of RAR-β2 expression suppressed esophageal cancer cell growth and induced apoptosis *in vitro* and tumor formation *in vivo*; these effects were correlated with decreased expression of activator protein 1 and cyclooxygenase-2 (COX-2) and phosphorylated extracellular-signal-regulated kinases (Erk1/2)[4]. Moreover, the novel retinoid receptor-induced gene-1 (RRIG1), which is a downstream gene of RAR-β2, participates in regulating the effects of RAR-β2 on cell growth and gene expression[5,6]. RRIG1 protein binds to and inhibits a small GTPase RhoA activity. Restoration of RRIG1 expression inhibits RhoA activation and, consequently, reduces tumor cell colony formation, invasion, and proliferation, which are correlated with inhibition of Erk1/2 phosphorylation, COX-2, and cyclin D1 expression[5,6]. These genes together may form a novel molecular pathway that involves RAR-β2-induced RRIG1 expression and suppression of RhoA/Erk1/2/AP-1/COX2[4]. Therefore, targeting this molecular pathway should translate into better control of esophageal cancer.

Cancer chemoprevention was defined as the use of natural, synthetic, or biologic chemicals (such as drugs and food supplements) in the prevention, suppression, or delay of the carcinogenesis process[6]. Several clinical trials of different drugs have been conducted in an attempt to prevent esophageal cancer. The first class of agents tried was the retinoids[7]. The use of retinoids was based on the fact that vitamin A deficiency was found in esophageal cancer patients and the fact that this deficiency induced hyperkeratotic changes in the esophageal mucosa of experimental animals[8,9]. A clinical trial using N-4-(ethoxycarbophenyl) retinamide demonstrated that cancer incidence in the treatment group with severe esophageal dysplasia was reduced by 43.2% compared with that in the placebo group[10]. However, the results from two other trials conducted in Linxian, China, by the National Cancer Institute were inconclusive[11,12]. Our *in vitro* study demonstrated that esophageal cancer cells which do not express RAR-β2 are resistant to all-trans retinoic acid[13]. In animal experiments, dietary N-(4-hydroxyphenyl) retinamide enhanced tumorigenesis in response to N-nitrosomethylbenzylamine in the rat esophagus by increasing tumor initiation events[14]. Moreover, 13-*cis* RA did not reduce NBMA-induced esophageal tumor multiplicity in rats[15].

Non-steroidal anti-inflammatory drugs (NSAIDs) were also tested for the prevention of esophageal cancer[16]. Epidemiological and experimental studies indicated that NSAIDs decreased esophageal cancer incidence[17-21]. Our *in vitro* data showed that aspirin and NS398 induced apoptosis in esophageal cancer cells, which correlated with their ability to inhibit COX-2 enzymatic activity and upregulate the expression of 15-LOX-1 and -2[22-25]. However, during clinical trials of these agents in the chemoprevention of colorectal cancer, it was reported that Vioxx (rofecoxib) and Celebrex (celecoxib) induced cardiovascular events, which raised safety concerns about the high-dose and long-term use of these drugs in cancer prevention[26,27].

However, to date, most preclinical and clinical chemoprevention studies of human cancers have been focused on targeting a single gene, which showed limited activities *in vitro* and *in vivo*[3,16]. In this study, we aimed to target multiple genes in a molecular pathway using combinations of drugs to determine whether this approach is more effective than single-drug treatments in the inhibition of esophageal cancer.

MATERIALS AND METHODS

Cell culture and drug treatment

The human esophageal cancer cell lines SKGT-4 and TE-8 used in our previous studies[13,22] were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% fetal bovine serum (FCS), at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2. For drug treatment, these cells were grown in monolayer overnight and then treated with or without curcumin, (-)-epigallocatechin-3-gallate (EGCG), lovastatin, and combinations of these agents for up to 5 days. The drugs were dissolved in dimethyl sulfoxide (DMSO) and then diluted before use. The concentration of lovastatin was 2 or 4 μmol/L, curcumin was 20 or 40 μmol/L, and EGCG was 20 or 40 μmol/L (all from LKT Laboratories, Inc., St. Paul, MN, United States). The concentrations for the drug combinations were the same as those used individually. For the methyl thiazolyl tetrazolium (MTT) assay, 20 μL of MTT (5 mg/mL, Sigma, St Louis, MO, United States) was added to each well of the 96-well plates and incubated for an additional 4 h. After the growth medium was removed, 100 μL of DMSO was added to the wells to dissolve the MTT crystal, and the optical densities were measured with an automated spectrophotometric plate reader at a single wavelength of 540 nm. The percentage of cell growth was calcu-

lated using the formula: % control = ODt/ODc × 100, where ODt and ODc are the optical densities for treated and control cells, respectively. The data were then analyzed statistically using the Student's *t* test.

Tumor cell invasion assay

Boyden chambers coated with Matrigel were obtained from BD Biosciences (Bedford, MA, United States) for assaying tumor cell invasion ability[6]. Esophageal cancer cells SKGT-4 and TE-8 were first starved in medium without FCS overnight, and the cells (5×10^4) were re-suspended in the FCS-free medium and placed in the top chambers in triplicate. The medium in the top chambers contained lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L), or their combinations. The lower chamber was filled with DMEM and 10% FCS as the chemoattractant and incubated for 48 h. The upper surface was then wiped with a cotton swab to remove the remaining cells. The cells which invaded the Matrigel and attached to the lower surface of the filter were fixed and stained with 1% crystal violet solution. The cells in the reverse side were photographed (5 microscopic fields at 100 × magnification per chamber). The cells in the photographs were then counted, and the data were summarized as mean ± SD and presented as a percentage of the controls (mean ± SD). The data were then analyzed statistically using the Student's *t* test.

Protein extraction and Western blotting

The cells were grown and treated with or without the drugs for 2 d. After that, total cellular protein was extracted as described previously[5,6,13,22-25]. Samples containing 50 μg of protein from each treatment were then separated by 10%-14% on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred electrophoretically to a Hybond-C nitrocellulose membrane (GE-Healthcare, Arlington Heights, IL, United States) at 500 mA for 2 h at 4 ℃. The membrane was subsequently stained with 0.5% Ponceau S containing 1% acetic acid to confirm that the proteins were loaded equally and to verify transfer efficiency. Next, the membranes were subjected to Western blotting by overnight incubation in a blocking solution containing 5% bovine skimmed milk and 0.1% Tween 20 in phosphate-buffered saline (PBS) at 4 ℃. The next day, the membranes were first incubated with primary antibodies and then with horse anti-mouse or goat anti-rabbit secondary antibodies (GE Healthcare) for enhanced chemiluminescence detection of antibody signals. The antibodies used were anti-Ki67 (Vector Laboratories, Burlingame, CA, United States), anti-phosphorylated Erk1/2 (Cell Signaling Technology, Danvers, MA, United States), anti-COX-2 (BD Transduction Laboratories, Lexington, KY, United States), and anti-β-actin (Sigma-Aldrich, St. Louis, MO, United States).

Animal experiments

An animal usage procedure was approved by our Institutional Animal Care and Usage protocol. Esophageal cancer SKGT-4 cells were grown and treated with or without these drugs for 3 d before injection (the doses were the same as above). Nu/nu nude mice (6-8 wk of age) were treated with or without curcumin (50 μg/kg per day), EGCG (50 μg/kg per day), lovastatin (50 μg/kg per day), and their combinations (the same doses used individually) orally for two days and then subcutaneously injected in the right flank through a 22-gauge needle with 2×10^6 tumor cells mixed with 50% Matrigel (BD Biosciences) for a total volume of 200 μL per mouse. The animals were then continuously treated with or without these drugs orally 5 d/wk for an additional 30 dand monitored for tumor formation and growth daily. The tumor mass volumes, measured weekly with a vernier caliper, were calculated as follows: length × width^2/2. At the end of the experiments, the tumor xenografts were taken excised, weighed and the results summarized.

Esophageal cancer tissue samples

Our institutional review board (IRB) approved our protocol for the use of patient tissue samples in this study, which included 156 consecutive patients with available paraffin blocks who had undergone esophagectomy without preoperative chemotherapy or radiotherapy between the years 1986 and 1997 at The University of Texas M.D. Anderson Cancer Center.

Immunohistochemistry

Human esophageal cancer tissue specimens and tumor xenografts from the nude mice were resected and subjected to tissue processing, these samples were embedded in paraffin and 4-μmol/L-thick sections were prepared for immunohistochemical analyses of Ki67, phosphorylated Erk1/2, and COX-2 expression. Briefly, the sections were de-paraffinized twice in xylene for 10 min each and rehydrated in a series of ethanol (100%-50%) and were then subjected to antigen retrieval by cooking in a pressure cooker with 0.01 mol/L citric buffer for 10 min and H_2O_2 treatment to eliminate endogenous tissue peroxidase activity. The tissue sections were then incubated with 100 μL of 20% normal horse or goat serum in PBS and anti-Ki67 (1:50), phosphorylated Erk1/2 (1:50), or COX-2 (1:50) diluted in PBS overnight. The next day, the sections were washed with PBS three times and once with PBS containing 0.1% Tween 20 and further incubated with a second antibody (Horse anti-mouse IgG or Goat-anti rabbit IgG from Vector, Laboratories) for 30 min. After washing with PBS, the sections were then incubated with ABC solution (Vector) in the dark for 30 min and 9-ethylcarbazol-3-amine buffer for 15 min for color development. The sections were counterstained with hematoxylin for 30 s, covered with a cover slip and then reviewed and scored under a microscope as positive or negative staining (10% or more tumor cells with positive staining were counted as positive staining).

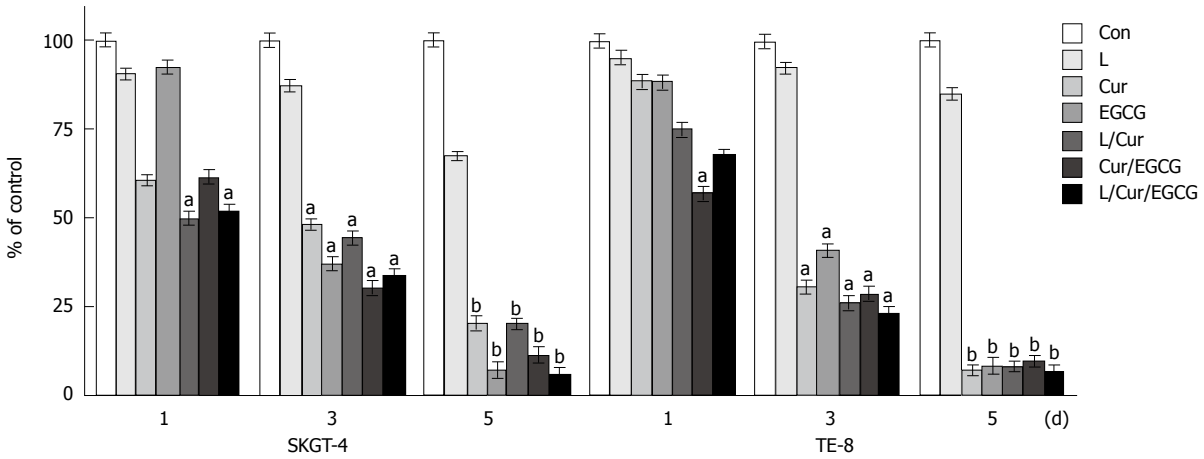


Figure 1 Suppression of esophageal cancer cell growth by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Esophageal cancer SKGT-4 and TE-8 cells were grown in monolayer overnight and then treated with or without curcumin (Cur) (40 μmol/L), (-)-epigallocatechin-3-gallate (EGCG) (40 μmol/L), lovastatin (L) (4 μmol/L) and their combinations for up to 5 d. Methyl thiazolyl tetrazolium assays were then carried out to detect changes in cell viability (see Methods section). The experiments were repeated three times and the results are summarized as a % of the control (Con) (mean ± SD) and analyzed statistically using the Student's *t* test. $^{a}P < 0.05$, $^{b}P < 0.01$.

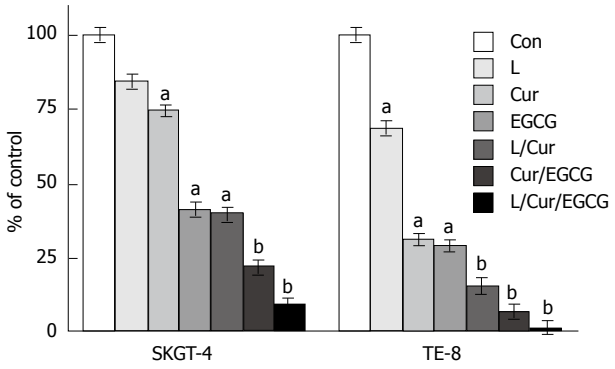


Figure 2 Suppression of tumor cell invasion by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Esophageal cancer SKGT-4 and TE-8 cells were grown and treated with or without curcumin (Cur) (40 μmol/L), (-)-epigallocatechin-3-gallate (EGCG) (40 μmol/L), lovastatin (L) (4 μmol/L) and their combinations in monolayer for 3 d and then subjected to cell invasion assays in Boyden chambers containing Matrigel for 48 h. The invasive cells were stained with 1% crystal violet solution, counted and the results are summarized as a % of the control (Con) (mean ± SD). The data were then analyzed statistically using the Student's *t* test. $^{a}P < 0.05$, $^{b}P < 0.01$.

RESULTS

Reduction of tumor cell viability by curcumin, EGCG, lovastatin and their combinations

We first determined the effects of curcumin, EGCG and lovastatin individually and their combinations on the suppression of esophageal cancer cell growth by treating esophageal cancer TE-8 and SKGT-4 cell lines with two different doses (curcumin at 20 and 40 μmol/L, EGCG at 20 and 40 μmol/L, lovastatin at 2 and 4 μmol/L and these individual drug doses were used for combination treatments) for up to 5 d. The doses selected were based on previous studies[28-37]. Our data showed that lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L) and their combinations significantly reduced tumor cell viability (Figure 1).

Suppression of tumor cell invasion by curcumin, EGCG, lovastatin and their combinations

We next determined the effects of these three drugs on the regulation of esophageal cancer cell invasion capacity and found that lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L), and their combinations significantly reduced tumor cell invasion (Figure 2).

Modulation of gene expression by curcumin, EGCG, lovastatin and their combinations

We then assessed the regulation of gene expression by these three drugs and found that lovastatin (4 μmol/L), curcumin (40 μmol/L), EGCG (40 μmol/L) and their combinations downregulated the expression of p-Erk1/2, c-Jun and COX-2, but upregulated activated caspase 3 expression in esophageal cancer SKGT-4 and TE-8 cells (Figure 3).

Suppression of tumor growth in nude mouse xenografts by curcumin, EGCG, lovastatin and their combinations

We then performed nude mouse xenograft assays of SKGT-8 cells to determine the effects of the drugs individually and their combinations. We found that lovastatin (50 μg/kg per day), curcumin (50 μg/kg per day), EGCG (50 μg/kg per day) and their combinations at the same doses inhibited tumor growth differently (Figure 4). Treatment with a single drug such as curcumin or lovastatin did not have any effects on tumor formation and growth (Figure 4), although these drugs individually and in combination inhibited expression of Ki67, p-Erk1/2 and COX-2 expression in xenograft tissues (Figure 5).

Expression of phosphorylated Erk1/2 and COX-2 in esophageal cancer tissue specimens

To determine the relevance of p-Erk1/2 and COX-2 expression in human esophageal cancer, we analyzed their expression in esophageal cancer tissue specimens

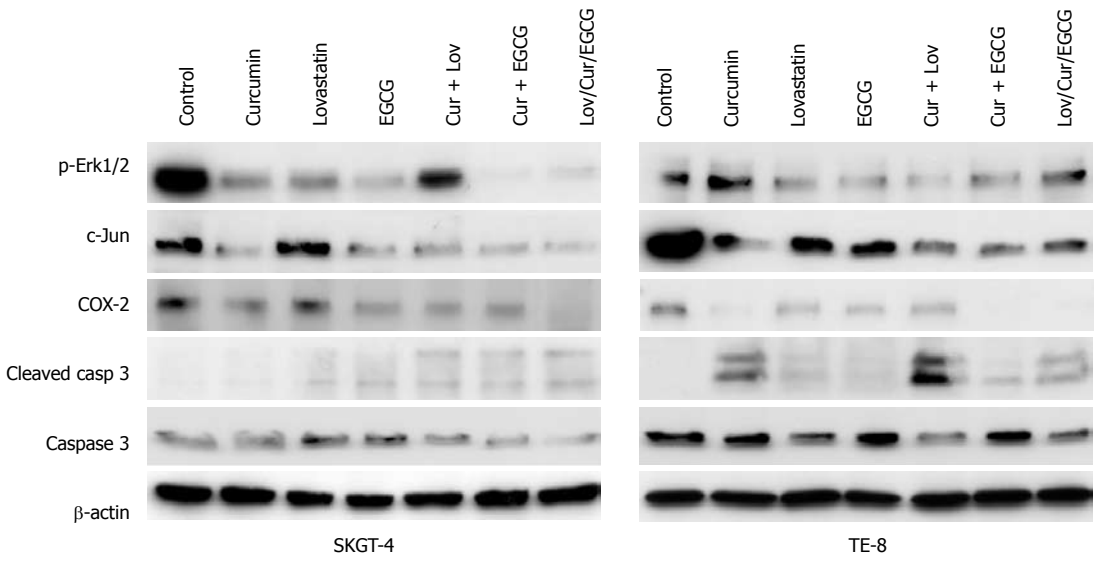


Figure 3 Modulation of gene expression by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Esophageal cancer SKGT-4 and TE-8 cells were grown in monolayer overnight and treated with or without curcumin (Cur) (40 μmol/L), (-)-epigallocatechin-3-gallate (EGCG) (40 μmol/L), lovastatin (Lov) (4 μmol/L) and their combinations for 2 d and total cellular protein was extracted from the cells and subjected to Western blotting analysis of gene expression. Erk1/2: Extracellular-signal-regulated kinases; COX-2: Cyclooxygenase-2.

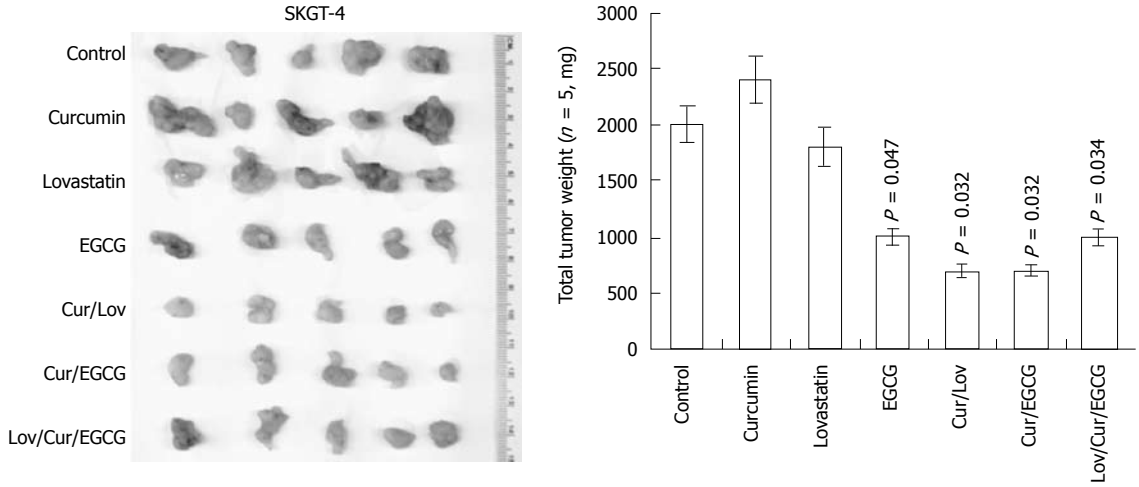
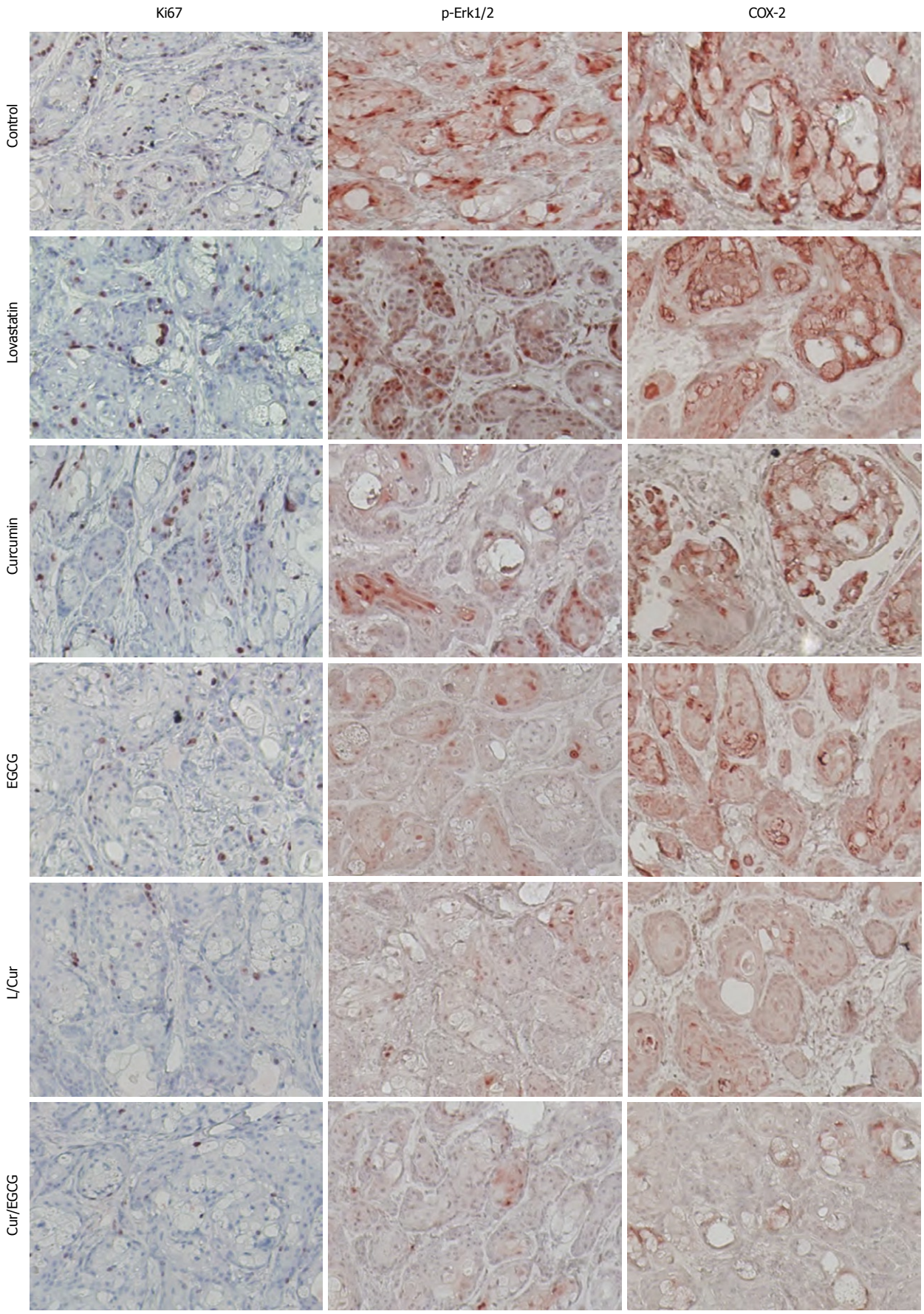


Figure 4 Inhibition of esophageal cancer cell growth by curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations in nude mouse xenografts. Esophageal adenocarcinoma SKGT-4 cells were inoculated subcutaneously into nude mice (5 per group). Two days before tumor cell injection, the mice started treatment with or without curcumin (Cur) (50 μg/kg per day), (-)-epigallocatechin-3-gallate (EGCG) (50 μg/kg per day), lovastatin (Lov) (50 μg/kg per day) and their combinations (the same doses as given individually) for 30 d (5 d/wk by oral gavage). At the end of the experiments, the xenograft tumor mass was isolated and weighed and summarized.

using immunohistochemistry. We found that 77 of 156 (49.4%) tumors expressed phosphorylated Erk1/2 and that 121 of 156 (77.6%) esophageal cancers expressed COX-2 protein. In particular, phosphorylated Erk1/2 was expressed in 23 of 50 (46%) esophageal squamous cell carcinoma (SCC) and in 54 of 106 (50.9%) adenocarcinoma, while COX-2 was expressed in 39 of 50 (78%) esophageal SCC and in 82 of 106 (77.4%) esophageal adenocarcinoma (Figure 6).

DISCUSSION

In the current study, we demonstrated that curcumin, EGCG, lovastatin, and their combinations can significantly reduce the viability and invasion capacity of esophageal cancer cells *in vitro*. Nevertheless, they were much less effective *in vivo* in nude mouse xenografts, especially curcumin and lovastatin individually. At the molecular level, these three agents individually or in combination inhibited the expression of phosphorylated Erk1/2, c-Jun, and COX-2 and induced caspase 3 expression in esophageal cancer cells *in vitro*. In nude mouse xenografts, the expression of p-Erk1/2 and COX-2 was downregulated by these three drugs, especially their combinations. We also analyzed the expression of phosphorylated Erk1/2 and COX-2 in tissue


Ki67
p-Erk1/2
COX-2
Control
Lovastatin
Curcumin
EGCG
L/Cur
Cur/EGCG

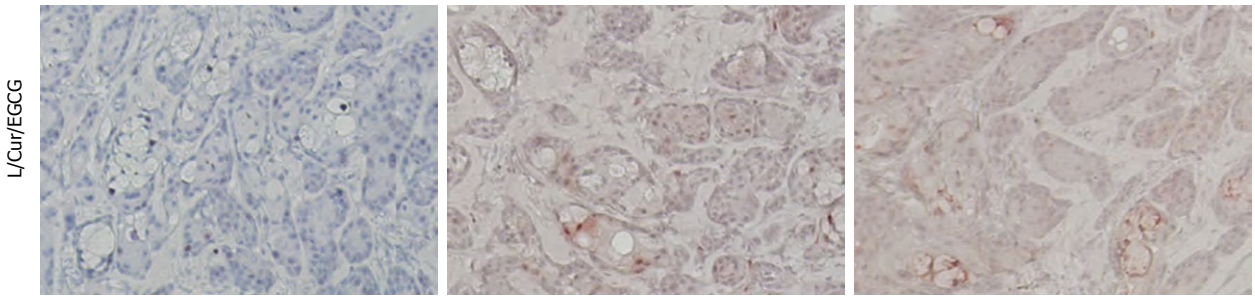


Figure 5 Reduced expression of Ki67, phosphorylated extracellular-signal-regulated kinases and cyclooxygenase-2 in xenografts following treatment of mice with or without curcumin, (-)-epigallocatechin-3-gallate, lovastatin and their combinations. Tumor cell xenografts obtained from the nude mouse experiments were processed and subjected to immunohistochemical analyses of Ki67, phosphorylated extracellular-signal-regulated kinases (Erk1/2) and cyclooxygenase-2 (COX-2) expression. Representative images were obtained in each treatment group. EGCG: (-)-epigallocatechin-3-gallate; L: Lovastatin; Cur: Curcumin.

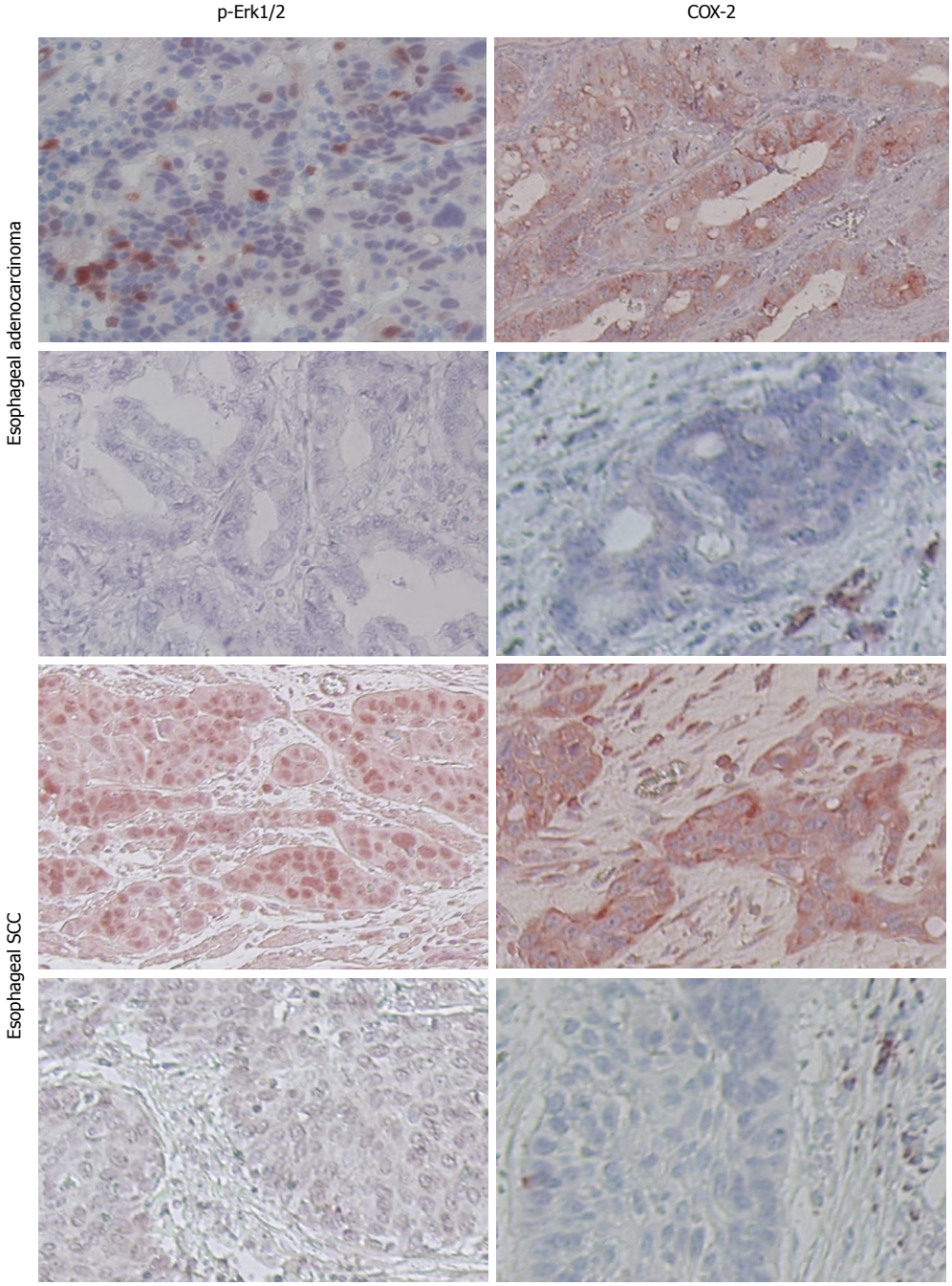


Figure 6 Expression of phosphorylated extracellular-signal-regulated kinases and cyclooxygenase-2 in esophageal cancer specimens. Paraffin sections of esophageal cancer tissues were immunostained with anti-phosphorylated extracellular-signal-regulated kinases (Erk1/2) or cyclooxygenase-2 (COX-2) antibody. Representative images were obtained from these tissue sections. SCC: Squamous cell carcinoma.

specimens from esophageal cancer patients. The data showed that 49.4% of esophageal cancers expressed phosphorylated Erk1/2 and that 77.6% of cancers expressed COX-2 protein. These data suggest that curcumin, EGCG, and lovastatin inhibit esophageal cancer cell growth *in vitro* and in nude mouse xenografts possibly through the suppression of phosphorylated Erk1/2, c-Jun and COX-2 expression.

Previous studies have shown the chemopreventive activity of EGCG in suppressing carcinogenesis in several organs, including the esophagus[29,34]. Molecularly, EGCG can suppress the mitotic signal transduction pathway, e.g., inhibit Erk1/2 phosphorylation and anti-AP-1 activity[38]. A recent study demonstrated that EGCG induced a concentration- and time-dependent reversal of hypermethylation of RAR-β2 in esophageal cancer cell lines, resulting in re-expression of RAR-β2[33]. Furthermore, curcumin has been shown to inhibit different cancers at the initiation, promotion, and progression stages in animal models[31,32,38]. Curcumin also suppressed growth and induced apoptosis in numerous types of cancer cells *in vitro*[38,39]. Although the defined mechanisms of its action require further study, its efficacy appears to be related to the induction of glutathione and glutathione-S-transferase activity, inhibition of lipid peroxidation and arachidonic acid metabolism, and suppression of oxidative DNA adduct formation[32,38,39]. Curcumin can inhibit the activation of NF-κB and the expression of c-Jun, c-Fos, c-Myc, Erk1/2, COX-2, PI3K, Akt, CDKs, and iNOS[31,35,38,39]. Curcumin was also able to suppress cigarette smoke-induced NF-κB activation and COX-2 expression in head and neck SCC and non-small-cell lung cancer cells[31,32]. In esophageal cancer, dietary curcumin can inhibit chemically-induced esophageal carcinogenesis in mice and rats[28,40]. In addition, the statin family of drugs has shown cancer chemopreventive effects[41]. Statins can trigger some tumor cells to undergo apoptosis *in vitro* and suppress tumor growth *in vivo*[30,37,41]. Statins also have an antimetastatic property, which is evident in their suppression of tumor cells invasiveness in Matrigel, as well as in animal experiments[42]. In addition, statins, especially at high concentrations, can inhibit capillary tube formation by endothelial cells *in vitro* and *in vivo*[35,44]. The effects of statins are thought to be mediated through inhibition of Ras and RhoA activity[41]. Based on these previous studies and reports, we determined the effects of their combinations on suppression of esophageal cancer cell growth *in vitro* and in nude mouse xenografts by targeting the RAR-β2/Erk1/2/AP1/COX-2 pathway[3]. Indeed, our current study has demonstrated the effects of their combinations *in vitro*. Molecularly, these three agents were able to regulate the expression of this gene pathway *in vitro* and *in vivo* in nude mice. Nevertheless, individually curcumin and lovastatin had no effect on tumor formation and growth in nude mice, even when the highest dose possible was used. This may be due to the bioavailability of curcumin and the induction of COX-2 expression by high dose lovastatin, reported previously[30,39]. However, the current study did not show the induction of COX-2 expression by high dose lovastatin in esophageal cancer *in vitro* and in nude mice, similar to that seen in prostate cancer[30].

However, there are some limitations in the current study. Firstly, we showed that these three drugs regulated gene expression of the RAR-β2/Erk1/2/AP1/COX-2 pathway, however, previous studies also showed that as chemoprevention agents, these drugs target multiple genes and their pathways in different cancers. Thus, further study is needed to determine the mechanisms of action of these drugs in human cancers. Furthermore, we used established esophageal cancer cell lines to determine the chemopreventive effects of these agents in this study, the results of which may be quite different in comparison to those in premalignant cells *in vivo*. The xenograft assay tested the effects of these agents in suppressing tumor initiation and growth, but not tumor development per se, although the xenograft assay did test the bioavailability of these agents *in vivo*. In addition, we did not test whether the doses of these three agents are clinically achievable, and to reduce costs, we utilized a single dose of each agent and their combinations. Thus, future studies are needed to test these agents in a clinical Phase I trial and in animal experiments where more doses and a time course study will be included.

ACKNOWLEDGMENTS

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COMMENTS

Background

Esophageal cancer remains a lethal disease, and is the least studied cancer in the United States. The overall 5-year survival rate for esophageal cancer is only 10%-15%, while the incidence of esophageal adenocarcinoma has significantly increased in the United States and other Western countries. These data indicate an urgent need for the development of novel strategies for prevention, early detection and management of esophageal cancer

Research frontiers

The authors found that the combination of curcumin, (-)-epigallocatechin-3-gallate and lovastatin was able to suppress esophageal cancer cell growth *in vitro* and in nude mouse xenografts.

Innovations and breakthroughs

These three agents inhibited phosphorylated Erk1/2, c-Jun and COX-2 expression.

Applications

Further clinical trials using these agents are warranted.

Peer review

This is the first report of the effects of combining these agents on esophageal cancer cells. The data suggest that these agents might be used individually or in combination for chemoprevention of esophageal cancer. Overall, the study is well designed and the data are convincing.

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ORIGINAL ARTICLE

Characterization of gastric cancer models from different cell lines orthotopically constructed using improved implantation techniques

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Abstract

AIM: To develop orthotopic gastric cancer mouse models from different cell lines and characterize the tumor features to assist further in preclinical trials and clinical treatment strategies.

METHODS: Human gastric cancer SGC-7901 and BGC-823 cell suspensions were injected subcutaneously into nude mice to develop solid tumors, and tumor tissue pieces were then implanted under the serous coat of the stomach. An autopsy was performed on all animals of the SGC-7901 and BGC-823 models to observe the primary tumor growth and metastases using pathological and immunohistochemical methods.

RESULTS: Both models showed large tumors *in situ* resulting in pressure and infiltration of the adjacent organs. The gastric cavity became smaller, along with stenosis of the cardia or pylorus. There were biological and statistical differences between the two models. The metastasis rate in involved organs (lymph nodes, kidney, spleen, testis) was significantly higher in the BGC-823 model compared to the SGC-7901 model ($P < 0.05$ or $P < 0.01$). The median survival of the BGC-823 model was shorter than that of SGC-7901 (23 d *vs* 84 d, $P < 0.05$). Histopathologically, the primary tumor and metastatic lesions of the two models showed obvious atypia and mucus in the cytoplasm. Compared with the SGC-7901 model, BGC-823 appeared more poorly differentiated (absence of adenoid structure), had a smaller volume, and richer capillary structure. Immunohistochemical staining revealed cytokeratin 20 and epithelial membrane antigen expression was positive in the SGC-7901 tumors, while negative in BGC-823 ones.

CONCLUSION: Models using the SGC-7901 and BGC-823 cell lines were established which could function in gastric cancer research on carcinogenesis mechanism and drug discovery. The two models showed different tumor behavior and the latter was more malignant than the former.

Key words: Gastric cancer; Orthotopic implantation; Mouse model; Metastasis; Cell line

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INTRODUCTION

Gastric cancer is still the second most common malignant tumor in the world, and patients with gastric cancer generally have metastasis when clinically examined. There is a low five-year survival rate and poor quality of life even after tumor resection. We need to develop animal models of gastric cancer for exploring the mechanisms of carcinogenesis and clinical treatment strategy. Since the 1990s, orthotopic implantation techniques have gained popularity in establishing animal models of various tumors[1-4]. Many researchers have constructed orthotopic models of gastric cancer with different methods[5-11]. Of these methods, orthotopic transplantation using tumor pieces is useful because it not only simulates clinical cancer behavior but also promotes metastasis[12]. In the past, researchers mainly employed the "sewing" method to develop stomach cancer models[6,13]. However, there are disadvantages; such as a difficult operation, a time-consuming process, and low survival rates, and the sewing technique is not commonly used. In recent years, the procedure has been improved using tissue adhesive adhering to tumor pieces, which greatly facilitates surgical performance and decreases the mortality rate resulting from surgical operation[5,14]. Based on previous research, we intended to develop gastric cancer models from the SGC-7901 and BGC-823 cell lines and observe their biological characteristics to aid preclinical trials and therapy strategies. The human gastric cell line SGC-7901 was first established from the metastatic lymph node of a 56-year-old female patient suffering gastric adenocarcinoma. The BGC-823 cell line was derived from a specimen from a male patient who was 62 years of age, which was conserved in the Tumor Institute of Tianjin in China. The two gastric cell lines are poorly differentiated. This is the first time that a gastric cancer model of BGC-823 cell line was orthotopically constructed with histological tumor tissue *via* the "adhering" method.

MATERIALS AND METHODS

Cell lines

Human gastric cancer cell lines SGC-7901 and BGC-823 were used for this study. The cells were purchased from the Centre of Cell Cultures of Chinese Academy of Medical Sciences, Shanghai, China, and cultured at 37 ℃ in a humidified atmosphere with 5% CO_2, in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine and 1 mmol/L sodium pyruvate. Cells were maintained by serial passaging after trypsinization with 0.1% trypsin.

Animals

Five- to six-week-old male Balb/c nu-nu mice (weight 18-20 g) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Medical Sciences, China. They were kept in cages in a pathogen-free environment (temperature 25 ℃-27 ℃, humidity 45%-50%) and supplied with food and water *ad libitum*. All animal experiments were approved by the ethical committee of the Chongqing Medical University, and conformed to National and International Policies on Human Care and Use of Laboratory Animals.

Subcutaneous tumor specimens

Subcutaneous tumors were grown and then tumor tissue pieces used in surgical orthotopic implantation (SOI). Two cell lines were collected in the log phase and injected subcutaneously at 10^7/0.2 mL into the bilateral croup of Balb/c nu-nu mice. After 2 wk, the resulting tumors from the croup were harvested under strict aseptic conditions following removal of necrotic tissue from the central tumor areas, and cut into small pieces of approximately 1 mm^3.

Orthotopic implantation of tumor fragments

Nude mice were divided into 2 groups of 12 animals each, and were explanted with tumor fragments[5] from the SCG-7901 and BGC-823 cell lines, respectively. All procedures were performed under anesthesia with Sumianxin Ⅱ (0.02 mL per animal; China Academy of Military Medical Science) at the benchtop. After a left-side upper abdominal incision was made, the stomach of the nude mouse was gently exteriorized. One small tissue pocket was prepared in the middle wall of the greater curvature using an ophthalmic scissor, and then one tumor piece was placed into the pocket following fixation with a drop of medical tissue adhesive (gifts from Shunkang Corporation of Biological Adhesive, Beijing, China). To avoid adhesion to adjacent normal tissue, the quantity of the tissue adhesive was strictly measured. The stomach was then returned to the peritoneal cavity, and the abdominal wall was closed with 4-0 absorbable sutures. The mice were given special care and fed in cages as usual after surgery.

Evaluation of tumor growth and metastasis

All mice of the two groups were closely observed. At time of death, an autopsy was carried out to examine the tumor growth. The volume of primary tumor was calculated by the following formula: $V = 0.4 \times ab^2$ (a: maximum diameter; b: minimum diameter)[15]. Primary tumor, lymph nodes (gastroepiploic plexus, hilus pulmonis and mesenterium), and other organs (liver, lung, kidney, testis, spleen, *etc.*) involving infiltration or metastasis were sampled. The sampled tissues were fixed in 10%

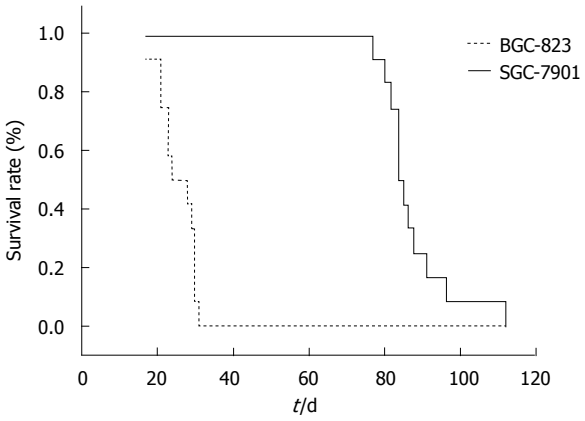

Figure 1 Survival curve of the SGC-7901 and BGC-823 gastric cancer models. The median survival in the BGC-823 model is significantly shorter than that in SGC-7901 (23 d *vs* 84 d, $P < 0.05$).

formalin, embedded in paraffin, sectioned to 3 μm thick, and stained with hematoxylin and eosin for microscopic examination. The ascitic fluid was centrifuged (1000 r, 5 min), and a cytologic smear was prepared for microscopic examination of malignant tumor cells.

Immunohistochemistry

The expression of cytokeratin (CK; High MW), cytokeratin 20 (CK-20) and epithelial membrane antigen (EMA) was studied by using mAbs 34βE12, KS20.8 and GP1.4 (MAB-0052, 0057, 0061; Maixin Inc., Fuzhou, China). An ultrasensitive SP kit (KIT-9710; Maixin Inc., Fuzhou, China) and a DAB kit (DAB-0031; Maixin Inc., Fuzhou, China) were employed according to the manufacturer's instructions. The expression of CK, CK-20 and EMA proteins was defined as positive if the stained area of tumor cells was predominant in their cytoplasm.

Statistical analysis

Data are presented as mean ± SD. The median survival was analyzed using the Wilcoxon Rank-Sum test. The volume of primary tumor was analyzed by the student *t* test. The incidence of metastasis in both groups was analyzed using Fisher exact test. Differences were judged statistically significant at a *P* value < 0.05.

RESULTS

The incidence of tumor growth and general condition

The tumor uptake rate after orthotopic implantation in both of the two groups was 100% (12/12). The median survival for the SGC-7901 and BGC-823 groups were 84 d and 23 d, respectively ($P < 0.05$, Figure 1). Mice of the SGC-7901 group had obvious cachexia (emaciation, retardation, ascites formation, *etc.*), whereas none of the BGC-823 group showed a decline in their general health.

Observation and evaluation of primary tumor growth

The average tumor volume of the BGC-823 group was 1.24 ± 0.73 cm^3, while it was 9.30 ± 3.62 cm^3 in the SGC-7901 group (Figure 2A and B). There was a statistical significance in the difference ($P < 0.05$, Figure 2C). In addition, observations of the primary tumor and related characteristics in the two groups are presented in Table 1 in detail.

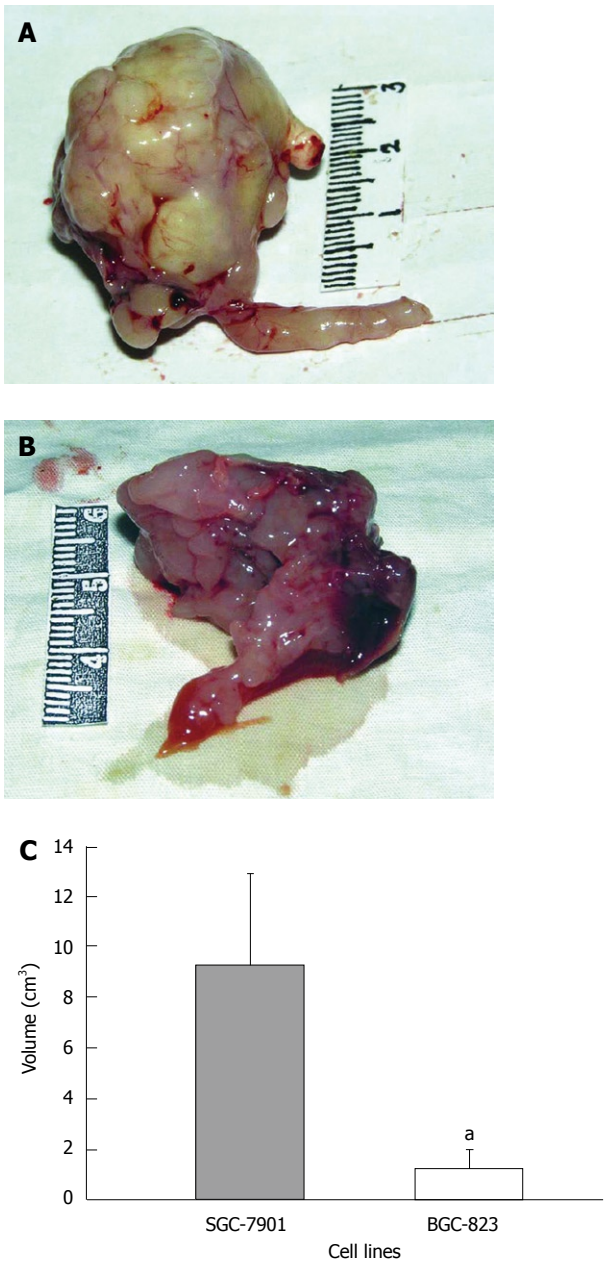

Figure 2 Macroscopic examination of the primary tumor in the two models. A: The tumor of SGC-7901 shows large volume (9.30 ± 3.62 cm^3), irregular lobular shape, and stenosis in the cardia or pylorus; B: The tumor volume of BGC-823 is 1.24 ± 0.73 cm^3, with irregular lobular appearance; C: Comparison of primary tumor volume in two models. The difference is statistically significant. $^aP < 0.05$, student *t* test.

Evaluation of metastases derived from orthotopic implantation tumors

Metastasis, which was always located in lymph nodes, liver, kidney, lung, peritoneum or diaphragm, occurred in both groups of gastric cancer models. Additionally, oth-

Table 1 Comparison of primary tumor and related characters in the two models

Items		SGC-7901 group	BGC-823 group
Primary tumor	Shape	Irregularly lobular	Irregularly lobular
	Color	Grayish yellow	Gray-white
	Texture	Stiffer	Softer
	Vascularity	Rich	Richer
Section of stomach tumor	Gastric cavity	Narrow or vanished	Narrow or vanished
	Greater and lesser curvature	Undistinguishable	Undistinguishable
	Pylorus	Partly or totally obstructed	Totally obstructed
Effects on adjacent organs	Abdominal cavity	Occupying whole abdomen	Occupying upper abdomen
	Adhering extent	Adhering to liver lobes	Adhering to many organs
	Compressed status	Severely compressed	Partly compressed
Ascites	Liquid quantity	Little	Much
	Property	Clear and yellowish	Bloody fluid

Table 2 Comparison of metastatic rates in models of SGC-7901 and BGC-823, *n*/*N* (%)

Groups	Site and incidence of metastasis						
	Lymph nodes[a]	Liver	Kidney[a]	Lung	Spleen[b]	Testicle[a]	Peritoneum or septum
SGC-7901	7/12 (58)	10/12 (83)	5/12 (42)	1/12 (8)	0	0	10/12 (83)
BGC-823	12/12 (100)	12/12 (100)	10/12 (83)	5/12 (42)	7/12 (58)	5/12 (42)	12/12 (100)

[a]$P < 0.05$, [b]$P < 0.01$.

er organs (spleen and testicle) were also found to have metastatic lesions in the BGC-823 group. The incidence of metastasis from various organs in the BGC-823 group was higher than that in the SGC-7901 group, and these differences (except for liver, lung, peritoneum or diaphragm) were considered statistically significant ($P < 0.05$ or $P < 0.01$, Table 2).

Histology

The two groups differed in histological appearance of subcutaneous tumor samples, though the cell lines forming tumors were both poorly differentiated adenocarcinomas. In the SGC-7901 group, tumor cells spread with nest-like structures, characterized by nuclear polymorphism, nuclear hyperchromatism, many red nucleoli, and rich pathological karyokinesis. Mucus was observed in the cytoplasm of part of the tumor cells which resulted in mucus lakes, and adenoid structures and rich blood vessels formed in tumor areas. In the BGC-823 group, histopathologic examination confirmed a different phenotype of the BGC-823 tumor compared to the SGC-7901 tumor. The tumor cells mostly showed medullary growth with characteristics of ample cells, fewer fibroblasts, and rich vascularity. The neoplasm revealed the signs of undifferentiated structure, small size, nuclear hyperchromatism, and rich pathological karyokinesis. Mucus was found in the cytoplasm of part of the neoplastic cells, but there was an absence of glandular differentiation.

Both primary tumors and metastases, whose tumor cells were microscopically identical to the subcutaneous tumor, of the two group models showed similarity in histopathologic characteristics. The stomach cancer of the two models infiltrated the various layers of gastric wall with disruption of the integrity of the mucous layer or muscularis mucosae (Figure 3A and B). Widespread infiltration of tumor cells was found in the subcapsular, the cortical and medullary areas of lymph nodes, while extension into the medullary parts the lymph sinus was diminished (Figure 3C). In some of the lymph nodes, the nodal parenchyma was even totally replaced by neoplastic tumor. Metastases were detected in the liver (Figure 3D) and the kidney (Figure 3E) with nest-like structures or a glandular appearance, occasionally in the adrenal gland or pancreas (not shown). Metastatic lesions were always separated from adjacent normal tissue by fibrous capsules, and lymphoid infiltration in peripheral tumor areas was also seen. Tumors metastasized to the lung and destroyed bronchi or bronchioles (Figure 3F). In addition, local invasion was observed within the abdominal muscle or diaphragmatic muscle. Organs such as the spleen and the testicle (Figure 3G and H) were not spared in the BGC-823 models. Histological examination revealed that the spleen parenchyma was infiltrated and the seminiferous tubules of the testis or ductus epididymidis, and the prostate, were invaded by the metastatic tumor.

Smears of cast-off cells from ascites in both groups confirmed that the malignant cells originated from the primary adenocarcinoma (not shown).

Immunohistochemistry analysis

The expression of CK-20 and EMA protein was positive within the primary tumor and metastases in the SGC-7901 group, whereas CK expression was negative in the same tissues (Figure 4). In the BGC-823 group, CK, CK-20 and EMA immunostaining were negative.

DISCUSSION

The purpose of our study was to develop gastric cancer models from different cell lines with intact tumor

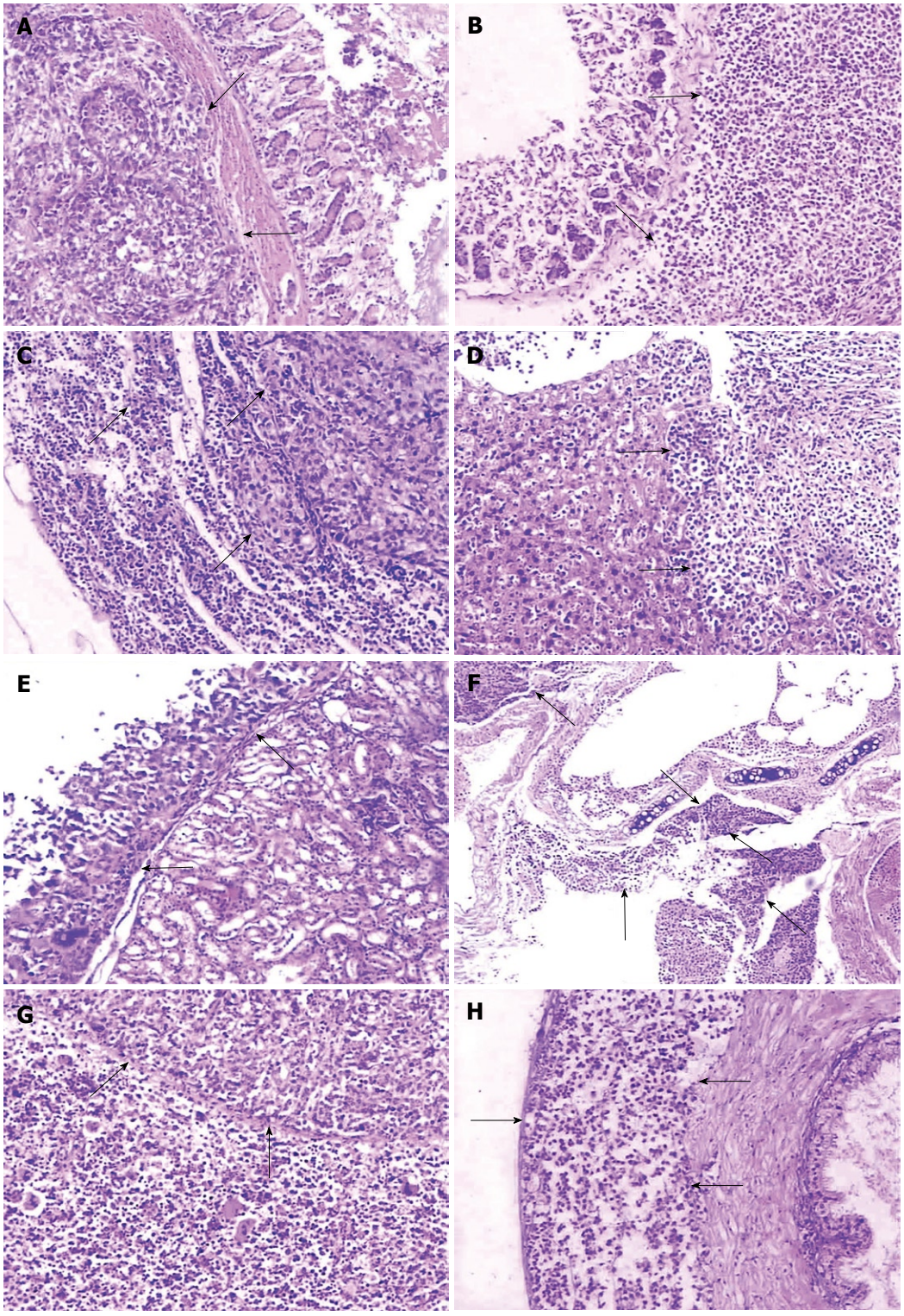

Figure 3 Histology of the primary tumor and metastasis in the two models by hematoxylin and eosin staining. The tumor cells of the SGC-7901 (A) and the BGC-823 (B) model infiltrate the submucosa of the stomach; C: Tumor cells (arrows) are found in the subcapsular and cortical areas of lymph node of the SGC-7901 model; D: Metastases (arrows) detected in the liver of the BGC-823 model; E: Tumor invades into the kidney of the SGC-7901 model, with a fibrous capsule surrounding relatively normal renal tissue (arrows); F: Tumor metastasizes to the lung and surrounds the bronchium in the BGC-823 model; Other organs (G: Spleen; H: Testicle) with metastasis involvement in the BGC-823 model. Tumors are marked with arrows. Hematoxylin and eosin stain; Magnification, × 100.

pieces orthotopically implanted into the stomach wall by tissue glue adhesion. In the present study, we could replicate similar results of the SCG-7901 orthotopic models which have been established by other researchers in China. This is the first time that an animal model has been created from the BGC-823 gastric cancer cell line with SOI of tumor tissue.

Interestingly, we find the two models reveal signifi-

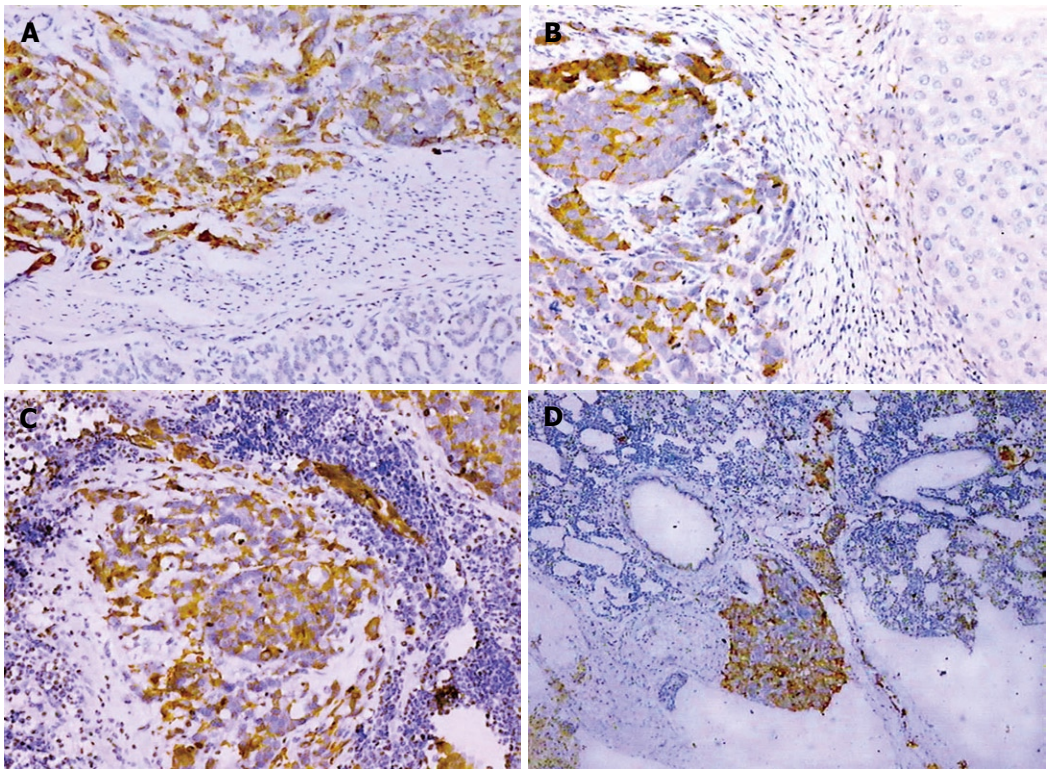

Figure 4 Immunohistochemistry of primary tumor and metastasis in the SGC-7901 model using the self-potential method. Cytokeratin 20 (A: Stomach; B: Liver) and epithelial membrane antigen expression (C: Lymph node; D: Lung) shows as positive, characterized by the brown-yellow stain in the cytoplasm. Visualized by using DAB reagent; Magnification, × 100.

cant differences in biological behavior. It has been reported that heterogeneity resulting from differences of histopathological type, grade, and individual factors reside in different tumor cell lines[5,16]. Thus, animal models derived from different tumor cell lines present different characteristics. Bhargava *et al*[5] orthotopically established gastric cancer models in two sites of the stomach using tumor fragments from three cell lines of differently differentiated extent. The findings showed that the tumor uptake rate and metastases were all different among the three cell lines, between two sites, and at various time points. Fujihara *et al*[13] developed a gastric cancer cell line subtype named OCUM-2M LN, characterized by high incidence of lymph node metastasis, which was obtained from the OCUM-2M cell line by serial passage selection in animals. The ability to metastasize to lymph nodes in subtype OCUM-2M LN was significantly higher than that in parental cell line OCUM-2M. In keeping with these authors above, we also found that heterogeneity among different cell lines contributed to differences of tumor behavior in both gastric cancer models.

Histopathological characteristics of malignant neoplasms were reflected in both cancer models, characterized by neoplastic atypia of varying degrees, and rich blood vessels promoting tumor growth and metastasis. However, the two types of tumor showed differences in pathologic phenotype. The SGC-7901 tumors showed nest-like structures and glandular differentiation, while the BGC-823 tumors presented medullary structures and no adenoid differentiation, which might be explained by the fact that the BGC-823 cell line develops variation and partly changed neoplastic properties during passage[9,13]. Microscopically, mucous secretion in both types of tumor cells, especially in the SGC-7901 tumor, was consistent with the characteristics of mucous adenocarcinoma. Meanwhile, the negative result of CK expression in the two models also excluded the differentiation of squamous carcinoma. The expression of CK-20 and EMA by immunohistochemical staining was positive in the SGC-7901 tumors, but negative in BGC-823. Histopathology and immunohistochemistry analysis all confirmed that the BGC-823 carcinoma had poorer differentiation than SGC-7901. In addition, all epithelial markers in BGC-823 tumors were negative which might suggest an epithelial-to-mesenchymal transition phase. We shall perform immunohistochemical staining with a mesenchymal marker, such as vimentin, to verify this presumption in our next experiments. It is reported that survival prognosis has close correlation with histopathologic grading of tumor[17-19]. The results in our study demonstrated that median survival course in the BGC-823 model was shorter than that in SGC-7901 ($P < 0.05$), which is compatible with histological examination.

Retrospective studies have suggested that there is correlation between tumor volume size and lymph node metastasis or five-year survival[19-22]. In our experimental results, the original tumor in both models presented a large-volume lesion invading adjacent organs and

destroying the stomach cavity. There is no theoretical significance regarding difference of the tumor volume in the SGC-7901 and BGC-823 groups, though the former is obviously larger than the latter ($P < 0.05$) mainly resulting from the different course of primary tumor growth. However, the SGC-7901 tumor texture was harder than that of BGC-823, which was possibly linked with rich stromal component in the former but ample tumor cells and rich blood vessels in the latter. Angiogenesis is a prerequisite for metastatic spread[15,23], which also explains why vascular metastasis is a frequent occurrence in the BGC-823 model.

Metastasis is not only linked with the prognosis of patients but also the dilemma of cancer therapy. In the field of experimental research, metastatic events are assumed to cover the factors of the anatomic site of implantation[12,13,24,25] or status (suspension or fragment) of tumor sample used in implantation[10,23]. Orthotopic transplantation in animal models of various cancers has played a key role in mimicking the clinical tumor behavior[5,23,24]. There has been variable success at achieving good tumor uptake rate and metastatic incidence with implantation procedures using suspension injections and tumor pieces[10,23]. Many studies indicate that the properties on the tumor cell surfaces are preserved by the implantation of tumor pieces, but destroyed after the cell suspensions have been treated by trypsinization resulting in changes of malignant character and further contributing to decline of tumor growth and metastatic rate[13,24]. In the present study, the models of orthotopic implantation into the gastric wall with intact tumor fragments showed not only high uptake rate but also metastasis through various pathways, which was consistent with the previous research. It is well known that the general metastasis in terminal patients with gastric cancer mainly involves direct infiltration, lymphatic metastasis, vascular spread, and implantation dissemination. The aggressive behavior in the two model groups resembles clinical patients suffering from gastric cancer. In the SGC-7901 group, direct infiltration and lymphatic metastasis were frequently observed. However, multiple steps were assumed to be involved in the metastases of the BGC-823 group. In BGC-823 models, we could not identify the accurate pathway of metastasis through local invasion in the liver, kidney, spleen and testicle. The total involvement of multiple steps facilitating metastases requires further studies.

Compared with the SGC-7901 group, metastasis occurrence is earlier ($P < 0.05$), metastasis incidence in different organs higher ($P < 0.05$), and the number of involved organs greater in the BGC-823 group. The deaths in the SGC-7901 group were considered as multi-organ failure caused by cachexia, while the BGC-823 mice mostly died of early metastases and high metastatic rates without visible cachexia. Taken together, the results in our study demonstrate that the BGC-823 gastric cancer model is more aggressive than the SGC-7901 one with the support of the comprehensive factors discussed above.

We have successfully developed two types of gastric cancer model with different tumor behavior by orthotopic implantation, which are characterized by advantages such as low cost and resemblance to clinical gastric cancer. The two models are both suitable for preclinical research on gastric cancer, and their different characteristics may aid with different needs of experimental research. Considering comprehensive factors, surgical orthotopic implantation is still a desirable technique compared with other methods of constructing gastric cancer models[26-29].

COMMENTS

Background

Gastric cancer is still the most common malignant tumor in the world, and detection is difficult before metastasis occurrence, so people need to develop animal models of gastric cancer for exploring the mechanisms of carcinogenesis and clinical treatment strategy. Gastric cancer models of orthotopic implantation with intact tumor tissue have been well established. Moreover, orthotopic implantation performance has been improved from the "sewing" method to the "adhering" one. SCG-7901 orthotopic models have been established by other researchers, whereas this is the first time that an animal model was created from the BGC-823 gastric cancer cell line with surgical orthotopic implantation of tumor tissue.

Research frontiers

Orthotopic implantation technique plays an important role in establishing an animal model of various tumors, including gastric cancer, which can not only simulate clinical cancer development but also facilitate metastasis occurrence. Moreover, the procedure of orthotopic implantation has been improved from the "sewing" method to the "adhering" one. The latter's performance has greatly simplified the surgical operation course. In addition, the BGC-823 gastric cancer model has not been constructed by others, and shows different tumor characteristics compares with the SCG-7901 model.

Innovations and breakthroughs

Orthotopic implantation with "glue paste technique" is a new, popular and convenient method used for the establishment of orthotopic animal models in recent years. The results regarding the BGC-823 gastric cancer model in this study are the first to be reported, and make it possible that researchers can learn the different biological characteristics of gastric cancer models from different cell lines.

Applications

Establishment of the BGC-823 gastric cancer model provides evidence for researchers to learn the biological differences between animal models from different gastric cancer cell lines, which can help them to choose an appropriate model to tailor their research.

Terminology

Surgical orthotopic implantation is defined as tumor cells in the form of suspension or fragments which are orthotopically implanted into the related organs.

Peer review

The study is aimed to develop gastric cancer models from different cell lines and analyze the difference of biological behavior in many aspects, in order to provide evidence for researchers to choose an appropriate experimental carrier and therapy target for clinical trials. The present research is valuable for clinical doctors and researchers.

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BRIEF ARTICLE

B1a lymphocytes in the rectal mucosa of ulcerative colitis patients

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Abstract

AIM: To assess B1a cell expression in the rectal mucosa of ulcerative colitis (UC) patients in comparison with healthy controls.

METHODS: Rectal mucosa biopsies were collected from 15 UC patients and 17 healthy controls. $CD5^+$ B cells were analysed by three colour flow cytometry from rectal mucosal samples after mechanical disaggregation by Medimachine®. Immunohistochemical analysis of B and T lymphocytes was also performed. Correlations between, on the one hand, rectal B1a cell concentrations and, on the other, erythrocyte sedimentation rate and C-reactive protein levels and clinical, endoscopic and histological disease activity indices were evaluated.

RESULTS: Rectal B-lymphocyte ($CD19^+/CD45^+$) rate and concentration were higher in UC patients compared with those in healthy controls (47.85% ± 3.12% *vs* 26.10% ± 3.40%, $P = 0.001$ and 501 ± 91 cells/mm^2 *vs* 117 ± 18 cells/mm^2, $P < 0.001$); Rectal B1a cell density ($CD5^+CD19^+$) was higher in UC patients than in healthy controls (85 ± 15 cells/mm^2 *vs* 31 ± 6.7 cells/mm^2, $P = 0.009$). Rectal B1a cell ($CD5/CD19^+$) rate correlated inversely with endoscopic classification (Rs = -0.637, $P < 0.05$).

CONCLUSION: B1a lymphocytes seem to be involved in the pathogenesis of UC, however, the role they play in its early phases and in disease activity, have yet to be defined.

Key words: B1 cell; CD5; Flow cytometry; Rectum; Ulcerative colitis

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INTRODUCTION

The aetiology of ulcerative colitis (UC) is still unknown, but several studies have demonstrated that there is an abnormal immunologic response to gut antigens[1,2]. One

of the mechanisms which protects the body against intestinal luminal antigens before a specific aggressive inflammatory response is unleashed, is the production of natural antibodies, in particular immunoglobulin A (IgA)[3,4]. A large proportion of IgA is produced in a T-independent way by B cells and in particular, according to several studies, by the B1 sub-population. The majority of B1 cells, also called B1a, express CD5 on their surface. In a previous study[5] we reported that B1a cell ($CD5^+CD19^+$) blood concentrations were reduced in UC patients with respect to those in healthy controls, and that the B1a cell rate was inversely correlated with erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels in UC patients. The aim of the present study was to analyse the rate and concentration of B1a cells in the rectal mucosa of UC patients, to compare these values with those found in healthy controls, and to assess any possible correlation with disease activity.

MATERIALS AND METHODS

Patients

The study population consisted of 15 UC patients and 17 healthy controls. The study protocol was drafted in accordance with the Declaration of Helsinki and all of the patients and controls who participated signed informed consent statements. Patients taking immunosuppressive drugs or corticosteroids were excluded from the study.

The disease activity of the UC patients, undergoing ordinary follow-up colonoscopy, was classified in accordance with the Seo clinical score[6], the modified endoscopic Baron classification[7] and Geboes' histological scoring[8]. Subjects who underwent colonoscopy as a cancer screening procedure and whose result was negative, were enrolled as healthy controls.

Methods

Blood samples and 6 rectal mucosal biopsy specimens, taken 10-15 cm from the anal verge during colonoscopy, were collected from each patient.

Flow-cytometry

A cellular suspension, obtained by fragmentizing 4 rectal biopsies from each patient using a Medimachine (Consul TS, Rivalta di Torino, Italy), was used for flow cytometry. The method utilized was as follows: the collected rectal biopsies were placed in physiologic solution (Na 0.9%) and immediately processed. The biopsies were first washed twice with physiologic solution for 2 min each wash. Each biopsy was minced into < 1 mm^3 pieces which were placed in a sterile microblade-equipped polyethylene chamber (Medicons, BD Biosciences, San Jose, CA, United States) with 1 mL phosphate buffered saline (PBS) 0.01 mol/L. The Medicons contain an immobile stainless steel screen with 100 hexagonal holes, each surrounded by six microblades. When the Medicons are inserted into the Medimachine the tissue comes into contact with the blades by means of a rotating element

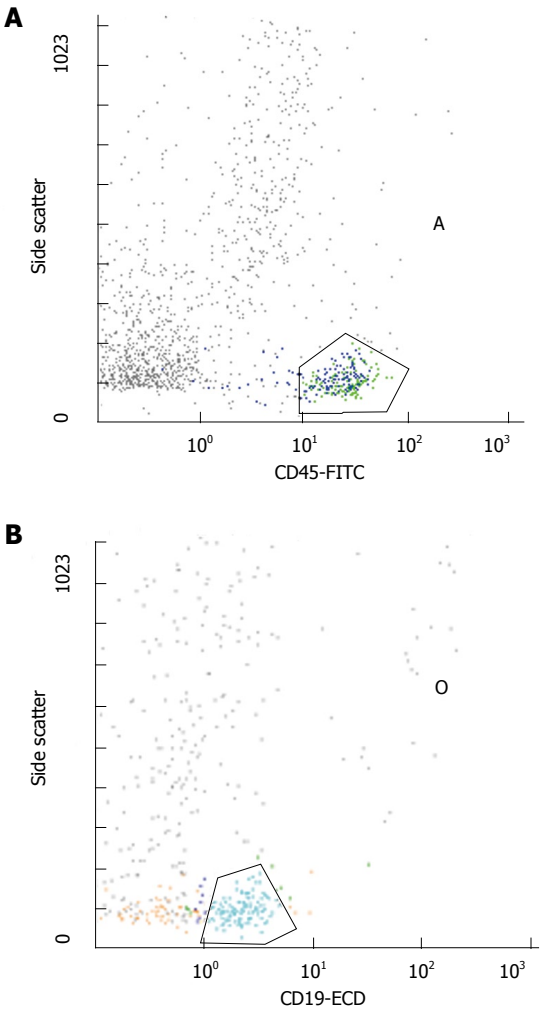

Figure 1 Flow cytometry. A: Immunologic gate for rectal $CD45^+$ cells; B: Immunologic gate for rectal $CD19^+$ cells.

and is disaggregated. A micropump positioned under the screen forces the liquid to pass through the bore-holes, ensuring that the bore-holes remain clean. Medicons with 35 μm separator screens were used.

Fragments were dissociated 4 times for 20 s at a constant speed of 100 r/min. The suspension was filtered using filters with 30 μm diameter holes (Flicons, BD Bioscences, San Jose, CA, United States) and then analysed by flow-cytometry (Figures 1 and 2).

A three-colour flow-cytometric analysis was performed using the following associations: CD45 fluorescein isothiocyanate (FITC), Isotype IgG1 (clone J.33, mouse) (Beckman Coulter Inc, Fullerton, CA, United States), CD5 RD-1 (phycoerythrin), Isotype IgG2a (clone SFCI24T6G12, mouse) (Beckman Coulter Inc.), CD19 ECD (Texas red), Isotype IgG1 (clone J4.119, mouse) (Beckman Coulter Inc.). Flow cytometry was performed as previously described[5].

All mAbs were used at optimal saturating concentrations as recommended by the manufacturers. The cells were washed in a FACS buffer containing PBS/5% FCS/0.05% and sodium azide, then incubated with 10 mg of human

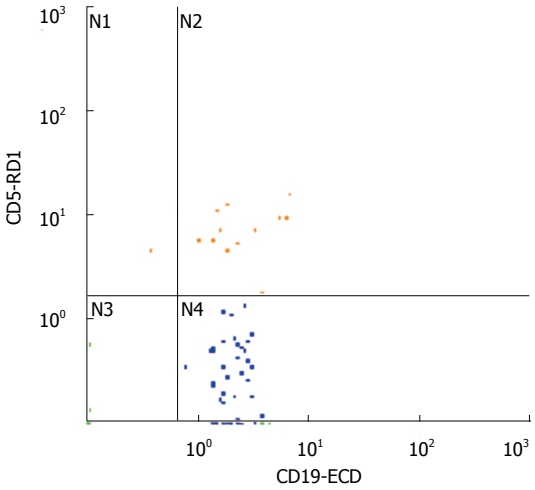

Figure 2 Flow cytometry. $CD5^+/CD19^+$ lymphocytes (B1a cells).

IgG (Sigma Chemical Co., St. Louis, MO, United States) for 30 min at 4 ℃-8 ℃ to block Fc receptors. Cells were washed to remove excess IgG and were triple-stained with either RD-1-conjugated mAb against CD5, or RD-1-control IgG mAb, ECD-conjugated mAb against CD19 or ECD-control IgG mAb and FITC-conjugated mAb against CD45 or FITC-control IgG mAb for 30 min at 4 ℃-8 ℃. Cells were washed twice, re-suspended in a FACS buffer and fixed with 1% paraformaldehyde. At that point, 2 mL of lysing solution (0.17 mol/L NH_4Cl) were added and following 15 min of incubation, samples were analyzed by flow cytometry using a Coulter EPICS XL-MCL (Beckman Coulter Inc). Mononuclear cells were gated depending on their CD45 expression characteristics. Different subsets of cells (CD19) were then gated on the basis of fluorescence 1 and fluorescence 2 staining. An immunologic gate was performed on $CD19^+$ cells to uncover CD5. Isotypic controls were used for all the samples. A two-colour flow cytometric analysis was similarly performed to study the T cells using anti-CD45 FITC mAb (Isotype IgG1, mouse, clone J.33, Beckman Coulter Inc.) and anti-CD3 Phycoerythrin-Cyanin 5 (Pc5) mAb (Isotype IgG1, mouse, clone UCHT 1, Beckman Coulter Inc.).

Immunohistochemistry

Two rectal biopsies from each patient studied underwent immunohistochemical analysis. Samples were fixed in 10% neutral buffered formalin, processed for embedding in paraffin wax, and cut into 5 μm-thick sections. Sections were dewaxed and rehydrated by routine protocols. For anti-CD3 immunohistochemistry, antigen unmasking was performed with 10 mmol/L sodium citrate buffer, pH 6.0, in a microwave oven at 96 ℃ for 30 min. Antigen unmasking was not necessary for anti-CD20 analysis. Sections were incubated in 0.3% hydrogen peroxide for 10 min at room temperature to remove endogenous peroxidase activity, and then in blocking serum for 30 min. Samples were incubated with primary antibodies at room temperature for 45 min. The primary antibodies used were: anti-CD3 (Polyclonal rabbit anti-Human, Dako, Milan, Italy) and anti-CD20 (Monoclonal mouse anti-human CD20cy, Dako) diluted 1:50 and 1:200, respectively. Sections were then washed three times in PBS for 5 min each wash, treated with secondary antibody (Envision System HRP, Dako) for 30 min at room temperature and developed in 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, Milan, Italy). Finally, the sections were counterstained with hematoxylin.

Sections were examined using a Leica DM4500B microscope (Leica Microsystems, Wetzlar, Germany) connected to a Leica DFC320 high-resolution digital camera (Leica Microsystems) and a computer equipped with software for image acquisition and analysis (QWin, Leica Microsystems). The densities of CD3- and CD20-positive cells were evaluated at a magnification of 40 ×, and 10 fields per section were examined. The densities were calculated for each section by dividing the number of positive cells by the area of the rectal mucosa analysed.

Table 1 Comparison of rectal B and T lymphocyte populations and B1 subpopulations in ulcerative colitis patients and controls

	Ulcerative colitis	Controls
$CD19^+/CD45^+$ (%)	47.8 ± 3.1[b]	26.1 ± 3.4
$CD20^+$ (cells/mm^2)	501 ± 91[b]	117 ± 18
$CD3^+/CD45^+$ (%)	53.5 ± 4.2[a]	68.3 ± 3.5
$CD3^+$ (cells/mm^2)	485 ± 100	445 ± 95
$CD5^+/CD19^+$ (%)	15.5 ± 2.0	23.5 ± 4.9
$CD5^+CD19^+$ (cells/mm^2)	85 ± 15[b]	31 ± 6.7

[a]$P < 0.05$, [b]$P < 0.01$ *vs* controls.

Calculation of rectal mucosa B1a cell concentration

The rectal mucosa B1a cell concentration ($CD5^+CD19^+$) was calculated by multiplying the $CD5^+/CD19^+$ ratio, obtained by flow-cytometry, by the B lymphocyte concentration, obtained by immunohistochemistry.

ESR and CRP analysis from blood test

ESR and CRP were measured using the Westergren method and immuno-nephelometry, respectively.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed using Mann-Whitney *U* test for the comparison between the UC patients and controls and by Spearman's Rank test for correlations. Statistical significance was set at $P < 0.05$.

RESULTS

Adequate material for flow-cytometry was obtained from 13/15 UC patients (8 males and 5 females, median age 54 years, range 19-71 years) and from 13/17 controls (8 males and 5 females, median age 61 years, range 37-88 years). Of the 13 UC patients included in the study, 5 were taking mesalazine and 8 were not.

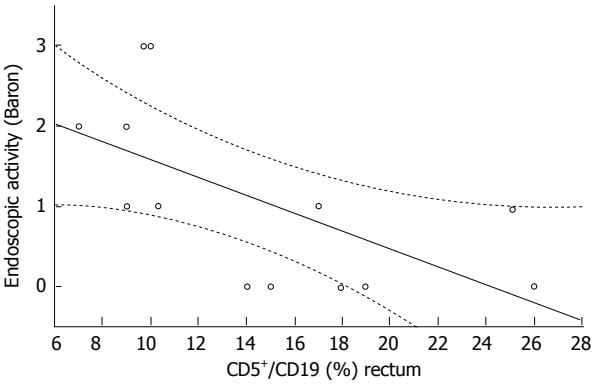

Figure 3 Correlation between B1a cells percentage in ulcerative colitis patients and endoscopic disease activity (*Rs* = -0.68, *P* = 0.01).

Ulcerative colitis was clinically active (Seo score > 150) in 5 patients and endoscopically active (Baron score > 1) in 4. The median histologic activity score was 3 (range 0-5).

Flow cytometry

The percentage of B lymphocytes ($CD19^+/CD45^+$) in the rectal mucosa was higher in UC patients with respect to healthy controls (47.8% ± 3.1% *vs* 26.1% ± 3.4%, *P* = 0.001); while the percentage of rectal T lymphocytes ($CD3^+/CD45^+$) was significantly lower in UC patients with respect to the controls (53.5% ± 4.2% *vs* 68.3% ± 3.5%, *P* = 0.02).

The rectal B1a cell rate ($CD5^+/CD19^+$) did not differ significantly in the two groups (Table 1), and was inversely correlated with endoscopic activity (Rs = -0.68, *P* = 0.01, Figure 3), but not with the clinical SEO disease activity index, ESR and CRP levels, or with age. The mean rectal B1a cell rate was higher, but not significantly different in patients with remission or mild histologic activity (score 0-1), with respect to patients with moderate-severe histologic activity (score 2-5) (22.0% ± 3.0% and 12.7% ± 2.5%, respectively, *P* = 0.1). The rectal B1a cell rate was not significantly different in the patient group taking mesalazine compared with those not taking mesalazine (11.0 ± 2.1 and 17.2 ± 3.0, respectively, *P* = 0.13).

Immunohistochemistry

Histological analysis confirmed that there was an increased concentration of B lymphocytes CD20+ in the rectal mucosa of ulcerative colitis patients with respect to that in controls (cell density 501 ± 91 cells/mm^2 *vs* 117 ± 18 cells/mm^2, *P* < 0.001). T cell density was not significantly different in the UC patients and controls (485 ± 100 *vs* 445 ± 95, *P* = 0.6).

Calculated rectal B1a cell concentration

The calculated B1a cell density was significantly increased in UC patients with respect to that in controls: 85 ± 15 cells/mm^2 *vs* 31 ± 6.7 cells/mm^2, *P* = 0.009.

DISCUSSION

More than 80% of the body's activated B cells are located in the gut, where a continuous interaction takes place between the immune system and the trillion bacteria that reside there[9].

IgA generation by B cells is an important mechanism that regulates this homeostasis, contributing to immune protection but without provoking inflammation. A large proportion of the intestinal IgA against cell wall antigens and proteins of commensal bacteria is specifically induced in response to their presence within the microflora, but is independent of T cells or germinal centre formation. This T cell-independent IgA production is derived from B1 lymphocytes which develop in the peritoneal compartment and are distributed diffusely in the intestinal lamina propria[10]. In mice, peritoneal B cells (B1 cells) do not differentiate during migration through the lymphoid organs and finally home to the gut lamina propria where they switch and differentiate to IgA+ plasma cells[11]. The physiological importance of B1 cells in the maintenance of homeostasis at the mucosal surface has been clearly demonstrated[12].

B cells in inflammatory bowel disease have not been as extensively studied as T cells[13], and data on the role of B1 cells in UC are particularly scanty. Except for a smaller sub-group called B1b, B1 cells are distinguishable from B2 cells by expressing CD5 on their surface[14]. Even in the absence of external antigen stimulation, B1 cells produce natural antibodies (Ab) that provide early, broad protection against pathogens[4]. B1 cells are also known to produce auto-reactive Ab, including Ab to cell membrane components, such as phosphorylcholine[15] and phosphatidylcholine[16] to immunoglobulins (rheumatoid factor) and to single-stranded DNA[17].

B1 cells were thus analysed for their role in autoimmunity and high circulating B1a lymphocyte levels have been reported in some autoimmune diseases, such as systemic lupus erythematosus, primary Sjogren's syndrome[18], rheumatoid arthritis[19], multiple sclerosis[20] and anti-phospholipid syndrome[21]. In the light of recent findings, these cells, and in particular the CD5 molecule on B cells, seem to play a role in preventing autoimmunity[22].

In a previous study we reported that B1a cell ($CD5^+/CD19^+$) concentrations are reduced in the blood of patients with UC even after restorative proctocolectomy[5]. Moreover, B1a cell rate was found to be inversely correlated with ESR and CRP levels in UC patients, indicating that these cells play a protective role against inflammation. Low $CD5^+/CD19^+$ blood percentages in UC and in Crohn's disease (CD) have also been reported by other authors[23,24].

The present study focused on the presence of B lymphocytes, and in particular the B1a sub-group, in the rectal mucosa of UC patients. An increased concentration of B lymphocytes was found in the rectal mucosa of these patients and their percentage within the leukocyte pool was also increased. B1a cell concentrations

in the rectal mucosa of UC patients were significantly higher than those in healthy controls, but not their percentage within the whole B lymphocyte pool ($CD5^+$/$CD19^+$). Senju *et al*[25], who analysed subsets of lamina propria lymphocytes using two-colour flow cytometry, also found no differences in the percentage of $CD5^+$ B lymphocytes in UC, CD patients and controls. They reported that the majority of B cells in the intestinal mucosa did not possess CD5 antigens on their cell surface. In the present study, performed using three colour flow cytometry, we found that the percentage of $CD5^+$ B cells in the rectum was small, but not negligible (between 15% and 23%). The use of anti-CD45 helps to restrict the flow cytometric analysis to leukocytes, excluding from the study the other types of cells homing in the rectal mucosa.

Finding an increased concentration of B1a cells, but not a higher percentage within the whole B lymphocyte population, means that other B lymphocyte subsets were increased in the rectal mucosa of these patients. In addition, finding an inverse correlation between $CD5^+$ B cells and endoscopic disease activity suggests that these cells are recruited to a relatively lower extent when the disease is more severe and possibly, at that point, a more specific immune reaction has already begun. As our findings seem to indicate that there is a loss of tolerance at this disease stage, further studies will clarify this point. The finding of an inverse correlation between rectal mucosa B1a cell rate and endoscopic disease activity is consistent with previous data concerning the inverse correlation of circulating B1a cell rate and blood ESR and CRP levels[5]. The former is a sign of local events, and the latter of a systemic imbalance.

Peterson *et al*[26] reported that the depletion of $CD5^+$ B1 cells has different effects on the induction phase with respect to the effector phase of experimental autoimmune encephalomyelitis. During the induction phase it increases disease incidence, while in the effector phase it reduces disease severity. The role of $CD5^+$ B cells in UC is not yet clear. It remains to be clarified if they play a protective role by producing polyspecific immunoglobulins, an unleashing role by producing autoreactive Ab, or if there is an even more complex modulating role that differs depending on the disease activity. The findings in this and other studies seem to indicate that their role in UC is not marginal, but more in-depth analyses are warranted to better define their function and to determine if and how their modulation has an impact on disease activity.

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COMMENTS

Background

The aetiology of ulcerative colitis (UC) is still unknown, but several studies have demonstrated that there is an abnormal immunologic response to gut antigens.

Research frontiers

B1 lymphocytes are important in maintaining mucosal surfaces in a state of homeostasis, and it has been found that B1 cell concentrations are reduced in the blood of patients with UC even after restorative proctocolectomy. In the present study, B1a cell expression was assessed in the rectal mucosa of UC patients and compared with that in healthy controls using three color flow-cytometry.

Innovations and breakthroughs

Rectal B1a cell density ($CD5^+CD19^+$) was increased in ulcerative colitis patients compared with healthy controls, but its rate correlated inversely with endoscopic disease score.

Applications

The findings in this and other studies seem to indicate that B1a lymphocytes play a role in the pathogenesis of ulcerative colitis and their modulation could have an impact on the control of disease activity.

Terminology

B1 lymphocytes are distinguishable from B2 lymphocytes because they express CD5 on their surface. Even in the absence of external antigen stimulation, B1 cells produce natural antibodies that provide early, broad protection against pathogens. B1 cells are also known to produce auto-reactive antibodies. CD5 is a 67 kD trans-membrane glycoprotein that interacts in the B lymphocyte with the B cell receptor, negatively regulating growth signaling.

Peer review

This study assessed B1a cell concentration in rectal mucosa of 13 UC patients as compared to controls. A possible correlation between these cells and either endoscopic (modified Baron classification) or clinical (Seo clinical score) activity index was also investigated. Data found that B1a cell concentration is increased in UC, but its distribution (i.e., percentage within leucocytes) did not differ between patients and controls. Moreover, a significant, inverse correlation between B1a cell concentration in rectal mucosa and endoscopic activity index was found.

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BRIEF ARTICLE

Human epidermal growth factor receptor-2 gene amplification in gastric cancer using tissue microarray technology

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Abstract

AIM: To assess human epidermal growth factor receptor-2 (HER2)-status in gastric cancer and matched lymph node metastases by immunohistochemistry (IHC) and chromogenic *in situ* hybridization (CISH).

METHODS: 120 cases of primary gastric carcinomas and 45 matched lymph node metastases from patients with full clinicopathological features were mounted onto multiple-punch and single-punch tissue microarrays, respectively, and examined for HER2 overexpression and gene amplification by IHC and CISH.

RESULTS: Twenty-four tumors (20%) expressed HER2 immunohistochemically. An IHC score of $\geqslant$ 2+ was observed in 20 tumors (16.6%). HER2 amplification was detected by CISH in 19 tumors (15.8%) and in their matched lymph node metastases. A high concordance rate was found between HER2 positivity (as detected by IHC) and *HER2* gene amplification (as detected by CISH), since 19 of the 20 IHC positive cases were amplified (95%). All amplified cases had 2+ or 3+ IHC results. Amplification was associated with intestinal phenotype ($P < 0.05$). No association with grading, staging or survival was found.

CONCLUSION: In gastric cancer, HER2 amplification is the main mechanism for HER2 protein overexpression and is preserved in lymph node metastases.

Key words: Human epidermal growth factor receptor-2; Immunohistochemistry; Chromogenic *in situ* hybridization; Gastric cancer

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INTRODUCTION

Alterations of the human epidermal growth factor receptor-2 (*HER2*) gene are implicated in the development and progression of many tumors[1-4]. In breast cancer, HER2 amplification has been found in about 20% of cases and was linked to poor prognosis[5,6]. Breast cancer patients with HER2 amplification have been effectively treated with the monoclonal antibody trastuzumab, a HER2 inhibitor[7-10]. Recently, a number of studies have suggested a response to trastuzumab therapy for other

cancers with HER2 amplification, including germ cell, endometrium and salivary duct carcinoma[11-13].

In gastric cancer, HER2 amplification has been found in 7% to 27% of tumors[14-19]. Reports of trastuzumab therapy in metastatic gastric cancer showed complete tumor regression and disappearance of the metastases in two cases[20,21]. A phase Ⅲ randomized study (Trastuzumab for HER2-positive metastatic gastric cancer) in patients with inoperable, metastasizing and/or recurring gastric cancer with HER2 overexpression or *HER2* gene amplification, documented that 47.3% of the patients who received trastuzumab, along with their chemotherapy, showed a significant regression of the primary tumor and/or the metastases. Moreover, trastuzumab caused a prolongation of the median survival time by 2.4 mo in all patients[22]. Based on these reports, gastric cancer patients with HER2 overexpression and/or amplification could be good candidates for trastuzumab therapy.

HER2 testing can be performed either by immunohistochemical evaluation of protein expression or by evaluating the gene copy number by *in situ* hybridization, most commonly using fluorescence *in situ* hybridization (FISH). However, while immunohistochemistry (IHC) is a relatively inexpensive, easy to perform method for most pathology laboratories, FISH is technically demanding, expensive and requires special equipment[23-25]. An alternative method, chromogenic *in situ* hybridization (CISH), is a combination of *in situ* hybridization with a detection system using a chromogen similar to IHC. Slides are visible under a light microscope and show correlation with morphology. A number of studies compared HER2 testing with IHC, FISH and CISH in breast carcinoma and have shown good correlation between CISH and FISH results[25-30].

We evaluated HER2 overexpression and gene amplification by IHC and CISH, respectively, in 120 cases of gastric carcinoma patients and 45 matched lymph node metastases mounted onto multiple-punch and single-punch tissue microarrays respectively. Our data suggests that, in gastric cancer, HER2 amplification is the main mechanism for HER2 protein overexpression and is preserved in lymph node metastases.

MATERIALS AND METHODS

Patients

The current study involved 120 non-consecutive patients with gastric carcinoma, surgically treated at the 3rd and 4th Departments of Surgery, University of Athens, between 2004 and 2007. Histomorphological data were reviewed from the corresponding hematoxylin and eosin stained slides. Clinical data were obtained from corresponding reports. Clinicopathological information included: gender, age, tumor diameter, histological subtype, tumor location, pT stage, pN stage, pM stage, vascular and lymphatic invasion, survival time, and information on post-operative therapy. Characteristics of patients are summarized in Table 1.

Table 1 Characteristics of patients with gastric cancer

Clinicopathological feature		Frequency *n* (%)
Patient age at diagnosis (yr)	Mean 69.6, min-max 27-96	
Tumor diameter (cm)	Mean 4.6, min-max 1.3-12	
Gender	Male	84 (70)
	Female	36 (30)
Histological type	Intestinal	80 (66.66)
	Diffuse	24 (20)
	Mixed	16 (13.33)
Tumor location	Cardia	37 (30.8)
	Corpus	39 (32.5)
	Antrum	44 (36.66)
pT stage	pT1	15 (12.5)
	pT2	65 (54.16)
	pT3-4	40 (33.33)
pN stage	pN0	36 (30)
	pN1	43 (35.8)
	pN2+3	41 (34.2)
pM stage	pM0	105 (87.5)
	pM1	15 (12.5)
Tumor grade	G1-2	42 (35)
	G3	78 (65)
Venous invasion	Present	37 (30.8)
	Absent	83 (69.2)
Lymphatic invasion	Present	84 (70)
	Absent	36 (30)
Adjuvant therapy	None	32 (26.6)
	Treated	88 (73.4)
	Chemotherapy	55 (62.5)
	Chemo/Radiotherapy	33 (37.5)
5-year survival (%)	(95% CI)	38.9 (25-52)

Specimen characteristics

Paraffin-embedded tissue blocks of primary tumors and matched positive lymph nodes were retrieved from the Department of Pathology, University of Athens. The use of this material was approved by the local Ethics committee. Two tissue microarrays (TMAs) were constructed. The first included punches from primary tumors. In order to exclude bias due to possible tumor heterogeneity, each patient had multiple tumor punches taken from formalin-fixed, paraffin-embedded blocks using a tissue cylinder with a diameter of 1 mm, which were subsequently transferred into one recipient paraffin block (3 cm × 2.5 cm) using a semiautomated tissue arrayer. Each patient had on average 5.1 tissue punches included on this array, including at least 4 tumor punches. The second TMA included single punches from matched metastatic lymph nodes in 45 patients.

Assay methods

IHC: Five μm TMA sections were dewaxed and rehydrated in distilled water. Endogenous peroxidase was blocked using 0.5% H_2O_2. To determine the HER2 expression immunohistochemically, the HercepTestTM (Dako, Glostrup, Denmark) was used according to the manufacturer`s protocol. Following pressure cooker-mediated antigen retrieval sections were incubated with the prediluted primary antibody. Control samples included normal gastric mucosa and breast cancer tissue. Immunostaining was scored by an experienced gastrointestinal pathologist following a 4-step

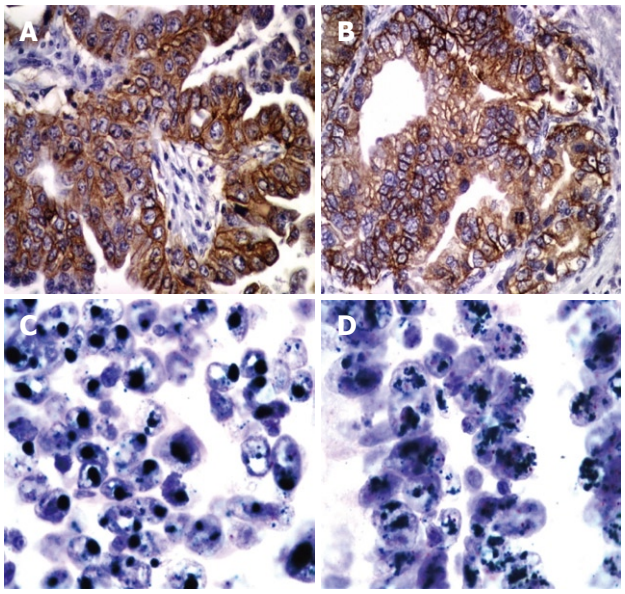

Figure 1 Examples of human epidermal growth factor receptor-2 immunohistochemical expression and amplification in primary and metastatic gastric cancer. Immunohistochemistry shows strong membranous staining of human epidermal growth factor receptor-2 (HER2) in intestinal type gastric cancer (A) and (B) (× 400). Chromogenic in situ hybridization assay shows amplification of HER2 in primary gastric cancer (C) and the corresponding lymph node metastasis (D). Clustered green signals represent the amplified *HER2* gene, while red signals represent centromere 17. Cell nuclei are counterstained with hematoxylin (× 1000).

score (0, 1+, 2+, 3+), according to the consensus panel recommendations on HER2 scoring for gastric cancer[31].

CISH: HER2 CISH was performed using a CISH HER2 probe and Immunodetection Kit (ZytoDot2C SPEC HER2/CEN 17 Probe Kit). TMA sections were deparaffinized and incubated for 5 min in 3% H_2O_2, followed by Heat Pretreatment Solution EDTA in a covered staining jar standing in a boiling water bath at 98 ℃ for 15 min. After washing in distilled water, Pepsin Solution (ES1) was applied and slides were incubated for 5 min at room temperature in a humidity chamber. Sections were then washed in distilled water, dehydrated in increasing ethanol, and air dried. ZytoDot2C SPEC HER2/CEN 17 Probe was applied and sections were covered with a coverslip sealed with a layer of hot glue. Samples were then denaturated at 80 ℃ for 5 min, transferred to a humidity chamber and left to hybridize overnight at 37 ℃. On day 2, immunodetection was performed according to the manufacturer's instructions and sections were counterstained with Hematoxylin and mounted.

Statistical analysis

χ^2 tests and contingency tables were used to analyze the relationship between IHC and CISH, and categorical parameters. Overall survival was estimated by the Kaplan-Meier method and evaluated by log-rank testing. All analysis were carried out using SAS (V9, The SAS Institute, NC, United States).

Table 2 Lauren phenotype, human epidermal growth factor receptor-2 immunohistochemistry and chromogenic *in situ* hybridization in gastric carcinoma

		Diffuse (n = 24)	Mixed (n = 16)	Intestinal (n = 80)
HER2 IHC	0	23	14	59
	1+	0	0	4
	2+	1	0	5
	3+	0	2	12
HER2 CISH	Non amplified	24	14	63
	Amplified	0	2	17

HER2: Human epidermal growth factor receptor-2; IHC: Immunohistochemistry; CISH: Chromogenic *in situ* hybridization.

RESULTS

HER2 immunohistochemistry

HER2 protein expression was observed in 24 of the 120 gastric carcinomas (20%). In more detail, one of the 24 diffuse type carcinomas (4.16%) and 23 of the 96 intestinal and mixed type carcinomas (23.95%) showed HER2 protein expression. Immunostaining was always membrane bound and showed basolateral predominance (Figure 1A and B). Immunostaining in mixed type carcinomas was restricted in the intestinal type component. Quantitative analysis of the immunostaining, according to the consensus panel recommendations on HER2 scoring for gastric cancer, resulted in fourteen 3+ cases (11.66%), six 2+ cases (5%) and four 1+ cases (3.33%)[31] (Table 2). IHC was interpretable in 652 of the 660 spots (98.8%). Reasons for non-interpretable results were missing tissue spots or absence of tumor tissue.

HER2 CISH

Tissue spots were scanned for possible intratumoral heterogeneity by using a 10× objective lens. CISH hybridization signals of the *HER2* gene appeared as dark green-colored dot-shaped signals. The chromosome 17 centromeric regions appeared as bright red-colored dot-shaped signals. Areas of necrosis and overlapping nuclei were avoided. Signal enumeration was performed using the 40× objective lens of a light microscope. HER2 amplification was observed in 19 of the 120 primary gastric carcinomas (15.8%). Amplified cases showed intratumoral heterogeneity with areas of low amplification where HER2 signals appeared as multiple dots or small clusters, and areas of high amplification with presence of large, green *HER2* gene signal clusters (Figure 1C and D). All amplified cases had 2+ or 3+ IHC results (Table 2). HER2 amplification showed significant association with histologic tumor type. Seventeen (21.25%) of the 80 intestinal and two (12.5%) of the 16 mixed type cancers, but 0 (0%) of the diffuse type cancers, were amplified ($P < 0.002$, Table 2). No association between *HER2* gene amplification and tumor grade, size, stage or localization was found.

Table 3 Comparison of human epidermal growth factor receptor-2 immunohistochemistry and chromogenic *in situ* hybridization in primary gastric carcinomas and matched lymph node metastases

	HER2 IHC			HER2 CISH		
	PT	LNM	Concordance	PT	LNM	Concordance
Positive	7	6	85.7%	6	6	100%
Negative	38	39	97.4%	39	39	100%

HER2: Human epidermal growth factor receptor-2; IHC: Immunohistochemistry; CISH: Chromogenic *in situ* hybridization; PT: Primary gastric cancer; LNM: Lymph node metastases.

HER2 alterations in lymph node metastases

Comparative analysis of primary tumors and corresponding lymph node metastases, performed in 45 cases, showed a high concordance in the presence of HER2 overexpression or amplification ($P < 0.0001$, Table 3).

Clinicopathological characteristics

Increasing tumor grade and stage were associated with reduced patient survival ($P < 0.0001$ each). No correlation was observed between patient survival and HER2 overexpression or amplification, even after including postoperative therapy of the patients and location of the tumors in the analysis (Figure 2).

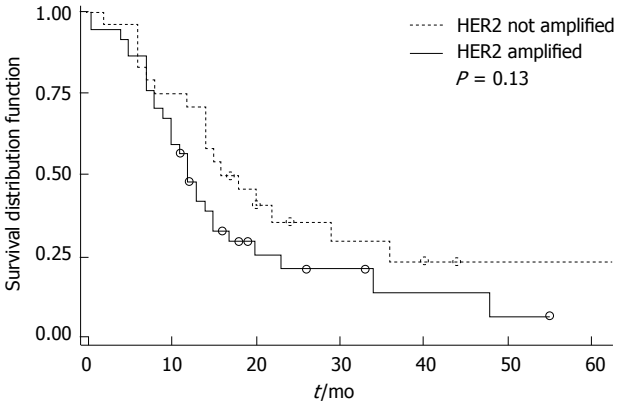

Figure 2 Kaplan-Meier curve for disease-specific survival and human epidermal growth factor receptor-2 amplification in gastric carcinomas. HER2: Human epidermal growth factor receptor-2.

DISCUSSION

The HER2 protein is a frequently analyzed gene product, especially in breast cancer. Recent studies have examined HER2 expression in other tumor types, including gastric cancer[18,32,33]. Immunohistochemical HER2 expression and protein positivity (≥ 2+) was observed in 20% and 14.58%, respectively, of the 120 gastric carcinomas in our study. These results are in keeping with previous reports demonstrating similar frequencies of HER2 overexpression in gastric cancer[18,32,33]. HER2 positivity was observed in 23% of the cases in the study by Yano *et al*[18] and in 22.6% of the cases in the study by Kim *et al*[33]. More recently, a TMA study of 166 gastric carcinomas by Marx *et al*[32] found a HER2 positivity rate of 17% and a strong correlation between IHC and *HER2* gene amplification detected by FISH. In our cases, comparable to others, HER2 overexpression and/or amplification were almost exclusively found in gastric cancers of the intestinal type. This finding supports the presence of different molecular characteristics between the main histologic tumor types that seem to develop through different molecular pathways.

No correlation between HER2 overexpression and/or amplification and tumor localization could be demonstrated in our study. This might be contradictory to previous studies where a high frequency of HER2 expression was reported in cardia carcinomas[34]. However, adenocarcinomas of the gastroesophageal junction, many of which are Barrett carcinomas, are known to have a high rate of HER2 amplification and cannot always be differentiated from cardia carcinomas. This may lead to an artificial increase in the rate of HER2 expression reported in cardia carcinomas.

A high concordance rate was found between HER2 positivity, as detected by IHC, and *HER2* gene amplification, as detected by CISH, since 19 of the 20 IHC positive cases (i.e., ≥ 2+) were amplified (95%). Many of them (73.6%) had high *HER2* gene amplification. However, one HER2 2+ case, detected in a diffuse carcinoma, was not found to be amplified, which may be attributed to a technical error of IHC associated with formalin fixation of the tissue. Alternatively, other mechanisms of HER2 protein overexpression can contribute to inconsistencies between IHC and ISH results.

In the present study we found a high concordance rate of HER2 status between primary tumors and their corresponding lymph node metastases. This finding is in keeping with previous published results, where HER2 amplification status was found to be almost identical in the primary gastric carcinomas and their corresponding lymph node metastases[32] and provides further evidence for the role of HER2 amplification in gastric cancer.

Our findings support gastric cancer HER2 amplification as being the main mechanism that leads to HER2 protein overexpression. Similar findings have previously been reported for esophageal cancer[35]. Moreover, Tapia *et al*[19], in a large-scale TMA study, examined more than 4000 samples from 120 different tumors and could not find any tumors with HER2 overexpression in the absence of gene amplification.

HER2 testing has developed over a number of years, and many retrospective studies using formalin-fixed, paraffin-embedded material and different methodologies have provided inconsistent results[36,37]. Correlation between technical methods can be used to obtain a high concordance rate and to better define the assays with the best ability to identify patient groups that would benefit from a targeted therapy. Although in breast cancer HER2 IHC is an acceptable method of predicting HER2 status, previous studies have shown marked variation in different diagnostic laboratories regarding the interpretation of positive staining[25]. On the other hand, FISH is

technically demanding, expensive and does not produce a permanent archival slide. In our study, CISH testing showed a good correlation to IHC, and therefore seems to represent a reliable methodology for HER2 testing. Moreover, the slides are visible under a light microscope and show good correlation with tumor morphology.

In this study, we used the tissue microarray technique using multiple tissue punches per case to account for possible heterogeneity in terms of protein expression or gene amplification in the primary tumor. Each patient had an average of 4 tumor punches taken. This is particularly important for HER2 testing in gastric cancer since considerable heterogeneity concerning gene amplification has been reported in many studies[38,39]. Such heterogeneity was also noted in our study, since in many amplified cases, areas with low and high amplification were found within the same tumor. Multiple sampling thus helped to minimize possible biases in evaluation, as suggested by Goethals *et al*[40], who recommended that at least four punches of primary tumor are required to account for possible heterogeneity. However, a single tissue punch was sampled in the case of lymph nodes since the issue of heterogeneity may be substantially less important. Our study also benefits from complete clinicopathological and follow-up characterization of patients. In contrast to previous studies[34,41] we could not demonstrate any association between HER2 positivity and clinical outcome.

Our study provides evidence supporting gastric cancer HER2 amplification as being the main mechanism for HER2 protein overexpression and that HER2 amplification is preserved in the lymph node metastases. Therefore, gastric cancer patients with HER2 overexpression and/or amplification seem to be good candidates for anti-HER2 therapy. Moreover, CISH is a reliable and inexpensive method that can be used for HER2 testing of gastric cancer.

COMMENTS

Background

Alterations of the human epidermal growth factor receptor-2 (*HER2*) gene are implicated in the development and progression of many tumors. Breast cancer patients with HER2 amplification have been effectively treated with the monoclonal antibody trastuzumab, a HER2 inhibitor. Recently, a number of studies have suggested a response to trastuzumab therapy for other cancers with HER2 amplification, including gastric cancer, where HER2 amplification has been found in 7% to 27% of the tumors. Reports of trastuzumab therapy in metastatic gastric cancer showed complete tumor regression and disappearance of the metastases in two cases. Based on these reports, gastric cancer patients with HER2 overexpression and/or amplification could be good candidates for trastuzumab therapy.

Research frontiers

The HER2 protein is a frequently analyzed gene product, especially in breast cancer. Recent studies have examined HER2 expression in other tumor types, including gastric cancer. The frequency and significance of HER-2/neu amplification in gastric carcinoma are investigated. In this study, immunohistochemical HER2 expression and protein positivity ($\geq$ 2+) was observed in 20% and 14.58%, respectively, of the 120 gastric carcinomas.

Innovations and breakthroughs

The authors evaluated HER2 overexpression and gene amplification by immunohistochemistry and chromogenic *in situ* hybridization (CISH) respectively, in 120 cases of gastric carcinoma patients and 45 matched lymph node metastases mounted onto multiple-punch and single-punch tissue microarrays respectively. The data suggest that, in gastric cancer, HER2 amplification is the main mechanism for HER2 protein overexpression and is preserved in the lymph node metastases.

Applications

This study provides evidence supporting gastric cancer HER2 amplification as being the main mechanism for HER2 protein overexpression and that HER2 amplification is preserved in the lymph node metastases. Therefore, gastric cancer patients with HER2 overexpression and/or amplification seem to be good candidates for anti-HER2 therapy. Moreover, CISH is a reliable and inexpensive method that can be used for HER2 testing of gastric cancer.

Peer review

The authors studied HER2/neu expression and gene amplification in a cohort of Greek patients with gastric cancer. The manuscript includes 2 aspects. Firstly, they describe the expression/amplification of HER2-neu in the study group. Secondly, they highlight the potential applicability of CISH as a routine method. Data are mostly well documented and conclusions drawn are appropriate.

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BRIEF ARTICLE

Protective effect of alcohol consumption for fatty liver but not metabolic syndrome

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Abstract

AIM: To investigate the effect of alcohol on the metabolic syndrome (MS) and fatty liver in Japanese men and women.

METHODS: A cross-sectional study was conducted in a medical health checkup program at a general hospital. This study involved 18 571 Japanese men and women, 18-88 years of age, with a mean body mass index of 22.6 kg/m^2. A standardized questionnaire was administered. The total amount of alcohol consumed per week was calculated, and categorized into four grades. Fatty liver was examined by ultrasound modified criteria of the revised National Cholesterol Education Program Adult Treatment Panel Ⅲ and the new International Diabetes Federation.

RESULTS: The prevalence of fatty liver decreased in men and women with light to moderate alcohol consumption, whereas the prevalence of MS was not so changed. The prevalence of fatty liver of any grade in men was lower than that in those with no or minimal alcohol consumption. In women with light to moderate alcohol consumption, prevalence of fatty liver was lower than that in women with no or minimal alcohol consumption. By logistic regression analysis, the odds ratio (OR) for MS in women with light alcohol consumption was decreased to < 1.0, but this change was not clear in men. The OR for fatty liver was clearly < 1.0 in men with any level of alcohol consumption and in women with light to moderate consumption.

CONCLUSION: Light to moderate alcohol consumption has a favorable effect for fatty liver, but not for MS in Japanese men and women.

Key words: Alcoholic hepatitis; Epidemiology; Fatty liver; Metabolic syndrome; Alcohol consumption

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INTRODUCTION

The metabolic syndrome (MS) is defined by abdominal

obesity, hypertension, elevated fasting blood glucose, and dyslipidemia[1]. Importantly, MS is a risk factor for the development of type 2 diabetes mellitus and coronary artery disease, and is associated with an increased risk of cerebrovascular disease and all-cause mortality[2]. The favorable effect of alcohol intake enhances insulin sensitivity, increases high-density lipoprotein-cholesterol (HDL-C), and contributes to a lower risk of type 2 diabetes mellitus[3-6] and cardiovascular disease[7-10]. Some reports have shown that the prevalence of MS is associated with alcohol consumption, irrespective of the amount consumed[11-13]. However, several studies have reported beneficial effects of alcohol consumption on MS[14-16]. Moreover, a study in Korean adults has indicated that light alcohol consumption is associated with a reduced prevalence of MS, whereas substantial alcohol intake leads to a dose-dependent increase in the risk of MS[17]. The effect of alcohol on MS in the general population has been inconsistent in the literature.

Fatty liver is closely associated with MS, and is considered the hepatic manifestation of MS[18]. Findings on the relation between alcohol consumption and fatty liver have also been inconsistent in the literature. Although alcohol consumption certainly may be a cause of fatty liver in some cases[19,20], it potentially plays a protective role against fatty deposition in the liver[21-25].

Therefore recent studies have implied the possibility that the effect of alcohol is different between fatty liver and MS, although fatty liver is closely associated with MS. However, the discrepancy of alcohol effect among the previous studies may be due to differences in the following cofactors: ethnicity, age, body mass index (BMI), drug usage, and lifestyle, such as alcohol consumption, smoking, and exercise. However, no large epidemiological study has investigated the effect of alcohol on fatty liver and MS at the same time.

We performed a cross-sectional study to investigate the effect of alcohol on fatty liver and MS at the same time. In this study, we separated the subjects according to the grade of alcohol consumption and compared the prevalence of fatty liver and MS in each grade. We focused on the discrepancy in the association between alcohol and MS and between alcohol and fatty liver. Additionally, we checked the impact of prevalence of MS without fatty liver, or fatty liver without MS.

MATERIALS AND METHODS

Study design

We performed a cross-sectional study of participants of a medical health checkup program, including abdominal ultrasonography. The study was approved by the ethics committee of Murakami Memorial Hospital, Gifu, Japan. The program was conducted in the Medical Health Checkup Center at Murakami Memorial Hospital. The purpose of the medical health checkup program was to promote public health through early detection of chronic diseases and the evaluation of their underlying risk factors. Known as a "human dock", medical services of this kind are very popular in Japan.

Study population

All of the subjects participating in such health checkup programs at Murakami Memorial Hospital between January 2004 and December 2009 were invited to join this study. Participants who tested positive for hepatitis B antigen or hepatitis C antibody and those who reported a history of known liver disease, including viral, genetic, autoimmune, and drug-induced liver disease, were excluded from the study[26]. We invited 22 119 participants in the health checkup program to enroll in the study. Of these, a total of 19 016 Japanese participants (11 295 men and 7721 women) were enrolled after giving informed consent. We excluded 123 participants (92 men and 31 women) with hepatitis C virus, 312 (214 men and 98 women) with hepatitis B virus, and nine (7 men and 2 women) who were diagnosed with other liver diseases. As a result, this study consisted ultimately of 18 571 participants (10 982 men and 7589 women). The mean age was 46.5 years (SD: 9.9; range: 18-88 years), and the mean BMI was 22.6 kg/m^2 (SD: 3.3; range: 14.0-58.3 kg/m^2).

Data collection

The health checkup programs that were used for the collection of data included the following tests: eye examinations, urinalysis, blood-cell counts, blood chemistry, electrocardiography, chest radiography, barium examination of the upper gastrointestinal tract, and abdominal ultrasonography. The medical history and lifestyle factors of all participants, including physical activity and habits pertaining to smoking and alcohol consumption, were surveyed by a standardized self-administered questionnaire. When the participants had difficulty completing the questionnaire, trained nurses provided assistance. We undertook blood and urine examinations with MODULAR ANALYTICS (Hitachi High-Technologies Corp. Ltd., Tokyo, Japan).

Standardized questionnaire for lifestyle factors

A standardized questionnaire was administered to all participants by the same trained team of interviewers. Habits regarding alcohol consumption were evaluated by asking the participants about the amount and type of alcoholic beverages consumed per week during the past month, then estimating the mean ethanol intake per week. The validity of information related to alcohol consumption was confirmed previously[27]. The total amount of alcohol consumed per week was calculated in grams, and then categorized into the following four grades: non or minimal alcohol consumption, < 40 g/wk; light alcohol consumption, 40-140 g/wk; moderate alcohol consumption, 140-280 g/wk; and excess alcohol consumption, > 280 g/wk[22,24]. Smoking status was also categorized into three groups (never smoker, ex-smoker, and current smoker). On the questionnaire, participants reported the type, duration and frequency of their partici-

pation in sports or recreational activities[28]. When participants performed any kind of sports at least once a week regularly, we categorized them as regular exercisers[29].

Definition of fatty liver

The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was done by trained technicians with Aloka SSD-650CL (Aloka Co., Ltd., Tokyo, Japan). All ultrasonographic images were stored as photocopies. Gastroenterologists reviewed the photocopies and made the diagnosis of fatty liver without reference to any of the participant's other individual data. Of four known criteria (hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring), the participants were required to have hepatorenal contrast and liver brightness to be given a diagnosis of fatty liver[30].

MS

There are several differing criteria for the MS worldwide[1,31-34]. In this study, we used the following two definitions: (1) the revised National Cholesterol Education Program Adult Treatment Panel Ⅲ (rATP Ⅲ) definition[34]; and (2) the new International Diabetes Federation (IDF) definition[32].

According to the rATP Ⅲ definition[1], subjects who had three or more of the following criteria were identified as having MS: (1) triglycerides ≥ 150 mg/100 mL; (2) HDL-C < 40 mg/100 mL for men, and < 50 mg/100 mL for women; (3) elevated blood pressure (systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg); (4) fasting glucose ≥ 100 mg/100 mL instead of ≥ 110 mg/100 mL; or (5) abdominal-obesity-modified waist circumference cutoffs (≥ 90 cm for men and ≥ 80 cm for women) were used instead of the waist circumference cutoffs (≥ 102 cm for men and ≥ 88 cm for women) proposed in the existing definition.

According to the new IDF definition, Japanese people were defined as having MS if the subjects had abdominal obesity (waist circumference cutoffs ≥ 90 cm for men and ≥ 80 cm for women) plus two or more of the following risk factors: (1) elevated triglyceride level ≥ 150 mg/100 mL or on treatment; (2) low HDL–C < 40 mg/100 mL for men and < 50 mg/100 mL for women or on treatment; (3) elevated blood pressure (systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg); and (4) high fasting glucose ≥ 100 mg/100 mL[32].

Sample size

Because preliminary studies indicated that the number in the excess alcohol consumption group was small (n = 22 and 24), we invited as many subjects as possible. Practically, we collected data for waist circumference from 2004. Then, we set the study period from 2004 to 2009.

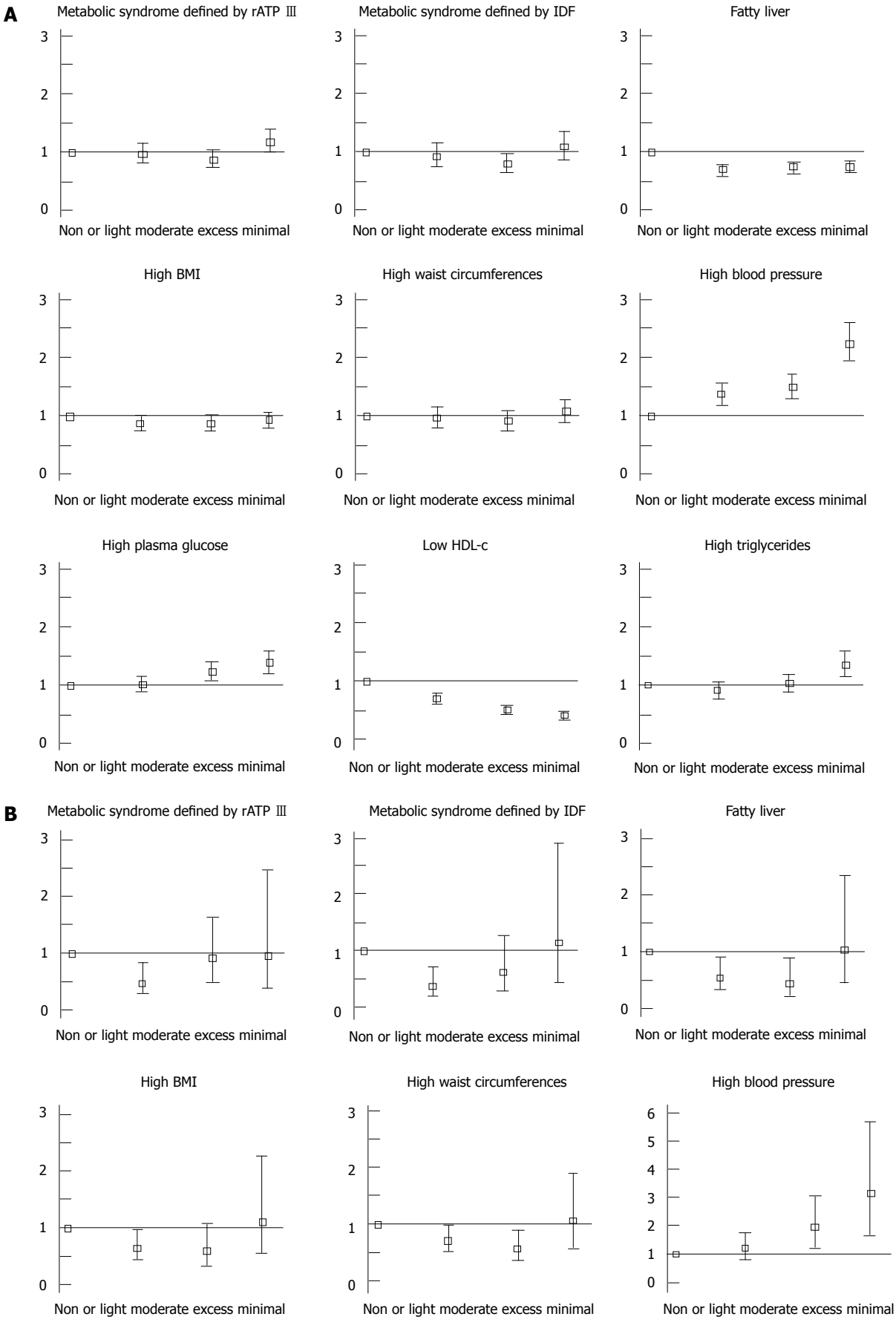
Statistical analysis

The R version 2.4.1 (available from http://www.r-project.org/) was used for statistical analyses. Data was expressed as mean (SD). Two groups of subjects were compared by χ^2 test. The significance of differences between non or minimal alcohol consumption and the others was determined by two-tailed, multiple χ^2 tests with Bonferroni correction ($P < 0.016$ for three comparisons in four groups). The linear association of alcohol consumption with several parameters associated with MS was evaluated by Spearman's rank correlation, and a P value < 0.05 was accepted as significant. We assessed the odds ratio (OR) of the alcohol consumption grade for MS and fatty liver using a multivariate logistic model while controlling for potential covariates. In a multivariate logistic model, we selected age, use of drugs that potentially affect MS, and lifestyle, such as alcohol consumption, regular exercise, and smoking as the potential covariates. The adjusted OR and 95% CIs were calculated.

RESULTS

We investigated the OR of alcohol consumption for MS defined by rATP Ⅲ and fatty liver using a logistic regression model (Figure 1). The OR for MS was decreased to < 1.0 in women with light alcohol consumption, but it was not clear in men. The OR for fatty liver was clearly < 1.0 in men with any level of alcohol consumption and in women with light to moderate consumption. In men and women, the ORs for high blood pressure and high fasting plasma glucose were increased as the level of alcohol consumption increased. Conversely, the OR for low HDL-C was decreased to < 1.0 in men and women. However, the OR for high triglycerides was increased to > 1.0 in men with excess alcohol consumption, and decreased to < 1.0 in women with light consumption. Moreover, the OR for high waist circumference was not significant, and was the same as the OR for high BMI. The actual adjusted ORs are shown in Table 1.

Table 2 indicates the basic characteristics of men and women in the four grades. And liner association between alcohol consumption and several factors associated with MetS were evaluated by Spearman's rank correlation (Table 3). BMI and waist circumferences were lowest in light consumption (Table 2), but Spearman's rank correlation coefficients were not significant (Table 3). Systolic blood pressure, diastolic blood pressure, and fasting plasma glucose were increased as the alcohol consumption increased (Tables 2 and 3). On the other hand, the associations of consumption with diastolic blood pressure, and that with fasting plasma glucose were not statistically significant in women, while systolic blood pressure was also increased as the consumption increased in women. In men and women, low-density lipoprotein (LDL) cholesterol, non HDL cholesterol, and LDL cholesterol/HDL cholesterol ratio were decreased and HDL cholesterol were increased as the consumption increased (Tables 2 and 3). Triglycerides were lower in light consumption and were higher in moderate and excess than those in non or minimal (Table 2). Over all, the trend of the associations between alcohol consumption and MetS


A
Metabolic syndrome defined by rATP Ⅲ
Metabolic syndrome defined by IDF
Fatty liver
High BMI
High waist circumferences
High blood pressure
High plasma glucose
Low HDL-c
High triglycerides
B
Metabolic syndrome defined by rATP Ⅲ
Metabolic syndrome defined by IDF
Fatty liver
High BMI
High waist circumferences
High blood pressure
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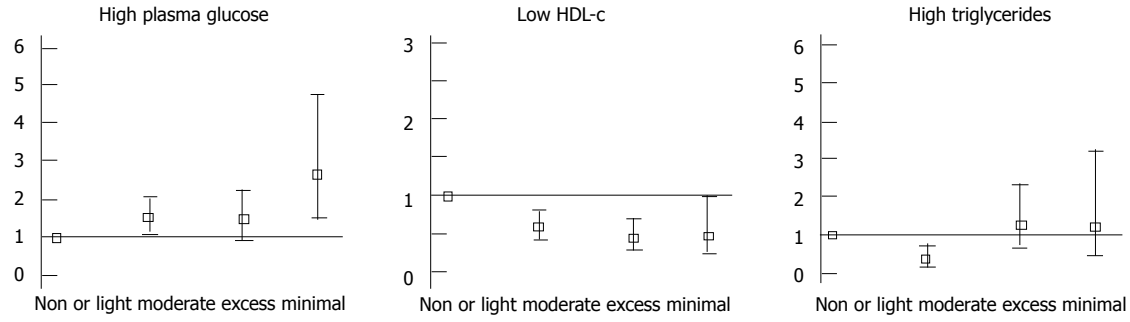

Figure 1 Adjusted odds ratio of the level of alcohol consumption for metabolic syndrome and fatty liver in men (A) and women (B). We assessed the odds ratio (OR) of the level of alcohol consumption for metabolic syndrome (MS) and fatty liver using a multivariate logistic model while controlling for potential covariates. In a multivariate logistic model, we selected age, usage of drugs that potentially affected MS, and lifestyle factors such as wine consumption, regular exercise, and smoking status, as the potential covariates. Bars mean the adjusted OR and 95% CIs. HDL-c: High-density lipoprotein-cholesterol; rATP Ⅲ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ; IDF: International Diabetes Federation; BMI: Body mass index.

Table 1 The adjusted odds ratio (95% CI) for the metabolic syndrome and fatty liver

	Men odds ratio (95% CI)	*P* value	Women odds ratio (95% CI)	*P* value
For MetS defined by rATP Ⅲ				
The grade of alcohol consumption				
Light	0.98 (0.83-1.15)	0.77	0.48 (0.27-0.82)	0.008
Moderate	0.88 (0.75-1.04)	0.14	0.9 (0.5-1.65)	0.74
Excess	1.18 (1.01-1.39)	0.043	0.96 (0.37-2.48)	0.93
Age	1.01 (1-1.02)	< 0.001	1.08 (1.05-1.1)	< 0.001
The usage of drugs	4.95 (4.3-5.69)	< 0.001	7.46 (4.96-11.24)	< 0.001
Wine consumers	1.13 (0.67-1.9)	0.66	0.52 (0.24-1.15)	0.11
Regular exercisers	0.6 (0.51-0.71)	< 0.001	0.6 (0.38-0.94)	0.027
Smoking states				
Ex smoker/non smoker	1.21 (1.03-1.41)	0.017	0.89 (0.48-1.65)	0.72
Current smoker/non smoker	1.13 (0.97-1.33)	0.12	1.07 (0.58-1.98)	0.83
For MetS defined by IDF				
The grade of alcohol consumption	0.93 (0.75-1.14)	0.46	0.36 (0.19-0.69)	< 0.001
Light	0.78 (0.63-0.97)	0.029	0.61 (0.3-1.25)	0.18
Moderate	1.08 (0.87-1.33)	0.48	1.14 (0.45-2.9)	0.78
Excess	1 (0.99-1.01)	0.66	1.08 (1.05-1.1)	< 0.001
Age	3.64 (3.05-4.34)	< 0.001	4.85 (3.09-7.63)	< 0.001
The usage of drugs	0.78 (0.37-1.65)	0.52	0.6 (0.26-1.35)	0.22
Wine consumers	0.64 (0.51-0.79)	< 0.001	0.55 (0.34-0.92)	0.021
Regular exercisers				
Smoking states	1.3 (1.06-1.59)	0.01	1.03 (0.54-1.97)	0.93
Ex smoker/non smoker	1.09 (0.89-1.35)	0.39	1.07 (0.54-2.1)	0.85
Current smoker/non smoker				
For fatty liver				
The grade of alcohol consumption				
Light	0.69 (0.6-0.79)	< 0.001	0.54 (0.34-0.88)	0.012
Moderate	0.72 (0.63-0.83)	< 0.001	0.43 (0.21-0.88)	0.021
Excess	0.74 (0.64-0.85)	< 0.001	1.02 (0.44-2.35)	0.97
Age	1 (0.99-1)	0.21	1.06 (1.04-1.08)	< 0.001
The usage of drugs	2.09 (1.83-2.38)	< 0.001	2.17 (1.4-3.38)	< 0.001
Wine consumers	0.85 (0.53-1.35)	0.48	0.59 (0.3-1.15)	0.12
Regular exercisers	0.67 (0.59-0.77)	< 0.001	0.76 (0.52-1.13)	0.17
Smoking states				
Ex smoker/non smoker	1.24 (1.09-1.41)	< 0.001	0.38 (0.18-0.79)	0.01
Current smoker/non smoker	0.92 (0.81-1.05)	0.21	1 (0.57-1.74)	1
For high BMI				
The grade of alcohol consumption				
Light	0.86 (0.75-0.99)	0.034	0.64 (0.43-0.97)	0.036
Moderate	0.85 (0.73-0.98)	0.026	0.59 (0.33-1.06)	0.077
Excess	0.9 (0.78-1.05)	0.18	1.1 (0.53-2.31)	0.8
Age	0.99 (0.98-0.99)	< 0.001	1.04 (1.02-1.06)	< 0.001
The usage of drugs	2.19 (1.91-2.51)	< 0.001	1.77 (1.15-2.74)	0.01
Wine consumers	0.79 (0.48-1.29)	0.34	0.68 (0.39-1.21)	0.19
Regular exercisers	0.93 (0.81-1.06)	0.29	0.69 (0.48-0.99)	0.047
Smoking states				
Ex smoker/non smoker	1.19 (1.04-1.36)	0.013	0.56 (0.32-0.97)	0.037

Current smoker/non smoker	0.99 (0.87-1.14)	0.92	0.95 (0.58-1.57)	0.85
For high waist circumferences				
The grade of alcohol consumption				
Light	0.96 (0.81-1.13)	0.61	0.71 (0.51-0.97)	0.03
Moderate	0.89 (0.75-1.06)	0.21	0.57 (0.36-0.9)	0.016
Excess	1.06 (0.89-1.27)	0.5	1.06 (0.58-1.93)	0.85
Age	1 (0.99-1.01)	0.85	1.07 (1.06-1.09)	< 0.001
The usage of drugs	2.35 (2.01-2.74)	< 0.001	1.64 (1.14-2.36)	0.007
Wine consumers	0.63 (0.32-1.23)	0.18	0.68 (0.43-1.07)	0.095
Regular exercisers	0.71 (0.6-0.84)	< 0.001	0.69 (0.52-0.92)	0.012
Smoking states				
Ex smoker/non smoker	1.26 (1.07-1.49)	0.005	0.93 (0.64-1.35)	0.69
Current smoker/non smoker	1.04 (0.88-1.23)	0.67	0.91 (0.6-1.37)	0.64
For high blood pressure				
The grade of alcohol consumption				
Light	1.33 (1.16-1.53)	< 0.001	1.22 (0.85-1.75)	0.27
Moderate	1.47 (1.27-1.7)	< 0.001	1.97 (1.26-3.07)	< 0.001
Excess	2.24 (1.93-2.59)	< 0.001	3.13 (1.71-5.72)	< 0.001
Age	1.04 (1.03-1.05)	< 0.001	1.07 (1.06-1.09)	< 0.001
The usage of drugs	6.16 (5.33-7.13)	< 0.001	16.09 (10.87-23.83)	< 0.001
Wine consumers	1.09 (0.68-1.73)	0.73	0.72 (0.42-1.23)	0.23
Regular exercisers	0.81 (0.71-0.93)	< 0.001	0.8 (0.57-1.12)	0.19
Smoking states				
Ex smoker/non smoker	1.01 (0.88-1.15)	0.93	0.78 (0.5-1.23)	0.28
Current smoker/non smoker	0.67 (0.59-0.77)	< 0.001	0.73 (0.45-1.18)	0.2
For high plasma glucose				
The grade of alcohol consumption				
Light	1 (0.88-1.13)	0.94	1.52 (1.11-2.08)	0.009
Moderate	1.23 (1.08-1.4)	< 0.001	1.46 (0.95-2.25)	0.085
Excess	1.38 (1.2-1.58)	< 0.001	2.66 (1.49-4.76)	< 0.001
Age	1.03 (1.03-1.04)	< 0.001	1.07 (1.06-1.09)	< 0.001
The usage of drugs	2.05 (1.79-2.33)	< 0.001	1.88 (1.29-2.72)	< 0.001
Wine consumers	1.78 (1.17-2.72)	0.007	0.56 (0.33-0.96)	0.034
Regular exercisers	0.76 (0.68-0.86)	< 0.001	0.92 (0.68-1.24)	0.58
Smoking states				
Ex smoker/non smoker	1.22 (1.08-1.38)	< 0.001	0.68 (0.44-1.05)	0.083
Current smoker/non smoker	0.94 (0.83-1.06)	0.31	0.67 (0.42-1.06)	0.087
For low HDL-c				
The grade of alcohol consumption				
Light	0.7 (0.61-0.8)	< 0.001	0.58 (0.42-0.79)	< 0.001
Moderate	0.49 (0.42-0.57)	< 0.001	0.43 (0.27-0.69)	< 0.001
Excess	0.41 (0.35-0.48)	< 0.001	0.48 (0.24-0.96)	0.039
Age	1.01 (1-1.01)	0.082	1.01 (1-1.02)	0.055
The usage of drugs	3.4 (2.96-3.9)	< 0.001	4.24 (2.97-6.03)	< 0.001
Wine consumers	0.79 (0.47-1.32)	0.37	0.9 (0.6-1.35)	0.61
Regular exercisers	0.71 (0.62-0.82)	< 0.001	0.68 (0.52-0.91)	0.009
Smoking states				
Ex smoker/non smoker	1.06 (0.92-1.22)	0.45	0.64 (0.43-0.95)	0.027
Current smoker/non smoker	1.65 (1.44-1.9)	< 0.001	1.47 (1.03-2.11)	0.036
For high triglycerides				
The grade of alcohol consumption				
Light	0.89 (0.76-1.03)	0.11	0.37 (0.19-0.74)	0.005
Moderate	1.01 (0.87-1.18)	0.88	1.27 (0.68-2.35)	0.45
Excess	1.34 (1.16-1.56)	< 0.001	1.2 (0.45-3.2)	0.72
Age	1 (0.99-1)	0.19	1.06 (1.04-1.09)	< 0.001
The usage of drugs	3.06 (2.67-3.51)	< 0.001	11.13 (7.07-17.53)	< 0.001
Wine consumers	0.83 (0.49-1.4)	0.48	0.92 (0.43-1.99)	0.83
Regular exercisers	0.68 (0.59-0.79)	< 0.001	0.73 (0.44-1.21)	0.22
Smoking states				
Ex smoker/Non smoker	1.26 (1.09-1.46)	< 0.001	0.99 (0.49-2.01)	0.98
Current smoker/Non smoker	1.44 (1.24-1.66)	< 0.001	1.91 (1.02-3.55)	0.042

BMI: Body mass index; HDL-c: High density lipoprotein cholesterol; rATP Ⅲ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ; IDF: International Diabetes Federation.

were not so changed between men and women.

This observed inverse association between alcohol consumption and fatty liver might be due to changed habits of alcohol use after previous detection of fatty liver. We analyzed the study population according to previous data. Among 10 981 men, 6547 were new participants and 4434 were repeat participants. Among 7573 women, 5138 were new participants and 2435 were

Table 2 The correlation between components for the metabolic syndrome, liver associated enzymes and alcohol consumptions in participants free from drugs

	Men				Women			
	Non or minimal	Light	Moderate	Excess	Non or minimal	Light	Moderate	Excess
No. of subjects	6154	1734	1616	1478	6893	406	207	84
Age, yr	46.3 (10)	47.5 (9.4)	49.3 (9.4)	49.4 (8.9)	45.1 (9.9)	45.2 (8.8)	45.5 (8.5)	45 (9.3)
Aspartate aminotransferase, IU/L	20.2 (8.7)	19.6 (7.1)	20.8 (10.2)	23.2 (11.6)	17.2 (9)	17.1 (4.9)	17.5 (5.8)	19 (6.2)
Alanine aminotransferase, IU/L	26.1 (16.9)	23.7 (13.3)	23.8 (14.1)	25.5 (14.7)	15.9 (13.1)	15.2 (6.7)	15.6 (6.7)	16 (7)
Gamma-glutamyltransferase, IU/L	24.3 (21.9)	28.9 (24.6)	36 (34.5)	48.8 (51)	13.8 (9.5)	15.8 (12.1)	17.1 (10.6)	22.6 (21.8)
BMI, kg/m^2	23.5 (3.3)	23.2 (2.9)	23.3 (2.7)	23.4 (2.9)	21.4 (3.2)	20.9 (2.7)	20.9 (3.1)	21.3 (3.2)
Waist circumference, cm	81.8 (8.6)	81.5 (7.7)	82.2 (7.5)	82.9 (7.7)	72.3 (8.8)	71.5 (7.9)	71.5 (8.6)	73.9 (9.4)
Systolic blood pressure, mmHg	120.1 (15.6)	121.7 (15.8)	123.9 (15.8)	127.4 (16.1)	111.2 (16.2)	111.2 (15.6)	114.3 (19.4)	116.7 (17)
Diastolic blood pressure, mmHg	75.9 (10.3)	77.2 (10.2)	79 (10.4)	81.3 (10.1)	69.1 (10.3)	69.6 (10.6)	72.2 (11.9)	72.9 (10.9)
Fasting plasma glucose, , mg/dL	100.5 (19.3)	100 (16.4)	102.4 (19.9)	103.5 (19.1)	91.3 (12.7)	91.8 (10.1)	91.5 (9.8)	96.1 (18.7)
HDL-c, mg/dL	46 (11.4)	49.6 (12.5)	52 (13.4)	53.3 (14.2)	59.9 (13.3)	65.7 (14.1)	69 (14.9)	68.6 (14)
LDL-c, mg/dL	128 (30)	122.8 (29.6)	121.5 (29.5)	116.6 (31.6)	118.1 (31.1)	108.4 (29.6)	105.6 (27.7)	102 (26.5)
nonHDL-c, mg/dL	155.4 (33.9)	150.1 (33.4)	150.6 (33)	149.2 (34.7)	139.3 (34.8)	129 (32.2)	127.3 (31.8)	124.4 (28.1)
LDL-c/HDL-c ratio	3 (1)	2.6 (0.9)	2.5 (0.9)	2.4 (0.9)	2.1 (0.8)	1.7 (0.7)	1.6 (0.6)	1.6 (0.6)
Triglycerides, mg/dL	109.5 (83)	108.6 (100.2)	117.7 (107.7)	135.5 (116.7)	64.6 (44.5)	60 (31.9)	69 (53.4)	67.2 (42.2)

Among 18 571 participants (10 982 men and 7589 women), we separate men and women into the following four grades: non or minimal alcohol consumption, < 40 g/wk; light alcohol consumption, 40-140 g/wk; moderate alcohol consumption, 140-280 g/wk; and excess alcohol consumption. Data was expressed as mean (SD). BMI: Body mass index; HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol.

Table 3 The linear association between the amount of alcohol consumption and several parameters associated with the metabolic syndrome

	Men		Women	
	ρ	*P* value	ρ	*P* value
Age, yr	0.11	< 0.001	-0.09	< 0.001
Aspartate aminotransferase, IU/L	0.09	< 0.001	0	0.77
Alanine aminotransferase, IU/L	-0.02	0.08	-0.02	0.05
Gamma-glutamyltransferase, IU/L	0.34	< 0.001	0.08	< 0.001
BMI, kg/m^2	-0.01	0.16	-0.02	0.1
Waist circumference, cm	0.04	< 0.001	-0.01	0.32
Systolic blood pressure, mmHg	0.14	< 0.001	-0.04	< 0.001
Diastolic blood pressure, mmHg	0.17	< 0.001	-0.02	0.18
Fasting plasma glucose, mg/dL	0.08	< 0.001	-0.01	0.28
HDL-c, mg/dL	0.23	< 0.001	0.13	< 0.001
LDL-c, mg/dL	-0.13	< 0.001	-0.13	< 0.001
nonHDL-c, mg/dL	-0.08	< 0.001	-0.13	< 0.001
LDL-c/HDL-c ratio	-0.25	< 0.001	-0.17	< 0.001
Triglycerides, mg/dL	0.06	< 0.001	-0.06	< 0.001

The liner association between alcohol consumption and several factors associated with the metabolic syndrome were evaluated by Spearman' s rank correlation in 10 982 men and 7589 women, respectively, and a *P* value of < 0.05 was accepted as a significant level. BMI: Body mass index; HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol.

Table 4 Alcohol consumptions of new and repeat participants

	Men			Women		
	n	Alcohol consumption (g/wk)	*P* value	*n*	Alcohol consumption (g/wk)	*P* value
New participants without fatty liver	4417	113.02 (161.03)	< 0.001[2]	4662	21.37 (67.57)	0.092[2]
New participants with fatty liver	2130	96.14 (157.95)	0.7[1]	476	13.99 (68.47)	0.841[1]
Repeat participants without fatty liver	2986	125.79 (157.24)	< 0.001[3]	2154	21.48 (58.33)	0.853[3]
Repeat participants with fatty liver	1448	102.02 (155.3)		281	18.09 (75.15)	

[1]New participants with fatty liver *vs* repeat participants with fatty liver; [2]New participants with fatty liver *vs* new participants without fatty liver; [3]Repeat participants with fatty liver *vs* repeat participants without fatty liver.

repeat participants. The alcohol consumption of repeat participants with fatty liver was the same as that of new participants with fatty liver (Table 4). Next, we analyzed repeat participants and separated them into four groups according to previous and present fatty liver. We assessed the changed habit of alcohol consumption. At first, the change in alcohol consumption was small in each group (Table 5). Moreover, the level of change was no different among the four groups of men and women.

We calculated the number of subjects with both MS and fatty liver, those who had only MS, and those who had only fatty liver (Table 6). Unexpectedly, more than half of the participants with fatty liver were not diagnosed with MS, even if the definition was changed. 42.5% or 25.5% of men with fatty liver were diagnosed with MS defined by rATP Ⅲ or IDF, respectively. Conversely, 66.0% or 78.8% of men with MS defined by rATP Ⅲ or IDF were diagnosed with fatty liver. The result was similar in women: 43.7% or 38.6% of women with fatty liver were diagnosed with MS defined by rATP Ⅲ or IDF, respectively. Conversely, 49.1% or 55.9% of women with MS defined by rATP Ⅲ or IDF were diagnosed with fatty liver. The prevalence of fatty liver among men with MS decreased along with level of alcohol consumption and prevalence of fatty liver among women with MS decreased in those with light or moderate consumption (Table 6).

DISCUSSION

As far as we know, the present study is the first to investigate the association of alcohol with ultrasonography-

Table 5 Changed alcohol consumption habits among four groups

Previous fatty liver	Present fatty liver	Men		Women	
		n	Change of alcohol consumption (g/wk)	*n*	Change of alcohol consumption (g/wk)
Negative	Negative	2784	–1.69 (109.95)	2112	–5.38 (48.13)
Negative	Positive	269	4.07 (116.14)	75	–20.69 (142.71)
Positive	Negative	202	6.07 (104.09)	42	–20.9 (56.96)
Positive	Positive	1179	–4.45 (100.32)	206	–5.56 (31.87)

Change in alcohol consumption (g/wk) was calculated by present alcohol consumption minus previous alcohol consumption, and expressed as mean (SD). We performed a Tukey test to investigate the statistical significance of the difference between two groups. No significant difference was identified.

Table 6 Positive prevalence of parameters for four levels of alcohol consumption *n* (%)

	Non or minimal	Light	Moderate	Excess	Non or minimal *vs* light	Light *vs* moderate	Moderate *vs* excess
Men							
Fatty liver	2248 (36.5)	457 (26.4)	449 (27.8)	424 (28.7)	< 0.001	< 0.001	< 0.001
MS defined by rATP Ⅲ	1282 (20.8)	331 (19.1)	317 (19.6)	373 (25.2)	0.12	0.33	< 0.001
MS defined by IDF	668 (10.9)	165 (9.5)	143 (8.8)	182 (12.3)	0.12	0.04	0.15
Fatty liver among men with MS defined by rATP Ⅲ	923 (72)	204 (61.6)	188 (59.3)	206 (55.2)	< 0.001	< 0.001	< 0.001
Fatty liver among men with MS defined by IDF	568 (85)	118 (71.5)	100 (69.9)	127 (69.8)	< 0.001	< 0.001	< 0.001
MS defined by rATP Ⅲ among men with fatty liver	923 (41.1)	204 (44.6)	188 (41.9)	206 (48.6)	0.17	0.79	0.005
MS defined by IDF among men with fatty liver	568 (25.3)	118 (25.8)	100 (22.3)	127 (30)	0.85	0.22	0.058
Components of MS							
High waist circumference	968 (15.7)	257 (14.8)	236 (14.6)	257 (17.4)	0.38	0.32	0.15
High blood pressure	1766 (28.7)	591 (34.1)	626 (38.7)	707 (47.8)	< 0.001	< 0.001	< 0.001
High fasting plasma glucose	2332 (37.9)	681 (39.3)	749 (46.3)	729 (49.3)	0.31	< 0.001	< 0.001
Low HDL-C	2133 (34.7)	425 (24.5)	333 (20.6)	284 (19.2)	< 0.001	< 0.001	< 0.001
High triglycerides	1385 (22.5)	360 (20.8)	394 (24.4)	459 (31.1)	0.13	0.14	< 0.001
High BMI (> 25 kg/m^2)	1731 (28.1)	417 (24)	388 (24)	379 (25.6)	< 0.001	< 0.001	0.07
Smoking status							
Current smoker	2012 (32.7)	602 (34.7)	657 (40.7)	740 (50.1)	0.12	< 0.001	< 0.001
Never smoker	2220 (36.1)	446 (25.7)	308 (19.1)	180 (12.2)	< 0.001	< 0.001	< 0.001
Ex smoker	3933 (63.9)	1288 (74.3)	1308 (80.9)	1298 (87.8)	< 0.001	< 0.001	< 0.001
Usage of drugs	938 (15.2)	249 (14.4)	307 (19)	326 (22.1)	0.38	0.00	0.00
Regular exerciser	1096 (17.9)	361 (20.9)	326 (20.3)	272 (18.5)	0.01	0.04	0.64
Wine consumer	45 (0.7)	18 (1)	17 (1.1)	14 (0.9)	0.26	0.30	0.52
Women							
Fatty liver	719 (10.5)	22 (5.4)	9 (4.3)	7 (8.3)	< 0.001	0.01	0.65
MS defined by rATP Ⅲ	632 (9.2)	19 (4.7)	17 (8.2)	6 (7.1)	< 0.001	0.73	0.65
MS defined by IDF	494 (7.2)	12 (3)	10 (4.8)	6 (7.1)	< 0.001	0.25	0.84
Fatty liver among women with MS defined by rATP Ⅲ	322 (50.9)	4 (21.1)	3 (17.6)	2 (33.3)	0.020	0.013	0.65
Fatty liver among women with MS defined by IDF	284 (57.5)	3 (25)	3 (30)	2 (33.3)	0.051	0.16	0.44
MS defined by rATP Ⅲ among women with fatty liver	322 (44.8)	4 (18.2)	3 (33.3)	2 (28.6)	0.024	0.73	0.63
MS defined by IDF among women with fatty liver	284 (39.5)	3 (13.6)	3 (33.3)	2 (28.6)	0.026	0.97	0.84
Components of MS							
High waist circumference	1257 (18.2)	61 (15)	26 (12.6)	16 (19)	0.12	0.05	0.96
High blood pressure	1026 (14.9)	65 (16)	45 (21.7)	22 (26.2)	0.58	0.01	0.01
High fasting plasma glucose	906 (13.1)	70 (17.2)	33 (15.9)	19 (22.6)	0.02	0.29	0.02
Low HDL-C	1772 (25.7)	58 (14.3)	25 (12.1)	10 (11.9)	< 0.001	< 0.001	0.01
High triglycerides	507 (7.4)	12 (3)	18 (8.7)	6 (7.1)	0.00	0.56	0.89
High BMI (> 25 kg/m^2)	792 (11.5)	30 (7.4)	14 (6.8)	9 (10.7)	0.01	0.05	0.96
Smoking status							
Current smoker	372 (5.4)	50 (12.3)	57 (27.5)	25 (29.8)	< 0.001	< 0.001	< 0.001
Never smoker	6086 (88.3)	291 (71.7)	106 (51.2)	37 (44)	< 0.001	< 0.001	< 0.001
Ex smoker	804 (11.7)	115 (28.3)	101 (48.8)	47 (56)	< 0.001	< 0.001	< 0.001
Usage of drugs	575 (8.3)	36 (8.9)	22 (10.6)	6 (7.2)	0.78	0.30	0.87
Regular exerciser	1179 (17.2)	82 (20.3)	34 (16.7)	19 (22.6)	0.13	0.91	0.25
Wine consumer	140 (2)	30 (7.4)	17 (8.2)	11 (13.1)	< 0.001	< 0.001	< 0.001

This table indicates the positive prevalence of parameters for four levels of alcohol consumption: non or minimal, < 40 g/wk; light, 40–140 g/wk; moderate, 140–280 g/wk; and excess, > 280 g/wk. Two groups of subjects were compared by using the unpaired *t* test and χ^2 test. The significance of differences between two side-by-side groups among the four groups was determined by two-tailed, multiple χ^2 tests with Bonferroni correction ($P < 0.016$ for three comparisons in four groups). Smoking status was also categorized into three groups; never smoker, ex smoker and current smoker. Regular exercisers were defined as participants who performed any kind of sports at least once a week. Usage of drugs was defined as participants who receive any drugs that potentially affected metabolic syndrome (MS). HDL-c: High-density lipoprotein-cholesterol; rATP Ⅲ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ; IDF: International Diabetes Federation; BMI: Body mass index.

proven fatty liver and MS at the same time in a general population. Our study clearly indicated that the effect of alcohol was different between fatty liver and MS, although they are correlated closely. The effect of alcohol consumption on MS was not consistent, because it differed among components of MS. Alcohol consumption was associated with lower risk for HDL-C, but was associated with higher risk for high blood pressure and high fasting plasma glucose. Waist circumferences were not affected by level of alcohol consumption. Moreover, these results were similar when we used the Japan Society for The Study of Obesity definition for MS[33]. Our study clearly indicated that light to moderate alcohol consumption was associated with lower risk of fatty liver. Moreover, any level of alcohol consumption could have a protective effect on fatty liver in men. Our study was cross-sectional, therefore, the findings might be due to changed alcohol consumption after previous detection of fatty liver. However, our sub-analysis indicated that changes in alcohol consumption were small and were not due to previous detection of fatty liver.

Previous studies have indicated that the presence of fatty liver is a strong predictor of MS[36], and fatty liver correlates with all the components of MS[37]. Among populations with no or light alcohol consumption, liver fat content in participants with MS is significantly increased up to fourfold higher than those without MS[37], and the incidence of fatty liver has been shown to be increased fourfold in men and 11-fold in women with MS[27]. Although fatty liver is considered to be a hepatic manifestation of MS, more than half of Japanese men and women with fatty liver were not diagnosed with MS.

Interpretations

Alcohol consumption is a lifestyle factor, and its effects on health range from beneficial to detrimental. The dose-response relationship between alcohol and all-cause mortality follows a J- or U-shaped curve, which points to lower all-cause mortality among those with light to moderate alcohol consumption compared to excess consumption[38]. This effect is thought to be due mainly to a reduction in cardiovascular disease[7]. This reduction in cardiovascular disease has been attributed to the beneficial impact of alcohol on plasma lipid levels, hemostatic factors[8], and insulin sensitivity[3,6]. Some studies have suggested that as much as half of the cardiovascular benefit attributable to alcohol consumption may be because it increases HDL-C level[7-10]. We found that HDL-C was increased as the quantity of alcohol consumption increased, which was consistent with previous reports[7-10]. On the other hand, alcohol consumption contributes to elevated blood pressure[39,40]. Then, we also found that blood pressure was increased as the quantity of alcohol consumption increased.

In fact, studies about the association between alcohol consumption and obesity have not been consistent. Waist-to-hip ratio increases as the quantity of alcohol consumption increases[41,42], and waist circumference increases with excess alcohol consumption (> 40 g/d)[43]. Alcohol consumption of 30 g/d or more significantly increases BMI and the risk of weight gain[44]. In contrast, another study has reported that light-to-moderate alcohol consumption reduces waist circumference[16]. Moreover, some studies have found no significant association between alcohol consumption and obesity[45,46]. We also found no significant association between alcohol consumption and high waist circumference and BMI (BMI > 25 kg/m^2).

Alcohol consumption may increase triglyceride concentrations[8]. Triglycerides have been reported to be higher in individuals with excess alcohol consumption, but lower in those with light to moderate consumption[8]. Similar results were found in our study, which indicated that the OR for high triglyceride levels was increased to > 1.0 in men with excess alcohol consumption, and was decreased to < 1.0 in women with light consumption. The favorable effect of alcohol consumption contributing to enhance insulin sensitivity has been reported[3-6]. However, another study has provided different evidence that insulin resistance is related to alcohol consumption in a U-shaped manner[47]. In our study, the OR for high fasting plasma glucose was increased as the level of alcohol consumption increased.

Overall, the present study proved that the effect of alcohol consumption for MS was not significant in the Japanese general population. In fact, studies regarding the association between alcohol consumption and MS have not been consistent[11-17], because the relationship heavily depends on the individual components of MS. Moreover, previous studies have claimed that the type of alcohol is important for the association of alcohol consumption and MS[23,48,49]. Modest wine consumption has been reported to be associated with reduced all-cause mortality[48,49] and fatty liver[23]. In our study, however, the association between wine consumption and MS and fatty liver was not significant. Similarly, Djousse *et al*[15] have reported that the association between alcohol consumption and MS is unrelated to the type of alcoholic beverage.

Findings on the relation between alcohol consumption and fatty liver have also been inconsistent[19-25]. Fat deposition in the liver has been shown to be primarily due to an increased influx of fatty acids to the liver; most likely as a result of the increased lipolysis associated with obesity and insulin resistance, and as a result of increased hepatic *de novo* lipogenesis[50]. Reduced fatty acid oxidation and mitochondrial dysfunction and decreased export of fat further contribute to the accumulation of liver fat[51,52]. Alcohol-dehydrogenase-mediated ethanol metabolism generates a reduced form of nicotinamide adenine dinucleotide, which promotes steatosis by stimulating the synthesis of fatty acids and opposing their oxidation[53]. The hepatic lipogenic pathway is activated after the consumption of 24 g/d ethanol[53]. Sterol regulatory element binding protein 1c (SREBP1c)[54] and peroxisome proliferator activated receptor (PPAR)α[55], are altered with alcohol consumption. The involvement

of AMP-activated protein kinase activity in the action of ethanol on the liver has been demonstrated in experimental models of ethanol-induced steatosis[56].

SREBP1c is upregulated, which potentially results in increased conversion of glucose to fatty acids and triglycerides in experimental models of obesity[52]. PPARα is a nuclear receptor that is important in fatty acid uptake and oxidation, and has been shown to be underexpressed in experimental models of non-alcoholic steatosis[57]. In addition, the administration of adiponectin reverses non-alcoholic steatosis in experimental models[57,58].

Thus, several pivotal factors in the pathogenesis of fatty liver may be common in both alcoholic and non-alcoholic subjects. Therefore, the possible mechanism by which alcohol has a protective effect against fatty deposition in the liver remains unclear. Our study also provides clear evidence that light to moderate alcohol consumption has a favorable effect on fatty liver.

Some limitations of our study should be noted. First, although ultrasonography has been validated for detecting fatty liver, it may give an incorrect diagnosis compared to liver biopsy[30]. Second, self-reported information regarding alcohol intake is frequently subject to underreporting, and misreporting could be a source of bias. However, the self-reported information regarding alcohol intake in our study was validated previously[27]. Third, the generalizability of our study to non-Japanese populations is uncertain.

In conclusion, the effect of alcohol consumption was different between MS and fatty liver. The relationship between alcohol consumption and MS depends on the individual components of MS given the inconsistency of the association between alcohol consumption and MS. Light to moderate alcohol consumption has a favorable effect on fatty liver in Japanese men and women. Moreover, any level of alcohol consumption may have a protective effect against fatty liver in men. Unexpectedly, more than half of Japanese men and women with fatty liver were not diagnosed with MS, although fatty liver is considered to be a hepatic manifestation of MS. However, our previous studies have implied that the risk of fatty liver for cardiovascular disease is independent of MS[59]. Thus, fatty liver without MS is an important disease in the general population.

A future longitudinal study is needed to clarify that alcohol consumption has true hepatoprotective effects. Furthermore, the protective mechanism of alcohol against fatty deposition in the liver remains unclear. Thus, basic research is also needed to clarify the mechanisms that underlie modest alcohol consumption and fatty liver.

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COMMENTS

Background

The effects of alcohol on the metabolic syndrome (MS) have been inconsistent in previous studies. Fatty liver is closely associated with MS, and is considered to be the hepatic manifestation of MS. Findings on the relation between alcohol consumption and fatty liver have also been inconsistent.

Research frontiers

The favorable effect of alcohol intake enhances insulin sensitivity, and increases high-density lipoprotein-cholesterol, which contributes to a lower risk of type 2 diabetes mellitus, and cardiovascular disease. Moreover, alcohol consumption plays a protective role against fatty deposition in the liver, although alcohol consumption certainly could be a cause of fatty liver in some cases.

Innovations and breakthroughs

The authors investigated the association of alcohol consumption with ultrasonography-proven fatty liver and the MS at the same time in a general population, and clearly indicated that light to moderate alcohol consumption has favorable and unfavorable effects for components of MS, but has a protective effect for fatty liver.

Applications

Light to moderate alcohol consumption has a favorable effect on fatty liver in Japanese men and women. Moreover, any level of alcohol consumption may have a protective effect in men. A future longitudinal study is needed to clarify that alcohol consumption has true hepatoprotective effects. Furthermore, the mechanism of alcohol protection against fatty deposition in the liver remains unclear. Thus, basic research is also needed to clarify the mechanisms that underlie the effect of modest alcohol consumption on fatty liver.

Peer review

The strengths of the study include its large sample size and validated questionnaire.

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BRIEF ARTICLE

^{18}F-fluoro-2-deoxyglucose uptake on PET CT and glucose transporter 1 expression in colorectal adenocarcinoma

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Abstract

AIM: To evaluate the correlation between the level of ^{18}F-fluoro-2-deoxyglucose (^{18}F-FDG) uptake and glucose transporter 1 (GLUT1) expression in colorectal adenocarcinoma (CRA).

METHODS: Forty four patients with resected CRA and preoperative ^{18}F-FDG positron emission tomography - computed tomography data were investigated in this study. Comparison of maximum standardized uptake value (SUVmax) of the lesion was made with GLUT1 expression by immunohistochemistry and various clinicopathologic factors including tumor volume, invasion depth, gross finding, and lymph node metastasis.

RESULTS: SUVmax was 14.45 ± 7.0 in negative GLUT1 expression cases, 15.51 ± 5.7 in weak GLUT1 expression cases, and 16.52 ± 6.8 in strong GLUT1 expression cases, and there was no correlation between between GLUT1 expression and SUVmax. SUVmax was significantly correlated with tumor volume ($P < 0.001$). However, there was no significant differences in SUVmax and GLUT1 expression among other clinicopathologic factors.

CONCLUSION: GLUT1 expression does not correlates significantly with ^{18}F-FDG uptake in CRA. ^{18}F-FDG uptake was increased with tumor volume, which is statistically significant.

Key words: ^{18}F-fluoro-2-deoxyglucose; Glucose transporter 1; Colorectal cancer

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INTRODUCTION

Cancer cell growth is an energy-related process supported by increased glucose metabolism[1]. This uptake is mediated by glucose transporter (GLUT) proteins, which are membrane proteins responsible for the transport of glucose across cellular membranes. A family of seven glucose transporters have been cloned[2]. Among these, GLUT1 is the best-known basic, high-affinity glucose transporter, which is restricted to erythrocytes and blood-tissue barriers such as the blood-brain and blood-nerve barriers, in most normal tissues[3,4]. It has long been recognized that cancer cells have increased rates of glucose

metabolism compared with normal cells[5]. Increased GLUT1 expression has been described in many cancers, including breast, lung, kidney, urinary bladder, stomach, colorectum, endometrium, thyroid, head and neck, liver, ovary, salivary gland, and prostate cancer[6] due to a high metabolic rate and fast growth environment, but, generally absent in benign epithelial tissues. The expression of GLUT1 thus would appear to be a potential marker for malignant transformation, and the degree of tumor GLUT1 expression might correlate with biologic behavior in individual patients[7].

Positron emission tomography (PET) using ^{18}F-fluoro-2-deoxyglucose (FDG) is a rapidly developing functional-imaging modality that has shown great promise in the fields of primary, recurrent and metastatic tumor detection, planning and monitoring therapy[8-12]. The cellular mechanism underlying the increased ^{18}F-FDG accumulation in malignant tumors is associated with a higher rate of phosphorylation and diminished rate of dephosphorylation of intracellular phosphorylated glucose, a higher rate of glucose transport across the cell membrane, and higher activity of hexokinase[13]. There have been several studies about possible associations of GLUT1 expression with other clinicopathologic parameters and ^{18}F-FDG PET findings in several cancers, such as carcinoma of lung, pancreas, and breast[1]. However, to the best of our knowledge, it has not been elucidated in colorectal adenocarcinoma (CRA). Therefore, we conducted a prospective study to determine the association between GLUT1 expression and the maximum standardized uptake values (SUVmax) obtained from ^{18}F-FDG PET scans. The relationship between GLUT1 and SUVs with other clinicopathologic factors was also evaluated. Additionally, we evaluated a difference in GLUT1 expression between adenoma and carcinoma in the colorectum.

MATERIALS AND METHODS

Patients

Among patients who had FDG-PET examination and underwent curative surgery for CRA at Chosun University Hospital from January 2007 to December 2010, the present study was conducted on a non-consecutive series of 44 patients where paraffin embedded tissues were relatively well preserved and complete medical records were present. Patients who underwent preoperative chemoradiotherapy and emergency surgery, and patients who had evidence of hereditary non-polyposis colorectal cancer or familial adenomatous polyposis were excluded from the study. The various clinicopathological parameters of the patients were confirmed by reviewing the patient medical records and pathology files. The relationship between clinicopathological parameters for the patients and the immunohistochemical findings with survival was investigated for all 44 patients. Additionally, there were 27 adenomatous cases, including 15 cases of tubular adenoma (TA), 7 villous adenomas (VA), and 5 tubulo-villous adenomas (TVA).

Histopathological analysis

Microscopic examination: each tumor was re-evaluated by retrospective analysis of the medical records and the tissue slide files of the Department of Pathology. Age, gender, tumor size, histological subtypes and the degree of differentiation, the depth of tumor invasion, the status of lymph node metastases and the presence of a distant metastasis were assessed. Stage was defined according to the TNM staging system of the American Joint Committee on Cancer[17]. The examined tissues were fixed in 10% neutral formalin, and the prepared paraffin embedded tissues were sectioned 4-5 μm in thickness. Hematoxylin and eosin staining was performed, and the sections were examined under a light microscope. A representative area suitable for the study purpose was selected, and slides were prepared for immunohistochemical analysis.

Immunohistochemical staining

All of the specimens in this study were tested using a gout polyclonal antibody against GLUT1 (Abcam) according to the manufacturer's protocol. Immunolocalization was performed using the mouse ImmunoCruz Staining System: sc-2050 (Santa Cruz Biotechnology), according to the manufacturer's protocol. The staining process was performed according to a standard protocol. Briefly, the 4 μm sections that were obtained after formalin fixation and paraffin embedding were deparaffinized in xylene and were then rehydrated with distilled water through a graded series of ethanol solutions. The sections were then placed in a glass jar with 10 mmol/L citrate buffer (pH 6.0) and were irradiated in a microwave oven for 15 min. The sections were allowed to cool in the jar at room temperature for 20 min. The slides were then rinsed with Tris buffered saline (TBS). A blocking reagent was added for 10 min after quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min. The slides were then washed as described previously, and the slides were subsequently subjected to the primary antibody reaction. Immunohistochemistry was performed on the Nexes ES (Ventana, Tucson, AZ). Slides were incubated with the antibodies for 32 min. The Ventana basic DAB detection kit (catalog No. 760-001) was the secondary detection method. This includes biotinylated immunoglobulin secondary antibody, containing affinity purified goat-antimouse IgG and IgM (b200 lg/mL) and goat-antirabbit IgG (b200 lg/mL) in phosphate buffer with preservative. Incubation was for 8 min. This was followed by conjugated streptavidin horseradish peroxidase. Slides were counterstained with hematoxylin (Ventana catalog No. 760-2021).

Analysis and interpretation of staining

GLUT1 immunostaining was quantified by grading the proportion of cells that were GLUT1 positive. Cells showing strong and distinctive membranous immunoreactivity for GLUT1 were considered positive. Cyto-

Table 1 Summary of clinicopathologic factors of adenocarcinoma

Characteristics	*n* (%)
Age (yr)	
≤ 50	7 (15.9)
51~59	7 (15.9)
60~69	10 (22.7)
≥ 70	20 (45.5)
Sex	
Male	24 (54.5)
Female	20 (45.5)
Pathologic tumor classification (pT)	
pT1	2 (4.5)
pT2	5 (11.4)
pT3	35 (79.6)
pT4	2 (4.5)
Pathologic lymph node classification	
pN0	26 (59.1)
pN1	17 (38.6)
pN2	1 (2.3)
Metastasis classification (M)	
M0	42 (95.5)
M1	2 (4.5)
Gross type	
Ⅰ (polypoid)	8 (18.2)
Ⅱ (ulcerative)	17 (38.6)
Ⅲ (infiltrative)	19 (43.2)

plasmic staining, including a supra nuclear dot pattern or nuclear staining, was regarded as negative[6]. The degree of GLUT1 immunostaining of a specimen was graded according to the proportion of GLUT1-positive cells in it (weakly positive, < 10%; moderately positive, 10%-50%; strongly positive, > 50%)[7].

Statistical analysis

The mean with standard deviation (SD) was calculated for the longest tumor diameter and SUVmax. Mann-Whitney *U* or Kruskal-Wallis test was used to assess differences in the levels of SUVmax and in the staining scores of GLUT1 between the groups. Correlations between SUVmax and GLUT1 expressions and between SUVmax and tumor diameter were analyzed by Spearman's rank test. A value of $P < 0.05$ was considered as statistically significant. The SPSS statistics 17.0 program (SPSS, Korea) was used for statistical evaluation.

RESULTS

The clinical characteristics of the patients are summarized in Table 1. The average age at the time of surgery was 65.73 years and the ratio of male to female participants was 24:20 (54.5%:45.5%). Mean tumor size was 18.92 cm, and mean SUVmax value was 15.47. In normal epithelium, specific GLUT1 expression was not observed. As expected, erythrocyte membranes were strongly GLUT1 positive. In adenoma cases, GLUT1 expression was absent in 23 cases (85.1%) and weakly positive in 4 cases, which were one VA and 3 TVAs. The positive rate of GLUT1 expression was significantly different ($P = 0.008$) among the TA, VA, and TVA. Of 44 cases of CRA, 91% had specific GLUT1 immunostaining in the plasma membrane. The extent of expression varied greatly. Of immunopositive cases, 13 cases (29.5%) showed weak staining (< 10% of tumor cells), 12 cases (27.3%) moderate staining (10%-50% of tumor cells), and 15 cases (34.1%) strong expression (> 50% of tumor cells), which were significantly different from adenomatous cases ($P < 0.001$). In cancer tissue, GLUT1 is usually strongly positive in the center of the necrotic and infiltrative areas (Figure 1). Concerning correlation between GLUT1 expression and SUVmax in PET, the mean SUVmax was 14.45 ± 7.0 in negative GLUT1 expression cases, 15.51 ± 5.7 in weak GLUT1 expression cases, and 16.52 ± 6.8 in strong GLUT1 expression cases, and there was no significant correlation between GLUT1 expression and SUVmax. SUVmax was significantly correlated with tumor volume ($P = 0.002$). However, GLUT1 expression did not correlate with tumor size. There was no significant difference in SUVmax and GLUT1 expression among other clinicopathologic factors including invasion depth, lymph node metastasis and gross type (Table 2).

Table 2 Relation between glucose transporter 1 expression/maximum standardized uptake values and clinicopathologic parameters

Clinicopathologic factors	*n*	GLUT1 expression[1]				SUVmax	
		0	1	2	*P* value	Medium	*P* value
T stage							
T1	2	2	0	0	0.282	6	0.108
T2	5	1	2	2		15.1	
T3	35	12	10	13		24.22	
T4	2	2	0	0		17.5	
N stage							
N0	26	10	7	9		20.3	
N1	17	6	5	6	0.795	25.09	0.346
N2	1	1	0	0		12	
Gross type							
Ⅰ (polypoid)	8	3	4	1		19.94	
Ⅱ (ulcerative)	17	7	3	7	0.473	24.94	0.496
Ⅲ (infiltrative)	19	7	5	7		20.39	
Tumor size (median)		19.59	28.71	20.83	0.14		0.002[2]

[1]GLUT1 expression; [2]Statistically significant, $P < 0.05$. 0: Negative or weak; 1: Moderate; 2: Strong expression. GLUT: Glucose transporter; SUVmax: Maximum standardized uptake values.

DISCUSSION

Among Gluts, Glut-1 and Glut-3 have been proven to show overexpression in both messenger RNA and protein in a variety of cancer cells[14-18]. Therefore, Glut-1 and Glut-3 may play an important role in glucose uptake by these cancers and could be useful biomarkers for malignant transformation[1]. We herein demonstrate that GLUT1 protein expression is a marker for malignant transformation in CRA. For CRA, an initial report showed increased expression of GLUT1 mRNA compared with normal colon[19], and GLUT1 immunostaining was subsequently demonstrated in seven of nine colorectal carci-

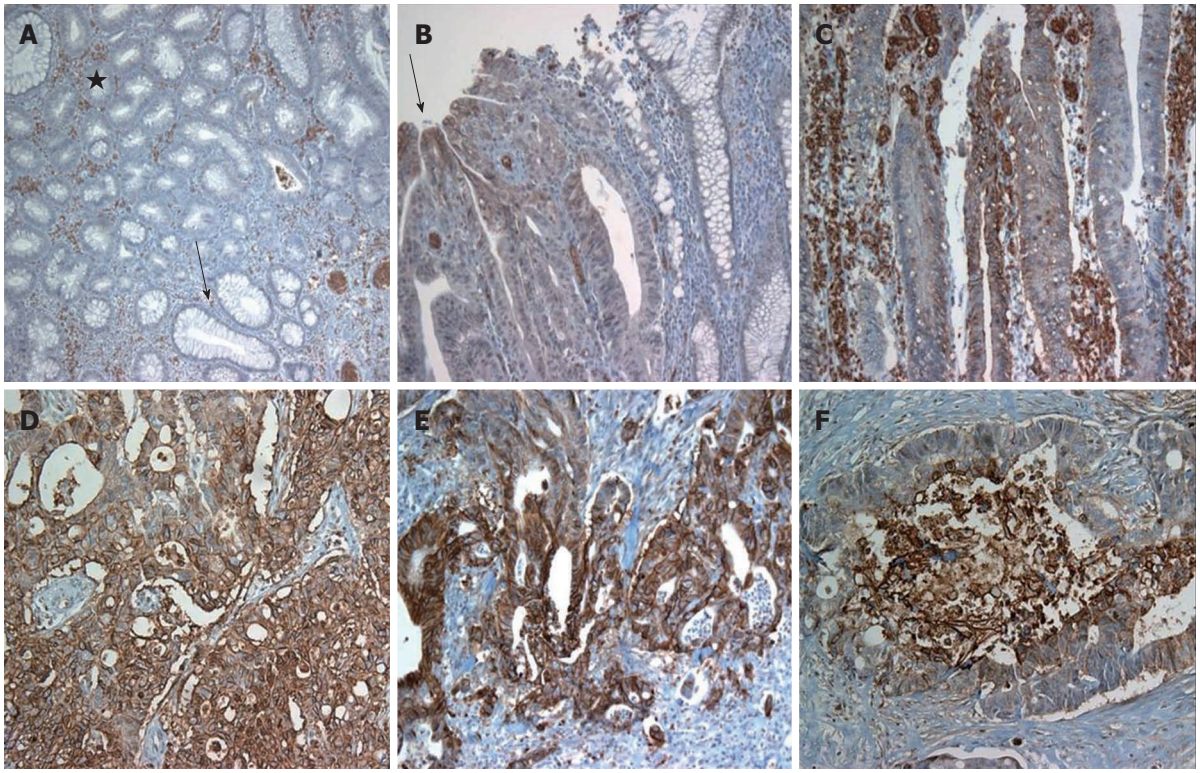

Figure 1 Glucose transporter 1 expression in normal colonic epithelium, adenomas and adenocarcinomas. A: No glucose transporter 1 (GLUT1) expression in tubular adenoma (star) and normal epithelium (arrow), while immunostaining in erythrocytes; B and C: GLUT1 immunostaining in the villous adenoma (B, arrow: Expression at the tip of villous frond); D: Colorectal adenocarcinoma with strong GLUT1 expression; E and F: More strong expression at the infiltrative border (E) and necrotic center (F).

nomas[20]. A recent study of 53 colon carcinomas demonstrated the presence of GLUT1 immunostaining in 83%, and a higher degree of GLUT1 expression correlated with the presence of lymph node metastases[21]. The greater degree of GLUT1 expression in these tumors most likely reflects a greater enhancement of glycolytic metabolism in the more malignant tumors. It has recently been reported that GLUT1 (and/or GLUT3 expression) correlates with poor prognosis and tumor aggressiveness in carcinomas of the lung and bladder, and in squamous cell carcinoma of the head and neck[22-24]. Although the present study did not show these results, these data suggest the possibility that tumors with absent GLUT1 staining might express another GLUT iso-form such as GLUT3, which also might be associated with poor prognosis[22].

In the present study, the normal and most adenomatous colorectal mucosa did not express GLUT1 protein. In benign colorectal neoplasms, GLUT1 expression was absent in TA, and in VA and TVA, there was only rare focal staining at the tips of villous fronds. These results are consistent with a recent report that some VAs have very limited focal GLUT1 expression[21]. GLUT1 expression in VA is consistent with the concept that GLUT1 is a marker of neoplastic progression in the colon, because it is this subtype of colonic adenoma that is believed to have the greatest potential for malignant transformation[7].

In cancer tissue, GLUT1 is usually strongly positive in the luminal border and center of the necrotic and infiltrative areas. Rapid proliferation relative to vascular support exposes tumor cells to persistent hypoxic conditions with potential necrotic or apoptotic effects[6]. Malignant cells, however, can undergo genetic and adaptive changes that allow them to avoid oxygen deprivation-induced death. One of these changes is an increased uptake of glucose and other sugars compared with normal cells[25]. In normal human small intestinal villi, the tips of villi may be a site of relative hypoxia[26]. Because hypoxia is known to stimulate glycolysis and GLUT1 expression[27], the localization of GLUT1 immunostaining to this site in VAs also might reflect an adaptation to enhanced local glycolytic demand[7].

Two possible mechanisms may explain the activation of *GLUT1* gene expression in CRA and other malignancies[7]. First, increased glycolysis and concomitant GLUT1 expression may be a constitutive feature of the malignant phenotype in many cancers. This is consistent with observations that transformation of cultured cells with ras or src oncogenes induces increased glucose uptake and GLUT1 expression[28,29]. Second, local hypoxia in the tumor microenvironment may result in an adaptive increase in glycolytic metabolism and GLUT1 expression[7]. The latter mechanism is also demonstrated in the present study; GLUT1 tended to be expressed stronger at the luminal border and center of tumor nests, increasing with distance from stromal blood vessels.

Higher levels of GLUT1 expression in neoplastic tis-

sue reflect an increased glycolytic metabolism[30]. In previous studies of CRA, a high level of GLUT1 expression was significantly associated with the presence of lymph node metastases[21] and poorer prognosis[7]. These studies suggested that the expression of GLUT1 could be a marker for malignant potential. In our study, the analysis of the association between GLUT1 expression and other clinicopathologic parameters did not show any significant correlation. These results differ from those of previously published data for other tumors[21,31,32], but they are compatible with the results of Avril *et al*[33] for breast cancer. In a study by Haber *et al*[7], the proportion of GLUT1 staining did not correlate with Dukes' stage of the CRA, but Sakashita *et al*[30] demonstrated that in T1 and T2 stage CRA, GLUT1 expression correlated with Duke stage. The discrepancy between the two studies could have been caused by differences in the clinical characteristics of the subjects enrolled. Haber's study included only 6 Dukes' A cases and all other cases were more advanced, while Sakashita's study analysed only T1 and T2 stage cases. So, Sakashita *et al*[30] speculated as follows: in early-stage carcinomas GLUT1 positivity is low, but correlates with the depth of the lesion. In contrast, in the more advanced stages, the tumor cells already show high GLUT expression, and no further increase of GLUT1 expression occurs, even when the cancer invades more deeply.

Cancer cells have higher rates of glucose metabolism than normal cells. Malignant tissues typically demonstrated higher ^{18}F-FDG uptake than benign lesions and normal tissue[34]. PET-CT using ^{18}F-FDG has been known to be a useful tool for several malignant tumors. Several immunohistochemical studies have demonstrated overexpression of GLUT1 in human malignancy and a correlation between GLUT1 expression and neoplastic progression[22]. The overexpression of GLUT1 in human cancers has been reported to be closely related to ^{18}F-FDG uptake on PET-CT[18]. Another report, however, showed no relation between GLUT1 expression and ^{18}F-FDG uptake on PET[33], and there is a controversial report that did not demonstrate a statistically significant correlation between GLUT1 expression and FDG uptake[35].

In present study ^{18}F-FDG uptake related to tumor size, whereas GLUT1 frequency did not. Brown *et al*[36] had mentioned that ^{18}F-FDG uptake and GLUT1 expression appeared to be associated with tumor size, but our data did not support their findings. Tumor size is one of the most important factors affecting the SUVmax[37]. The ^{18}F-FDG uptake might be influenced by the total amount of glucose uptake into the tumor. Therefore, the larger a carcinoma is, the higher is the ^{18}F-FDG uptake by the carcinoma shown on the PET scan. It is well known that SUVmax has a lower than "real" value when the tumor size is < 20 mm because of the limited resolution of current PET scanners[37,38]. In contrast, GLUT1 staining is examined through a microscope, and GLUT1 frequency is determined microscopically. Therefore, GLUT1 frequency shows microscopic activity of glucose uptake into the tumor and is influenced by cell type, cellularity, and pathological structure[39]. At the result, GLUT1 frequency would not be related to tumor size. GLUT1 expression could become strongly positive even in small carcinomas with high cellular density or metabolic activity.

In conclusion, in contrast to other malignant tumor such as lung cancer[39], squamous cell carcinoma of the cervix[1] and head and neck cancer[40], and cholangiocarcinoma[41], GLUT1 expression did not correlate significantly with ^{18}F-FDG uptake and other clinicopathologic parameters in CRA, which suggests that overexpression of GLUT1 cannot fully explain the biologic behavior of CRA. The ^{18}F-FDG uptake was significantly correlated with tumor size only. We identified that GLUT1 is usually strongly positive in the center of the necrotic and infiltrative areas in colorectal cancer. Although overexpression of GLUT1 is very important for ^{18}F-FDG uptake in cancer cells, further investigations should evaluate the contributions of other factors concerning tumor hypoxia and glucose metabolism.

COMMENTS

Background

Cancer cell growth is an energy-related process supported by increased glucose metabolism. This uptake is mediated by glucose transporter (GLUT) proteins, which are membrane proteins responsible for the transport of glucose across cellular membranes. Positron emission tomography (PET) using ^{18}F-fluoro-2-deoxyglucose (FDG) is a rapidly developing functional-imaging modality that has shown great promise in the fields of primary, recurrent and metastatic tumor detection, planning and monitoring therapy. Therefore, the authors conducted a prospective study to determine the association between GLUT1 expression and the maximum standardized uptake values (SUVmax) obtained from ^{18}F-FDG PET scans. The relationship between GLUT1 and SUVs with other clinicopathologic factors was also evaluated. Additionally, the authors evaluated the difference in GLUT1 expression between adenoma and carcinoma in the colorectum.

Research frontiers

This article may present information to the colorectal oncologist for further study about glucose metabolism of colorectal adenocarcinoma (CRA), and to the colorectal oncologist the usefulness of PET CT in evaluating colorectal cancer patients.

Innovations and breakthroughs

In contrast to other malignant tumors such as lung cancer, squamous cell carcinoma of cervix and head and neck, and cholangiocarcinoma, GLUT1 expression did not correlate significantly with ^{18}F-FDG uptake and other clinocopathologic parameters in CRA, which suggests that overexpression of GLUT1 cannot fully explain the biologic behavior of CRA. The ^{18}F-FDG uptake was significantly correlated with tumor size only. The authors identified that GLUT1 is usually strongly positive in the center of the necrotic and infiltrative areas in colorectal cancer. Although overexpression of GLUT1 is very important for ^{18}F-FDG uptake in cancer cells, further investigations should evaluate the contributions of other factors concerning tumor hypoxia and glucose metabolism.

Peer review

Overall the manuscript is reasonably well written and provides additional information on GLUT1 expression and FDG uptake.

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BRIEF ARTICLE

Prognostic relevance of circulating CK19 mRNA in advanced malignant biliary tract diseases

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Abstract

AIM: To determine the role of circulating tumor cells (CTCs) in prediction of the overall survival of patients with advanced malignant biliary tract obstruction.

METHODS: We investigated the prognostic value of CTCs by examining two markers, cytokeratin (CK) 19 and human telomerase reverse transcriptase (hTERT) mRNA, in 40 patients diagnosed with advanced malignant biliary tract diseases. Quantitative real-time reverse transcription polymerase chain reaction was used to detect CK19 and hTERT mRNA in the peripheral blood of these patients. Overall survival was analyzed using the Kaplan-Meier method and Cox regression modeling.

RESULTS: Positive CK19 and hTERT mRNA expression was detected in 45% and 60%, respectively, of the 40 patients. Univariable analysis indicated that positive CK19 mRNA expression was significantly associated with worse overall survival (P = 0.009). Multivariable analysis determined that positive CK19 mRNA expression, patient's age and serum bilirubin were each independently associated with overall survival.

CONCLUSION: CK19 mRNA expression levels in peripheral blood appear to provide a valuable marker to predict the overall survival of patients with advanced malignant biliary tract obstruction.

Key words: Circulating tumor cells; Cytokeratin 19; Human telomerase reverse transcriptase; Malignant biliary tract obstruction; Overall survival

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INTRODUCTION

Malignant biliary tract obstruction is a condition that can

result from tumors of the biliary tract, ampulla of vater, duodenum or head of the pancreas. In Thailand, cholangiocarcinoma (CCA) is the most common cause of malignant biliary tract obstruction[1]. Despite recent advances in the diagnosis and treatment of this disease, patient outcome remains poor. The high mortality rate arising from malignant biliary tract obstruction is due to the aggressiveness of tumors that are often discovered at a late stage of disease progression[2]. Palliative therapeutic approaches to endoscopic biliary drainage, such as the use of endoprosthesis stents, are generally recommended for these patients. The two major types of endoprosthesis stents are plastic or polyethylene (PE) stents and self-expanding metal stents (SEMS). Previous studies have demonstrated that partial or total occlusion of PE stents usually occurs 3-4 mo after insertion[3]. Four randomized controlled studies demonstrated that SEMS exhibited a significantly higher patency rate as compared with the PE stents (9 mo *vs* 1.5 mo)[4-7]; however, SEMS is much more expensive than PE stents (1500 USD *vs* 80 USD, in Thailand). A recent study indicated that patients who have a predicted survival duration of more than 4.5 mo should use SEMs for their palliative biliary drainage[8]. In this instance, the higher cost of the SEMs is balanced by a decreased need for repeat intervention that is often necessary in patients who have received PE stents. Therefore, identification of reliable prognostic factors that allow for an accurate prediction of survival duration in patients with advanced malignant biliary tract obstruction is extremely important.

One of the major mechanisms for tumor metastasis is the dissemination of tumor cells from the primary tumor into circulating blood[9]. Previous studies have indicated that detection of circulating tumor cells (CTCs) in the peripheral blood can be used in staging and prognosis stratification for breast and colon cancer patients[10-12]. Until now, however, there has been no study concerning the role of the detection of CTCs as a prognostic factor in patients with malignant biliary tract diseases.

To date, the most common CTCs detection method is quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), a process that can detect mRNA expression levels of the genes coding for these tumor antigens[13]. A high-quality detection marker is required for efficient quantitative real-time RT-PCR-mediated detection of CTCs. Therefore, identification of a good target marker is of the utmost importance for CTC detection. Several gene markers, such as cytokeratin (CK) 19 and human telomerase reverse transcriptase (hTERT), have been evaluated as tumor-specific markers for the detection of CTCs in gastrointestinal cancers[14,15].

hTERT mRNA can be detected in 85% of all cancer cells, including cholangiocarcinoma cells[16]. This is in contrast to most normal cells, which exhibit little or no expression. Our previous study demonstrated that high levels of hTERT mRNA can be detected in the blood circulation of cholangiocarcinoma patients, and it has also been suggested that hTERT mRNA is a promising marker for the detection of cancer cells[17].

CK19 is generally expressed in ductal epithelium (bile ducts, pancreas, and renal collecting tubules) and in the mucosa of the gastrointestinal tract. CK19 immunohistochemistry is used in diagnostic pathology mainly to confirm epithelial immunophenotype in undifferentiated tumors or to establish biliary, pancreatic or renal ductular origin[18]. Most adenocarcinomas arising from the gastrointestinal tract are CK19 positive, including cholangiocarcinoma and pancreatic cancer[18]. Many investigators have used the detection of CK19 mRNA in peripheral blood as a target gene to investigate CTCs[14,19,20]; however, until now there has been no study focusing on the detection of hTERT and CK19 in the peripheral blood of patients with malignant biliary tract diseases.

This study was aimed to evaluate if the levels of CTCs could be used to predict the overall survival of patients with advanced malignant biliary tract obstruction. *CK-19* and *hTERT* were selected as the target genes for CTCs. In addition, this study was performed in accordance with the REporting recommendations for tumor MARKer prognostic studies[21] to ensure the standardization and transparency of the study.

MATERIALS AND METHODS

Patients and samples

We prospectively included the patients with advanced malignant biliary tract diseases who underwent palliative endoscopic retrograde cholangiopancreatography or percutaneous transhepatic biliary drainage at Department of Surgery, Rajavithi Hospital from January 2008 to December 2009. The cutoff date for data analysis was December 31, 2010. The inclusion requirements included patients present with malignant bile duct obstruction that was not amenable to curative resection and patients who were followed up for at least one month after biliary tract drainage. All blood and clinical information was obtained with patient informed consent after approval by the Rajavithi Hospital Ethics Committee.

Pre-treatment fasting blood samples were collected from the peripheral vein into ethylenediaminetetraacetic acid-containing tubes. The first 3 mL blood was discarded to prevent epidermal contamination (2-syringe technique). Sample processing was performed within 1 h of blood withdrawal. Blood was transferred into a 30-mL falcon tube and centrifuged at 1800 r/min at room temperature for 20 min. Plasma was removed, and the peripheral blood mononuclear cell (PBMC) fraction was stored at -80 ℃ until use.

RNA extraction and cDNA synthesis

The total RNA of PBMC fraction samples was extracted using the RNeasy mini kit (Qiagen, GmbH, Germany) following the protocol provided by the manufacturer. RNA integrity was checked by electrophoresis and quantified by absorption at 260 and 280 nm using a spectrophotometer (Beckman Coulter Du® 800, Fullerton, CA). Total RNA was reversely transcribed using random primers and

Table 1 Primer sequences

Primer	Forward	Reverse
hTERT	GCGGAAGACAGTGGTGAACT	AGC TGGAGTAGT CGCTCT GC
CK19	CCCGCGACTACAGCCACTA	GCTCATGCGCAGAGCCT
β-Actin	GTGGGGCGCCCCAGGCACCA	GTCCTTAATGTCACGCACGATTTC

hTERT: Human telomerase reverse transcriptase; CK19: Cytokeratin 19.

Table 2 Clinical characteristics of patients with advanced malignant biliary tract obstruction

	Parameters	*n* (%)
Gender	Male	23 (57.5)
	Female	17 (42.5)
Age (yr)	< 60	18 (45.0)
	> 60	22 (55.0)
Type of cancers	Hilar cholangiocarcinoma	28 (70.0)
	Pancreatic cancer	6 (15.0)
	Common bile duct cancer	2 (5.0)
	Ampullary cancer	2 (5.0)
	Gall bladder cancer	2 (5.0)

the IscriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, United States) following the protocol provided by the manufacturer. cDNA was stored at -80 ℃ until use.

Detection of CK-19 and hTERT mRNA by quantitative real-time PCR

Expression of *CK19* and *hTERT* genes was analyzed using specific primers (Table 1). In this assay, the housekeeping gene *β-actin* was used as an internal control to normalize variations in integrity and the total amount of cDNA. Quantitative real-time PCR assays were performed in triplicate using SYBR Green master mix (Superarray, Frederick, MD, United States) on the Chromo 4™ System (MJ Opticon Monitor ver. 3.1) (Bio-Rad, United States) for 20 min at 50 ℃. After this, 42 cycling steps for amplification of PCR products were performed (15 s, 94 ℃ for denaturation; 30 s, 60 ℃ for annealing; and 30 s, 72 ℃ for extension). Melting curve analysis was used to assess the specificity of the amplified products. The expression levels of *CK19* and *hTERT* genes from the cDNA were measured by quantitative real-time PCR using the relative quantification method ($2^{-\Delta\Delta Ct}$ method)[22]. The fold-change in gene expression was normalized to a housekeeping gene (*β-actin*) and relative to a calibrator sample. A pool of cDNA derived from the PBMCs of 30 cases of benign (common bile duct stone and gall stone) biliary tract diseases was used as the calibrator source[23]. Evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator. In this study, we set the positive value as a fold change in gene expression that was greater than 1.5 times relative to the calibrator and the negative value was set as a fold change in gene expression that was lesser than or equal to 1.5 times relative to the calibrator.

Determination of blood chemistries in serum samples

Biochemical studies of serum samples, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin, alkaline phosphatase, carcinoembryonic antigen (CEA) and cancer antigen (CA)19-9, were measured using routine automated methods in the Pathological Laboratory at Rajavithi Hospital.

Cell lines and cell spiking experiments

The human cholangiocarcinoma cell line RMCCA1[24] was incubated in Ham's F12 medium (Invitrogen-Gibco, Carlsbad, CA, United States) containing 10% fetal calf serum (Euroclone-Celbio, Pero, MI) at 37 ℃ in 5% CO_2. To determine the sensitivity of quantitative real-time PCR for detecting cancer cells in PBMCs, cell spiking experiments was performed. The PBMCs obtained from healthy volunteers were counted and diluted in Ham's F12 medium. RMCCA1 cells were serially diluted from 1 × 10^6 cells/mL to 1 cell/mL and added to the PBMCs. Quantitative real-time PCR was then performed to detect CK19 and hTERT mRNA.

Statistical analysis

The primary endpoint of this study was the overall survival of the patients. Survival curves were estimated using the Kaplan-Meyer method, and univariable survival comparisons were calculated according to the log rank test. Multivariable survival analysis was performed using the Cox proportional hazards regression model. The quantitative variables were compared using Mann-Whitney *U* or Student's *t* test, as appropriate. Qualitative variables were reported as counts, and comparisons between independent groups were performed using the Pearson χ^2 test. All tests of significance were two sided and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Forty-two patients with malignant biliary tract disease were included in this study. Two patients were excluded because of the poor quality of the RNA extracted from their peripheral blood. The average age of these patients was 62 years (range, 41-82 years). With regard to cancer type, 6 (15.0%) were pancreatic head cancer, 2 (5.0%) were ampullary cancer, 2 (5.0%) were gall bladder cancer, 2 (5.0%) were middle and distal common bile duct cancer and 28 (70.0%) were hilar cholangiocarcinoma. The clinical characteristics of the patients are shown in Table 2.

Cell spiking assay

CK19 and hTERT mRNA levels were elevated in the RMCCA1 cell line (Figure 1); therefore, we decided to use this cell line as a positive control for our study. Detection sensitivity of the quantitative real-time PCR assay was determined by serial 10-fold dilutions of RMCCA1 cells in PBMCs. The results demonstrated that CK19 and hTERT mRNA could be detected at levels up to 1000 cells per 10^{10} PBMC dilutions.

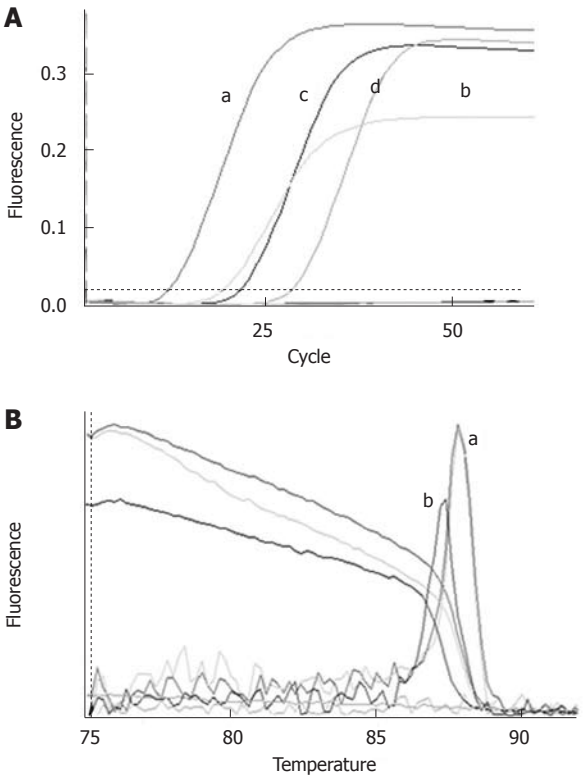

Figure 1 Gene expression levels of cytokeratin 19 and human telomerase reverse transcriptase in RMCCA1 cells (as measured by quantitative real time polymerase chain reaction). A: Amplification plot of cytokeratin (CK)19 mRNA from 10 000 RMCCA1 cells. (a) 1000 RMCCA1 cells; (b) amplification plot of human telomerase reverse transcriptase (hTERT) mRNA from 10 000 RMCCA1 cells; (c) 1000 RMCCA1 cells; and (d) are demonstrated; B: SYBR Green melting curve for quantitative real time reverse transcription polymerase chain reaction (RT-PCR). The melting curves from quantitative real time PCR for CK19 (a) and hTERT (b) consistently gave a single peak with no evidence of non-specific amplification or primer-dimerisation.

Detection of hTERT and CK19 mRNA in PBMCs of malignant biliary tract disease patients

PBMC samples from 40 patients were evaluated for the expression of hTERT and CK19 mRNA. The expression was positive (gene expression more than 1.5 times relative to the calibrator) in 45% (18/40) of samples for CK19 mRNA and 60% (24/40) of samples for hTERT. Figure 2 illustrates the distribution of CK19 mRNA and hTERT mRNA expression in the peripheral blood of these patients.

Relationship between CK19 and hTERT mRNA expression in peripheral blood and clinic pathological features of patients

No statistically significant difference was found among the data obtained from the patients considered as negative or those who were positive for hTERT or CK19 mRNA expression in PBMCs. Factors evaluated included gender, age, serum albumin, globulin bilirubin AST and ALT and alkaline phosphatase levels (Table 3).

Survival analysis

At the time of data analysis, only 1 patient was alive and 39 patients had died. The median overall survival for these patients was 4.0 mo (95% CI: 2.56-4.56). The median survival time was 1.7 mo in patients with positive CK19 mRNA expression, whereas the survival time was 5.3 mo in patients with negative CK19 mRNA expression (Log Rank, $P = 0.009$). We found that the median survival time in patients with a negative hTERT mRNA expression was not significantly different from patients with positive hTERT mRNA expression (5.9 mo *vs* 3.2 mo, Log Rank, $P = 0.183$) (Figure 3).

To identify variables that could be of potential prognostic significance in patients with advanced malignant biliary tract disease, univariable and multivariable analyses were performed using the Cox proportional hazard model to compare the impact of the mRNA expression levels of CK19 and hTERT. Other clinical parameters such as positive or negative hTERT expression, CK19 expression (positive or negative), CEA (cut-off value = 5 ng/mL), CA19-9 (cut-off value = 500 U/mL), total bilirubin (cut-off value = 15 mg/dL) and albumin (cut-off value = 3 g/dL) were also examined. Univariable analysis indicated that only CK19 mRNA expression showed significance as a prognostic factor. Multivariable analysis demonstrated that CK19 mRNA expression ($P = 0.024$), age ($P = 0.026$) and serum bilirubin ($P = 0.002$) were all independent risk factors for survival. The relative risk for CK19 mRNA positive patients was 3.2 times greater than that for CK19 mRNA negative patients (Table 4).

Table 3 Clinical characteristics of patients with negative and positive cytokeratin 19 and human telomerase reverse transcriptase gene expression

	CK19 gene		*p*	*hTERT* gene		*p*
	Negative	Positive	value	Negative	Positive	value
Age (yr)	63.80	61.38	0.42	65.26	60.88	0.16
Sex (male: female)	11:11	12:6	0.35[1]	8:8	15:9	0.52[1]
Total bilirubin (mg/dL)	16.21	16.42	0.95	17.73	15.47	0.53
Albumin (g/dL)	2.88	2.97	0.62	2.83	2.99	0.36
Globulin (g/dL)	3.98	3.76	0.42	3.92	3.84	0.79
AST (U/L)	84.57	112.80	0.23	69.27	117.48	0.12
ALT (U/L)	42.47	65.85	0.12	34.87	66.68	0.28
ALP (IU/L)	449.68	528.85	0.56	436.88	421.91	0.98
BUN (mg/dL)	13.77	26.58	0.13	12.25	23.52	0.18
Creatinine (mg/dL)	0.75	1.30	0.20	0.61	1.19	0.22
CA19-9 (U/mL)	570.20	594.30	0.62[2]	1818.00	259.15	0.11[2]
CEA (ng/mL)	7.47	5.68	0.50[2]	7.21	5.82	0.23[2]

Quantitative variables are presented as the mean value, with the exception of cancer antigen (CA)19-9 and carcinoembryonic antigen (CEA), which are presented as median values. [1]Pearson χ^2 was used to compare two groups; [2]Mann-Whitney *U* test was used to compare groups. CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.

DISCUSSION

The highest incidence of cholangiocarcinoma occurs in Thailand, and the majority of patients included in this study were diagnosed with this disease[1]. In this study,

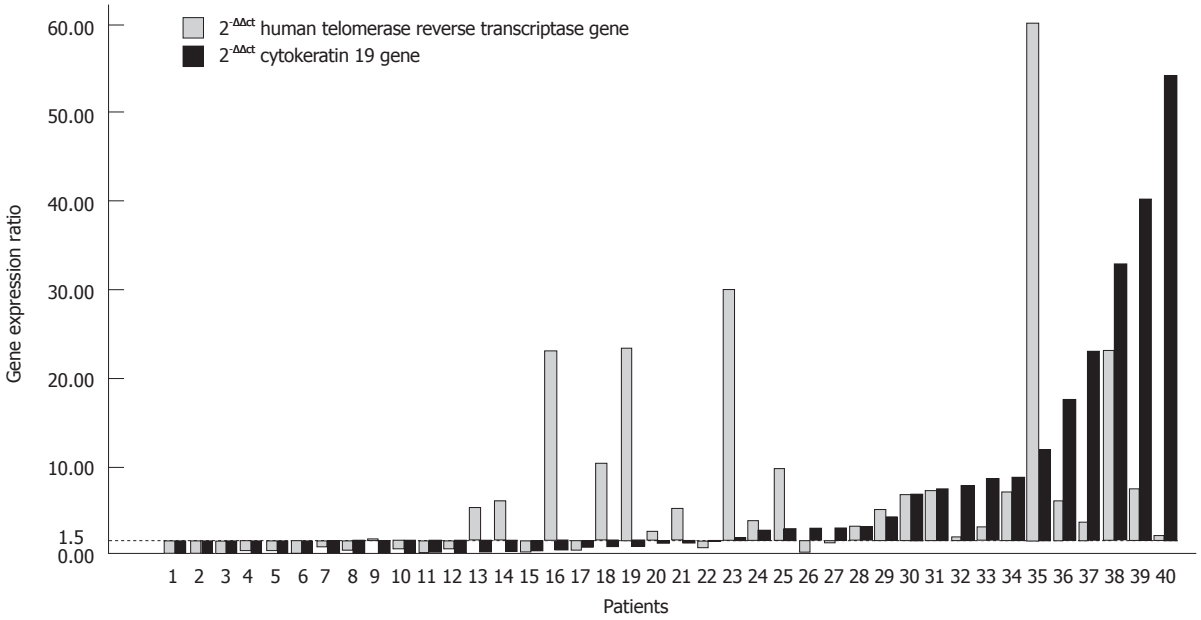

Figure 2 The distribution levels of cytokeratin 19 and human telomerase reverse transcriptase genes in the peripheral blood of 40 patients. The positive value is determined as a fold change in gene expression of more than 1.5 times relative to the calibrator.

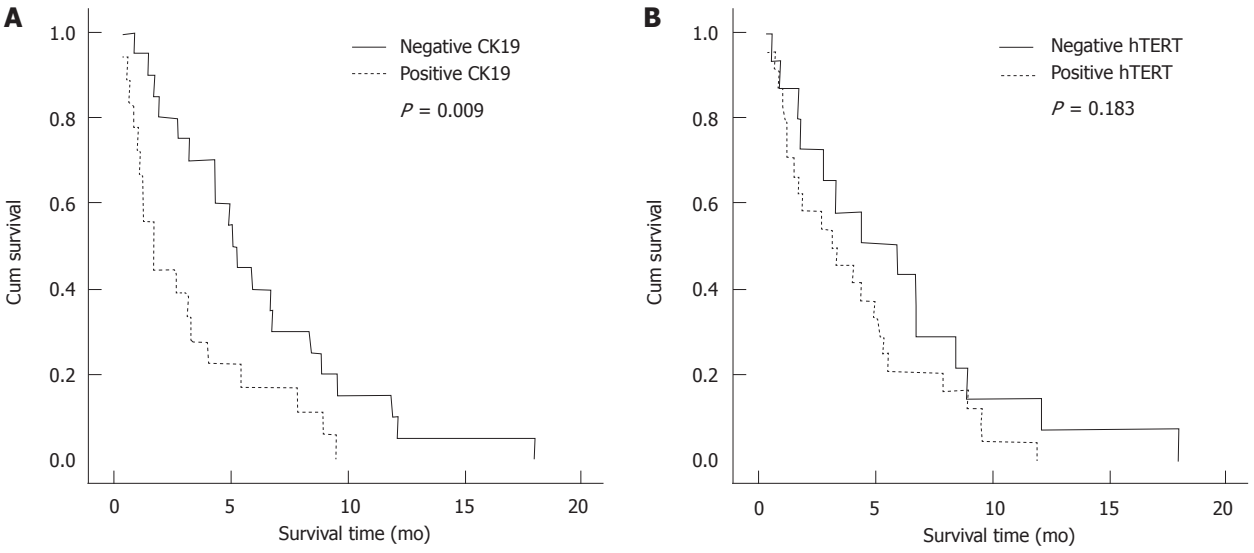

Figure 3 Kaplan-Meier survival curves of patients with positive or negative expression of cytokeratin 19 (A) and human telomerase reverse transcriptase (B) genes measured in the peripheral blood. CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase.

the median survival was 4.0 mo, a finding that is not significantly different from the survival time (3.6-5.0 mo) observed in previous studies of advanced malignant biliary tract disease where the majority of patients were diagnosed with pancreatic cancer[7,8,25]. These results indicate that all cancers that lead to malignant biliary tract obstruction are highly lethal. Most advanced malignant biliary tract disease patients can only be treated with palliative biliary tract drainage.

The choice of stents (PE or SEMs) for endoscopic palliation of jaundice due to malignant biliary tract obstruction is dependent upon the estimation of patient survival[8]. Therefore, there is a need for more accurate tests to predict the survival of patients with advanced malignant biliary tract diseases, as this could significantly improve the treatment outcome for these patients. This is the first cohort paper that studies the level of CTCs as a prognostic factor for overall survival of patients with advanced malignant biliary tract obstruction.

We used quantitative real-time RT-PCR to detect CTCs. As a result of the PCR-based methods, we cannot identify exactly the cell source of the measured markers. Quantitative real-time RT-PCR assesses the expression of target genes from mRNA extracted from the lysates of cells harvested from the peripheral blood of patients. As such, these samples contain not only CTC but

Table 4 Survival analysis using clinical parameters measured by univariable and multivariable analysis

Variables	Univariable analysis		Multivariate analysis	
	P value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)
CK19 expression	0.011[1]	2.42 (1.22-4.78)	0.024[1]	3.20 (1.17-8.75)
hTERT expression	0.188	1.60 (0.80-3.22)	0.580	1.34 (0.47-3.82)
Age	0.541	0.82 (0.42-1.57)	0.026[1]	0.38 (0.16-0.89)
Total bilirubin	0.106	1.72 (0.89-3.31)	0.002[1]	3.97 (1.69-9.36)
Albumin	0.360	0.73 (0.37-1.35)	0.213	0.59 (0.26-1.35)
CA19-9	0.374	0.73 (0.37-1.43)	0.478	0.74 (0.32-1.70)
CEA	0.381	1.39 (0.68-2.88)	0.124	1.86 (0.84-4.12)

[1]Statistically significant. CI: Confidence interval; CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase; CA: Cancer antigen; CEA: Carcinoembryonic antigen.

also PBMC, circulating endothelial cells and skin cells (e.g., keratinocytes, fibroblasts and melanocytes) that contaminate the sample during blood withdrawal and provide alternate potential sources for the PCR-detected genes[13,26]. Therefore, strict selection of target genes for detection of CTCs is very important. In this study, we used *CK19* and *hTERT* genes as targets for the detection of CTCs. Previous studies have suggested that CTCs are likely to be the principal cell source for *CK19* gene expression, as CK19 expression is mainly restricted to epithelial cells and is limited in normal peripheral blood cells[20,27]. Additionally, we used the 2-syringe technique during blood collection to avoid epithelium contamination from injected site.

In our study, the patients with positive CK19 expression exhibited significantly shorter overall survival compared with the patients with negative CK19 expression (5.3 mo *vs* 1.7 mo; $P = 0.009$). Additionally, multivariable analysis using the Cox regression model also demonstrated that the levels of CK19 expression in peripheral blood, the levels of serum total bilirubin and the age of the patients can all function as independent prognostic factors in patients with advanced malignant biliary tract disease. This is consistent with previously published studies that reported that positive CK19 mRNA expression in peripheral blood was independently associated with a reduction in disease-free survival in patients with breast cancer[20]. In addition, positive CK19 mRNA expression in peripheral blood following chemoradiation was an independent, unfavorable prognostic factor for both overall survival and progression-free survival in patients diagnosed with non-small cell lung cancer[19].

In this study, there were more patients with positive hTERT mRNA expression in peripheral blood than the patients with positive CK19 mRNA expression (60% *vs* 45%); however, detection of hTERT mRNA in the peripheral blood was not identified as an independent prognostic factor in this study. We suggested that the detection of hTERT mRNA expression levels was not a good candidate as a prognostic factor for patients with advanced malignant biliary tract disease. It may be suitable for the distinction between malignant and benign biliary tract diseases in combination with other tumor specific markers.

In this study, neither univariable nor multivariable analysis indicated that serum levels of CA19-9 could be used as a prognostic factor for patients with advanced malignant biliary tract disease. This finding is inconsistent with a previous study that indicated that the levels of serum CA19-9 are of prognostic relevance in patients with biliary tract cancer[28,29]. We suggested that differences among the patients should be considered. Overall survival in a previous study was 16.1 mo[29], whereas the overall survival in our study was 4.0 mo. In addition, the majority of patients in the previous study received chemotherapy, while only two patients in our study received this treatment. This finding reflects a higher disease severity in the patients examined in our study.

Our study demonstrated that the expression of CK19 mRNA in PBMCs prior to palliative procedures was significantly associated with overall survival of the patients with advanced malignant biliary tract disease. We therefore recommend PE stents for patients with positive CK19 mRNA expression in their peripheral blood. The more expensive SEMS should be reserved for patients with negative peripheral blood expression of CK19 mRNA. Further cost-effectiveness studies should be conducted to evaluate the benefit of using CK19 mRNA in helping the physician make decisions regarding the selection of stent-type and the need for endoscopic repeat intervention in patients with advanced malignant biliary tract disease.

COMMENTS

Background

In Thailand, cholangiocarcinoma is the most common cause of malignant biliary tract obstruction. Despite recent advances in the diagnosis and treatment of this disease, patient outcome remains poor. Palliative therapeutic approaches to endoscopic biliary drainage, such as the use of endoprosthesis stents, are generally recommended for these patients. Identification of reliable prognostic factors that allow for an accurate prediction of survival duration in patients suffering from advanced malignant biliary tract obstruction is extremely important.

Research frontiers

This study demonstrated that the levels of circulating tumor cells (CTCs) could be used to predict the overall survival of patients with advanced malignant biliary tract obstruction.

Innovations and breakthroughs

The expression of cytokeratin (CK)19 mRNA in peripheral blood mononuclear cells prior to palliative procedures was significantly associated with overall survival of patients with advanced malignant biliary tract disease.

Applications

This study recommends polyethylene stents for patients with positive CK19 mRNA expression in their peripheral blood. The more expensive self-expanding metal stents should be reserved for patients with negative peripheral blood expression of CK19 mRNA.

Terminology

CTCs is the dissemination of tumor cells from the primary tumor into circulating blood. The detection of CTCs can be used in staging and prognosis stratification for cancer patients.

Peer review

This study provides evidences that high levels of CTCs in advanced malignant biliary tract obstruction patients might be used as a prognostic factor. This is a well performed and clearly presented study.

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BRIEF ARTICLE

Quality of life and psychological outcome of donors after living donor liver transplantation

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Abstract

AIM: To investigate the health related quality of life (HRQoL) and psychological outcome of donors after living donor liver transplantation.

METHODS: Participants were 92 consecutive liver transplant donors who underwent hepatectomy without middle hepatic vein at West China Hospital of Sichuan University between January 2007 and September 2010. HRQoL was measured using the Chinese version of the Medical Outcomes Study Short Form-36 (SF-36), and psychological symptoms were measured using the Symptom Checklist-90-Revised (SCL-90-R). Data collected from donors were compared to previously published data from the general population. Clinical and demographic data were collected from medical records and questionnaires.

RESULTS: The general health score of the SF-36 was significantly lower in females (59.78 ± 12.25) than in males (75.83 ± 22.09). Donors more than 40 years old scored higher in social functioning (85.71 ± 14.59) and mental health (82.61 ± 20.00) than those younger than 40 (75.00 ± 12.13, 68.89 ± 12.98; social functioning and mental health, respectively). Donors who had surgery more than two years prior to the study scored highest in physical functioning (P = 0.001) and bodily pain (P = 0.042) while those less than one year from surgery scored lowest. The health of the liver recipient significantly influenced the general health (P = 0.042), social functioning (P = 0.010), and role-emotional (P = 0.028) of donors. Donors with full-time employment scored highest in role-physical (P = 0.005), vitality (P = 0.001), social functioning (P = 0.016), mental health (P < 0.001), the physical component summary scale (P < 0.001), and the mental component summary scale (MCS) (P < 0.001). Psychological measures indicated that donors were healthier than the general population in obsessive-compulsive behavior, interpersonal sensitivity, phobic anxiety, and paranoid ideation. The MCS of the SF-36 was significantly correlated with most symptom scores of the SCL-90-R.

CONCLUSION: HRQoL and psychological outcome were favorable in living liver transplant donors after donation. Specifically, gender, age, time since operation, recipient health condition, and employment after donation, influenced postoperative quality of life.

Key words: Health related quality of life; Psychology; Living donor liver transplantation; Donor

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INTRODUCTION

The rapid growth of living donor liver transplantation (LDLT) is attributable to the continual improvement in recipient survival and the shortage of deceased donor liver grafts[1,2]. Evidence supports a significant reduction in mortality of recipients listed for liver transplantation[3,4]. However, the donor of LDLT is exposed to risks inherent to a surgical procedure, and may suffer a considerable psychological burden[5]. Therefore, the safety of the donor operation and the health related quality of life (HRQoL) of the donor after surgery is critical while maintaining graft viability.

In the transplant center at West China Hospital of Sichuan University, liver recipient survival rates at one, three, and five years were 87.4%, 80.5% and 72.7%[6], respectively, which are similar to that reported elsewhere. Since 2001[7] over 250 cases of LDLT have been performed in our center, accounting for 30% of total transplant volume and this ratio is expected to increase in the future. However, the HRQoL and psychological outcome of donors remain unclear. The aim of the current cross-sectional study was to explore the HRQoL and the psychological outcome of donors after LDLT. To our knowledge, this is the first study of HRQoL and psychological outcome for the living liver transplant donor in mainland China. The results of the study may better guide adult-to-adult LDLT practice.

MATERIALS AND METHODS

Patients

From January 2007 to September 2010, 92 consecutive liver donors at West China Hospital of Sichuan University were approached for participation. The investigation extended from September 2010 to March 2011. Inclusion criteria were: age ⩾ 18 years, an understanding of Chinese, and greater than 6 mo recovery from surgery. Exclusion criteria were: severe medical complications and limited ability to self-express. Clinical and demographic data were collected from medical records and self-report questionnaires (completed by interview or mail).

Instruments

HRQoL was assessed using the Chinese version (2002)[8] of the Medical Outcomes Study Short Form-36 (SF-36)[9,10]. The SF-36 is a valid, self-administered questionnaire used internationally to measure 8 domains of health: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health during the last 12 mo. The raw scores of each subscale were transformed into scores that ranged from 0 to 100, with higher scores indicating higher levels of functioning or well-being. The level of HRQoL was assessed by comparing the mean value for the study sample with the mean value for a representative sample of the general population of Sichuan province in China[11]. Scores representing overall physical functioning and mental functioning were calculated from the subscales and presented as the physical component summary scale (PCS) and mental component summary scale (MCS).

The Symptom Checklist-90-Revised (SCL-90-R)[12] is a 90-item self-report symptom inventory used to measure the psychological symptoms patterns of community, medical, and psychiatric respondents. It is a simple questionnaire that has been validated in a number of languages. The Chinese version was adapted by Wang[13]. Each of the items is rated on a five-point scale of distress ranging from "not at all" (1) to "extremely" (5). The nine primary symptom dimensions were labeled as: somatization, obsessive-compulsive behavior, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism. We assessed the level of psychological health of our sample and compared it with the Chinese norm[14].

Ethical considerations

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the West China Hospital of Sichuan University Ethics Committee. All participants were asked to sign an informed consent form.

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 13.0. Between-group differences in HRQoL and psychological health were examined with independent sample *t* tests, analysis of variance, or nonparametric tests, as appropriate. Multiple comparisons for observed means were tested using the Student-Newman-Keuls procedure when equal variances could be assumed, and by the Games-Howell procedure when equal variances could not be assumed. Pearson correlation analysis were used to analyze the relationships between HRQoL and psychological symptoms. Statistical significance was set at $P < 0.05$.

RESULTS

Donor characteristics

Informed consents for participation were obtained from 92 donors. In the end, 71 (77.2%) validated questionnaires were returned. The results of SF-36 and SCL-90-R completed by interview or mail were not statistically different. All donors received a right hepatectomy without middle hepatic vein, and the vast majority of them reported that they would donate again. All donor relationships with liver recipient and recipient families

Table 1 Donor characteristics

Factors	Frequency	mean ± SD/percent (%)
Age (yr)		38.94 ± 10.44
≤ 40	42	59.2
> 40	29	40.8
Marital status (married/unmarried)	62/9	87.3/12.7
Gender (male/female)	40/31	56.3/43.7
Educational status		
Elementary school	15	21.1
Middle school	46	64.8
University	10	14.1
Occupation		
Worker	12	16.9
Peasant	36	50.7
Civil servant	7	9.9
Others[2]	16	22.5
Complication (yes/no)	5/66	7.0/93.0
Time since operation		
≤ 1 yr	16	22.5
> 1 yr, ≤ 2 yr	34	47.9
> 2 yr, ≤ 3 yr	21	29.6
Employment after donation		
Full-time	53	74.6
Part-time	9	12.7
No employment	9	12.7
Recipients		
Parenthood	9	12.7[1]
Children	9	12.7[1]
Couples	7	9.9[1]
Brothers and sisters	30	42.3[1]
Distant relatives	16	22.5[1]
Recipient health well-being		
Fine	56	78.9
Deterioration or death	15	21.1

[1]The sum of percentages is not equal to 100% due to rounding error; [2]Includes students, unemployed, *etc*.

were improved after donation. The demographics and clinical characteristics of the study population are shown in Table 1. The mean age of participants was 38.94 ± 10.44. Most donors were married (87.3%). More than half of the donors were male (56.3%), peasants (50.7%), and had achieved a secondary education level (64.8%). A total of 7.0% of donors experienced early or late complications including slight biliary leakage, pulmonary infection, and bodily pain. Many (47.9%) donor operations occurred 1-2 years before completing the questionnaires. Most donors worked full- or part-time after donation (87.3%). All donors were related to recipients, and most of them were close relatives (77.6%). The majority of recipients (78.9%) were in good health at the time of investigation.

HRQoL and psychological outcomes

The majority of scores on SF-36 domains did not significantly differ between donors and a representative sample (n = 1603) from the general population of Sichuan province in China (Table 2). Only scores in bodily pain (t = -2.387, P < 0.05) and social functioning (t = -2.246, P < 0.05) were significantly lower in donors compared to the general population, while the average donor physical functioning score was significantly higher than the general population (t = 2.230, P < 0.05).

Table 2 Health related quality of life after donation

SF-36 domains	Donors (71) mean ± SD	General population mean ± SD	t value	P value
Physical functioning	93.66 ± 7.26	90.80 ± 15.07	2.230	0.033
Role-physical	80.88 ± 33.18	79.51 ± 34.70	0.241	0.811
Bodily pain	71.29 ± 27.15	82.41 ± 21.25	-2.387	0.023
General health	67.33 ± 19.11	67.30 ± 21.97	0.010	0.992
Vitality	67.22 ± 18.72	71.44 ± 15.81	-1.234	0.227
Social functioning	79.69 ± 14.11	85.29 ± 18.06	-2.246	0.032
Role-emotional	76.47 ± 39.81	76.45 ± 38.47	0.003	0.998
Mental health	74.13 ± 17.12	73.52 ± 15.68	0.196	0.846

SF-36: Short Form-36.

Table 3 Psychological symptoms after donation

SCL-90-R dimensions	Donors (71) mean ± SD	Chinese norm mean ± SD	t value	P value
Somatization	1.41 ± 0.39	1.37 ± 0.48	0.600	0.553
Obsessive-compulsive behavior	1.50 ± 0.30	1.62 ± 0.58	-2.119	0.042
Interpersonal sensitivity	1.42 ± 0.32	1.65 ± 0.51	-4.183	< 0.001
Depression	1.39 ± 0.35	1.50 ± 0.59	-1.741	0.092
Anxiety	1.35 ± 0.37	1.39 ± 0.43	-0.708	0.485
Hostility	1.54 ± 0.44	1.48 ± 0.56	0.797	0.432
Phobic anxiety	1.11 ± 0.13	1.23 ± 0.41	-5.312	< 0.001
Paranoid ideation	1.25 ± 0.29	1.43 ± 0.57	-3.472	0.002
Psychoticism	1.25 ± 0.34	1.29 ± 0.42	-0.660	0.514

SCL-90-R: Symptom Checklist-90-Revised.

The average SCL-90-R scores of the general population were significantly greater than average donor scores in the areas of obsessive-compulsive behavior (t = -2.119, P < 0.05), interpersonal sensitivity (t = -4.183, P < 0.001), phobic anxiety (t = -5.312, P < 0.001), and paranoid ideation (t = -3.472, P < 0.01) (Table 3). These results indicate that the psychological well-being of liver transplant donors was higher than the general population in these dimensions.

Analysis of HRQoL

The general health domain of the SF-36, was significantly lower for female donors compared to male donors (t = 2.661, P < 0.05). Donors more than 40 years old scored higher in social functioning (t = 2.269, P < 0.05) and mental health (t = 2.184, P < 0.05). Donors who underwent surgery more than two years before the current study scored highest in physical functioning (F = 9.394, P = 0.001) and bodily pain (F = 3.513, P < 0.05), while those undergoing surgery less than one year prior to the study scored lowest. Quality of life differed significantly depending on donor employment status. Donors with full-time employment scored highest in role-physical (F = 5.790, P = 0.005), vitality (F = 9.018, P = 0.001), social functioning (F = 4.786, P < 0.05) and mental health (F = 11.051, P < 0.001). Interestingly, recipient health condi-

Table 4 Donor health related quality of life

Factors SF-36 domains	Groups mean ± SD	Groups mean ± SD	Groups mean ± SD	*t*/*F* value	*P* value
Gender	Male	Female			
General health	75.83 ± 22.09	59.78 ± 12.25		t = 2.661	0.012
Age (yr)	≤ 40	> 40			
Social functioning	75.00 ± 12.13	85.71 ± 14.59		t = 2.269	0.031
Mental health	68.89 ± 12.98	82.61 ± 20.00		t = 2.184	0.038
Time since operation (yr)	≤ 1	> 1, ≤ 2	> 2, ≤ 3		
Physical functioning	82.50 ± 2.89	94.42 ± 6.66[1]	98.33 ± 2.58[1]	F = 9.394	0.001
Bodily pain	52.67 ± 24.35	70.91 ± 27.57[2]	91.33 ± 13.43[1,2]	F = 3.513	0.042
Employment after donation	Full-time	Part-time	No employment		
Role-physical	93.64 ± 6.93	76.67 ± 12.91[3]	75.00 ± 22.36[3]	F = 5.790	0.005
General health	69.45 ± 18.01[4]	76.22 ± 18.54[4]	50.67 ± 16.03	F = 3.538	0.041
Vitality	74.24 ± 15.52	46.67 ± 8.12[3]	48.33 ± 14.38[3]	F = 9.018	0.001
Social functioning	84.09 ± 12.31	75.00 ± 14.43	66.67 ± 12.91[3]	F = 4.786	0.016
Mental health	81.82 ± 11.89	52.00 ± 5.24[3]	53.33 ± 11.50[3]	F = 18.137	< 0.001
PCS	58.51 ± 5.31	52.31 ± 5.01[3,4]	43.59 ± 5.52[3]	F = 11.051	< 0.001
MCS	54.31 ± 6.00	44.56 ± 3.42[3,4]	34.92 ± 2.66[3]	F = 32.748	< 0.001
Recipient health well-being	Well	Poor or death			
General health	71.57 ± 19.10	55.42 ± 9.03		t = 2.121	0.042
Social functioning	82.69 ± 11.77	66.67 ± 17.08		t = 2.763	0.010
Role-emotional	87.18 ± 31.38	41.67 ± 46.29		t = 2.603	0.028

Only statistically significant data are displayed. [1]Compared with group "≤ 1 yr", $P < 0.05$; [2]Compared with group "> 1, ≤ 2 yr", $P < 0.05$; [3]Compared with group "Full-time", $P < 0.05$; [4]Compared with group "No employment", $P < 0.05$. SF-36: Short Form-36; PCS: Physical component summary scale; MCS: Mental component summary scale.

Table 5 Correlation analysis between health related quality of life and psychological symptoms

SCL-90-R dimensions	SF-36			
	PCS		MCS	
	r value	*P* value	*r* value	*P* value
Somatization	0.200	0.290	-0.246	0.190
Obsessive-compulsive behavior	0.173	0.362	-0.421	0.020
Interpersonal sensitivity	-0.067	0.726	-0.545	0.002
Depression	-0.306	0.114	-0.557	0.002
Anxiety	-0.222	0.238	-0.393	0.032
Hostility	-0.335	0.071	-0.456	0.011
Phobic anxiety	0.118	0.535	-0.201	0.266
Paranoid ideation	0.035	0.853	-0.157	0.407
Psychoticism	0.028	0.881	-0.209	0.267

SCL-90-R: Symptom Checklist-90-Revised; SF-36: Short Form-36; PCS: Physical component summary scale; MCS: Mental component summary scale.

tion also influenced donor general health (t = 2.121, P < 0.05), social functioning (t = 2.763, P = 0.010), and role-emotional (t = 2.603, P < 0.05) (Table 4). Marital status, educational status, categories of occupation, complications, or donor-recipient relationship did not significantly affect quality of life.

To reduce the number of outcome variables regarding HRQoL, outcomes among donors were also compared using the PCS and the MCS of the SF-36. PCS (F = 11.051, P < 0.001) and MCS (F = 32.748, P < 0.001) scores were highest in donors with full-time employment and lowest in unemployed donors (Table 4). Other demographic and clinical factors did not affect PCS or MCS scores.

Table 5 presents the correlation coefficients between PCS and MCS scores of the SF-36 and the scores on the SCL-90-R subscales. MCS scores were significantly (all P < 0.05) correlated with obsessive-compulsive behavior (r = -0.421), interpersonal sensitivity (r = -0.545), depression (r = -0.557), anxiety (r = -0.393), and hostility (r = -0.456). There were no significant correlations between PCS scores and SCL-90-R scores.

DISCUSSION

Overall, donors reported a positive experience. The vast majority of donors stated that they would donate again, and almost all believed they had benefited from the donation. All donors were able to return to their (pre-donation) job a few months after donation (while some donors chose to quit their previous job). There were few significant differences in quality of life domains between the donors in the current study and the general population. Interestingly, donors reported a higher level of physical functioning than the general population. This observation has been previously described[15-17].

Female donors scored lower than male donors in the general health domain of the SF-36. This difference may be due to social and psychological factors[18,19]. The rates of psychological distress and physical illness are higher in women probably due to gender roles. Gove[20] points out that the highly structured roles of men tend to be causally linked to good mental health and low rates of morbidity, while the typical nurturing roles of women tend to be associated with a high level of social demand and lack of privacy. Furthermore, occupying a nurturing role impairs one's ability to effectively adopt a patient role[20].

Employment status, a measurement indicative of the

donor's ability to resume societal roles, was significantly related to quality of life. Previous research[21,22] has found that employed liver recipients reported better HRQoL than those unemployed after liver transplantation. However, the relationship between employment and HRQoL of donors remains elusive. While previous research[23,24] reported that most donors are able to return to pre-donation employment status within a few months, the direct relationship between employment status and HRQoL was not detailed until the current study.

Other factors impacting the quality of life included age of donor and time since operation. Older donors reported a significantly higher quality of life in domains such as social functioning and mental health. In addition, quality of life of donors more than one year after surgery was greater than that of donors who had undergone surgery during the previous time. These results suggest that HRQoL recovers with time post operation. In agreement, a study by Chan *et al*[25] found that donor quality of life, particularly the physical component, was most significantly affected during the first three postoperative months while physical and mental quality of life returned to pre-operation levels by a 6 to 12 mo period.

Despite previous reports[15,17] showing no relationship between recipient outcome and donor quality of life, the current study found that recipient well-being was an important factor influencing donor quality of life. The donors in the present study were all genetically and emotionally related to recipients. Throughout the donation process, donors were strongly concerned about the recipients. These emotional ties resulted in a strengthening of the relationships between donors and recipients and their families.

The resection of the right hepatic lobe is a safe operation and resulted in a good psychological outcome for most donors, irrespective of donation-related potential risks. The majority of donors were not anxious, did not feel coerced, and did not consider donation dangerous prior to the operation. Some donors reported excitement in facing a new experience and some said they could handle any consequences of the surgery. Only a few donors reported being anxious, being unsure about the operation, and experiencing increased stress prior to the operation. Some donors verbalized feelings of gratefulness and increased maturity post surgery. Most aspects of donor mental quality of life were significantly related to psychological symptoms. These results indicate the necessity of providing support to donors who experience negative feelings.

In conclusion, LDLT donors were healthy and the overall quality of life and psychological outcome were favorable. Employment after donation is an important factor significantly related to quality of life. Gender, age of donor, time since operation, and recipient health were all found to influence aspects of the quality of life of donors. Right hepatectomy is an acceptable procedure, with encouraging donor outcomes. Donor HRQoL and psychological status should continue to be monitored.

The current study has limitations that should be addressed. The data were collected at a single transplant center and the study design was a cross-sectional analysis which can be less informative than a longitudinal analysis. Nevertheless, the present study yielded important preliminary findings in mainland China. Longer follow-up periods and prospective studies will be necessary to identify long-term quality of life and psychosocial consequences of adult LDLT donors.

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COMMENTS

Background

Living-donor liver transplantation (LDLT) is an effective treatment for end-stage liver disease in selected recipients, especially in Asian countries where the cadaveric graft supply is markedly limited. The graft and recipient survival rates are excellent and equivalent to those after deceased-donor liver transplantation. However, the donor of LDLT is exposed to risks inherent to a surgical procedure, and may suffer a considerable psychological burden. Therefore, the safety of the donor operation and the health related quality of life (HRQoL) of the donor after surgery is critical while maintaining graft viability.

Research frontiers

Currently, Not much researches about HRQoL and psychological well-being of the living liver donors have been reported after LDLT. There is a lack of comprehensive and systemic assessment data on donors' HRQoL and psychological well-being after LDLT. So the present study evaluated the HRQoL and psychological well-being on the LDLT donors and identified some potential factors affecting their HRQoL.

Innovations and breakthroughs

This article is one of the few literatures on the quality of life and psychological well-being of the living liver donors after LDLT. The study is well constructed and well planned, and is a comprehensive and systemic assessment data on donors' HRQoL and psychological well-being after LDLT.

Applications

The present study yielded important preliminary findings on reseaches of the donors' quality of life and psychological well-being. The findings will have a significant impact on future clinical strategies.

Terminology

LDLT is a procedure in which a living person donates a portion of his or her liver to another. HRQoL is a multi-dimensional concept that includes domains related to physical, mental, emotional and social functioning.

Peer review

This is a good study contributing to this important area of liver transplantation. The authors have shown that the right donor hepatectomy is an acceptable procedure.

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CASE REPORT

Central nervous system vasculitis and polyneuropathy as first manifestations of hepatitis C

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Abstract

Sensory or motor peripheral neuropathy may be observed in a significant proportion of hepatitis C virus (HCV)-infected patients. However, central nervous system (CNS) involvement is uncommon, especially in cryoglobulin-negative subjects. We describe a case of peripheral neuropathy combined with an ischemic CNS event as primary manifestations of chronic HCV infection without cryoglobulinemia. Significant improvement was observed after antiviral therapy. We discuss the spectrum of neurological manifestations of HCV infection and review the literature.

Key words: Hepatitis C; Central nervous system; Polyneuropathy; Interferon-α

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INTRODUCTION

Hepatitis C virus (HCV) infection has become a major cause of liver disease with approximately 170 million people infected worldwide[1]. The severity of the disease varies widely, ranging from asymptomatic carrier state to cirrhosis and hepatocellular carcinoma. HCV chronic infection is often associated with abnormal immunological responses that can result in several extrahepatic conditions such as membranoproliferative glomerulonephritis, Sjögren's syndrome, idiopathic thrombocytopenic purpura, lichen planus, porphyria cutanea tarda, and mixed cryoglobulinemia[2]. Even though these conditions occur relatively infrequently, they significantly increase morbidity and mortality among HCV patients. Although sensory or motor peripheral neuropathy may be observed in a significant proportion of HCV-infected patients, central nervous system (CNS) involvement is uncommon, especially in cryoglobulin-negative subjects[3]. Here, we describe a patient with peripheral neuropathy combined with CNS vasculitis as primary manifestations of chronic HCV infection.

CASE REPORT

A previously healthy 37-year-old Caucasian woman presented to the emergency department in May 2003, with a 9-mo history of malaise, loss of appetite, and substantial weight loss (19.96 kg). Over the previous month, she had developed fatigue and muscle weakness, and become

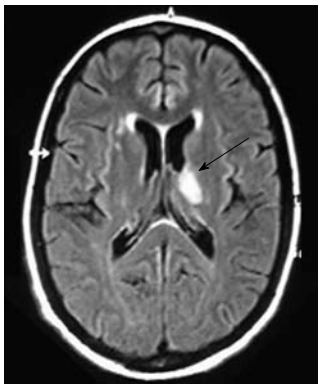

Figure 1 Magnetic resonance imaging of the head showing a 1.5-cm, high-signal lesion in the left thalamus, suggestive of ischemic injury (arrow).

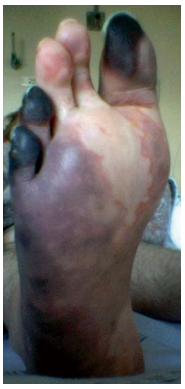

Figure 2 Necrotic lesions on the right forefoot due to severe vasculitis.

unable to perform several activities of daily living, such as hair brushing, climbing stairs and doing household chores. There was no history of blood transfusions or intravenous drug abuse. The patient was conscious and oriented to time and place. On examination, atrophy of the dorsal interosseous muscles, flaccid quadriparesis with hyporeflexia, and symmetrical distal sensory loss were noted. An electroneuromyographic study revealed sensorimotor polyneuropathy.

Over the next 24 h, she became increasingly disoriented. Magnetic resonance imaging of the head showed a T1 low, T2 and fluid-attenuated inversion recovery (FLAIR) high-signal lesion in the left thalamus, approximately 1.5 cm in diameter (Figure 1), which probably represented ischemic injury. In addition, small foci of increased signal intensity at the semioval center and subcortical white matter were identified on T2 and FLAIR sequences. A rheumatologic panel including antinuclear antibody, rheumatoid factor, anti-DNA and cardiolipin antibodies was negative. Thyroid-stimulating hormone, vitamin B12, and aminotransferases levels were within normal limits. Further testing showed negative serology for hepatitis B virus, HIV, syphilis, cytomegalovirus, and human T-lymphotropic virus 1/2. Enzyme immunoassay to detect HCV antibody was positive, as well as serum HCV-RNA by polymerase chain reaction (PCR). A liver biopsy confirmed chronic hepatitis with mild necroinflammatory activity and no fibrosis. We then considered that the diagnosis of CNS vasculitis and peripheral polyneuropathy was probably related to chronic HCV infection. Serum cryoglobulins were persistently negative after seven determinations. The patient was initially treated with intravenous methylprednisolone followed by oral prednisone, with resolution of her symptoms. Subsequently, standard interferon-α (3 mU three times per week) plus ribavirin (1 g/d) were added to steroid maintenance therapy. During HCV treatment, an attempt to reduce prednisone dose resulted in the development of necrotic lesions on the right forefoot (Figure 2), which led to its amputation. In spite of permanent discontinuation of antiviral drugs and the need for increasing corticosteroid dosage, the patient showed sustained virological response, with HCV RNA persistently undetectable in serum by sensitive PCR-based assay. She remains asymptomatic, until last seen, under low dose prednisone.

DISCUSSION

Although the precise frequency of peripheral neuropathy in HCV-infected patients is unknown, it is considered the most common neurological complication in this setting. In a French cohort of 321 subjects with chronic hepatitis C, symptomatic peripheral neuropathy was observed in 9% of the cases[4]. Even though the neurological findings were more frequent among cryoglobulin-positive patients, in this study, a significant proportion of cryoglobulin-negative individuals presented with peripheral nervous system involvement (17% *vs* 8%). Other reports of peripheral neuropathy in HCV-infected patients without detectable cryoglobulins[5-7] indicate that, although the presence of cryoglobulins seems to be an important feature in these cases, there are possibly other factors contributing to the development of peripheral neuropathy. In a study including 51 patients with HCV infection and neuropathy, Nemni *et al*[7] showed that 22% of the subjects had undetectable serum cryoglobulins. Cryoglobulin-negative individuals were more likely to have mono- or multiple neuropathy. Interestingly, the morphological findings in the sural nerve from cryoglobulin-negative and -positive patients are consistent with an ischemic mechanism of nerve damage. The authors stated that the vasculitic process in cryoglobulin-negative HCV subjects was probably secondary to complement pathway activation by HCV itself, or by an interaction between the virus and the host immune system. A direct role of HCV in the pathogenesis of peripheral neuropathy was also proposed, based on the finding of HCV RNA in nerve biopsy specimens[8]; however, this association remains to be confirmed.

Specific CNS involvement is more rarely reported in HCV-infected patients. CNS involvement, however, may present different facets, such as fatigue, depression, cognitive impairment and vasculitis. Although it may be the initial extrahepatic manifestation of HCV infection, well-documented reports on CNS involvement in patients with HCV-associated vasculitis are rare and include mostly cryoglobulin-positive patients[9-11]. Stroke

episodes, transient ischemic attacks, progressive reversible ischemic neurological deficits, lacunar infarctions, or encephalopathic syndrome, commonly attributed to ischemia or rarely to hemorrhage, may occur[12]. Similar to HCV-related peripheral neuropathy, the mechanism behind the CNS vasculitic process in HCV infection is poorly understood, but it has been postulated that recurrent cryoglobulin precipitation with complement fixation and/or HCV-related induction of the innate mechanism of complement activation might be involved in ischemic and inflammatory tissue damage[7]. Although the exact pathway is not known, HCV-induced vasculitis without cryoglobulinemia by the other mechanisms previously discussed for peripheral neuropathy may be responsible for the CNS findings in this case.

The treatment of HCV-associated peripheral neuropathy in cryoglobulin-positive individuals is based on anti-HCV drugs. Combination therapy with interferon (pegylated or not) plus ribavirin may induce a complete clinical response in a significant proportion of patients with HCV-related systemic vasculitis, and consequently, in those with cryoglobulin-related peripheral neuropathy[13,14]. The role of HCV therapy in subjects with cryoglobulin-negative peripheral neuropathy is unclear. Lidove *et al*[5] have reported significant neurological improvement in two cryoglobulin-negative patients treated with interferon monotherapy. However, long-term follow-up was not reported and the possibility of development or worsening of peripheral neuropathy in interferon-based treatments is a major concern in this setting[15]. Data about the safety and efficacy of interferon-based regimens in the treatment of HCV-associated CNS vasculitis are even scarcer. There are a few case reports showing favorable outcomes in cryoglobulinemic subjects treated with corticosteroids or interferon for CNS involvement[12,16,17]. However, such reports cannot support a solid recommendation, especially for those patients with cryoglobulin-negative CNS vasculitis. In addition, it should be emphasized that for cases of severe cryoglobulinemia-associated vasculitis (as those with rapidly progressive renal failure or neurological involvement), it is recommended that antiviral therapy should be delayed for 2-4 mo, while they are submitted to aggressive therapy with plasmapheresis, corticosteroids (intravenous methylprednisolone followed by oral prednisone), and either cyclophosphamide or rituximab[18].

In this report, we describe a patient with peripheral neuropathy combined with an ischemic CNS event as primary manifestations of chronic HCV infection. The absence of other classical risk factors for ischemic stroke, the association with peripheral vasculitis and the improvement observed after steroid therapy suggests a vasculitic origin for the neurological findings. Although this report cannot prove a definite cause-and-effect of HCV infection and the neurological manifestations observed, an important role of HCV is suggested by the significant improvement observed after the HCV sustained virological response. Another interesting finding in the present case was the achievement of sustained viral clearance in spite of the prolonged use of steroids. Although we have not been able to evaluate viral load during therapy sequentially, previous studies have shown that exposure to steroids increases HCV viral load, both in liver transplant patients and in the non-transplant setting[19,20].

In conclusion, our case highlights the need for clinicians to broaden consideration of differential diagnoses, with particular attention to atypical features of common diseases. Testing for HCV should be performed in all cases of neurological signs of uncertain origin, even in the absence of usual risk factors for hepatitis C. Successful antiviral therapy may lead to a significant improvement of neurological manifestations and should be considered in these cases.

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CASE REPORT

Concurrent systemic AA amyloidosis can discriminate primary sclerosing cholangitis from IgG4-associated cholangitis

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Abstract

Chronic hepatobiliary inflammatory diseases are not widely acknowledged as underlying disorders of systemic AA amyloidosis, except epidemic schistosomiasis. Among them, primary sclerosing cholangitis (PSC) might initiate amyloid A protein deposition in diverse tissues, giving rise to systemic amyloidosis, due to a progressive and unresolved inflammatory process, and its possible association with inflammatory bowel diseases. Nevertheless, only one such case has been reported in the literature to date. We report a 69-year-old Japanese woman with cirrhosis who was diagnosed with PSC complicated with systemic AA amyloidosis, without any evidence of other inflammatory disorders. As a result of cholestasis in conjunction with biliary strictures and increased serum IgG4, the presence of $IgG4^+$ plasma cells was examined systemically, resulting in unexpected documentation of Congo-red-positive amyloid deposits, but not $IgG4^+$ plasma cells, in the liver, stomach and salivary glands. Elevated serum IgG4 is the hallmark of IgG4-related disease, including IgG4-associated cholangitis, but it has also been demonstrated in certain patients with PSC. Amyloid A deposits in multiple organs associated with an indolent clinical course that progresses over many years might have a diagnostic value in discriminating PSC from IgG4-associated cholangitis.

Key words: Primary sclerosing cholangitis; IgG4-associated cholangitis; AA amyloidosis

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is an intractable fi-

bro-inflammatory disease of the bile ducts that is characterized by biliary strictures without any underlying insults, e.g., immunodeficiency, ischemia, and biliary toxins[1]. PSC usually follows an indolent but progressive course, which results in eventual death or liver transplantation in the majority of patients. A single center study in Germany demonstrated that the estimated median survival from the time of diagnosis to death or time of liver transplantation was 9.6 years; 39.6% of patients underwent liver transplantation, while 14.3% of them developed hepatobiliary malignancies[2]. Definite diagnosis is thus required in cases with suspected lesions, especially to discriminate PSC from IgG4-associated cholangitis (IAC); a recently defined disorder with better prognosis[3-5].

IAC consists of a biliary stricture that responds to or improves with corticosteroid therapy[6], and is recognized as one of a variety of IgG4-related disease that exhibits a wide range of clinical manifestations. The clinical diagnostic criteria for IgG4-related disease consists of three parts: namely, enlarged/thickened lesions in one or more organs; elevated serum IgG4 levels (≥ 135 mg/dL); and histopathological findings[5]. Although IgG4 levels are usually higher in patients with IAC than in those with PSC, raised serum IgG4 levels have been recently reported in 9%-36% of patients with PSC[7,8]. Therefore, the identification of $IgG4^+$ plasma cell infiltrates in the bile duct as well as in other organs[5,9] is important in making a diagnosis.

In this report, we describe a patient with cirrhotic PSC who had elevated levels of serum IgG4. Multiple organ biopsies were performed to obtain a definitive diagnosis and rule out IAC. Unexpectedly, Congo-red-positive amyloid deposits, but not $IgG4^+$ plasma cells, were demonstrated in the liver, stomach and salivary glands. Subsequently, raised levels of serum amyloid A protein (SAA) were confirmed, resulting in a diagnosis of PSC complicated with systemic AA amyloidosis, despite the absence of known genetic susceptibility[10]. This is the second report describing the concurrence of systemic AA amyloidosis in PSC. A sustained acute phase response involving the overproduction of SAA over a period of many years is likely to characterize indolent hepatobiliary inflammation in PSC, but not in IAC.

CASE REPORT

A 69-year-old Japanese woman was referred to our hospital with progressive elevation of cholestatic liver enzymes in October 2009. She had a history of endoscopic sphincteropapillotomy for choledocholithiasis at age 47 years, at which time, PSC was also suspected due to the irregularity of the extra- and intrahepatic bile duct walls, as revealed by endoscopic retrograde cholangiopancreatography (ERCP). Her cholestatic liver tests subsequently remained abnormal despite removal of all gallstones, indicating the presence of PSC. She had never been immunocompromised. Fatigue, pruritis, and abdominal fullness had worsened even after administration of ursodeoxycholic acid (600 mg/d; 13.2 mg/kg body weight) and she was therefore admitted to our hospital in October 2010. On admission, mild jaundice was apparent, and blood tests revealed elevated liver enzymes along with increased acute phase proteins, i.e., aspartate aminotransferase 109 IU/L (normal < 37 IU/L), alanine aminotransferase 80 IU/L (normal < 39 IU/L), alkaline phosphatase 871 IU/L (normal < 359 IU/L), γ-glutamyltranspeptidase 72 IU/L (normal < 75 IU/L), total bilirubin 3.2 mg/dL (normal < 1.2 mg/dL), C-reactive protein 2.66 mg/dL (normal < 0.3 mg/dL), and SAA protein 303.9 mg/L (normal < 8.0 mg/L). With a Child-Pugh score of 10, her functional hepatic reserve was reduced and she had moderate ascites. Anti-nuclear, anti-smooth muscle, anti-mitochondrial, and perinuclear anti-neutrophil cytoplasmic antibodies were negative. Serum IgG was 2890 mg/dL (normal < 1700 mg/dL), including elevated IgG4 level of 251 mg/dL (normal < 105 mg/dL). Although serum IgE was 15 400 IU/mL (normal < 361 IU/mL), antibodies against parasites, including liver flukes, were negative. Viral markers for hepatitis B and C were both negative. Abdominal contrast-enhanced computed tomography showed biliary strictures from common hepatic duct to the second to third branches, accompanied by dilatation of the distal bile ducts. Atrophy of the right hepatic lobe in conjunction with collateral vessel formation around the lower esophagus confirmed cirrhosis. The size and contours of the pancreas were normal. ERCP revealed irregularities of the walls of the lower common, hilar and intrahepatic bile ducts, which were accompanied by multiple strictures and a "pruned-tree" appearance in the intrahepatic bile ducts (Figure 1). Deterioration of liver function likely resulted from progression of PSC, but given the equivocal ERCP results and elevated IgG4 levels, we elected to rule out IgG4-related disease, particularly IAC. The biopsied specimen from the distal common bile duct contained only erosive duct mucosa, preventing the visualization of $IgG4^+$ lymphoplasmacytic infiltration. We therefore performed biopsies of the major duodenal papilla[9], the salivary glands, and the gastric mucosa in order to investigate the possibility of multiple organ infiltration of $IgG4^+$ plasma cells.

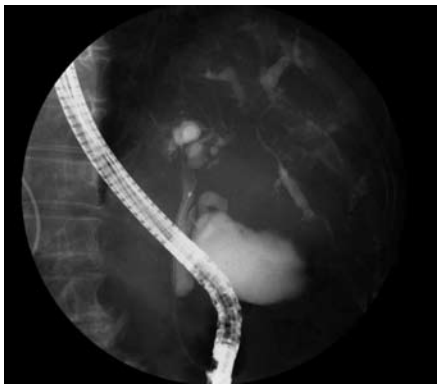

Figure 1 Endoscopic retrograde cholangiopancreatography revealed multiple strictures of the hilar and intrahepatic bile ducts, with a "pruned-tree" appearance, accompanied by dilatation of the distal bile ducts.

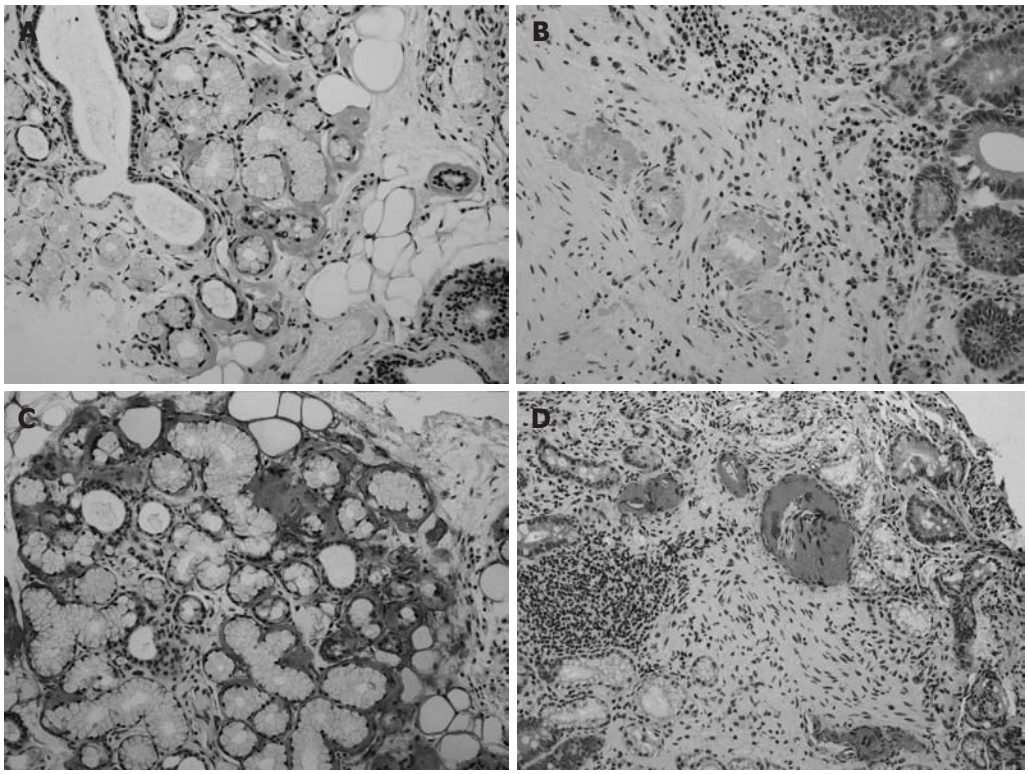

Figure 2 Histology of the salivary glands and the stomach by hematoxylin and eosin stain (200 ×). Amorphous eosinophilic materials in the subglandular and stroma of the salivary glands (A) and submucosal stroma of the stomach (B) were demonstrated. They were found to be Congo-red-positive. Immunohistochemical staining with anti-human amyloid A antibody (200 ×) confirmed a positive AA stain in the salivary glands (C) and stomach (D).

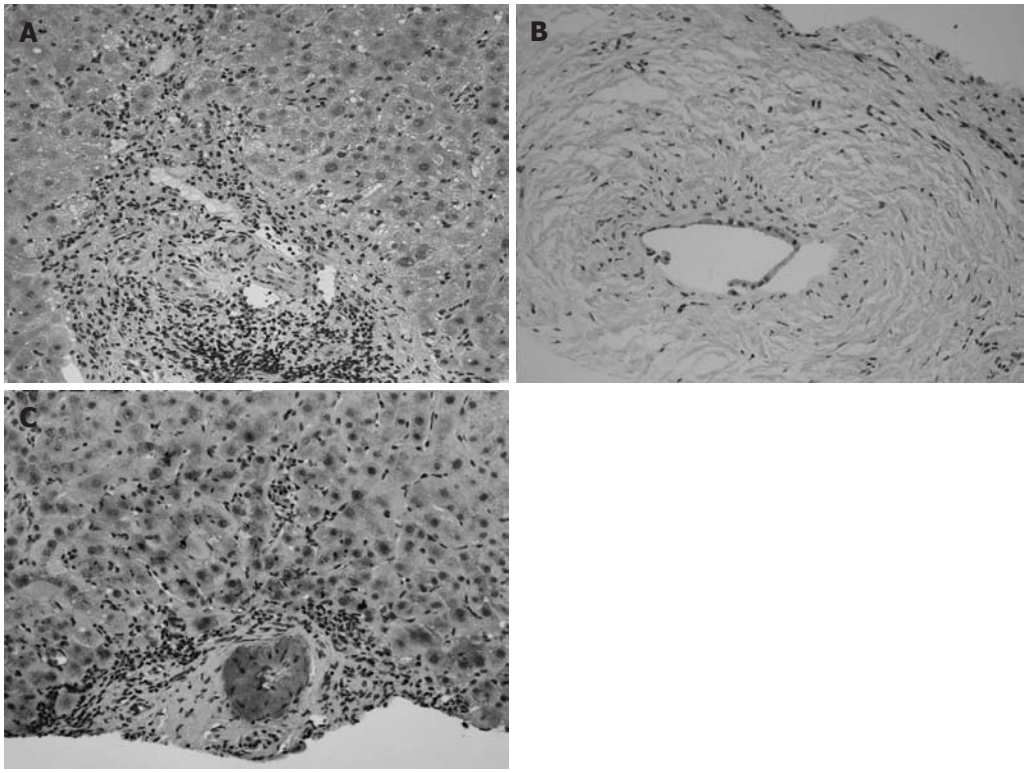

Figure 3 Histology of the liver by hematoxylin and eosin stain. An increase in lymphoneutrophilic infiltrates in the portal tracts, interface hepatitis with ductular proliferation, cholate stasis (400 ×, A), and damaged interlobular bile ducts with collagenous periductal thickening (200 ×, B) were revealed. Amyloid deposition in the vessel walls of the portal tracts was also apparent in immunohistochemical staining (200 ×, C).

Unexpectedly, IgG4$^+$ plasma cells were scarcely found by immunohistochemistry, while Congo-red-positive, amorphous eosinophilic materials were demonstrated in the subglandular and stroma of the salivary glands and in the submucosal stroma of the stomach (Figure 2A and B). Potassium permanganate sensitivity and positive AA immunohistochemical staining confirmed that these materials were AA amyloid deposits (Figure 2C and D). Subsequent liver histology revealed an increase in lymphoneutrophilic infiltrates with some eosinophils in the portal tracts, interface hepatitis and bridging fibrosis, damaged interlobular bile ducts with collagenous periductal thickening, marked ductular proliferation, and cholate stasis (Figure 3A and B). Amyloid deposition in the vessel

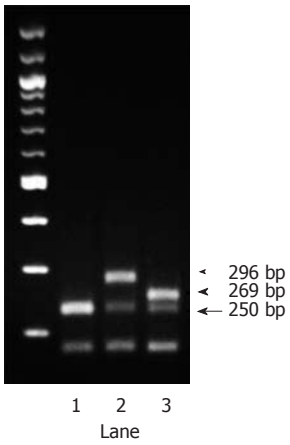

Figure 4 Serum amyloid A1 genotyping by polymerase chain reaction-restriction fragment length polymorphism analysis (2% agarose gel electrophoresis). Arrow (250 bp), arrow head (269 bp) and small arrow head (296 bp) correspond to serum amyloid A1 allele 1.5, 1.3 and 1.1, respectively. Lane 1: 1.5/1.5 homozygosity (our patient); Lane 2: 1.1/1.5 heterozygosity (control patient); Lane 3: 1.3/1.5 heterozygosity (another control patient).

walls of the portal tracts was also apparent (Figure 3C). Oral administration of 30 mg/d prednisolone for 1 wk failed to show any beneficial effect on cholestasis, therefore, a diagnosis of IAC turned out to be implausible. Abnormal uptake was not found in positron emission tomography; again, excluding underlying IgG4$^+$-related lymphoproliferative disease, as well as IgE myeloma. The normal colonoscopic appearance of the colonic mucosa, as well as the histology that only indicated nonspecific inflammation of the intestine, excluded a concurrent diagnosis of inflammatory bowel disease. Currently known patterns of genetic susceptibility to systemic AA amyloidosis were found to be absent[10,11]. SAA1 genotyping by polymerase chain reaction-restriction fragment length polymorphism analysis[12] revealed homozygosity of SAA1.5/1.5 (Figure 4), and sequencing of whole Mediterranean fever (MEFV) exons demonstrated no amino acid substitution mutations (data not shown). Based on all of the above data, we diagnosed the patient with PSC complicated by systemic AA amyloidosis.

DISCUSSION

In this study, we presented a patient with PSC complicated by systemic AA amyloidosis. To the best of our knowledge, this is the second reported case of concurrent PSC and systemic AA amyloidosis, and it included detailed information on pathology as well as on genetic susceptibility to AA amyloidosis.

Sustained overproduction of SAA in association with chronic unresolved inflammation, as demonstrated in our case, is essential for the development of amyloidosis[13]. Nevertheless, susceptibility to AA amyloidosis differs among various diseases. According to Lachmann et al, the most prevailing underlying inflammatory disorder is chronic inflammatory arthritis, followed in descending order by chronic sepsis, periodic fever syndromes, and Crohn's disease[13]. Although PSC is indolent, progressive inflammatory hepatobiliary disease results in cholestatic cirrhosis. Nonetheless, only one other case of PSC has been reported as a cause of AA amyloidosis[14]. Even assuming that about 6% of AA amyloidosis patients do not have underlying inflammatory disorders[13], AA amyloidosis in our case was likely to be secondary to PSC, as coexisting inflammatory bowel disease was excluded. The patient had neither the SAA locus conferring susceptibility to AA amyloidosis in Japanese rheumatoid arthritis, namely SAA1.3, nor MEFV amino acid substitution mutations that are responsible for familial Mediterranean fever, an autoinflammatory disease. Multiple factors affecting amyloid deposition in tissues, such as amyloid P and apolipoprotein E, might have cooperatively contributed to the pathogenesis of AA amyloidosis in this case.

Regarding the diagnosis of PSC, discrimination of IAC is of primary importance owing to better prognosis of the latter with corticosteroid therapy. Serum IgG4 levels in conjunction with cholangiographic features have clinical relevance in this process. In our case, while the elevated serum IgG4 level favored diagnosis of IAC, the "pruned-tree" appearance of the intrahepatic bile ducts coupled with stenosis of the lower common bile duct were equivocal. Moreover, the fact that 9%-36% of patients with PSC also show mildly elevated IgG4[7,8] indicates the need for additional parameters to facilitate differential diagnosis. The presence of IgG4$^+$ plasma cells in the bile ducts and in other organs is suggestive of IgG4-related disease and thus has more diagnostic specificity[5]. In our case, PSC was confirmed by the absence of IgG4$^+$ plasma cells in the examined organs. On the other hand, unexpected demonstration of Congo-red-positive amyloid deposition in the salivary glands, stomach and liver prompted us to consider the distinct implications of these findings on the differential diagnosis, because AA amyloid found in various organs might have diagnostic specificity for PSC. To the best of our knowledge, there have been no case reports describing coexistence of AA amyloid deposits with IgG4$^+$ plasma cells in IgG4-related diseases. A sustained acute phase response in PSC might be a sufficient cause for AA amyloid deposition. Alternatively, mechanistically distinct and/or mutually exclusive inflammatory processes occurring in PSC and IAC, in the latter case reportedly Th-2-dependent[5], might be responsible for the phenomenon. At any rate, estimation of the incidence of AA amyloidosis in PSC[15], as well as in IAC, in a large cohort is necessary to verify our hypothesis.

The proper treatment of PSC complicated by systemic AA amyloidosis remains to be determined. The aim of treatment of AA amyloidosis is generally considered to be the suppression of underlying inflammatory conditions, thereby reducing SAA concentrations[13]. Immunosuppressive agents including anti-tumor necrosis factor therapies are often administered for this purpose, with the exclusion of conditions involving chronic sep-

sis, such as bronchiectasis. Renal dysfunction has been reported as a predominant disease manifestation, and progression to end-stage renal failure has been linked to increased mortality of systemic AA amyloidosis[13]. Regression of AA amyloid deposits (as evaluated by serum amyloid P scintigraphy), that is associated with median SAA concentration during anti-inflammatory therapy, is inversely correlated to the outcomes of renal dysfunction[13]. Although the first case of coexisting PSC and systemic AA amyloidosis reported in the literature experienced relief of symptoms and biochemical improvement as a result of the combination of corticosteroid and azathioprine, the patient experienced progression of amyloidosis-induced nephrotic syndrome followed by its reversal after liver transplantation[14]. Maintaining SAA in a low-normal range through use of anti-inflammatory agents was also difficult in our case; short-term use of prednisolone showed no benefit and colchicine did not decrease serum SAA (data not shown). Administration of tocilizumab, a humanized anti-interleukin 6 receptor antibody that strongly suppresses SAA levels, might be promising for patients with secondary AA amyloidosis who are not responding adequately to treatment for underlying inflammation[16].

In summary, we presented a female PSC patient who had concurrent systemic AA amyloidosis. Is AA amyloidogenesis more specific to PSC than IAC? Could AA amyloid deposition discriminate PSC from IAC? Reexamination of AA amyloid deposition in PSC cases in a large cohort will help answer these and other relevant questions.

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1361 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article *via* online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

Aims and scope

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing
Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

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Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Statement of human and animal rights
When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

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Title: Title should be less than 12 words.

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Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Abstract

There are unstructured abstracts (no less than 256 words) and structured abstracts (no less than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no less than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections.

AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of *P* values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of *P* values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Pleased provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

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Authors: the name of the first author should be typed in boldfaced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

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

Organization as author

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

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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Patent (list all authors)

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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

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