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doi:10.3748/wjg.14.5454

World J Gastroenterol 2008 September 21; 14(35): 5454-5460
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Polymorphisms of microsomal triglyceride transfer protein in different hepatitis B virus-infected patients

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Supported by F.Hoffmann-La Roche Ltd Switzerland and the National High Technology Research and Development Program of China (863 Program), No. 2006AA02A411
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Received: May 19, 2008 Revised: July 7, 2008
Accepted: July 14, 2008
Published online: September 21, 2008

Abstract

AIM: To identify the two polymorphisms of microsomal triglyceride transfer protein (*MTP*) gene in the Chinese population and to explore their correlation with both hepatitis B virus (HBV) self-limited infection and persistent infection.

METHODS: A total of 316 subjects with self-limited HBV infection and 316 patients with persistent HBV infection (195 subjects without familial history), matched with age and sex, from the Chinese Han population were enrolled in this study. Polymorphisms of MTP at the promoter region -493 and at H297Q were determined by the allele specific polymerase chain reaction (PCR).

RESULTS: The ratio of males to females was 2.13:1 for each group and the average age in the self-limited and chronic infection groups was 38.36 and 38.28 years, respectively. None of the allelic distributions deviated significantly from that predicted by the Hardy-Weinberg equilibrium. There was a linkage disequilibrium between H297Q and -493G/T (D' = 0.77). As the χ^2 test was used, the genotype distribution of *MTP*-493G/T demonstrated a significant difference between the self-limited infection group and the entire chronic group or the chronic patients with no family history (χ^2 = 8.543, P = 0.015 and χ^2 = 7.199, P = 0.019). The allele distribution at the *MTP*-493 position also demonstrated a significant difference between the study groups without family history (χ^2 = 6.212, P = 0.013). The T allele emerged as a possible protective factor which may influence the outcomes of HBV infection (OR: 0.59; 95% CI: 0.389-0.897).

CONCLUSION: The polymorphism of the *MTP* gene, T allele at -493, may be involved in determining the HBV infection outcomes, of which the mechanism needs to be further investigated.

Key words: Hepatitis B virus; Microsomal triglyceride transfer protein; Single nucleotide polymorphism; Self-limited HBV infection; Chronic hepatitis B; Clinical outcomes

Peer reviewer: Eva Herrmann, Professor, Saarland University, Kirrberger Str., Homburg/Saar 66421, Germany

Yang ZT, Zhang XX, Kong XF, Zhang DH, Zhang SY, Jiang JH, Gong QM, Jin GD, Lu ZM. Polymorphisms of microsomal triglyceride transfer protein in different hepatitis B virus-infected patients. *World J Gastroenterol* 2008; 14(35): 5454-5460 Available from: URL: http://www.wjgnet.com/1007-9327/14/5454.asp DOI: http://dx.doi.org/10.3748/wjg.14.5454

INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide, especially in several areas of Asia and Africa. Most infected individuals can clear the virus, while only 5%-10% develop chronic hepatitis and remain in a persistent viral state[1,2]. The reasons for viral persistence are poorly understood, but host genetic factors are likely to influence the disease outcome[3].

Molecular genetics methods have increased our

ability to discover variations in the human genome and to correlate them with disease. Single nucleotide polymorphisms (SNPs) are used to characterize gene variations. Genetic associations can provide clues to fundamental questions about the pathogenesis of diseases and lead to new therapeutic avenues[4]. For chronic hepatitis B, this approach may help determine the basis for viral persistence and the development of end-stage complications such as cirrhosis or hepatocellular carcinoma. Initial genetic studies of viral hepatitis focused on human leukocyte antigen (*HLA*) associations[5] and polymorphisms in the promoter or coding region of several genes, such as interleukin-10 (*IL*-10), interferon-γ (*IFN*-γ), vitamin-D receptor (*VDR*), *etc*[6-10], and demonstrate some relationships with the outcome of HBV infection.

The liver is the major organ for the production of plasma lipoproteins, their uptake from plasma and their catabolism[11]. The production of apolipoprotein B (apoB)-containing lipoproteins by the liver is required for the assembly and secretion of very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs)[12-16]. The microsomal triglyceride transfer protein (MTP) also plays a key role in apoB secretion by catalyzing the transfer of lipids to the nascent apoB molecule as it is co-translationally translocated across the endoplasmic reticulum membrane[17,18]. Recent studies have shown that the polymorphism at *MTP*-493 is responsible for a change in the *MTP* gene at the transcription level, and that this is prone to influence the intrahepatic triglyceride content[19-21].

Hepatic steatosis frequently occurs during chronic hepatitis B and C. In a transgenic mouse model, hepatitis C virus (HCV) core protein has been shown to inhibit the MTP activity and to modify the hepatic VLDL assembly and secretion[22]. However, no data are available to demonstrate the functional polymorphism of *MTP*-493T/G in HBV-infected patients. The aim of this pilot study was to identify the two polymorphisms of the *MTP* gene in the Chinese population by SNP and to explore their correlation with both HBV self-limited infection and persistent infection.

PATIENTS AND METHODS

Human subjects

In China, 90% of Chinese people are Han and the other 10% derive from 55 minority populations. We enrolled 632 Han Chinese subjects from Ruijin Hospital of Shanghai Jiaotong University Medical School. Among them, 316 had persistent HBV infection (including 195 patients with no family history of chronic hepatitis B) and 316 had previously self-limited HBV infection with no family history. Age and sex were matched between these groups.

The diagnostic criteria for persistent HBV infection were based on the presence of hepatitis B surface antigen (HBsAg) and anti-core IgG-antibody (Anti-HBc), and the absence of anti-hepatitis B surface antibody (Anti-HBs) for more than 6 mo. The mean time from the presumed onset of HBV infection was defined as the first documented seropositivity for HBsAg with or without elevated serum liver enzyme.

Self-limited hepatitis B virus infection was defined as being positive for anti-HBs and anti-HBc, in the absence of previous HBV vaccination, and a negative family history of chronic hepatitis B. Serum HBV-DNA was analyzed to exclude patients with occult HBV infection.

Subjects negative for all HBV markers were not included in the study as these subjects were unlikely to have been exposed to HBV. If they had been exposed to the virus, it would be impossible to predict their outcome.

Patients with concurrent hepatitis A, C, D, E or human immunodeficiency virus (HIV) infection were excluded from the study. Patients with liver disease caused by other factors, such as excess alcohol consumption and autoimmune hepatitis, were also excluded from the study. Our study conforms to the ethical guideline of the 2004 Declaration of Helsinki.

Serological test

Five milliliters of whole blood samples was collected from each subject, the sera were stored at -20℃. Serology for HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc was conducted in accordance with the manufacturer's protocol (Axsym, Abbott).

Genomic DNA extraction

Genomic DNA was isolated using a genomic DNA purification kit (PUREGENE) according to its manufacturer's instructions. DNA samples were quantified with a biophotometer (Eppendorf) and subjected to allele specific real-time polymerase chain reaction (PCR).

Genotyping of gene polymorphisms

Polymorphisms of MTP, including *MTP*-493G/T and H297Q were analyzed. We used the Allele Specific PCR Primer Design Program provided by Roche to design primers (Table 1).

All amplifications were performed on ABI-7000 (real-time PCR) with a 50 μL reaction mixture containing 30 ng of genomic DNA, 0.2 μmol/L per primer, PCR buffer, 0.2 μmol/L of each dNTP (Promega), 4% DMSO (Fisherbrand), 2.4% glycerine, 5 units of Delta Z05 DNA polymerase (Roche), 1 × SYB green (Cambrex). Each genotyping contains 2 amplifications, with one common primer and two specific primers, respectively. To genotype the polymorphisms at the promoter region-493 and at the coding region of MTP at amino acid position 297, primers *MTP*-493-1, *MTP*-493-2, *MTP*-493-cp and MTP H297Q-1, MTP H297Q-2, MTP H297Q-cp were used to analyze the *MTP*-493 G/T and MTP H297Q polymorphisms, respectively (Table 1). Amplification was performed with activation and denaturation at 94℃ and at 95℃ and an annealing temperature of 60℃, respectively.

Genotypes were determined by the difference in cycling numbers (ΔCT) of 2 amplification curbs with the same genomic DNA and the melting curbs, according to the manufacturer's (Roche) instructions.

Statistical analysis

The frequencies of *MTP*-493G/T and *MTP* H297Q alleles were compared between the chronic infection and self-limited infection groups by the χ^2 test. Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotype frequencies using the χ^2 test. To analyze the linkage disequilibrium (LD), pair wise LD was analyzed between two loci on MTP by evaluating the measurement of D'. The difference between the probabilities of observing the alleles independently in the population is: $f(D) = f(A_1B_1) - f(A_1)f(B_1)$, where A and B refer to two genetic markers and *f* is their frequency. D' is obtained from D/Dmax and a value of 0.0 suggests independent assortment, whereas 1.0 means that copies of an allele occur exclusively with one of the possible alleles of the other marker. Analysis of D' was performed using HAPLOVIEW 3.0. The odds ratio with a 95% confidence interval, *P* values and Mantel-Haenszel test were calculated using SAS 8.0 to explore the SNP which may independently influence the outcome of HBV infection.

Table 1 Positions of analyzed SNP and primers used in this study

Genes (Ref.)	SNP	Primers	Sequences (5'-3')
MTP H297Q rs#2306985[23]	C/G	-1	CAGGTCTTCCAGAGCCAC
		-2	CAGGTCTTCCAGAGCCAG
		-cp	ATTTGTCTGCACTTACAGAAGGA
MTP-493[20]	G/T	-1	ATTTAAACTGTTAATTCATACCACA
		-2	TTTAAACTGTTAATTCATACCACC
		-cp	CTTTAACATTATTTTGAAGTGATTGG

Table 2 General characteristics of 632 subjects

	Self-limited HBV infection (*n* = 316)	Chronic hepatitis B (*n* = 316)	
		With family history (*n* = 121)	No family history (*n* = 195)
Male	215	80 (25.3%)	135 (42.7%)
Female	105	41 (13.0%)	60 (19.0%)
Age (yr)	38.28 ± 11.44	36.93 ± 12.28	39.12 ± 10.84
Age range (yr)	9-75	9-69	16-75
ALT level (× ULN)	Normal	3.62 ± 3.48	3.12 ± 2.62
HBV-DNA level (Log)	Undetectable	5.45 ± 2.34	

RESULTS

Demographic characteristics of subjects

In the 2 groups matched for age and sex, the male to female ratio was 2.13:1 (215:101) in each group. The distribution of age in the chronic hepatitis B and self-limited groups, calculated by SAS respectively, was normal ($P = 0.07$ and 0.182). The mean age of subjects in the two groups was 38.28 years and 38.36 years, respectively, with no significant deviation (STDEV was 11.44 and 11.12). In the chronic hepatitis B subgroups, 121 patients (80 males and 41 females) had a family history while 195 (135 males and 60 females) had no family history of liver disease. Serum alanine aminotransferase (ALT) levels in the self-limited group were normal, and 3 times higher than the upper normal limit in the chronic hepatitis B group. Serum HBV-DNA was detectable in each study subject but undetectable (< 3 $\log_{10}$) in the self-limited group, whereas it was positive in the chronic hepatitis B group (5.45 ± 2.34 $\log_{10}$).

The general characteristics of our study subjects are summarized in Table 2.

Allele frequencies and linkage disequilibrium

The polymorphisms of *MTP* H297Q and *MTP*-493G/T were analyzed in 632 subjects of the Chinese Han population in Shanghai. The T minor allele frequency of promoter polymorphisms-493 in the *MTP* gene was 0.123, whereas the G frequency of missense polymorphism H297Q was 0.668. The 2 SNPs of the *MTP* gene showed a statistically significant linkage disequilibrium (D' = 0.77, $P < 0.05$). None of the allelic distributions deviated significantly from that predicted by the Hardy-Weinberg equilibrium (calculated by SAS, Chi-Square $P > 0.05$).

Association of SNP genotypes with outcomes of HBV infection

The genotype distribution of *MTP*, depending on the outcome of HBV infection, is shown in Table 3. The genotype frequencies of *MTP* H297Q, CC, CG and GG were 0.104, 0.468 and 0.428 in the self-limited group while 0.104, 0.443 and 0.453 in the chronic group, respectively. The frequencies of the TT, TG and GG genotypes of *MTP*-493G/T were 0.013, 0.253 and 0.734 in the self-limited group, and 0.025, 0.165 and 0.810 in the chronic group, respectively. The χ^2 test was used to analyze the association of genotype distribution with HBV infection outcomes. The distribution of *MTP*-493G/T was significantly different between the self-limited and chronic hepatitis B groups, both before and after adjustment for family history ($\chi^2 = 8.543$, $P = 0.015$; $\chi^2 = 7.199$, $P = 0.019$). The genotype distributions of *MTP* H297Q demonstrated no significant difference between the two groups and subgroups (i.e. with and without family history).

A significant difference was demonstrated in the allele distribution of *MTP*-493G/T between the self-limited and chronic hepatitis B groups without familial history ($\chi^2 = 6.212$, $P = 0.013$, Table 4). As calculated by Mantel-Haenszel, the T allele emerged as a potential protective factor positively influencing the HBV infection outcomes in the self-limiting group compared with the chronic hepatitis B group without family history ($P = 0.013$, OR = 0.59 < 1).

DISCUSSION

Several studies suggested that HBV-associated chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) are more common in men than in women[24-26], showing that the relative risk for chronic HBV infection is increased in men when compared to that in women. In China, most HBV infections occur during the neonatal or perinatal period, following materno-foetal transmission where the mothers are HBeAg-positive

Table 3 Genotype distributions of *MTP* H297Q and *MTP*-493G/T *n* (%)

SNP	Genotype	Self-limited HBV infection (*n* = 316)	Chronic hepatitis B total (*n* = 316)	Chronic hepatitis B without family history (*n* = 195)	χ^2 test			
					SLHBV *vs* CHB total		SLHBV *vs* CHB no FH	
					Value	*P*	Value	*P*
MTP H297Q	CC	33 (10.4)	33 (10.4)	20 (10.3)	0.452	0.798	2.588	0.274
	CG	148 (46.8)	140 (44.3)	78 (40.0)				
	GG	135 (42.8)	143 (45.3)	97 (49.7)				
MTP-493G/T	GG	232 (73.4)	256 (81.0)	163 (83.6)	8.543	0.015	7.199	0.019[1]
	GT	80 (25.3)	52 (16.5)	30 (15.4)				
	TT	4 (1.3)	8 (2.5)	2 (1.0)				

[1]Fisher's exact test (2-Tail). SLHBV: Self-limited HBV infection group; CHB total: Chronic hepatitis B total group; CHB no FH: Chronic hepatitis B without chronic hepatitis B family history group.

Table 4 Allele Distributions of *MTP* H297Q and *MTP*-493G/T *n* (%)

SNP	Allele	Self-Limited HBV infection (*n* = 632)	Chronic hepatitis B total (*n* = 632)	Chronic hepatitis B without family history (*n* = 390)	χ^2 test and Mantel-Haenszel logit					
					SLHBV *vs* CHB total			SLHBV *vs* CHB no FH		
					P	OR	95% CI	*P*	OR	95% CI
MTP H297Q	C	214 (33.9)	206 (32.6)	118 (30.3)	0.52	0.903	0.659-1.236	0.232	1.18	0.899-1.549
	G	418 (66.1)	426 (67.4)	272 (69.7)						
MTP-493G/T	G	544 (86.1)	564 (89.2)	356 (91.3)	0.023	0.645	0.442-0.940	0.013	0.59	0.389-0.897
	T	88 (13.9)	68 (10.8)	34 (8.7)						

SLHBV: Self-limited HBV infection group; CHB total: Chronic hepatitis B total group; CHB no FH: Chronic hepatitis B without chronic hepatitis B family history group.

and the infants subsequently become chronic HBV carriers. The predominant mode of HBV spread is intra-familial from mothers to infants or siblings to siblings[25]. This should mean an equal exposure rate of males and females to HBV. The mechanism underlying such a male predominance is unknown. As age and sex were matched when the subjects were enrolled in our study, there was no significant difference in sex and age ($P > 0.05$). We also studied the family history of 632 subjects, in which all of the self-limited subjects and 195 chronic hepatitis B patients had no family history of the disease.

The majority of published studies on HBV persistence correlate to the role of the major histo-compati-bility complex (MHC) in determining the infection outcomes. The most convincing evidence refers to the association between HBV carriage and MHC class Ⅱ and Ⅰ molecules, such as human leukocyte antigen allele *DRB1*1302*[27-29] and allele A*0301[30], which are associated with viral clearance, whilst B*08 is associated with persistent infection[30]. Non-MHC genes have also proved interesting and successful candidates for association studies of hepatitis B viral infection. It was reported that SNPs in the *VDR*[31,32] and *TNF-α* genes, at position-857[33] as TT, are associated with the clearance of HBV in Gambians and the Chinese population, respectively. Two studies showed that the *TNF-α* SNP at position-238 may be associated with persistent infection[33,34]. Several population-based studies also revealed that *IFN-γ* with its +847 and CA repeat allele[35], cytotoxic T-lymphocyte antigen 4 (*CTLA4*) with its -318[36] and *IL-18* with its -607 and -137[37], interferon alpha receptor 1 with its -568C and -408T[38], CC chemokine receptor 5 (*CCR5*) with its 59029G and 59353, heterozygosity of *CCR5* delta 32[31,39], and mannose binding lectin (*MBL*)[40,41] are all associated with chronic HBV infection or HBV clearance.

MTP catalyzes the transport of triglyceride, cholesteryl ester, and phospholipids on phospholipid surfaces[42]. The large subunit of the human *MTP* gene is situated on chromosome 4q22-q24[43]. It has a key function in intracellular apolipoprotein (apo) B lipidation and secretion of VLDL[44]. Abundant MTP has been found on the luminal side of the endoplasmic reticulum and in the liver, intestine, and heart[45]. In the present study, we investigated the two polymorphisms of *MTP*; one is located at -493 of the promoter, the other at the 297th amino acid of the coding region. The polymorphism of *MTP*-493 G-to-T substitution affects the promoter activity of the *MTP* gene[19,20]. It was recently reported that the G allele, which decreases the *MTP* gene transcription, increases intrahepatic triglyceride content[21]. The T allele is associated with an increased expression of the MTP gene[19]. There is linkage disequilibrium between the 2 SNPs[46]. In our study, the genotype of *MTP*-493G/T distribution was significantly different between the two study groups, with different outcomes of HBV infection ($P = 0.015$). This significant difference was observed in allele distribution after adjustment for sex, age and family history ($P = 0.013$), indicating that the T allele may be one of the protective factors against HBV infection, especially against postnatal infection (OR: 0.59 < 1; 95% CI: 0.389-0.897).

A recent study showed that hepatitis C, as a metabolic disease, is associated with liver steatosis involving accumulation of intracytoplasmic lipid droplets[47].

The function of MTP is to lipidate the growing apoB polypeptide chain during translation, allowing apoB to fold correctly and assemble a lipoprotein with a neutral lipid core before secretion[48,49]. It appears to be obligatory for hepatic secretion of apoB[50,51]. It has been shown that the G allele in *MTP*-493 G/T influences the transcriptional activity and is associated with low plasma levels of LDL cholesterol in healthy middle-aged men, and the T allele is associated with an increased expression of the *MTP* gene *in vitro*, and may enhance hepatic secretion of larger VLDL[19]. It was reported that TT and TG in *MTP*-493 increase the *MTP* gene expression and hepatic secretion of VLDL. However, one French study demonstrated that the functional G/T *MTP* polymorphism does not play a role in the development of steatosis in chronic hepatitis C[52].

Brozovic S[53] recently showed that CD1d, a MHC class I-related molecule that functions in glycolipid antigen presentation to distinct subsets of T cells that express natural killer receptors and an invariant T-cell receptor-α chain (invariant NKT cells), is regulated by MTP in hepatocytes. MTP deletion affects the ability of hepatocytes CD1d to activate invariant NKT cells.

In self-limited infections, HBV-DNA falls by more than 90% within 2-3 wk following the viral replication peak and before the peak of antigen-specific CD8 response and liver damage[54]. The role of NK cells in the initial viral containment is confirmed by the observation that the NK cell peak in the circulation of patients infected with HBV precedes the decline of HBV replication[55]. This maximal elevation of the NK cell frequency is then followed by the peak of HBV-specific CD8 cells a few weeks later[55]. Experiments of T cell depletion with anti-CD4 or anti-CD8 antibodies injected into infected chimpanzees showed that NK and NKT cells can contribute substantially to early viral containment[56]. MTP may influence the outcomes of HBV infection by a mechanism mediated by CD1d regulation and NKT cells' activation during the early period of infection.

In summary, the 2 SNPs in MTP have linkage disequilibrium and the T at *MTP*-493G/T may be associated with the clearance of HBV leading to self-limited infection. This might be mediated by CD1d regulation and activation of NKT cells during the early period of infection. The mechanism needs to be further investigated. With the development of SNP detection technology, more SNPs in different genes are likely to be found to be associated with HBV infection. The combination of several SNPs can serve as a predictor for the HBV infection outcomes, leading to new therapeutic methods for HBV infection.

ACKNOWLEDGMENTS

The authors thank Dr. Alexendre Alcais and Dr. Emmanuelle Jouanguy, Faculte de Medecine Necker Paris France for their statistical analysis and helpful discussion.

COMMENTS

Background

Most infected individuals infected with hepatitis B virus (HBV) can clear the virus, while only 5%-10% develop chronic hepatitis and remain in a persistent viral state. The reasons for viral persistence are poorly understood, but host genetic factors are likely to influence the disease outcome.

Research frontiers

This study looked for the potential host genetic factors which may influence the outcomes of HBV infection.

Innovations and breakthroughs

The results show, for the first time, that single nucleotide polymorphisms (SNPs) of microsomal triglyceride transfer protein (*MTP*)-493G/T, which is responsible for MTP transcription level, might be involved in determining the outcomes of HBV infection.

Applications

Based on the results of our study, further investigation should be focused on the mechanism underlying the association between MTP-dependent lipid metabolism and HBV infection, which may lead to new therapeutic methods.

Peer review

The manuscript reports results of an interesting clinical trial. The authors analyzed the polymorphisms of *MTP*-493 at 297 positions in correlation with self-limited and persistent HBV infection in 632 Han Chinese patients. Such an analysis is of importance. The study is well designed and the paper is written in rather good English.

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