Association between polymorphisms of the E-selectin gene, hepatitis B virus DNA copies and preS1 antigen in patients with chronic hepatitis B infection

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Abstract. The aim of this study was to investigate the relationship between E-selectin +G98T, +A561C polymorphisms and the levels of hepatitis B virus (HBV) DNA and preS1 antigen (preS1Ag) in patients with chronic hepatitis B (CHB) infection. Polymorphisms of the E-selectin gene in 150 CHB patients and 150 healthy controls of two different nationalities were detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Real-time quantitative PCR was used to detect the levels of HBV DNA. preS1Ag and five items of hepatitis B were detected by enzyme-linked immunosorbent assay. Two genotypes, GG (94%, 96%) and GT (6%, 4%) of the E-selectin +G98T polymorphism, and AA (78.67%, 80.67%) and AC (21.33%, 19.33%) of the +A561C polymorphism, were found in these patients. There were also significant differences in the two nationalities in the genotypic frequencies in +A561C polymorphisms between patients and healthy subjects (χ^2=5.489, χ^2=5.653; P<0.05). In the patients studied, the relative risk of suffering from CHB in genotype AC was 2.122 and 2.313-fold higher for the two nationalities, respectively, than that of the AA genotype (OR=2.122, 95% CI 1.121-4.019; OR=2.313, 95% CI 1.002-5.360). There was also significant over-representation in the C allele frequency between the two groups (χ^2=5.000, χ^2=5.30; P<0.05), and the levels of HBV DNA and preS1Ag in the AC genotype patients were higher than those in the AA genotype (P<0.01 and P<0.05). E-selectin +A561C and +G98T polymorphisms were present in the populations studied. Therefore, there is a correlation between E-selectin +A561C polymorphisms and CHB. Allele C may be one of the predisposing factors, and mutation of this locus may impact the virus copy number.

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Introduction

Chronic hepatitis B (CHB) is a chronic infectious disease induced by a complex interaction between polygenes and multiple factors. A previous study showed that this variability of the disease was partially attributed to the host immunological and genetic factors (1). Evidence has shown that the gene polymorphisms of certain cytokines, such as tumor necrosis factor and interleukin, are involved in the occurrence and development of CHB. Ben-Ari *et al* showed that there is a correlation between the occurrence and development of CHB with cytokines and the network regulation of cytokines (2).

E-selectin, known as human leukocyte differentiation antigen CD62E, belongs to the selectin family of adhesion molecules. As the body of a pro-inflammatory cytokine, it is involved in many white cell and $CD4^+$ memory T-cell subsets in adhesion and aggregation, and plays a pivotal role in the process of immune and inflammatory responses (3). In a previous study, as a result of HBV long-term stimulation and circulation of lymphocyte and macrophage activation, a variety of inflammatory cytokines was released which induced the increase of E-selectin gene transcription and stimulated liver endothelial cells to express and release E-selectin (2).

The G98 to T transversion in the E-selectin gene occurs in the untranslated region of exon 2, and the A561 to C transversion occurs in the epidermal growth factor-like domain of exon 4; these were previously reported to be associated with atherosclerosis in a German population (4). Few studies have been carried out on the association between the E-selectin gene polymorphisms and CHB. Yan *et al* (5) studied a Han population of Shandong in 2006 and demonstrated that the serum levels of E-selectin had an impact on the occurrence and development of CHB. In 2007, Hajilooi *et al* (6) studied a population of Iran and found that the E-selectin gene polymorphisms of Ser128Arg and Leu554Phe were not considered a risk factor for CHB infection. In 2009, a study of a Hubei Han population showed that the E-selectin +A561C polymorphisms may be associated with liver cirrhosis in patients with chronic

Table I. Amplified fragment sequences.

Gene locus	Amplified product size (bp)
+G98T F: 5'-TGCCCAAAATCTTAGGATGC-3' R: 5'-AAGCCCAGGGAAGAACACAT-3'	332
+A561C F: 5'-ATGGCACTCTGTAGGACTGCT-3' R: 5'-GTCTCAGCTCACGATCACCAT-3'	357

F, forward; R, reverse.

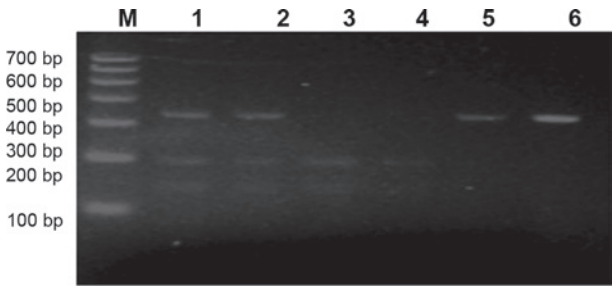

Figure 1. E-selectin +G98T gene polymorphism analyzed by PCR-RFLP. Lanes 1 and 2, GT genotype; lanes 3 and 4, GG genotype; lanes 5 and 6, PCR products.

hepatitis B virus (HBV) infection, and the +G98T polymorphism may be associated with fibrotic severity (7).

Data from China and other countries have shown that E-selectin gene polymorphisms and allele frequencies are present in different races and nations (8,9). Luo *et al* examined different ethnic groups in China that suffered from HBV infection. Their results showed the following percentages for positive HbsAg: Han, 15.3%; Tibetan, 26.2%; Yao, 24.0%; Li, 7.0% and Uygur, 5.3%. Different rates of HBV infection were observed among these ethnic groups (10). In Xinjiang, a typical multiracial province, minority populations are higher, thereby providing a valuable heredity resource. E-selectin is known to affect the occurrence and development of CHB, however, no study has focused on the E-selectin gene polymorphisms in Han and Uygur populations with liver diseases. Therefore, the aim of the present study was to investigate the association of E-selectin polymorphisms with disease progression in chronic HBV-infected patients and to determine the HBV DNA copies and levels of preS1 antigen (preS1Ag) in the circulation of individuals in Chinese Han and Uygur populations. Additionally, we investigated diagnostic factors in Xinjiang Han and Uygur CHB patients to determine susceptibility and prognosis in order to provide guidance for the management and treatment of CHB.

Materials and methods

Study subjects. From January to July 2010, 150 patients with CHB infection (mean age, 37.6±10.7 years) and 150 control subjects (mean age, 34.5±11.2 years) were recruited sequentially from the Chinese Han populations in Xinjiang Shihezi. From July to September 2010, a further 150 patients (mean age 39.6±9.7 years) and 150 control subjects (mean age 34.5±11.2 years) were recruited sequentially from the Uygur population in Xinjiang Akesu. Patients were diagnosed according to the 'Guideline on Prevention and Treatment of Chronic Hepatitis B' by the Chinese Society of Hepatology (11). None of the patients had any other types of liver diseases, such as chronic hepatitis C, alcoholic liver diseases, autoimmune liver diseases or metabolic liver diseases. None of the control subjects suffered from liver, kidney, incretion, cardiovascular or cerebrovascular diseases. The study was carried out with the approval of the local hospital's ethics committee. All participants gave their informed consent to participate.

Genomic DNA. DNA was extracted from blood samples of the study subjects using a blood genome DNA extraction kit (GeneCore BioTeke Co., Ltd., Beijing, China). The primers, designed as previously described (12), are shown in Table I. The primers were synthesized and purified by the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

Polymerase chain reaction (PCR) amplification of the +G98T and +A561C polymorphisms. PCR was performed in a total volume of 25 μl containing 3.0 μl DNA, 0.5 μl of each primer, 12.5 μl PCR mix (including dNTPs and $MgCl_2$; Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.) and 8.5 μl distilled water. Amplification was carried out using the British TC-512 gradient automatic PCR amplification instrument (negative controls without DNA template). The reaction was performed as follows: an initial denaturation at 94˚C for 5 min, followed by 35 cycles of denaturing at 94˚C for 30 sec, annealing at 59˚C for 30 sec, with extension for 45 sec at 72˚C and a final extension at 72˚C for 10 min. The +A561C PCR product (357 bp) was then digested by *Pst*I restriction endonuclease (Fermentas, Vilnius, Lithuania). The +G98T PCR product (332 bp) was digested by *HPh*I restriction endonuclease (Fermentas), and the digested products were run on a 3% agarose gel and visualised under UV light by ethidium bromide staining.

Laboratory determination. For HBV DNA copies, PE7300 real-time fluorescent quantitative PCR analyzer (DAAN Gene Co. Ltd. of Sun Yat-sen University, Shenzhen, China) was used. Experimental clinical trusted sensitivity was $1x10^3$ copies/ml. For plasma preS1Ag and five items of hepatitis B, the ELISA kit was used. The critical value of preS1Ag was average absorbance of negative controls multiplied by 2.5.

Statistical analysis. Statistical analysis was performed using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Each result was calculated as the mean ± SD. The Hardy-Weinberg balance was used to check the sample with group representation. Differences in genotypic and allelic frequency between ethnic groups were evaluated using the Fisher's exact test or the χ^2 test as appropriate, involving HBV DNA variables after log transformation analysis. The odds ratio (OR) was calculated together with its 95% confidence interval (CI). $P<0.05$ was considered to indicate statistically significant differences.

Table II. Comparison of the E-selectin +G98T genotypes and alleles in patients and controls [n (%)].

Groups	No.	Genotype		Allele	
		GG	GT	G	T
Han					
Cases	150	141 (94.0)	9 (6.0)	291 (97.0)	9 (3.0)
Controls	150	138 (92.0)	12 (8.0)	288 (96.0)	12 (4.0)
χ^2 value		0.512		0.444	
P-value		>0.050		>0.050	
OR (95% CI)		0.734 (0.300-1.798)		0.743 (0.308-1.790)	
Uygur					
Cases	150	140 (93.3)	10 (6.7)	290 (96.7)	10 (3.3)
Controls	150	137 (91.3)	13 (8.7)	287 (95.7)	13 (4.3)
χ^2 value		0.423		0.407	
P-value		>0.050		>0.050	
OR (95% CI)		0.753 (0.319-1.775)		0.761 (0.328-1.763)	

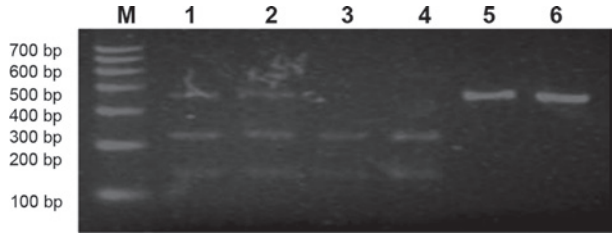

Figure 2. E-selectin +A561C gene polymorphism analyzed by PCR-RFLP. Lanes 1 and 2, AC genotype; lanes 3 and 4, AA genotype; lanes 5 and 6, PCR products.

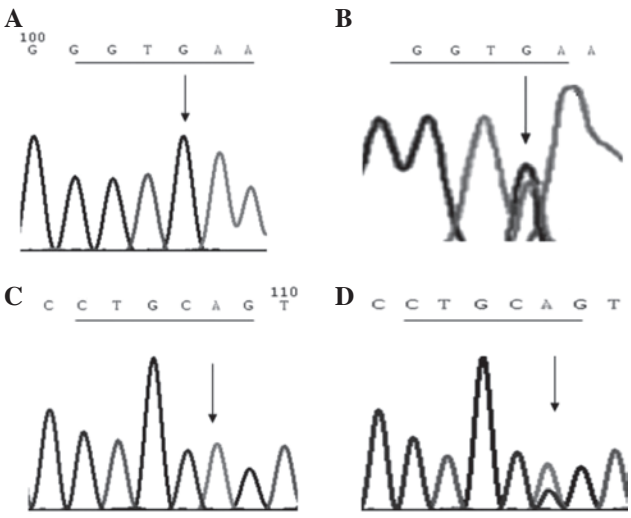

Figure 3. DNA sequencing of the E-selectin +A561C and +G98T polymorphisms. (A) Sample 163, position 104 to G. (B) Sample 16, position 104 to G/T. (C) Sample 133, position 109 to A. (D) Sample 501, position 109 to A/C.

Results

E-selectin polymorphism detection. The E-selectin +G98T gene polymorphism is shown in Fig. 1. The PCR amplified fragment was 332 bp. By restriction mapping, we found that this transversion abolished the recognition site for *Hph*I. There were two types of genotypes: homozygous wild-type GG (194 and 138 bp), and heterozygous genotype GT (332, 194 and 138 bp). Sequencing results are shown in Fig. 3A and B.

The E-selectin +A561C PCR amplified fragment was 357 bp. Using restriction endonuclease *Pst*I, two genotypes were evident: homozygous wild-type AA (219 and 138 bp) and heterozygous genotype GT (357, 219 and 138 bp) (Fig. 2). Sequencing results are shown in Fig. 3C and D.

Discussion

Using the Hardy-Weinberg balance test, the E-selectin +G98T and +A561C genotypes were shown to achieve a genetic balance in the Han and Uygur populations with regard to the distribution frequency, which had a group representation. Results of the χ^2 test and correlation analysis showed that E-selectin +A561C and +G98T polymorphisms were present in the Xinjiang Han and Uygur populations. Significant differences were found in the allele and genotype frequencies of the +A561C polymorphism between patients and controls in the Han and Uygur populations (P<0.05). In the Han population, the relative risk of suffering from CHB in the AC genotype was 2.122-fold higher than that of the AA genotype (OR=2.122, 95% CI 1.121-4.019). Results of the C allele relative risk analysis were OR=1.988 and 95% CI 1.079-3.666. In the Uygur population, the relative risk for CHB for the AC genotype was 2.313-fold higher than that of the AA genotype (OR=2.313, 95% CI 1.002-5.360). Findings of the C allele relative risk analysis were OR=2.183 and 95% CI 1.104-4.318. These results suggest that the changes observed for the E-selectin +A561C gene polymorphisms are one of the genetic factors of CHB, thus the C allele may be a risk factor (Table III).

Investigations into the gene polymorphisms of CHB and HBV DNA copies and the levels of preS1Ag found that these

Table III. Comparison of the E-selectin +A561C genotypes and alleles in patients and controls [n (%)].

Groups	No.	Genotype		Allele	
		AA	AC	A	C
Han					
Cases	150	118 (78.67)	32 (21.33)	268 (89.33)	32 (10.67)
Controls	150	133 (88.67)	17 (11.33)	283 (94.33)	17 (5.67)
χ^2 value		5.489		5.000	
P-value		<0.025		<0.050	
OR (95% CI)		2.122 (1.121-4.019)		1.988 (1.079-3.666)	
Uygur					
Cases	150	123 (82.00)	27 (18.00)	273 (91.00)	27 (9.00)
Controls	150	137 (91.30)	13 (8.70)	287 (95.70)	13 (4.30)
χ^2 value		5.653		5.300	
P-value		<0.025		<0.025	
OR (95% CI)		2.313 (1.002-5.360)		2.183 (1.104-4.318)	

Table IV. Comparison of the +A561C genotypes and HBV DNA copies (Log10 copies/ml) and the levels of preS1Ag [n (%)] in the Han and Uygur patients with chronic hepatitis B.

Genotype	Han			Uygur		
	No.	HBV DNA	preS1Ag-positive	No.	HBV DNA	preS1Ag-positive
AA	118	1.91±1.58	24 (20.33)	123	2.01±1.34	27 (21.95)
AC	32	6.35±2.46	17 (53.13)	27	4.79±2.31	12 (44.44)
Statistical value		t=12.31	χ^2=13.62		t=8.413	χ^2=5.822
P-value		<0.01	<0.01		<0.01	<0.025

HBV DNA variables using log transformation.

Table V. E-selectin +G98T polymorphism in populations of different countries and nationalities.

Countries/nationalities	No.	Genotype			Allele		χ^2 value	P-value
		GG	GT	TT	G	T		
USA	50	40 (80.0)	10 (20.0)	0 (0.0)	90 (90.0)	10 (10.0)	6.9980	<0.05
Germany	71	57 (80.3)	14 (19.7)	0 (0.0)	128 (90.1)	14 (10.9)	9.7260	<0.05
Italy	300	220 (73.3)	77 (25.7)	3 (1.0)	517 (86.2)	83 (13.8)	27.060	<0.05
China Gansu Hui	50	41 (82.0)	9 (18.0)	0 (0.0)	91 (91.0)	9 (9.0)	5.2090	<0.05
China Guangxi Zhuang	162	145 (89.5)	16 (9.9)	1 (0.6)	306 (94.4)	18 (5.6)	2.5880	>0.05
China Guangzhou Han	170	154 (90.6)	16 (9.4)	0 (0.0)	324 (95.3)	16 (4.7)	1.2879	>0.05
China Xinjiang Uygur	150	139 (92.7)	11 (7.3)	0 (0.0)	289 (96.3)	11 (3.7)	0.2140	>0.05
China Xinjiang Han	150	141 (94.0)	9 (6.0)	0 (0.0)	291 (97.0)	9 (3.0)		

polymorphisms were significantly higher in AC genotype patients than in AA genotype patients (P<0.01, P<0.05). By means of logistic regression analysis and using the AA and AC genotypes as variables, a correlation was found between the AC genotype and the HBV DNA copies and levels of preS1Ag (P<0.01). E-selectin may be an endothelial cell injury marker of patients with CHB. A significant increase of HBV DNA and S1 antigen in the AC genotype may induce the release of

Table VI. E-selectin +A561C polymorphism in populations of various countries and nationalities.

Countries/nationalities	No.	Genotype			Allele		χ^2 value	P-value
		AA	AC	CC	A	C		
UK	57	45 (78.9)	11 (19.3)	1 (1.8)	101 (88.6)	13 (11.4)	5.022	<0.05
USA	608	427 (70.2)	175 (28.8)	6 (1.0)	1,029 (84.6)	187 (15.4)	19.490	<0.05
Germany	103	87 (84.5)	14 (13.6)	2 (1.9)	188 (91.3)	18 (8.7)	3.290	>0.05
Turkey	96	75 (78.1)	20 (20.8)	1 (1.1)	170 (88.5)	22 (11.5)	5.844	<0.05
Japan	301	276 (91.7)	25 (8.3)	0 (0.0)	577 (95.8)	25 (4.2)	1.087	>0.05
Iran	150	104 (69.3)	40 (26.7)	6 (4.0)	248 (82.7)	52 (17.3)	18.830	<0.05
Italy	300	225 (75.0)	72 (24.0)	3 (1.0)	522 (87.0)	78 (13.0)	11.960	<0.05
China Guangxi Zhuang	162	139 (85.8)	20 (12.3)	3 (1.9)	298 (92.0)	26 (8.0)	2.910	>0.05
China Guangzhou Han	170	158 (92.9)	11 (6.5)	1 (0.6)	327 (96.2)	13 (3.8)	3.196	>0.05
China Xinjiang Uygur	150	137 (91.3)	13 (8.7)	0 (0.0)	287 (95.7)	13 (4.3)	0.593	>0.05
China Xinjiang Han	150	133 (88.7)	17 (11.3)	0 (0.0)	283 (94.3)	17 (5.7)		

more inflammatory factors and stimulate the immune system to generate a strong response, leading to activation of the HBV replication *in vivo* of the AC genotype (Table IV).

No correlation was found between the E-selectin +G98T gene polymorphism and alleles in the Xinjiang Han and Uygur patients. Thus, the T allele is probably not a predisposing factor (Table II). Analysis of the single-nucleotide polymorphism using factors such as sample selection, sample size and ethnic background showed that a larger cohort of samples and the application of different race case-control studies are required to better understand the pathogenesis of CHB.

In addition, this study compared the E-selectin +G98T polymorphisms and allele frequencies in populations of various countries and nationalities. Table V shows that the +G98T genotype and allele frequencies of the Xinjiang Han population were significantly different from the American (13), German (4), Italian (14) and Gansu Hui populations of China (15) ($P<0.05$). By contrast, in the China Guangxi Zhuang (9), Guangzhou Han (9) and Uygur of Xinjiang populations, the differences were not statistically significant ($P>0.05$). A comparison of the E-selectin +A561C polymorphism in populations of various countries and nationalities was performed (Table VI). A comparison of the British (16), American (17), Turkish (18), Iranian (6) and Italian (14) populations showed the differences to be statistically significant ($P<0.05$). However, a comparison of German (19), Japanese (20), Guangxi Zhuang (9), Guangzhou Han (9) and Uygur of Xinjiang populations showed no statistically significant differences ($P<0.05$). Therefore, we conclude that E-selectin gene polymorphisms and allele frequencies exist in different races and nationalities, and this difference may affect certain E-selectin gene polymorphism-related diseases.

In conclusion, the preliminary results of our study have shown that the E-selectin +A561C and +G98T polymorphisms exist in the Han and Uygur populations and that the +A561C polymorphisms may have an impact on the occurrence, development and levels of HBV replication. However, the exact mechanism of HBV infection in the Xinjiang Han and Uygur populations remains to be determined in future studies.

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