Human Molecular Genetics, 2003, Vol. 12, No. 19 **2541–2546**
DOI: 10.1093/hmg/ddg262

Association of *TNF-α* promoter polymorphisms with the clearance of hepatitis B virus infection

Yoon Jun Kim[1], Hyo-Suk Lee[1,*], Jung-Hwan Yoon[1], Chung Yong Kim[1], Myoung Hee Park[2], Lyoung Hyo Kim[3], Byung Lae Park[3] and Hyoung Doo Shin[3]

[1]Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, Korea, [2]Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, Korea and [3]Department of Genetic Epidemiology, SNP Genetics Inc., Seoul, Korea

Received May 27, 2003; Revised July 17, 2003; Accepted July 30, 2003

The mechanisms underlying the resolution of hepatitis B virus (HBV) infection remain undetermined. Tumor necrosis factor-α (TNF-α) plays a pivotal role in host immune response to HBV, and the capacity for cytokine production in individuals has a major genetic component. The aim of this study was to examine whether *TNF-α* promotor polymorphisms are associated with the clearance of HBV infection. A total of 1400 Korean subjects were enrolled in two different groups: 'chronic carrier group' (CC; $n = 1109$), who were repeatedly hepatitis B surface antigen (HBsAg)-positive, and 'subjects who spontaneously recovered' (SR; $n = 291$), who were HBsAg-negative with antibodies to HBsAg and hepatitis B core antigen. *TNF-α* promoter polymorphisms at positions *−1031T>C*, *−863C>A*, *−857C>T*, *−376G>A*, *−308G>A*, *−238G>A* and *−163G>A* were determined and the genotype distributions of the CC and SR groups were compared. The *TNF-α* promoter alleles that were previously reported to be associated with higher plasma levels, i.e. the presence of the *−308A* allele (*TNF-α-308A/G* or *A/A*) or the absence of the *−863A* (*TNF-α-863C/C*) variant, were strongly associated with the resolution of HBV infection in three alternative analyzing models, i.e. *TNF-α-308G>A* ($P = 0.01$) and *TNF-α-863C>A* ($P = 0.003$–0.14), respectively. Haplotype analysis also revealed that *TNF-α haplotype 1* [*−1031T*; *−863C*; *−857C*; *−308G*; *−238G*; *−163G*] and *haplotype 2* [*−1031C*; *−863A*; *−857C*; *−308G*; *−238G*; *−163G*] were significantly associated with HBV clearance, showing protective antibody production and persistent HBV infection, respectively ($P = 0.003$–0.02). Our findings imply that variations in the genes governing the levels of constitutive and inducible TNF-α might be an important factor, which might explain the variable outcome of HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem, as HBV infects more than 350 million people worldwide (1). The clinical course of HBV infection varies from spontaneous recovery after acute hepatitis to a chronic persistent infection that may progress to cirrhosis or hepatocellular carcinoma. The mechanisms underlying resolution of acute HBV infection or its progression to chronicity remain undetermined. Age at infection has the most significant impact on the clinical outcome, evidenced by the fact that chronic infection occurs in ~90% of infants infected at birth, in 25–50% of children infected between the ages of 1 and 5 years, and in less than 5% of those infected during adult life (2–4). It is well known that the major mode of infection in HBV-endemic areas, including Korea, is perinatal transmission (2,5). The mechanisms underlying resolution of acute HBV infection or its progression to chronicity at each age group remain undetermined. When determining the chronicity of HBV infection within a group of patients who are presumed to have been infected at the same age, i.e. perinatally, in Korea, it is apparent that the outcome of the infection does not appear to be determined by variations in virulence of the viral strains (6,7), but that host factors are likely to influence disease outcome (8,9). Thus, it is conceivable that genetic differences play an additional role.

Cell-mediated immune responses directed toward infected liver cells have been considered to be the main inducer of hepatic injury and mediators of HBV clearance (8,10). On the other hand, recent evidence also suggests that antiviral

*To whom correspondence should be addressed at: Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, 28 Yungun-dong, Chongno-gu, Seoul 110-744, Korea. Tel: +82 27457557; Fax: +82 27448243; Email: hsleemd@snu.ac.kr

Table 1. Genotype distribution of *TNF*-α polymorphisms in the CC and SR groups

Loci	Genotype	CC	SR	Analyzing models					
				Codominant		Dominant		Recessive	
				OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
TNF-α-1031	*T/T*	655 (63.1%)	196 (68.2%)						
	C/T	331 (31.9%)	82 (28.6%)	1.25 (0.97–1.61)	0.08	1.25 (0.92–1.68)	0.15	1.76 (0.86–3.61)	0.13
	C/C	52 (5.0%)	9 (3.3%)						
TNF-α-863	*C/C*	684 (65.9%)	209 (74.8%)						
	A/C	317 (30.5%)	65 (23.3%)	**1.52 (1.16–2.00)**	**0.003**	**1.58 (1.16–2.15)**	**0.004**	2.05 (0.79–5.32)	0.14
	A/A	37 (3.6%)	5 (1.9%)						
TNF-α-857	*C/C*	719 (69.1%)	208 (74.2%)						
	C/T	298 (28.7%)	66 (23.5%)	1.23 (0.93–1.63)	0.14	1.30 (0.95–1.77)	0.10	0.99 (0.39–2.49)	0.98
	T/T	23 (2.2%)	6 (2.3%)						
TNF-α-308	*G/G*	971 (93.4%)	251 (88.7%)						
	A/G	68 (6.5%)	32 (11.3%)	**0.57 (0.37–0.89)**	**0.01**	**0.56 (0.35–0.87)**	**0.01**	—	—
	A/A	1 (0.1%)	0 (0%)						
TNF-α-238	*G/G*	915 (88.0%)	261 (91.6%)						
	A/G	115 (11.1%)	22 (7.7%)	1.41 (0.93–2.13)	0.10	1.47 (0.93–2.33)	0.10	1.64 (0.36–7.58)	0.52
	A/A	10 (1.0%)	2 (0.7%)						
TNF-α-163	*G/G*	1016 (97.1%)	267 (95.1%)						
	A/G	24 (2.3%)	7 (2.4%)	0.57 (0.21–1.56)	0.28	0.54 (0.12–2.41)	0.42	0.25 (0.03–2.27)	0.22
	A/A	6 (0.6%)	7 (2.4%)						
Ht1 [*T*; *C*; *C*; *G*; *G*; *G*]	*−/−*	411 (39.6 %)	86 (31.2 %)						
	−/Ht1	325 (31.3 %)	90 (32.6 %)	**0.78 (0.67-0.92)**	**0.003**	**0.67 (0.50–0.89)**	**0.006**	**0.71 (0.54–0.94)**	**0.02**
	Ht1/Ht1	302 (29.1 %)	100 (36.2 %)						
Ht2 [*C*; *A*; *C*; *G*; *G*; *G*]	*−/−*	704 (67.8 %)	207 (75.0 %)						
	−/Ht2	299 (28.8 %)	64 (23.2 %)	**1.42 (1.09–1.87)**	**0.01**	**1.46 (1.08–1.98)**	**0.02**	1.97 (0.76–5.11)	0.16
	Ht2/Ht2	35 (3.4 %)	5 (1.8 %)						
Ht3 [*T*; *C*; *T*; *G*; *G*; *G*]	*−/−*	998 (96.2 %)	265 (96.0 %)						
	−/Ht3	18 (1.7 %)	5 (1.8 %)	1.00 (0.65–1.52)	0.99	0.99 (0.50–1.98)	0.98	1.00 (0.40–2.51)	0.99
	Ht3/Ht3	22 (2.1 %)	6 (2.2 %)						

Logistic regression models were used for calculating the odds ratios (95% confidential intervals) and corresponding *P*-values, controlling for age (continuous) and sex as covariates. In an analyzing model in which a codominant (additive) effect of the variant (*V*) allele was assumed, the genotypes *Wild (W)/W*, *W/V* and *V/V* were coded as 0, 1 and 2, respectively; when a dominant effect was assumed, genotype *W/W* was coded as 0, and *W/V* and *V/V* combined were coded as 1. Accordingly, scores of 0 for *W/W* and *W/V* combined and of 1 for *V/V* were used in a model that assumed a recessive effect. Haplotypes with frequencies greater than 5% were used for analysis (Fig. 1B). Significant associations ($P < 0.05$) are in bold.

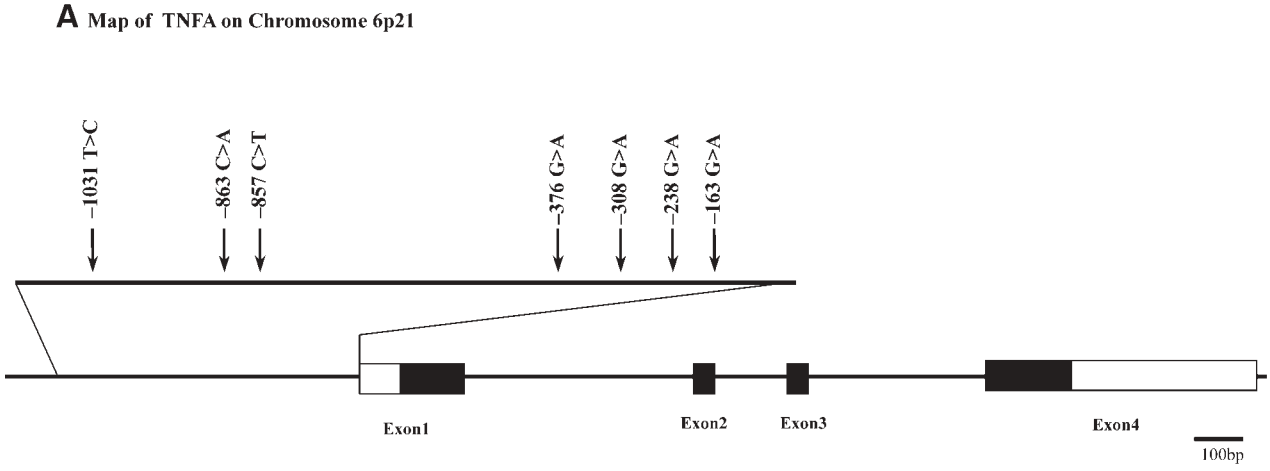
cytokines, such as tumor necrosis factor-α (TNF-α) and interferon gamma, released by the activated effector cells of innate and adoptive immune systems in the region of their targets, can induce the noncytolytic suppression of HBV expression and replication in the liver (11–13). TNF-α inhibits the transcriptional activity of the HBV core promoter *in vitro* (14). In an HBV transgenic mouse model and acutely infected chimpanzees, only a minority of infected hepatocytes were eliminated by direct contact with cytotoxic T cells (12,13). In the vast majority of infected cells, HBV appears to be suppressed and eliminated by antigen-non-specific cytokines (12,13).

The capacity for cytokine production in an individual has a major genetic component, and striking differences exist among individuals in terms of their ability to produce cytokines, which have been ascribed to polymorphisms within the regulatory regions or signal sequences of cytokine genes (15). Several biallelic polymorphisms have been described within the *TNF*-α gene, including seven in the promoter region at positions *−1031T>C*, *−863C>A*, *−857C>T*, *−376G>A*, *−308G>A*, *−238G>A* and *−163G>A* base pairs from the transcription start site (16,17). Moreover, a number of studies have shown that the *TNF*-α promoter polymorphism has a significant effect on transcriptional activity (18,19).

We hypothesized that genetic variation in the *TNF*-α gene could affect the clearance of HBV, and compared the prevalence of polymorphisms associated with the *TNF*-α gene in subjects with chronic HBV infection with that of those with serologic evidence of spontaneous recovery, to determine whether polymorphisms of the *TNF*-α gene are associated with the clearance of HBV infection.

RESULTS

The genotype distribution of *TNF*-α promoter polymorphisms at the positions *−1031T>C*, *−863C>A*, *−857C>T*, *−376G>A*, *−308G>A*, *−238G>A* and *−163G>A* were not significantly different between the healthy unrelated control and study groups (data not shown). The genotype frequencies of the 'chronic HBV carriers' (CC) and the 'subjects who spontaneously recovered' (SR) groups for each polymorphism of *TNF*-α are showed in Table 1. No evidence of a departure from the Hardy–Weinberg equilibrium was apparent. The frequencies of minor alleles of the *TNF*-α promoter polymorphisms at positions *−1031T>C*, *−863C>A*, *−857C>T*, *−376G>A*, *−308G>A*, *−238G>A* and *−163G>A* were 0.14, 0.18, 0.15, 0.001, 0.04, 0.06 and 0.02, respectively ($n = 1400$).



B TNFA Haplotypes

Haplotype	-1031 T>C	-863 C>A	-857 C>T	-308 G>A	-238 G>A	-163 G>A	Freq.
Ht1 [T; C; C; G; G; G]	T	C	C	G	G	G	0.560
Ht2 [C; A; C; G; G; G]	C	A	C	G	G	G	0.168
Ht3 [T; C; T; G; G; G]	T	C	T	G	G	G	0.156
Ht4 [T; C; C; A; G; G]	T	C	C	A	G	G	0.044
Ht5 [C; C; C; G; A; G]	C	C	C	G	A	G	0.044
Ht6 [T; C; C; G; G; A]	T	C	C	G	G	A	0.006
Ht7 [T; A; C; G; G; G]	T	A	C	G	G	G	0.006
Ht8 [C; C; C; G; A; A]	C	C	C	G	A	A	0.005
Ht9 [T; C; C; G; A; G]	T	C	C	G	A	G	0.004
others	-	-	-	-	-	-	0.007

C LD coefficients (|D'|) among TNFA SNPs

		|D'|					
		-1031 T>C	-863 C>A	-857 C>T	-308 G>A	-238 G>A	-163 G>A
r^2	-1031T>C	-	0.956	1	1	0.840	0.267
	-863C>A	0.682	-	1	1	1	1
	-857C>T	0.053	0.040	-	0.859	0.887	1
	-308G>A	0.013	0.010	0.007	-	1	1
	-238G>A	0.148	0.012	0.009	0.003	-	0.470
	-163G>A	0.004	0.003	0.003	0.001	0.053	-

Figure 1. TNF-α gene map, haplotypes, and LD coefficients. (**A**) Gene map and SNPs in TNF-α on chromosome 6p21. Coding exons are marked by black blocks and 5′- and 3′-UTR by white blocks. First base of the transcriptional site was denoted as nucleotide +1. (**B**) Haplotypes of TNF with frequency >0.004 were presented (out of 14 haplotypes inferred). (**C**) Linkage disequilibrium coefficient ($|D'|$ and r^2) among TNF-α SNPs.

The frequency of *TNF-α-376G>A* was extremely rare in the Korean population and was excluded from further analysis. Fourteen haplotypes were identified among the single nucleotide polymorphisms (SNPs; Fig. 1B). In the initial analysis, a significant increase of homozygous and heterozygous individuals for the *TNF-α-863A* allele (*TNF-α-863A/C* or *A/A*) was observed in the CC group than the SR group (OR = 1.52–1.58, $P = 0.003$–0.004; Table 1). In contrast, the *−308A* allele (*TNF-α-308A/G* or *A/A*) was significantly associated with HBV clearance and protective antibody production (OR = 0.56–0.57, $P = 0.01$; Table 1). All other loci showed no significant associations.

Among 14 haplotypes, three common haplotypes with frequency greater than 5% were used for analysis (Fig. 1B). *TNF-α haplotype 1* [*−1031T*; *−863C*; *−857C*; *308G*; *−238G*; *−163G*] was associated with HBV clearance (OR = 0.67–0.78, $P = 0.003$–0.02; Table 1), whereas *haplotype 2* [*−1031C*; *−863A*; *−857C*; *−308G*; *−238G*; *−163G*] was associated with chronic HBV infection, when a codominant or dominant effect of *haplotype 2* was assumed (OR = 1.42–1.46, $P = 0.01$–0.02; Table 1).

We further evaluated whether *TNF-α-863C>A* or *−308G>A* polymorphisms are associated with HLA-DRB1*13, which has been linked with protection against chronic HBV infection (7,20,21). HLA-DR13 was only associated with *TNF-α-308A* in the 107 healthy unrelated Korean subjects (Table 2). Significantly associated human leukocyte antigen (HLA) alleles with *TNF-α haplotypes* in Korean populations are shown in Table 2. *TNF-α haplotypes 1*, *2* and *3* were not associated with HLA-DR13.

DISCUSSION

In the present study, the *TNF-α* promoter allele associated with higher-plasma TNF-α levels, i.e. the presence of the *−308A* allele (*TNF-α-308A/G* or *A/A*) or the absence of the *−863A* (*TNF-α-863C/C*) variant, was found to be strongly associated with the resolution of HBV infection. Haplotype analysis revealed that *TNF-α haplotype 1* [*−1031T*; *−863C*; *−857C*; *−308G*; *−238G*; *−163G*] and *haplotype 2* [*−1031C*; *−863A*; *−857C*; *−308G*; *−238G*; *−163G*] are significantly associated with HBV clearance with subsequent protective antibody production, and with the persistence of HBV infection, respectively.

The *TNF-α* gene is located within the class III region of the major histocompatibility complex (MHC) between HLA-B and -DR, and its expression is tightly controlled at the transcriptional and post-transcriptional level. Although several polymorphic sites have been described in the *TNF-α* gene promoter region, *TNF-α-308G>A* has been shown to be associated with elevated

Table 2. Significant positive associations between *TNF*-α promoter polymorphisms and HLA alleles

Polymorphisms	Positive association ($P < 0.05$) with		
	HLA-A	HLA-B	HLA–DRB1[a]
TNF-α-863C		B62	
TNF-α-863A	A26	B44, B51, B61	**DRB1*0901**
TNF-α-308G			
TNF-α-308A	**A33**	**B58**	**DRB1*0301**, ***1302**
Ht1 [*T*; *C*; *C*; *G*; *G*; *G*]	A11	B7, B13, B46, B52, **B62**	DRB1*0101, *0406, *1405
Ht2 [*C*; *A*; *C*; *G*; *G*; *G*]	A26	B44, **B51**, B61	**DRB1*0901**, *1501
Ht3 [*T*; *C*; *T*; *G*; *G*; *G*]	A2	**B35**, **B54**, **B59**	DRB1*0405, ***1201**
Ht4 [*T*; *C*; *C*; *A*; *G*; *G*]	**A33**	**B58**	**DRB1*0301**, ***1302**
Ht5 [*C*; *C*; *C*; *G*; *A*; *G*]	A2		

[a]HLA alleles showing strong positive associations ($P < 0.001$) are in bold.

TNF-α transcriptional activity (18,19). On the other hand, polymorphisms at position *−863C>A* in the promoter region have been reported to be associated with reduced *TNF*-α promoter activity and lower TNF-α plasma levels (22). The present study demonstrates that the resolution of HBV infection is associated with TNF-α promoter alleles, which were reported to be related with higher circulating levels of TNF-α, and thus suggests the importance of *TNF*-α promoter polymorphisms in the noncytolytic clearance of HBV in addition to cytolytic clearance.

In a former study, an association was found between the *TNF*-α-*238A* promoter variant and chronic HBV infection, but no such association was found for the variant at position −308 (23). However, in the present study, chronic HBV infection was not associated with the *TNF*-α-*238A* allele, but, rather, spontaneous HBV clearance was found to be associated with the *TNF*-α-*308A* allele. The exact cause of this contradiction is unknown. However, differences in the mode of viral transmission (perinatal versus horizontal in adulthood) and ethnic difference between Asians and Caucasians might be involved. In addition, the number of ∼200 subjects enrolled in the previous work, involving statistical analysis corrected for small numbers, might not have been adequate for an association study.

Undoubtedly, it is possible that the associations we observed are caused by other confounding genetic or environmental factors. An inappropriate viral antigen presentation to T-lymphocytes, controlled by the MHC gene, may induce persistent HBV infection. Indeed, other groups have identified that DRB1*13 has a protective effect against the development of chronic hepatitis B in Africans, Caucasians, and Koreans (7,20,21). Moreover, the exact mechanism of an association between individuals carrying HLA–DRB1*13 and HBV clearance is unclear. The beneficial effect of the HLA–DRB1*13 allele on the outcome of HBV infection may be the result of more proficient antigen presentation by HLA–DRB1*13 molecules themselves, or of a linked polymorphism in a neighboring immunoregulatory gene, such as a *TNF*-α promoter gene. We showed that *TNF*-α-*863C*, which was associated with HBV clearance in the present study, is not in linkage disequilibrium with HLA–DRB1*13 in Koreans. Therefore, the protective effects of *TNF*-α-*863C* against HBV are not caused by HLA–DRB1*13 in Koreans and vice versa. *TNF*-α *haplotypes 1* and *2* were found not to be associated with HLA–DRB1*13 either. On the other hand, the *TNF*-α-*308A* variant was found to be in strong linkage disequilibrium with HLA–DRB1*13 in this study. Therefore, it is not clear which genetic factor, HLA–DRB1*13 or *TNF*-α-*308A*, is primarily associated with the protective effect against HBV. It is also possible that the *TNF*-α promoter polymorphisms and HLA–DRB1*13 are independent factors encoded within the MHC that influence HBV infection outcome, as another group of researchers has suggested (23). To solve this question, a functional and quantitative study capable of defining the respective effects of the *TNF*-α polymorphism and HLA–DRB1*13 should be carried out.

Table 3. Characteristics of subjects in the CC and SR groups

Group	Sex	*n*	Age [mean (± SD)]
CC	Male	825	49.9 (± 10.4)
	Female	284	50.8 (± 10.6)
SR	Male	191	54.7 (± 10.9)
	Female	100	53.6 (± 11.0)

The allele frequencies of *TNF*-α promoter polymorphisms *−863A* and *−308A* were 0.18 and 0.04 in the present study, respectively. In healthy Swedish men, Swedish women, and Japanese, the allele frequencies of *−863A* were 0.17, 0.14, and 0.14 (16,22,24), respectively, and in Taiwanese, Swedes, Gambians, Caucasians in the USA and in Japanese, the frequencies of *−308A* were 0.14, 0.20, 0.16, 0.14 and 0.017, respectively (16,22,25–27). Thus, the *TNF*-α-*308G>A* polymorphism is relatively rare in Koreans and Japanese. Therefore, it might be anticipated that *−863C>A* would play a more dominant role in determining the natural course of HBV infection than the *−308G>A* polymorphism in the Korean population. Accordingly, most of the Korean haplotypes contained the *−308G* allele and not the *−308A* allele, and the outcome of HBV infection seemed to be determined by the presence of the *−863C>A* polymorphism in the present study.

In summary, the *TNF*-α promoter alleles associated with higher plasma levels, i.e. the presence of the *−308A* allele (*TNF*-α-*308A*/*G* or *A*/*A*) or the absence of the *−863A* (*TNF*-α-*863C*/*C*) variant, were strongly associated with the resolution of HBV infection. Haplotype analysis revealed that *TNF*-α *haplotype 1* [*−1031T*; *−863C*; *−857C*; *−308G*; *−238G*; *−163G*] and *haplotype 2* [*−1031C*; *−863A*; *−857C*; *−308G*; *−238G*; *−163G*] were significantly associated with HBV

Table 4. Sequences of amplifying and extension primers for *TNF-α* promoter SNP genotyping by single base extension method

Loci	Primer	Sequences
TNF-α-1031T>C	Forward	5′-GCTTGTGTGTGTGTCTGG-3′
	Reverse	5′-GGACACACAAGCATCAAGG-3′
	Extension	5′-GATTATATAATCATGATTATAATCAATGATGATAAGCAAAGGAGAAGCTGAGAAGA-3′
TNF-α-863C>A	Forward	5′-TGACCACAGCAATGGGTAGGA-3′
	Reverse	5′-GCTCTCACTTCTCAGGGCCC-3′
	Extension	5′-CTCTACATGGCCCTGTCTTCGTTAAG-3′
TNF-α-857C>T	Forward	5′-TGACCACAGCAATGGGTAGGA-3′
	Reverse	5′-GCTCTCACTTCTCAGGGCCC-3′
	Extension	5′-ATCCTCTACATGGCCCTGTCTTC-3′
TNF-α-376G>A	Forward	5′-TCTCCCTCAACGGACTCAGCT-3′
	Reverse	5′-GTAGGACCCTGGAGGCTGAAC-3′
	Extension	5′-AATCAATGATGATAGGTCTGTGGTCTGTTTCCTTCTAA-3′
TNF-α-308G>A	Forward	5′-CTGAAGCCCCTCCCAGTTCT -3′
	Reverse	5′-CGGTTTCTTCTCCATCGCG -3′
	Extension	5′-ATATAATCATGATTATAATCAATGATGATCAATAGGTTTTGAGGGGCATG-3′
TNF-α-238G>A	Forward	5′-GGAGGCAATAGGTTTTGAGGG-3′
	Reverse	5′-GGTTTCTTCTCCATCGCGG-3′
	Extension	5′-TAATCAATGATGATAAGACCCCCCTCGGAATC-3′
TNF-α-163G>A	Forward	5′-AATCGGAGCAGGGAGGATG-3′
	Reverse	5′-CGGAAAACTTCCTTGGTGGA-3′
	Extension	5′-ATCATGATTATAATCAATGATGATTCTCGGTTTCTTCTCCATCG-3′

clearance and with the persistence of HBV infection, respectively. Our findings imply that variations in the genes governing the level of constitutive and inducible TNF-α are an important factor, which might explain the variable outcome of HBV infection and offer an approach to elucidating the molecular mechanisms of HBV clearance. This finding might also provide a new perspective on antiviral approaches for the treatment of patients with chronic hepatitis B, via direct TNF-α delivery or *TNF*-α-based gene therapy.

MATERIALS AND METHODS

Study subjects

A total of 1400 Korean subjects having either present or past evidence of HBV infection were prospectively enrolled from the outpatient clinic of the liver unit or from the Center for Health Promotion of Seoul National University Hospital between January 2001 and August 2001. Subjects were placed in two different groups: the CC and SR, according to serologic markers. The CC and SR cohorts consisted of 1109 and 291 subjects, respectively (Table 3). The diagnoses of the CC and SR subjects were established by repeated seropositivity for the hepatitis B surface antigen (HBsAg; Enzygnost® HBsAg 5.0; Dade Behring, Marburg, Germany) over a 6-month period, and for both anti-HBs (antibody to hepatitis B surface antigen; Enzygnost® Anti-HBs II; Dade Behring, Marburg, Germany) and anti-HBc (antibody to hepatitis B core antigen; AB-Corek; DiaSorin s.r.l., Saluggia, Italy) of the IgG type without HBsAg, respectively. We excluded subjects who were positive for anti-HBs alone but not for anti-HBc, and those positive for anti-HCV (antibody to hepatitis C antigen, HCV®3.2; Dong-A Pharmaceutical Co., Seoul, Korea) or anti-HIV (Genedia®; Greencross Life Science Corp., Yongin-shi, Korea). The patients who had any other types of liver disease such as autoimmune hepatitis, toxic hepatitis, primary biliary cirrhosis or Budd–Chiari syndrome were also excluded. No patients had a previous history of immunosuppression or anti-viral treatment. The normal control population consisted of 107 healthy unrelated Koreans. All healthy controls had been tested previously for HLA-A, -B, -DRB1 (28). HLA-A, -B and HLA-DRB1 were genotyped serologically using PCR-sequence specific oligonucleotides or PCR–single-strand conformational polymorphism technique. Informed consent was obtained from each patient, and the Institutional Review Board of Human Research at Seoul National University Hospital approved the study protocol.

Genotyping of the SNPs in TNF-α promoter region

The seven SNPs in the *TNF*-α promoter region at positions *−1031T>C*, *−863C>A*, *−857C>T*, *−376G>A*, *−308G>A*, *−238G>A* and *−163G>A* were genotyped by single-base extension methods (29). The PCR primer sequences used for the amplification and extension of the TNF-α SNPs by the single-base extension methods are listed in Table 4. PCR was performed in a mixture of 1.25 pmol of each primer, 50 ng of genomic DNA, 250 μM dNTPs, and 0.15 U *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA) in the buffer provided by the manufacturer. Amplification was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). To clean up the PCR reaction for the primer extension reaction, one unit of shrimp alkaline phosphatase (SAP; Amersham Life Sciences, Cleveland, OH, USA) and two units of *Exo*I (Amersham Life Sciences) were added to the PCR products. The mixture was then incubated at 37°C for 1 h, and at 72°C for 15 min to inactivate the enzymes. Primer extension reactions were performed with a SNaPshot ddNTP Primer Extension Kit (Applied Biosystems) according to the manufacturer's instructions. To clean up the primer extension reaction, one unit of SAP was added to the reaction mixture, which was then incubated at 37°C for 1 h, and then at 72°C for 15 min to inactivate the enzymes. The DNA samples, containing extension products, and Genescan 120 Liz size

standard solution (Applied Biosystems) were added to Hi-Di formamide (Applied Biosystems) according to the manufacturer's instructions. The mixture was then incubated for 5 min at 95°C, placed on ice for 5 min, and electrophoresed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Results were analyzed using the ABI Prism GeneScan and Genotyper (Applied Biosystems) software.

Statistics

Chi-squared tests were used to compare the observed numbers of each genotype with those expected for the population by the Hardy–Weinberg equilibrium. We calculated widely used measures of linkage disequilibrium between all pairs of biallelic loci, namely, Lewontin's D' ($|D'|$) (30) and the r^2 measures. Haplotypes and their frequencies were inferred using the algorithm developed by Stephens *et al.* (31). For exact haplotypes construction, data with missing genotypes were excluded. Odds ratios with a 95% confidence interval, and *P*-values of the logistic regression models controlling for the effects of age (as a continuous variable) and sex (male = 0, female = 1), were computed using SAS to analyze the categorized phenotypes based on the assumption that most patients, if not all, were infected with HBV perinatally (2,5).

ACKNOWLEDGEMENTS

We greatly acknowledge the study participants and their families, who participated in HBV cohort study of Seoul National University. This work was supported by the 21C Frontier Functional Human Genome Project (grant number FG-4-16), operated under the auspices of the Korean Ministry of Science and Technology.

REFERENCES

1. Purcell, R.H. (1993) The discovery of the hepatitis viruses. *Gastroenterology*, **104**, 955–963.
2. Stevens, C.E., Beasley, R.P., Tsui, J. and Lee, W.C. (1975) Vertical transmission of hepatitis B antigen in Taiwan. *New Engl. J. Med.*, **292**, 771–774.
3. Coursaget, P., Yvonnet, B., Chotard, J., Vincelot, P., Sarr, M., Diouf, C., Chiron, J.P. and Diop-Mar, I. (1987) Age- and sex-related study of hepatitis B virus chronic carrier state in infants from an endemic area (Senegal). *J. Med. Virol.*, **22**, 1–5.
4. Tassopoulos, N.C., Papaevangelou, G.J., Sjogren, M.H., Roumeliotou-Karayannis, A., Gerin, J.L. and Purcell, R.H. (1987) Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. *Gastroenterology*, **92**, 1844–1850.
5. Lok, A.S., Lai, C.L., Wu, P.C., Wong, V.C., Yeoh, E.K. and Lin, H.J. (1987) Hepatitis B virus infection in Chinese families in Hong Kong. *Am. J. Epidemiol.*, **126**, 492–499.
6. Cacciola, I., Cerenzia, G., Pollicino, T., Squadrito, G., Castellaneta, S., Zanetti, A.R., Mieli-Vergani, G. and Raimondo, G. (2002) Genomic heterogeneity of hepatitis B virus (HBV) and outcome of perinatal HBV infection. *J. Hepatol.*, **36**, 426–432.
7. Thursz, M.R., Kwiatkowski, D., Allsopp, C.E., Greenwood, B.M., Thomas, H.C. and Hill, A.V. (1995) Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. *New Engl. J. Med.*, **332**, 1065–1069.
8. Chisari, F.V. and Ferrari, C. (1995) Hepatitis B virus immunopathogenesis. *A. Rev. Immunol.*, **13**, 29–60.
9. Lin, T.M., Chen, C.J., Wu, M.M., Yang, C.S., Chen, J.S., Lin, C.C., Kwang, T.Y., Hsu, S.T., Lin, S.Y. and Hsu, L.C. (1989) Hepatitis B virus markers in Chinese twins. *Anticancer Res.*, **9**, 737–741.
10. Curry, M.P. and Koziel, M. (2000) The dynamics of the immune response in acute hepatitis B: new lessons using new techniques. *Hepatology*, **32**, 1177–1179.
11. Guidotti, L.G. and Chisari, F.V. (1996) To kill or to cure: options in host defense against viral infection. *Curr. Opin. Immunol.*, **8**, 478–483.
12. Guidotti, L.G., Ishikawa, T., Hobbs, M.V., Matzke, B., Schreiber, R. and Chisari, F.V. (1996) Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity*, **4**, 25–36.
13. Guidotti, L.G., Rochford, R., Chung, J., Shapiro, M., Purcell, R. and Chisari, F.V. (1999) Viral clearance without destruction of infected cells during acute HBV infection. *Science*, **284**, 825–829.
14. Romero, R. and Lavine, J.E. (1996) Cytokine inhibition of the hepatitis B virus core promoter. *Hepatology*, **23**, 17–23.
15. Danis, V.A., Millington, M., Hyland, V.J. and Grennan, D. (1995) Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. *Clin. Exp. Immunol.*, **99**, 303–310.
16. Higuchi, T., Seki, N., Kamizono, S., Yamada, A., Kimura, A., Kato, H. and Itoh, K. (1998) Polymorphism of the 5′-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. *Tissue Antigens*, **51**, 605–612.
17. Juszczynski, P., Kalinka, E., Bienvenu, J., Woszczek, G., Borowiec, M., Robak, T., Kowalski, M., Lech-Maranda, E., Baseggio, L., Coiffier, B. *et al.* (2002) Human leukocyte antigens class II and tumor necrosis factor genetic polymorphisms are independent predictors of non-Hodgkin lymphoma outcome. *Blood*, **100**, 3037–3040.
18. Kroeger, K.M., Carville, K.S. and Abraham, L.J. (1997) The −308 tumor necrosis factor-alpha promoter polymorphism effects transcription. *Mol. Immunol.*, **34**, 391–399.
19. Wilson, A.G., Symons, J.A., McDowell, T.L., McDevitt, H.O. and Duff, G.W. (1997) Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc. Natl Acad. Sci. USA*, **94**, 3195–3199.
20. Hohler, T., Gerken, G., Notghi, A., Lubjuhn, R., Taheri, H., Protzer, U., Lohr, H.F., Schneider, P.M., Meyer zum Buschenfelde, K.H. and Rittner, C. (1997) HLA-DRB1*1301 and *1302 protect against chronic hepatitis B. *J. Hepatol.*, **26**, 503–507.
21. Ahn, S.H., Han, K.H., Park, J.Y., Lee, C.K., Kang, S.W., Chon, C.Y., Kim, Y.S., Park, K., Kim, D.K. and Moon, Y.M. (2000) Association between hepatitis B virus infection and HLA-DR type in Korea. *Hepatology*, **31**, 1371–1373.
22. Skoog, T., van't Hooft, F.M., Kallin, B., Jovinge, S., Boquist, S., Nilsson, J., Eriksson, P. and Hamsten, A. (1999) A common functional polymorphism (C→A substitution at position −863) in the promoter region of the tumour necrosis factor-alpha (TNF-alpha) gene associated with reduced circulating levels of TNF-alpha. *Hum. Mol. Genet.*, **8**, 1443–1449.
23. Hohler, T., Kruger, A., Gerken, G., Schneider, P.M., Meyer zum Buschenefelde, K.H. and Rittner, C. (1998) A tumor necrosis factor-alpha (TNF-alpha) promoter polymorphism is associated with chronic hepatitis B infection. *Clin. Exp. Immunol.*, **111**, 579–582.
24. Wennberg, P., Nordstrom, P., Lorentzon, R., Lerner, U.H. and Lorentzon, M. (2002) TNF-alpha gene polymorphism and plasma TNF-alpha levels are related to lumbar spine bone area in healthy female Caucasian adolescents. *Eur. J. Endocrinol.*, **146**, 629–634.
25. Chen, C.J., Yen, J.H., Tsai, W.C., Wu, C.S., Chiang, W., Tsai, J.J. and Liu, H.W. (1997) The TNF2 allele does not contribute towards susceptibility to systemic lupus erythematosus. *Immunol. Lett.*, **55**, 1–3.
26. McGuire, W., Hill, A.V., Allsopp, C.E., Greenwood, B.M. and Kwiatkowski, D. (1994) Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature*, **371**, 508–510.
27. Czaja, A.J., Cookson, S., Constantini, P.K., Clare, M., Underhill, J.A. and Donaldson, P.T. (1999) Cytokine polymorphisms associated with clinical features and treatment outcome in type 1 autoimmune hepatitis. *Gastroenterology*, **117**, 645–652.
28. Park, M.H., Hwang, Y.S., Park, K.S., Tokunaga, K., Akaza, T., Juji, T. and Kim, S.I. (1998) HLA haplotypes in Koreans based on 107 families. *Tissue Antigens*, **51**, 347–355.
29. Makridakis, N.M. and Reichardt, J.K. (2001) Multiplex automated primer extension analysis: simultaneous genotyping of several polymorphisms. *Biotechniques*, **31**, 1374–1380.
30. Hedrick, P.W. (1987) Gametic disequilibrium measures: proceed with caution. *Genetics*, **117**, 331–341.
31. Stephens, M., Smith, N.J. and Donnelly, P. (2001) A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.*, **68**, 978–989.