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Association of transforming growth factor-β1 gene polymorphisms with a hepatocellular carcinoma risk in patients with chronic hepatitis B virus infection

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Abbreviations: anti-HBc, antibody to hepatitis B core antigen; anti-HCV, antibody to hepatitis C virus antigen; anti-HIV, antibody to human immunodeficiency virus antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; SBE, single base extension; SNP, single nucleotide polymorphism; TGF-β, transforming growth Factor-β

Abstract

Transforming growth factor-β1 (TGF-β1) can act as both a tumor suppressor and a stimulator of tumor progression. We have examined the relationship between polymorphisms of the TGF-β1 gene and the risk of hepatocellular carcinoma (HCC) in patients with chronic hepatitis B virus (HBV) infection. A total of 1,237 Korean subjects were prospectively enrolled; 1,046 patients with chronic HBV infection and 191 healthy controls with no evidence of recent or remote HBV infection. The patients were divided into two groups: those without (n = 809) and those with HCC (n = 237). Single nucleotide polymorphisms (SNPs) of TGF-β1 were searched for and genotyped using the single base extension method. In Korean subjects, only two SNPs were found among the seven known polymorphisms of TGF-β1, at position -509 and in codon 10. The risk of HCC was significantly lower in patients with the T/T or C/T genotypes than in those with the C/C genotypes at position -509 (P < 0.02), and also lower among those with the Pro/Pro or Leu/Pro genotypes than in those with the Leu/Leu genotypes in codon 10 (P < 0.007). Haplotype analysis revealed that the possession of [-509C > T; L10P] conferred a decreased likelihood of HCC (OR = 0.74; 95% CI, 0.59-0.93; P = 0.008). In conclusion, the presence of the TGF-β1 -509C > T promoter or of the L10P polymorphism, and the combination of both [-509C > T; L10P] as a haplotype were strongly associated with a reduced risk of HCC in patients with chronic HBV infection.

Keywords: chronic hepatitis; HBV; genetic susceptibility; hepatocellular carcinoma; single nucleotide polymorphism; transforming growth factor-β1

Introduction

Chronic hepatitis B virus (HBV) infection is a global public health problem as this virus is known to infect more than 350 million people worldwide and is one of the major risk factors of hepatocellular carcinoma (HCC) (Purcell, 1993). HCC is etiologically associated with HBV in 80% of cases (Yu *et al.*, 2000a), and is the dominant cause of death among HBV carriers, with a lifetime risk of 27% for males and 4% for females (Dickinson *et al.*, 2002). Among patients with chronic HBV infection, family history is a known risk factor for the development of HCC (Yu *et al.*, 2000b); therefore, genetic factors are likely to modify the risk of HCC. However, the genetic factors that determine progression to HCC remain unknown.

Single nucleotide polymorphisms (SNPs) has emerged as one of the important tools for tracking down the genes responsible for conferring susceptibility to common diseases, such as heart disease and cancer. Among those genes that may be involved in oncogenesis, transforming growth factor-β (TGF-β) has been identified as both a tumor suppressor during the early stages of tumorigenesis and a significant stimulator of tumor growth, invasion and metastasis during tumor progression (Cui *et al.*, 1996). In most epithelial and endothelial cells, TGF-β causes arrest of the cell cycle, and potently suppresses the prolif-

eration of hepatocytes (Wollenberg *et al.,* 1987). A recent case-control study showed that a polymorphism in the type I receptor for TGF-β that leads to decreased biological activity may be associated with increased risk of colon cancer (Pasche *et al.,* 1999). Changes in the expression of one of the TGF-β ligands, TGF-β1, have also been implicated in oncogenesis in transgenic mice; deletion of one copy of the TGF-β1 gene leads to increased cell turnover and susceptibility to liver and lung tumors when induced by carcinogens (Tang *et al.,* 1998). The production of TGF-β1 is under genetic control (Awad *et al.,* 1998; El-Gamel *et al.,* 1999; Grainger *et al.,* 1999), and seven polymorphisms have been described in the coding and promoter region of the TGF-β1 gene (Cambien *et al.,* 1996). The present study was to investigate the relationships between the known seven SNPs of the TGF-β1 gene and the risk of HCC occurrence in patients with chronic HBV infection in Korea.

Materials and Methods

Subjects

A total of 1,237 Korean subjects were prospectively enrolled; 1,046 patients with chronic HBV infection from a liver unit and 191 IgG anti-HBc negative healthy subjects from the Center for Health Promotion at Seoul National University Hospital between January 2001 and August 2001. Among the 1,046 HBV infected patients, 791 were male and 255 female. Patient ages ranged from 20 to 75 years with a mean age of 50.4 (SD = 10.4) years. Patients with chronic HBV infection were further divided into 2 groups: without (n = 809) and with HCC (n = 237) according to the absence or presence of concurrent HCC. HCC was diagnosed as described (Bruix *et al.,* 2001). Among patients with chronic HBV infection, patients with HCC were older than those without HCC (Table 1). The inclusion criteria for chronic HBV infection were repeated seropositivity for hepatitis B surface antigen by ELISA (Enzygnost® HBsAg 5.0; Dade Behring, Marburg, Germany) for a period of at least 6 months. Patients who were positive for anti-HCV or anti-HIV by ELISA (HCV®3.2; Dong-A Pharmaceutical Co., Seoul, Korea, GENEDIA®; Greencross Life Science Corp., Yongin-shi, Korea, respectively) and whose average alcohol consumption assessed by interview was ≥10 g/day were excluded. The consumption and types of alcohol drinking were asked and the alcohol content of each drink was calculated. Weekly alcohol intake was converted into alcohol grams/day. The patients who have any other types of liver disease such as autoimmune hepatitis, toxic hepatitis, primary biliary cirrhosis or Budd-Chiari syndrome were also excluded. We also excluded subjects who had a previous history of immunosuppression or anti-viral treatment.

Informed consent was obtained from each patient, and the Institutional Review Board of Seoul National

Table 1. The characteristics of patients with chronic HBV infection.

Group	No. of patients			Age (Mean ± SD)
	Total	Males	Females	
Without HCC	809	598	211	49.0 ± 10.5
With HCC	237	193	44	55.6 ± 8.8
Total	1,046	791	255	50.4 ± 10.4

Table 2. Sequences of amplifying primers used for direct sequencing of TGF-β1 gene.

Primer	Sequence
Promoter-1 (forward)	5'-ATGCCAGGTGGAAGGTGGAT-3'
Promoter-1 (reverse)	5'-TGCCAACTGTTCTCGCCAAC-3'
Promoter-2 (forward)	5'-CTTGCAGGCTATGGATTTTGC-3'
Promoter-2 (reverse)	5'-ATCCAGATGCGCTGTGGCTT-3'
Promoter-3 (forward)	5'-GAGGCCCCCATGTTGACAGA-3'
Promoter-3 (reverse)	5'-CCCAGCGGCAACGGAAAAGT-3'
5' UTR (forward)	5'-GAGGAAGGAGTCGCCGAGGA-3'
5' UTR (reverse)	5'-CGATAGTCTTGCAGGTGGATAG-3'
Exon1 (forward)	5'-CCCACCACACCAGCCCTGTTC-3'
Exon1 (reverse)	5'-GTGTCCTCTTCCTCCAGCCAGT-3'
Exon2 (forward)	5'-GGTATCAGAGACTGACTCCA-3'
Exon2 (reverse)	5'-AGTTCTAGGATTGTATGGTTTG-3'
Exon3 (forward)	5'-ACACCTTCGGCTGAGCTGT-3'
Exon3 (reverse)	5'-AGATTAGCCAATCACTCAGGT-3'
Exon4/5 (forward)	5'-TGCAGTGAGAGGGCAGAGT-3'
Exon4/5 (reverse)	5'-GCTAAAGGAGACAGATGCTC-3'
Exon6 (forward)	5'-GAGGAAGTGCTTAATCTGTGT-3'
Exon6 (reverse)	5'-GTGGGTCTTCATAGCTCATC-3'
Exon7 (forward)	5'-ACCGAATTGGAGATGGGAAGA-3'
Exon7 (reverse)	5'-AGAGATAGGGTCTCACTATGTT-3'

Table 3. Sequences of amplifying and extension primers for TGF-β1 single nucleotide polymorphism genotyping by the single base extension method.

Primer	Sequence
TGF-β1 -800G > A (forward)	5'-CTTGCAGGCTATGGATTTTGC-3'
TGF-β1 -800G > A (reverse)	5'-ATCCAGATGCGCTGTGGCTT-3'
TGF-β1 -800G > A (extension)	5'-TGAGGGACTCTGCCTCCAAC-3'
TGF-β1 -509C > T (forward)	5'-CTTGCAGGCTATGGATTTTGC-3'
TGF-β1 -509C > T (reverse)	5'-ATCCAGATGCGCTGTGGCTT-3'
TGF-β1 -509C > T (extension)	5'-TAATCAATGATGATGGGCAACAGGACACCTGA-3'
TGF-β1 L10P (forward)	5'-CCCACCACACCAGCCCTGTTC-3'
TGF-β1 L10P (reverse)	5'-GTGTCCTCTTCCTCCAGCCAGT-3'
TGF-β1 L10P (extension)	5'-ACAGCAGCGGTAGCAGCAGC-3'
TGF-β1 R25P (forward)	5'-CCCACCACACCAGCCCTGTTC-3'
TGF-β1 R25P (reverse)	5'-GTGTCCTCTTCCTCCAGCCAGT-3'
TGF-β1 R25P (extension)	5'-CATGATTATAATCAATGATGATGTGGCTACTGGTGCTGACGCCTGGCC-3'
TGF-β1 T263I (forward)	5'-TGCAGTGAGAGGGCAGAGT-3'
TGF-β1 T263I (reverse)	5'-GCTAAAGGAGACAGATGCTC-3'
TGF-β1 T263I (extension)	5'-ATCAATGATGATCCGGCCTTTCCTGCTTCTCATGGCCA-3'

University Hospital approved the study protocol.

Identification and validation of the SNPs of the TGF-β1 gene in the Korean population

Polymorphisms of TGF-β1 were identified in 24 unrelated Korean individuals by direct sequencing of the 7 exons and the promoter region. Genomic DNA was prepared from each blood sample using a QIA amp blood kit (QIAGEN Inc., Valencia, CA). The primers used for direct sequencing are listed in Table 2. To clean up the PCR products, we used a Montage PCR96 Cleanup Kit (Millipore Corp., Bedford, MA) and sequencing was carried out using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing data was aligned and analyzed using the Vector NTI® Suite (Informax, Bethesda, MD) and results were further validated in 340 unrelated Koreans by SNP genotyping as described below.

Genotyping for the SNPs of the TGF-β1 gene in the study population

The two SNPs, at TGF-β1 -509 and in codon 10, the presence of which have been confirmed in this study were genotyped in 1,046 patients with chronic HBV infection and in 191 healthy controls by the single base extension (SBE) method. The PCR primer sequences used for the amplification and extension of the TGF-β1 SNPs by the SBE method are listed in Table 3. 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) 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) 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 incubated at 95°C for 5 min, and then 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.

Statistical analyses

χ^2 tests were used to compare the observed numbers of each genotype with those expected for the population using the Hardy-Weinberg equilibrium. Heterozygosity for each locus with allele frequencies p and q = 1-p was calculated using $H = 1-p^2-q^2 = 2p(1-p)$. We examined several widely used measures of linkage disequilibrium between all pairs of biallelic loci, namely, the value of the χ^2 tests (corresponding *P*-values), Lewontin's D' (|D'|) and the d^2 measure. Odds ratios with a 95% confidence interval and *P*-values of the logistic regression models, controlling for the effects of age (as continuous variables) and sex, were computed using SAS for the analysis of categorized phenotypes based on the assumption that most patients, if not all, were infected with HBV perinatally (Stevens *et al.,* 1975; Lok *et al.,* 1987).

Results

SNPs of the TGF-β1 gene found in unrelated Korean individuals

When polymorphisms were searched for in 24 unrelated Korean individuals by direct sequencing of the 7 exons and the promoter region of TGF-β1 gene, only two known polymorphisms, -509C>T and L10P (29T>C) were found at positions -509 in promoter region and codon 10 in exon 1. No polymorphisms were found at positions -988, -800, +72, or at codon 25 or codon 263 in the 24 unrelated Korean individuals, and this was further confirmed by single base extension reactions and electrophoresis for the polymorphisms at positions -800, codon 25, and codon 263 in 340 unrelated Korean individuals. Consequently we tested TGF-β1 -509 and codon 10 polymorphisms in the present study.

Genotype distribution

The genotype distribution of TGF-β1 -509 and codon 10 polymorphisms were not significantly different between the control and patient groups (data not shown). The distributions of the genotypes of the polymorphisms at -509 and in codon 10 in patients with and without HCC are shown in Table 4. No evidence of a departure from the Hardy-Weinberg equilibrium was apparent. The genotype distribution of the TGF-β1 -509 and codon 10 polymorphisms were significantly different in those with HCC and without HCC; the risk of HCC was significantly lower among patients with the T/T or C/T genotypes at position -509 than among patients with the C/C genotype and was also lower among patients with the Pro/Pro or Leu/Pro genotypes in codon 10 than in those with the Leu/Leu genotype ($P < 0.02$ and $P < 0.007$, respectively).

Haplotype analyses were performed and the possible four haplotype frequencies are shown in Table 5. Two major haplotypes account for more than 97% of these four haplotypes. TGF-β1 -509C>T and the L10P polymorphisms showed strong linkage disequilibrium (|D'| = 0.985, $d^2 = 0.919$). By haplotype analysis, we found the possession of [-509T; L10P-Pro] conferred a decreased likelihood of HCC (OR = 0.74; 95% CI, 0.59-0.93; $P = 0.008$), while the [-509C; L10P-Leu] haplotype was associated with an increased likelihood of HCC (OR = 1.37; 95% CI, 1.09-1.72; $P = 0.006$) (Table 6).

Table 5. The Haplotype Frequencies in Korean patients with chronic HBV infection.

Haplotypes	TGF-β1-509	TGF-β1 codon 10	Frequencies
1	C	Pro	0.017
2	C	Leu	0.537
3	T	Pro	0.442
4	T	Leu	0.003

Table 4. Differential distribution of the TGF-β1 genotypes in Korean patients with chronic HBV infection. Statistical analysis. Logistic regression models were used for calculating the odds ratios (95% confidential interval) and corresponding P-values, controlling for age (continuous) and sex as covariates. Age was found to be highly associated with the occurrence of HCC ($P < 0.0001$) as expected. Sex was associated with the occurrence of HCC in patients with chronic HBV infection ($P < 0.001$).

Loci	Genotype	With HCC	Without HCC	OR (95% CI)	*P*
TGF-β1 -509	C/C	76 (33.3%)	187 (24.2%)	—	
	C/T, T/T	152 (66.7%)	586 (75.8%)	0.67 (0.48-0.93)	0.02
TGF-β1 codon 10	Leu/Leu	77 (35.3%)	183 (24.4%)	—	
	Leu/Pro, Pro/Pro	141 (64.7%)	568 (75.6%)	0.62 (0.45-0.88)	0.007

Table 6. Differential distribution of TGF-β1 haplotypes in Korean patients with chronic HBV infection.

Haplotypes	With HCC ($2n$ = 416)	Without HCC ($2n$ = 1,430)	OR (95% CI)	*P*-values
1 [C; Pro]	0.012	0.018	0.66 (0.22-1.81)	0.517
2 [C; Leu]	0.591	0.514	1.37 (1.09-1.72)	0.006
3 [T; Pro]	0.389	0.464	0.74 (0.59-0.93)	0.008
4 [T; Leu]	0.007	0.004	1.72 (0.34-7.75)	0.430

Discussion

This study shows that among the 7 known SNPs of TGF-β1 in the global populations, only two polymorphisms, at position -509 and in codon 10 were identified in the Korean population. No polymorphisms were found at positions -988, -800, +72, codon 25, or codon 263. The presence of the TGF-β1 -509C > T or of the L10P polymorphism, and the combination of [-509C > T; L10P] as a haplotype were found each to be strongly associated with a reduced risk of HCC occurrence in patients with chronic hepatitis B infection.

Previous studies in the Caucasian population have shown that the frequencies of the less frequent alleles at positions -988, -800, +72, codon 25, and codon 263 are 0.002, 0.088, 0.078, 0.082 and 0.030, respectively (Cambien *et al.,* 1996). On the other hand, the frequencies at positions -509 and codon 10 were found to be 0.343 and 0.416, respectively (Cambien *et al.,* 1996). The allele frequencies of TGF-β1 -509T and Pro allele in codon 10 in Koreans were 0.48 and 0.47 respectively but uncommon polymorphisms were not found in this study in the Korean population. Differences in allele frequencies of polymorphisms of the TGF-β1 gene in Caucasians and Koreans might be explained by ethnic difference. Lee *et al.* recently screened 300 SNPs selected from the public database in 24 Korean individuals and found that approximately 23% of the SNPs were not found (Lee *et al.,* 2001). The results of the present study suggest that ethnic and population based differences should be considered in the selection of SNPs because highly frequent population-specific alleles are particularly useful for mapping the genes responsible for disease susceptibility and other traits in the population (Parra *et al.,* 1998; Stephens *et al.,* 2001). Further studies are needed to estimate the allele frequencies of these SNPs in other populations.

The -509 polymorphism is located in a potential regulatory region and recently it was found that the -509T allele is related to a higher plasma concentration of TGF-β1 and a higher level of transcriptional activity than the -509C allele (Grainger *et al.,* 1999; Luedecking *et al.,* 2000). A gene dose effect was observed for the T allele; individuals homozygous for -509T/T had higher plasma concentrations of TGF-β1 than heterozygous C/T and homozygous C/C individuals (Grainger *et al.,* 1999). The Pro allele of the codon 10 polymorphism in the Japanese population was also found to be associated with higher serum levels of TGF-β1 than the Leu allele (Yamada *et al.,* 1998; Yokota *et al.,* 2000). A gene dose effect was also found for the Pro allele (Yamada *et al.,* 1998). These relationships might be expected by the strong linkage disequilibrium between the two polymorphisms of the TGF-β1 -509T promoter and Pro allele of the codon 10, shown by this study ($|D'|$ = 0.985) and a study in the Caucasian population ($|D'|$ = 0.95) (Cambien *et al.,* 1996). Therefore, polymorphisms which are associated with variations in the level of TGF-β1 expression may alter susceptibility to cancer in humans (Tang *et al.,* 1998). This hypothesis has been confirmed by a report that the Pro/Pro genotype in codon 10 of the TGF-β1 gene is associated with a markedly reduced risk of breast cancer (Ziv *et al.,* 2001). The present study shows that the possession of the -509T allele (T/T or C/T) or of the Pro allele (Pro/Pro or Leu/Pro) in codon 10 is associated with a decreased risk of HCC as compared with patients having the C allele or the Leu allele only (C/C or Leu/Leu). The haplotype [-509C > T; L10P] also showed a significant association with a reduced risk of HCC occurrence. TGF-β1 -509C/T genotype and Leu/Pro heterozygote genotype in codon 10 appear to be associated with a slightly higher level of TGF-β1 than the C/C or the Leu/Leu homozygote genotypes, respectively (Yamada *et al.,* 1998; Grainger *et al.,* 1999; Yokota *et al.,* 2000). These findings might suggest that TGF-β1 induced suppression of HCC could be augmented by only a slight increase of the TGF-β1 level by these genetic factors. These findings are consistent with the results of studies in transgenic mice and in hepatoma cell lines (Kim *et al.,* 2002). Transgenic mice with a single TGF-β1 gene deletion are more susceptible to liver and lung tumors induced by carcinogens (Tang *et al.,* 1998), while the increased expression of TGF-β1 under the control of a murine mammary tumor virus promoter reduces the risk of induced mammary carcinoma (Pierce *et al.,* 1995). In our study, the

[-509C>T; L10P] haplotype was found to be associated with a reduced risk of HCC with an odds ratio of 0.74. These findings suggest that the TGF-β1 polymorphism is one of the most important genetic factors in the control of HCC occurrence. These findings might be applied in clinical practice to stratify the risk groups for the occurrence of HCC in patients with chronic HBV infection. Further studies are needed to find polymorphisms in other genes that are more closely associated with HCC occurrence to be integrated into HCC surveillance programs in the future.

In conclusion, we found that the presence of the TGF-β1 -509C>T promoter or of the L10P polymorphism, and the combination [-509C>T; L10P] as a haplotype was strongly associated with a reduced risk of HCC occurrence in patients with chronic HBV infection. This finding might be useful in clinical practice in the design of HCC surveillance programs in patients with chronic HBV infection, if further genetic susceptibilities are identified.

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