

Matrix Metalloproteinase-3 Genotypes Influence Recovery from Hepatitis B Virus Infection

The reasons for the viral persistence of hepatitis B virus (HBV) infection are unknown, but are probably related to host immune factors. Several matrix metalloproteinases (MMPs) can regulate an inflammatory response. The aim of this study was to assess the effects of the single nucleotide polymorphisms (SNPs) of MMP-3 and -9 genes on the susceptibility to persistent HBV infection. We studied 489 Korean patients with HBV infection (144 inactive carriers, 182 chronic hepatitis, and 163 liver cirrhosis) and 174 healthy individuals who had recovered from HBV infection. MMP-3 gene SNPs were identified at two polymorphic sites (codon 45 [E45K] and codon 96 [D96D]) and MMP-9 gene SNPs at three polymorphic sites (codon 279 [R279Q], codon 607 [G607G], and codon 668 [Q668R]) in study subjects. The frequency of T allele at third position of codon 96 in the MMP-3 gene was higher in HBV persistence patients when analyzed by co-dominant model (age- and sex-adjusted OR=1.242, 95% CI=1.001-1.540, $p=0.049$). In conclusion the T allele at the third position of codon 96 in the MMP-3 gene might be associated with persistent HBV infection.

Key Words : Hepatitis B; Matrix Metalloproteinase; Polymorphism; Single Nucleotide

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INTRODUCTION

Hepatitis B virus (HBV) infection affects about 400 million people worldwide and is the most common cause of acute and chronic liver disease especially in several areas of Asia including Korea. Three factors thought to influence the outcome of HBV infection have been virus itself, host genetic factors, and environmental factors. Initially, the majority of host genetic studies with HBV infection have focused on human leukocyte antigen associations (1, 2). Recent studies showed that cytokine genetic polymorphisms have an association with the development of chronic HBV infection (3) and the progression of the infection (4).

The matrix metalloproteinases (MMPs) are thought to play key roles in embryonic development, morphogenesis, reproduction, and tissue remodeling (5). Although the main function of MMPs is the removal of extracellular matrix (ECM) during tissue resorption, their roles in infectious disease are deemed to be considerable. The exact role of most MMPs in inflammatory conditions has not yet been elucidated, even to the extent of understanding whether they function to improve or worsen inflammation. Several MMPs were reported to regulate an inflammatory response by controlling the activity and

mobilization of chemokines (6).

MMP-3, an important member of metalloproteinase family, is produced by various types of cells; fibroblast, smooth muscle cells, and macrophages. MMP-3 is known to mediate the release of macrophage chemotactic activity from chondrocytes (7). MMP-9 is one of the important MMP family members in terms of inflammation. Activated neutrophils release MMP-9, which degrades and neutralizes the serine protease inhibitor $\alpha 1$ -antitrypsin, a potent inhibitor of neutrophil elastase (8).

The aim of this study was to clarify whether *MMP3* or *MMP9* gene single nucleotide polymorphisms (SNPs) could predict the likelihood of viral persistence after HBV infection in a single ethnic Korean population.

MATERIALS AND METHODS

Study subjects

The case-control population included 663 Korean adults who were enrolled from the outpatient clinics of the Department of Gastroenterology, Ajou University Hospital (Suwon,

Korea) between March 2002 and December 2003. Of these, 489 patients who had been positive for both HBsAg and anti-HBc IgG for more than 6 months were defined as the persistent HBV infection (M=364, F=125, aged 16-77 yr, mean \pm SD; 41.84 ± 10.98). They were regularly followed up with blood tests for serum transaminase, HBeAg/anti-HBe and alpha-fetoprotein (AFP), and with ultrasonography or computed tomography of the liver at an interval of every 6 months for more than 12 months. Of the 489, 144 patients were considered to be inactive HBsAg carriers based on sustained normal alanine aminotransferase (ALT) levels and positivity for anti-HBe and an undetectable level of HBV DNA in serum. A total of 182 patients were found to have chronic hepatitis, manifested by elevated ALT (≥ 2 times the upper limit of normal) at least once during the follow-up period and positivity for HBeAg and HBV-DNA. A total of 163 patients were diagnosed as liver cirrhosis based on the typical morphologic findings on computed tomography/ultrasound and corresponding laboratory features or evidence of portal hypertension. None of them had hepatocellular carcinoma. In addition, we evaluated 174 healthy individuals (M=128, F=46, aged 26-75 yr, mean \pm SD; 48.73 ± 9.13) who had recovered from HBV infection (HBsAg [-], anti-HBc IgG [+], anti-HBs [+]) and visited the Center for Health Promotion of Ajou University Hospital during the period of the study as a control group.

Patients who were positive for anti-HBs and negative for anti-HBc IgG, and patients with other types of chronic liver disease such as alcoholic liver disease, chronic hepatitis C, steatohepatitis, and Wilson's disease were excluded from the study. All the subjects were Korean, a single ethnic group. Informed consent was obtained from each subject, and the Institutional Review Board of Human Research of Ajou University Hospital approved the study protocol.

Genotyping

We screened 40 Koreans to identify polymorphisms in the *MMP3* and *MMP9* genes. Among the identified polymorphisms, five were selected for larger scale genotyping (n=663) for the HBV association study based on frequencies and/or loca-

tion. We assessed SNPs at two polymorphic sites in the *MMP3* gene (the first position of codon 45 [E45K, G to A, rs679620] and the third position of codon 96 [D96D, C to T, rs602128]) and at three polymorphic sites in the *MMP9* gene (the second position of codon 279 [R279Q, G to A, rs17576], third position of codon 607 [G607G, G to A, rs13969], and the second position of codon 668 [Q668R, A to G, rs2274756]) in study subjects.

Genomic DNA was extracted from 300 μ L whole blood using a DNA Purification kit (GENTRA, Minneapolis, MN, U.S.A.), according to the manufacturer's instructions. The SNPs were detected by polymerase chain reaction (PCR) amplification. The sequence of the primers and probes used in the assays are provided in Table 1. The parameters for thermocycling were as follows: an initial activation step of 95°C for 10 min preceded the cycling program, followed by 35 cycles of denaturation for 95°C, annealing at 72°C for 1 min, and final extension at 72°C for 7 min. Each PCR product was purified by a Qiagen PCR purification kit, and the polymorphisms were detected by single base primer extension assay (SNP-IT™) using the method as previously described (9). Briefly, the genomic DNA region spanning the polymorphic site was PCR-amplified using one phosphorothiolated primer and one regular PCR primer. The amplified PCR products were digested with exonuclease. The 5'-phosphorothioates protect one strand of the PCR product from exonuclease digestion, resulting in the generation of a single-stranded PCR template. The single-stranded PCR template is overlaid onto a 384-well plate that contains covalently attached SNP-IT™ extension primer designed to hybridize immediately adjacent to the polymorphic site. The SNP-IT™ primer is extended for a single base with DNA polymerase and mixture of appropriate acycloclerminator that is labeled with either FITC or biotin and complementary to the polymorphic nucleotide. The identity of the incorporated nucleotide is determined with serial colorimetric reactions with anti-FITC-AP and streptavidin-HRP, respectively. The results of yellow and/or blue color developments were analyzed with an ELISA reader, and the final genotype calls were made with the QCR-eview™ program.

All genotyping results were quality controlled by includ-

Table 1. Sequences of PCR-amplifying primers and extension primers used in the SNP-IT assays

Gene	PCR-amplifying primers	Extension primers
<i>MMP3</i> -E45K	F: 5'-ATATTCAGACATTCACAATTGATT-3' R: 5'-TATTATTATACGAGCTTTAAAAGATAGTTC-3'	TTTATXCTTACAACACAAAATCAAATC
<i>MMP3</i> -D96D	F: 5'-ACAGACCCCGGTGACGGA-3' R: 5'-ACGGTCGCTGGCTCCAAC-3'	CGAGCGCCTGCGGGACCAGCCCAGC
<i>MMP9</i> -R279Q	F: 5'-AGTGTTCCTCCACTGGAGAGG-3' R: 5'-ATCTTTTCACCCTCCTGGC-3'	CGGCAAGTGCCCGGGCGATCCCCTC
<i>MMP9</i> -G607G	F: 5'-AGTGTTCCTCCACTGGAGAGG-3' R: 5'-ATCTTTTCACCCTCCTGGC-3'	CGGCAAGTGCCCGGGCGATCCCCTC
<i>MMP9</i> -Q668R	F: 5'-AGTGTTCCTCCACTGGAGAGG-3' R: 5'-CTCCTGGGGCCATCTTTT-3'	CCCTCACCTGCGTTCXGGAGATATACC

SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; MMP, matrix metalloproteinases.

ing duplicate samples. We included 6 replicate samples for each of 96 well plates and checked concordances. We accepted data from a plate only if they have less than one mismatches per each plate. Overall concordances were over 99.9% throughout the experiments.

Statistical analysis

For univariate analysis, the χ^2 test was used for Hardy-Weinberg equilibrium of alleles at individual loci and independent sample t-test for normally distributed continuous variables. For multivariate analysis, multiple logistic regression analysis was performed to determine which factor (s) was the most discriminating for HBV persistence after HBV infection, where age, sex, cytokine, and its related gene polymorphisms were independent variables. Odds ratios (ORs) with 95% confidence intervals were computed by logistic regression using SPSS version 11.0 software (Chicago, IL, U.S.A.). All p values were two-tailed, and a p value <0.05 was considered to indicate statistical significance throughout the study.

RESULTS

The clinical and demographic characteristics of the case-control populations were compared. There was no significant difference between HBV persistence and clearance subjects in terms of sex (M:F=364:125 vs. 128:46, respectively). Although an effort was made to obtain an age-matched cases and controls, the age was younger in the persistence group than that in the clearance group (41.84 ± 10.98 vs. 48.73 ± 9.13 , mean \pm SD, respectively, $p=0.00$).

Table 2 shows the summarized data regarding the geno-

type distributions in the HBV persistence and HBV clearance groups. In an analyzing model in which a co-dominant (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.

Genotype frequencies in the *MMP3* and *MMP9* exon regions were analyzed in patients with HBV persistence and HBV clearance subjects. Among the five SNP sites analyzed, T allele carriers at the third position of codon 96 in the *MMP3* gene had a weak correlation with HBV persistence in a co-dominant model (age- and sex-adjusted ORs; 1.242, $p=0.049$).

On the basis of unconditional logistic regression analysis with adjustment for age and sex, no statistically significant association was observed with other SNP sites in terms of the susceptibility to persistent HBV infection.

DISCUSSION

We examined the polymorphisms in exon regions of *MMP3* and *MMP9* genes in 663 Korean subjects and analyzed the association of these polymorphisms with HBV persistence after HBV infection. The T allele at the third position of codon 96 in the *MMP3* gene was associated with HBV persistence in a co-dominant model ($p=0.049$). This is the first study to demonstrate that genetically determined differences in the *MMP3* gene may be a determinant of recovery from HBV infection.

Recovery after HBV infection is known to be dependent on

Table 2. Association between *MMP3/MMP9* gene polymorphisms and HBV persistence whilst controlling age and sex

Loci	Genotype	HBV persistence (n=489)	HBV clearance (n=174)	Co-dominant		Dominant		Recessive	
				OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
<i>MMP3</i> -E45K	GG	201 (41.10%)	74 (42.53%)	1.199 (0.964-1.490)	0.103	1.227 (0.926-1.625)	0.154	1.331 (0.827-2.143)	0.239
	AG	237 (48.47%)	88 (50.57%)						
	AA	51 (10.43%)	12 (6.90%)						
<i>MMP3</i> -D96D	CC	210 (42.94%)	83 (47.70%)	1.242 (1.001-1.540)	0.049	1.306 (0.988-1.726)	0.061	1.331 (0.827-2.143)	0.239
	CT	228 (46.63%)	79 (45.40%)						
	TT	51 (10.43%)	12 (6.89%)						
<i>MMP9</i> -R279Q	GG	215 (43.97%)	72 (41.38%)	0.906 (0.722-1.136)	0.392	0.930 (0.703-1.230)	0.609	0.746 (0.435-1.277)	0.285
	AG	243 (49.69%)	86 (49.43%)						
	AA	31 (6.34%)	16 (9.20%)						
<i>MMP9</i> -G607G	AA	275 (56.24%)	110 (63.22%)	1.140 (0.898-1.447)	0.281	1.118 (0.845-1.481)	0.435	1.484 (0.759-2.901)	0.249
	AC	189 (38.65%)	59 (33.91%)						
	CC	25 (5.11%)	5 (2.87%)						
<i>MMP9</i> -Q668R	GG	382 (78.12%)	131 (75.29%)	0.988 (0.754-1.296)	0.931	0.947 (0.680-1.318)	0.746	1.183 (0.552-2.535)	0.666
	AG	87 (17.80%)	40 (22.98%)						
	AA	20 (4.09%)	3 (1.72%)						

MMP, matrix metalloproteinases; HBV, hepatitis B virus; OR, odds ratio; CI, confidence interval.

the integrated activities of the patients' immune systems and the cytokine network. Recent studies have shown that several immunoregulatory cytokines such as interferon- γ and tumor necrosis factor- α (TNF- α) inhibit HBV replication through the non-cytolytic process (10). We previously reported that the association between various cytokines and HBV persistence. We showed specific interleukin-10 and TNF- α promoter SNPs were associated with HBV persistence (11), but there was no association between mannose binding lectin gene SNPs and HBV infection (12).

MMPs represent a family of at least 20 zinc-dependent proteolytic enzymes that are important in physiological and disease-related ECM remodeling (5). As our knowledge of this family continues to grow, we are learning that their activities are not limited to matrix degradation (6). MMPs directly modulate chemokine activity. Processing by MMP-9 leads to a loss of chemotactic activity of various chemokines, such as CXCL5, CXCL6, and mouse CXCL5 (13). By contrast, the processing of CXCL8 by MMP-9 markedly increases its chemotactic activity (14). MMP-2, -3, and -9 can cleave and activate the IL-1 β precursor (15). Furthermore, after activating IL-1 β , MMP-3 degrades the biologically active cytokine, which can also be inactivated in vitro by MMP-1, -2, and -9. These data indicate a dual role for MMPs in biphasic modulation of inflammatory mediator activity (16).

Currently available data suggest that MMPs may have important roles in liver fibrosis and cirrhosis development, but a comprehensive description of MMP regulation in chronic hepatitis B is still lacking. Recent investigation suggested that the replication of HBV might cause increased secretion of MMP-2 that might, in turn, enhance the inflammation of liver tissue and lead to chronic hepatitis, since an elevated activity of MMP-2 has been related to inflammation in several systems (17). Previous reports showed that plasma activities of MMP-9 were elevated in hepatocellular carcinoma patients (18). Although a few investigators discussed the role of MMPs in liver disease, most of them had focused on their participation in cancer development and metastasis, some on liver cirrhosis, and few on chronic hepatitis.

Although the correlation was not strong, the results of this study suggest that the genotype of codon 96 in the MMP3 gene might be a host genetic factor affecting immunity against HBV infection. MMP-3 has no proven role in HBV pathogenesis. MMPs play a role in modulating chemokine activity, and Ahn et al. reported that genetic variations in CCR5 and its ligand, RANTES with clearance of hepatitis B virus in a Korean population (19). Thus, these data suggest either a novel mechanism of immune response to HBV involving MMP-3 or an alternative role for MMP-3 in HBV pathogenesis. Further work is needed to determine the precise contribution of MMP-3 to the control of HBV infection.

It would have been helpful to be able to correlate the precise levels of MMP-3 in our subjects. However, the MMP levels in sera would not be expected to be the representative of

serum levels at the time when HBV persistence or recovery from infection was determined. A meaningful measurement of serum MMP levels in our cohort would require a sample taken prior to HBV infection, which was not available.

In conclusion, the carriers of T allele at the third position of codon 96 in the MMP3 gene have higher risk of viral persistence after HBV infection. The information about SNPs in MMP genes might be useful for host genetic studies, especially in HBV infection.

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