Association of *GNLY* Genetic Polymorphisms with Chronic Liver Disease in a Korean Population

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Granulysin (GNLY) is found in cytotoxic granules of cytolytic T lymphocytes and natural killer (NK) cells, which are critical for hepatitis B virus (HBV) clearance. GNLY cytotoxicity plays an important role in the defense against viruses or intracellular bacteria. We hypothesized that genetic variation in the *GNLY* gene could affect the resistance of hosts against HBV infection. We compared the distribution frequencies of *GNLY* polymorphisms between an HBV-induced chronic liver disease (CLD) group and a spontaneous recovery (SR) control group to determine whether *GNLY* polymorphisms play a role in HBV clearance. A total of 117 patients in the SR group and 230 patients in the CLD group were enrolled. Samples derived from complex infections, including hepatitis C and human immunodeficiency virus, and those associated with insufficient clinical information (10 samples in SR and 24 samples in CLD) were excluded from the study. The final analysis included 107 SR and 206 CLD samples. DNA was extracted from peripheral blood, and *GNLY* genotypes were determined by the GoldenGate[®] method. The genotype distribution of the single-nucleotide polymorphisms (SNPs) rs2886767 (C>T), rs1561285 (G>C), and rs11127 (T>C) were significantly different between the SR and CLD groups in a recessive model ($p < 0.015$). These three SNPs were in a complete linkage disequilibrium (LD) block. Diplotype distributions of haplotype (HT) 1 (C-G-T) and HT2 (T-C-C) were significantly different between the SR and CLD groups in a recessive model ($p = 0.025$) and a dominant model ($p = 0.008$). All p -values remained significant after multiple comparisons. *GNLY* polymorphism genotypes and diplotypes were associated with the chronicity of HBV. These data suggested that genetic variation of *GNLY* may be an important factor in HBV clearance through the CD8⁺ T or NK cell-mediated removal of HBV-infected cells from the host.

Introduction

BETWEEN 350 AND 400 million people worldwide are chronically infected with the hepatitis B virus (HBV) (Lee, 1997; Lok and McMahon, 2007). While most HBV-infected patients show spontaneous recovery (SR) mediated by the host immune system, 5%–10% of patients fail to recover and develop chronic liver disease (CLD), including chronic hepatitis and cirrhosis (Thio *et al.*, 2003). HBV-induced CLD is a major risk factor for hepatocellular carcinoma (HCC) (Cha and Dematteo, 2005), which is one of the most common causes of cancer-related mortality. Although the effects of HBV infection have been studied extensively, the factors involved in the progression from acute hepatitis B to CLD have not been clearly defined. The chronicity of HBV is not only affected by environmental factors, viral strains, and gender and age at infection but also by the genetic variation of the host.

The Lin group (Thio *et al.*, 2003) reported that age- and sex-matched monozygotic twins and dizygotic twins showed

significant differences in the concordance rates for HBV infection. Moreover, the Carrilho group (Carrilho *et al.*, 2005) measured the frequency of HBV markers in genetically related (consanguineous) and nongenetically related (non-consanguineous) Brazilian families of Asian and Western origins and reported significantly higher ($p < 0.001$) HBsAg levels in Asian than in Western family members. These results suggest that genetic variations can result in different outcomes of HBV infection. Single-nucleotide polymorphisms (SNPs) are important sources of genetic variation, and the pathophysiology of HBV infection has been shown to be affected by individual genetic variants, including SNPs (Frodsham, 2005). The results of an SNP association study on the chronicity of HBV infection showed a variety of innate immunity-associated genes, including human leukocyte antigens (Guo *et al.*, 2011), NKG2D (Ma *et al.*, 2010), tumor necrosis factor- α (TNF- α) (Kim *et al.*, 2003), mannose-binding lectin (Thio *et al.*, 2005), interleukin-18 (IL-18) (Cheong *et al.*, 2010), and cytotoxic T lymphocyte antigen 4 (Thio *et al.*, 2004). In Korea, which is an HBV endemic area

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(Cheong, 2008; Park *et al.*, 2010), the annual mortality of HBV-induced HCC is an important problem (Lavanchy, 2004). The study of variation in innate immunity-associated genes is important to better understand the development of HBV chronicity in the Korean population.

A chronicity of HBV study that analyzed the recovery mechanisms leading to the host innate immune responses against HBV infection demonstrated differences in innate immunity between chimpanzees and transgenic mice (Litsov, 1975; Kagi *et al.*, 1994). A strong cytolytic T lymphocyte (CTL) response is an important factor in the chronicity and pathogenesis of HBV infection. In particular, CD8⁺ T cells play a significant role in the inhibition of HBV gene expression and prevention of HBV RNA-containing capsid assembly. In addition, they contribute to the induction of apoptosis in virus-affected hepatocytes via two general pathways of cytotoxicity, the TNF receptor super family, member 6 (Fas)/Fas ligand apoptotic pathway, and the granule exocytosis pathway with granzyme B and perforin (Kagi *et al.*, 1994; Lowin *et al.*, 1994; Stenger *et al.*, 1999; Robek *et al.*, 2002; Thimme *et al.*, 2003).

Natural killer (NK) cells also play an important role in HBV infection. NK cells do not require activation by specific virus antigens, and can therefore remove the infecting virus soon after infection. Moreover, similar to antigen-specific CD8⁺ T cells, NK cells can kill the infected cells directly through a physical cell–cell contact and indirectly through interferon- γ and TNF- α , as well as IL-3, granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor (Guidotti and Chisari, 2001). In chronic hepatitis B patients, hepatic NK cells have enhanced cytolytic activity against target cells compared to peripheral NK cells (Zhang *et al.*, 2010).

GNLY, which is found in cytotoxic granules in both CTL and NK cells, is a member of the saposin-like protein (SAPLIP) family. It is released together with the pore-forming protein, perforin, and granzymes upon antigen stimulation, and its expression is regulated by IL2, IL15, and chemokines (Stefanovic *et al.*, 1977; Jongstra *et al.*, 1987). GNLY is encoded by the GNLY gene in chromosome 2p11.2, which encodes a 15-kDa protein that is cleaved after synthesis at its amino- and carboxytermini to produce a 9-kDa protein (Jongstra *et al.*, 1987; Donlon *et al.*, 1990; Yabe *et al.*, 1990). The alternative splicing of GNLY generates NKG5, which is secreted into the extracellular space, and the 15-kDa protein designated as 519. Recombinant 9-kDa GNLY is lytic to tumors and broadly antimicrobial, and it kills gram-positive and gram-negative bacteria, yeast, fungi, and parasites (Stenger *et al.*, 1998).

The reported functions of GNLY suggest that SNPs of GNLY could be determining factors in the chronicity of hepatitis B. Therefore, the present study evaluated the association between GNLY genetic polymorphisms and the chronicity of HBV by comparing GNLY gene polymorphism frequencies between CLD patients and an SR control group in the Korean population using a genotype and diplotype analysis.

Materials and Methods

Case-control study subjects

A total of 347 Korean blood samples were obtained from the outpatient clinic of the Gastroenterology Department and

from the Center for Health Promotion of the Ajou University Hospital (Suwon, South Korea) without gender or age restrictions between March 2002 and February 2006. Samples were derived from genetically unrelated Korean patients. The experimental protocol was approved by the institutional review board. The SR group consisted of 117 subjects as controls, and HBV patient samples obtained from 230 CLD patients were divided into two groups according to serologic markers.

All samples were infected with HBV and classified into one of the two groups by a pathologist according to their HBV infection status, clinical data, and serological profile. Every 6 months, for more than 12 months, the 347 patients were subjected to serological tests for serum levels of the hepatitis B core antibody (HBcAb) (Anti-HBc II Reagent Kit; Abbott Laboratories, South Pasadena, CA), hepatitis B surface antigen (HBsAg) (Anti-HBs; Abbott Laboratories), and hepatitis B surface antibody (HBsAb) (HBsAg; Abbott Laboratories). Liver function tests for aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, and bilirubin levels were performed using commercially available assays. HBV DNA was detected using an HBV-branched DNA assay (Bayer Diagnostics, Tarrytown, NY). All samples showed elevated ALT at least once during the follow-up period and were positive for HBV DNA, irrespective of hepatitis B e antigen (HBeAg) positivity. The patients in the HBV clearance groups were HBsAg negative, HBeAg negative, anti-HBs positive, anti-HBc positive, and had recovered from HBV infection. The patients in the CLD group were HBsAg positive for more than 6 months with elevated ALT and AST (more than 2-times the normal upper limit) levels.

Samples testing positive for the presence of antibodies against complex infections such as hepatitis C (Genedia HCV ELISA 3.0; GreenCross, Gyeonggi, Korea) or anti-human immunodeficiency virus (HIV Ag/Ab combo; Abbott Laboratories), and samples with insufficient clinical information were excluded. The final analysis included 107 SR and 206 CLD samples.

Sample preparation

Blood samples were stored at -80°C for the handling of human genomic DNA. Genomic DNA was purified using G-DEX blood genomic DNA (gDNA) purification kits (Intron Biotechnology, Inc., Seungnam, Korea). The gDNA was quantified using the picogreen dsDNA quantification reagent, according to a standard protocol (Molecular Probes, Eugene, OR). The plates were read using a VICTORTM 3 1420 Multilabel counter, and a standard curve for gDNA concentration was created using known concentrations of lambda DNA.

SNP selection and genotyping

A total of three SNPs were selected from a public SNP database (www.ncbi.nlm.nih.gov/snp/) based on their location, suitability for the GoldenGate assay, and frequency among the tagging SNPs for the genotyping assay. The SNPs were rs2886767, which is located in the 5' flanking region, rs1561285, which is located in the 3th intron, and rs11127, which is located in the fourth exon. Genotyping was performed using the GoldenGate[®] kit according to a standard protocol (Illumina, Inc., San Diego, CA). Oligos were amplified by

allele-specific primer extension. After hybridization to a Sentrix array matrix, signal intensities were read by a BeadArray Reader (Illumina). GenomeStudio software (Version 1.5.16; Illumina) was used for genotyping analysis.

Statistical analysis

The genetic models for the association test were divided according to the minor allele, and the genetic model was considered additive (AA vs. Aa vs. aa), dominant (AA vs. Aa plus aa), or recessive (AA plus Aa vs. aa). Linkage disequilibrium (LD) blocks were checked by the Gabriel method using Haploview software. The χ^2 -test was used to assess the Hardy-Weinberg Equilibrium (HWE) between the SR group and the CHB group. The difference between groups was determined by the odds ratio (OR). ORs were presented with 95% confidence intervals (95% CIs) and were adjusted for age and sex. All statistical tests were performed using SAS software (SAS 9.1; SAS Institute, Cary, NC), and the significance level was set at $p < 0.05$. The probability values obtained were corrected for multiple testing by using Bonferroni's correction and permutation tests. The Bonferroni's p -value for reaching significance was 0.01 (0.05/5). The Plink program (<http://pngu.mgh.harvard.edu/~purcell/plink/>) was used to confirm the results and permutation test ($N = 10,000$).

Results

Clinical characterization

The fate of patients infected with HBV is determined by several factors, including host immune reactions. T cells and NK cells, which play an important role in protection against chronic hepatitis in HBV infections, express GNLY in response to the hepatitis B virus. In the present study, the effect of GNLY polymorphisms on the chronicity of hepatitis B was investigated by analyzing 3 GNLY SNPs in samples from an SR group (HBsAg-, HBcAb+, and HBsAb+), which consisted of 107 control patients who had recovered from HBV infection without treatment, and a CLD group (HBsAg+, HBcAb-, and HBsAb-), which consisted of 206 HBV-induced CLD patients who were still at risk of HBV infection. The age and sex distribution of the patients in the CLD and SR groups was statistically different (data not shown). The characteristics of the study subjects are summarized in Table 1.

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY SUBJECTS

Variable	SR	CLD
No. of subjects	107	206
Sex (Male/Female)	80/27	158/48
Age (mean $\pm$ SD)	46.04 $\pm$ 8.40	42.86 $\pm$ 9.62
Aspartate aminotransferase (U/L, mean $\pm$ SD)	30.62 $\pm$ 25.45	93.67 $\pm$ 161.74
Alanine aminotransferase (U/L, mean $\pm$ SD)	39.40 $\pm$ 29.40	107.36 $\pm$ 170.25
Albumin (g/dL, mean $\pm$ SD)	4.40 $\pm$ 0.26	3.96 $\pm$ 0.61
Bilirubin (mg/dL, mean $\pm$ SD)	0.90 $\pm$ 0.35	1.71 $\pm$ 2.88

Age, aspartate aminotransferase, alanine aminotransferase, albumin, and bilirubin are summarized and expressed as the mean $\pm$ SD (standard deviation).

SR, spontaneous recovery; CLD, chronic liver disease induced by hepatitis B virus.

Genotype distribution and genotype analysis

Genetic variants of rs2886767, rs1561285, and rs11127 did not show evidence of departure from HWE. Three genotypes had minor allele frequencies (MAFs) $> 1\%$ (Table 2). The results of the genotype analysis showed that the CC, GG, and TT genotypes were the most common in the rs2886767, rs1561285, and rs11127 polymorphisms in all groups (Table 2).

To analyze the genetic association between GNLY polymorphisms and clearance from HBV infection, genetic distributions were statistically analyzed. The SR and CLD samples were compared by a multiple logistic regression analysis with adjustment for gender and age. The GNLY SNP rs2886767 in the promoter region was a risk type associated with the homozygous variant TT genotype in a recessive model (OR = 2.44; 95% CI = 1.19–5.01; $p = 0.015$). The intronic SNP rs1561285 CC genotype was a risk type, and significant differences in this SNP were detected between the SR and CLD groups in a recessive model (OR = 3.78; 95% CI = 1.41–10.14; $p = 0.008$). In the nonsynonymous SNP rs11127, the CC genotype was also a risk type and was significantly different between the SR and CLD groups in a recessive model (OR = 3.26; 95% CI = 1.45–7.30; $p = 0.004$). After performing Bonferroni's correction and the permutation test, rs1561285 and rs11127 SNPs still showed significant correlations (Table 2).

Diplotype analysis

LD blocks of all SNPs in GNLY were constructed by the Gabriel method using Haploview software, which set the cutoff values at MAF > 0.1 and HWE p -value > 0.05 . A single haplotype block of the pairwise $D' > 0.984$ and $r^2 > 0.826$ is identified in the Table 3. Haplotype (HT) 1 (C-G-T) and HT2 (T-C-C) were the most common (frequency > 0.05) and accounted for 98.0% of all haplotypes (Table 4).

We noted that in this haplotype characterization. Based on three genetic models, the estimated HTs were used for a diplotype analysis by using logistic regression followed by adjustments for age and sex. The HT1 frequency reached a significant divergence in the recessive model between the SR and CLD groups (OR = 2.23; 95% CI = 1.11–4.48; $p = 0.025$). Analysis of the HT2 diplotype showed a significant difference between the SR and CLD groups in the dominant model (OR = 0.26; 95% CI = 0.10–0.71; $p = 0.008$) (Table 4). All diplotype p -values remained significant after the permutation test (HT1; $p = 0.035$, HT2: $p = 0.011$), and the HT2 of the diplotype p -values remained significant after Bonferroni's correction ($p = 0.042$).

Discussion

GNLY has been associated with a variety of infectious diseases, and it is a critical effector molecule for a broad spectrum of pathogenic microorganisms, including *Listeria monocytogenes*, *Trypanosoma cruzi*, *Salmonella typhimurium*, *Escherichia coli*, and *Mycobacterium tuberculosis* (Pena *et al.*, 1997; Stenger *et al.*, 1998; Krensky, 2000; Deng *et al.*, 2005). The cell-killing effect of GNLY has been demonstrated using the 9-kDa recombinant GNLY *in vitro*. GNLY is involved in the removal of viruses such as varicella zoster and Epstein-Barr. Expression of GNLY was associated with the recovery from infection by the herpes simplex virus and HBV (Hata *et al.*, 2001; Sun *et al.*, 2002; Morizane *et al.*, 2005; Li *et al.*,

TABLE 2. GENOTYPE FREQUENCIES AND ASSOCIATIONS BETWEEN HEPATITIS B VIRUS-INDUCED DISEASE IN *GNLY* SINGLE-NUCLEOTIDE POLYMORPHISMS

SNP	HWE	MAF	Genotype	Frequency (%)		Model	SR vs. CLD			p ^a	p ^b	p ^c
				SR	CLD		OR	Lower CI	Upper CI			
rs2886767	0.523	0.421	CC	29 (7.27)	73 (18.3)	Additive	1.05	0.75	1.49	0.763		
C>T			CT	67 (16.79)	90 (22.55)	Dominant	0.66	0.39	1.11	0.117		
Flanking_5UTR			TT	11 (2.76)	43 (10.78)	Recessive	2.44	1.19	5.01	0.015	0.027	0.074
rs1561285	0.121	0.381	GG	32 (8.02)	83 (20.8)	Additive	0.99	0.68	1.43	0.951		
G>C			CG	70 (17.54)	92 (23.05)	Dominant	0.61	0.36	1.01	0.055		
Intron			CC	5 (1.25)	31 (7.77)	Recessive	3.78	1.41	10.14	0.008	0.017	0.042
rs11127	0.434	0.411	TT	29 (7.27)	77 (19.3)	Additive	1.05	0.74	1.49	0.790		
T>C			CT	70 (17.54)	88 (22.05)	Dominant	0.60	0.36	1.01	0.055		
Exon			CC	8 (2.01)	41 (10.28)	Recessive	3.26	1.45	7.30	0.004	0.007	0.020

The CIs and p^a values were obtained from logistic regression with additive, dominant, and recessive models.

The p^b values were calculated by the permutation test.

The p^c values were calculated by Bonferroni's correction.

SNP, single-nucleotide polymorphisms; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

2010). However, an association study between infectious diseases and genetic variation of *GNLY* has not been performed to date.

The present study investigated the possible association between *GNLY* SNPs and the chronicity of HBV. The *GNLY* polymorphism frequency in the Korean population was compared between 107 controls in the SR group and 206 patients in the CLD group using statistical analyses. According to the SNP database, the rs2886767, rs1561285, and rs11127 SNPs are located in the 5' flanking region, intron, and the 4th exon of *GNLY*, respectively, and their genotype frequencies were significantly different between the SR and CLD groups. The risk genotype of rs2886767 was TT, and it was associated with a 2.44-fold increased susceptibility to CLD compared to the CC and CT types. The CC risk genotype of rs1561285 was associated with a 3.78-fold increased OR compared to the CC and CG genotypes. The CC risk type of rs11127 increased OR by 3.26-times compared to the TT and CT genotypes.

Li *et al.* (2010) reported that the expression of the 9-kDa form of *GNLY* is significantly increased in response to high levels of HBV DNA in a normal hepatic function group. In the present study, significant differences in the rs2886767 (C>T) genotype in the promoter region of *GNLY* between the SR and CLD groups led us to screen transcription factors that bind to rs2886767. The binding of several transcription factors, including E1A-binding protein p300 (EP300), IKARPS family zinc-finger (IKZF)-2, GATA-binding protein factor 1 (GATA-1), and GATA-2, to the protective allele (C)

of rs2886767 was predicted by TFSEARCH (version 3.1; www.cbrc.jp/research/db/TFSEARCH.html). Interestingly, the binding of EP300 to the region containing the rs2886767 risk allele (T) was not predicted. EP300 is a histone acetyltransferase that regulates the transcriptional machinery through chromatin remodeling and functions as an adaptor molecule. EP300 interacts with several transcription factors and enhances the expression of numerous gene regulatory elements (Chan and La Thangue, 2001; Asahara *et al.*, 2002). Our results suggest the possibility that the genotype-dependent interaction of EP300 with rs2886767 might affect the expression of *GNLY*.

GNLY is expressed via a toll-like receptor 2-dependent signaling pathway, and *GNLY* expression is regulated by specific transcription factors, including CCAAT/enhancer-binding protein beta (C/EBPβ), and activator protein 1 in *Acholeplasma laidlawii* stimulates THP-1 cells (Kida *et al.*, 2001; Kida *et al.*, 2002). C/EBPβ is a member of the basic region/leucine zipper class of transcription factors that initiates the opening of the chromatin structure and recruits the basal transcriptional machinery. C/EBPβ can recruit so-called coactivators such as the CREB (cAMP-responsive-element-binding)-binding protein (CBP) (Ramji and Foka, 2002). EP300 binds to the rs2886767 protective allele (C) through 5 protein interaction domains, the structures of which are similar to that of CBP. The function of C/EBPβ is strongly inhibited by the E1A protein. After the coactivation of EP300 and C/EBPβ, the inhibitory effect of E1A on C/EBPβ is blocked by EP300 binding. Moreover, C/EBPβ and EP300 enhance the expression of target genes up to 18.4-fold in the presence of E1A deficiency *in vivo* (Janknecht and Hunter, 1996; Mink *et al.*, 1997). The interaction between C/EBPβ and EP300 mediated by the rs2886767 protective allele may enhance *GNLY* expression. A genetic model derived from the current statistical analysis suggests that the combination of the protective genotypes CC and CT may efficiently eliminate HBV-infected cells by increasing *GNLY* expression. Therefore, polymorphisms of rs2886767 may help SR from HBV infection.

GNLY rs11127, which is located in the 4th exon, changes the amino acid sequence of the protein product as a

TABLE 3. LINKAGE DISEQUILIBRIUM COEFFICIENT BETWEEN SINGLE-NUCLEOTIDE POLYMORPHISMS IN *GNLY*

	SNPs	D'		
		rs2886767	rs1561285	rs11127
r ²	rs2886767	-	0.988	0.984
	rs1561285	0.826	-	1
	rs11127	0.929	0.882	-

TABLE 4. DIPLTYPE FREQUENCIES AND ASSOCIATIONS BETWEEN SPONTANEOUS RECOVERY AND CHRONIC LIVER DISEASE IN *GNLY* HAPLOTYPES

SNP	Diplotype	Frequency (%)		Model	SR vs CLD			p ^a	p ^b	p ^c
		SR	CLD		OR	Lower CI	Upper CI			
HT1	HT1/HT1	28 (8.95)	73 (23.32)	Additive	1.03	0.73	1.46	0.854		
C-G-T	HT1/-	67 (21.41)	90 (28.75)	Dominant	0.65	0.38	1.10	0.109		
	-/-	12 (3.83)	43 (13.74)	Recessive	2.23	1.11	4.48	0.025	0.035	0.123
HT2	HT2/HT2	5 (1.6)	31 (9.9)	Additive	0.99	0.68	1.43	0.953		
T-C-C	HT2/-	69 (22.04)	92 (29.39)	Dominant	0.26	0.10	0.71	0.008	0.011	0.042
	-/-	33 (10.54)	83 (26.52)	Recessive	1.57	0.95	2.61	0.080		

The CI and p^a values were obtained from logistic regression with additive, dominant, and recessive models.

The p^b values were calculated by the permutation test.

The p^c values were calculated by Bonferroni's correction.

nonsynonymous SNP through allelic exchange. To determine the effect of amino acid changes, we used the SIFT (<http://sift.jcvi.org>) and PolyPhen (<http://genetics.bwh.harvard.edu/pph/>) software programs, which were reported to have a high rate of correct predictions by functional testing. These programs showed that rs11127 amino acid changes did not affect the structure of *GNLY* (Luoma *et al.*, 2010). We therefore examined the SNP database for possible protein structural changes in *GNLY*. *In silico* assay results showed that changes in the genotype of rs35146240 may have a damaging effect. *GNLY* rs10181739 and rs71806663 are a predicted stop codon and essential splice site, respectively, according to a genetic database (<http://asia.ensembl.org>). Amino acid changes caused by genetic variants, including nonsynonymous SNPs, stop codons, and frame shifts, can induce structural changes.

As a member of the SAPLIP family, *GNLY* is positively charged and is targeted to negative charges on the surface of cells. The crystal structure of *GNLY* shows 5 helix bundles, and it has been reported to lyse the membrane by using helices 1, 2, and 3 in a scissoring motion, and by twisting and tearing as it enters deeper into the membrane. The formation of a pore in the target via perforin is followed by an increase in intracellular calcium levels as a result of an influx of extracellular calcium and the release of calcium from intracellular stores (Kaspar *et al.*, 2001; Anderson *et al.*, 2003). Nonsynonymous genetic variation of *GNLY* possibly induces the formation of an abnormal structure that does not recognize HBV-infected cells or results in the loss of the lytic function mediated by the five-helix bundle. This may alter the formation of the pore in HBV-infected cells, thereby enabling the interaction with apoptosis initiation molecules and preventing the early elimination of HBV-infected cells. Therefore, modifications in the structure of *GNLY* may affect the chronicity of HBV.

The association of SNPs rs2886767, rs1561285, and rs11127 with the chronicity of HBV was characterized by a strong LD ($r^2=0.826$). These SNPs formed C-G-T, T-C-C, T-G-C, and T-G-T HTs in the LD block, and the C-G-T and T-C-C HTs had more than a 95% distribution in all HTs. The LD block contained almost every region in *GNLY*, including the promoter region to the 4th exon. Diplotype analysis of HT1, which consists of a protective allele combination (C-G-T), showed that HT1 inclusion was associated with a 2.23-time higher chance of SR than HT1 exclusion. HT2 consisted of a risk

allele combination, and diplotype analysis of HT2 (T-C-C) showed that the OR toward HBV clearance was 0.26-fold that of the HT2 inclusion type in the HT2 exclusion type. Moreover, according to the Korea association resource dataset (Cho *et al.*, 2009) and the IMPUTE program (Marchini *et al.*, 2007), 24 SNPs, including rs2886767, rs1561285, and rs11127 in the *GNLY* gene, showed strong LD ($D'>0.904$). These data suggest that causative variants responsible for HBV clearance may be present in rs2886767, rs1561285, and rs11127 that form an LD block. Therefore, rs2886767, rs1561285, and rs11127 are markers of causative variants associated with the chronicity of HBV.

Our study was limited by a small sample size and few genetic variants. However, this is the first study to investigate the association between *GNLY* polymorphisms and HBV-induced CLD. In addition, SR patients were used as a control group instead of normal healthy controls to show the effect of genomic background on the chronicity of HBV infection. Distribution of genotypes and diplotypes from both groups remained significant after multiple testing using Bonferroni's correction and permutation tests. The current results are in support of the conjecture that genetic variation in the *IFI6* gene affects the clearance of HBV. Further studies should focus on investigating the effect of *GNLY* expression on HBV clearance. Our results suggest that the genetic variation of *GNLY* in HBV may play a role in the induction of CLD. In future studies, causal variants that affect *GNLY* molecular function should be identified through next-generation sequencing of the *GNLY* gene.

Conclusion

The C allele-containing genotype of rs2886767, the G allele-containing genotype of rs1561285, and the T allele-containing genotype of rs11127 were associated with HBV clearance. These data suggested that genetic variation of *GNLY* may be an important factor in HBV clearance through the removal of HBV-infected cells from the host by CD8⁺ T cells or NK cells.

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Disclosure Statement

No competing financial interests exist.

References

- Anderson, D.H., Sawaya, M.R., Cascio, D., Ernst, W., Modlin, R., Krensky, A., *et al.* (2003). Granulysin crystal structure and a structure-derived lytic mechanism. *J Mol Biol* **325**, 355–365.
- Asahara, H., Tartare-Deckert, S., Nakagawa, T., Ikehara, T., Hirose, F., Hunter, T., *et al.* (2002). Dual roles of p300 in chromatin assembly and transcriptional activation in cooperation with nucleosome assembly protein 1 *in vitro*. *Mol Cell Biol* **22**, 2974–2983.
- Carrilho, F.J., Ono-Nita, S.K., Cardoso, R.A., Cancado, E.L., Pinho, J.R., Alves, V.A., *et al.* (2005). A prospective study of hepatitis B virus markers in patients with chronic HBV infection from Brazilian families of Western and Asian origin. *Braz J Med Biol Res* **38**, 1399–1408.
- Cha, C., and Dematteo, R.P. (2005). Molecular mechanisms in hepatocellular carcinoma development. *Best Pract Res Clin Gastroenterol* **19**, 25–37.
- Chan, H.M., and La Thangue, N.B. (2001). p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci* **114**, 2363–2373.
- Cheong, J.Y. (2008). [Management of chronic hepatitis B in treatment-naïve patients]. *Korean J Gastroenterol* **51**, 338–345.
- Cheong, J.Y., Cho, S.W., Oh, B., Kimm, K., Lee, K.M., Shin, S.J., *et al.* (2010). Association of interleukin-18 gene polymorphisms with hepatitis B virus clearance. *Dig Dis Sci* **55**, 1113–1119.
- Cho, Y.S., Go, M.J., Kim, Y.J., Heo, J.Y., Oh, J.H., Ban, H.J., *et al.* (2009). A large-scale genome-wide association study of Asian populations uncovers genetic factors influencing eight quantitative traits. *Nat Genet* **41**, 527–534.
- Deng, A., Chen, S., Li, Q., Lyu, S.C., Clayberger, C., and Krensky, A.M. (2005). Granulysin, a cytolytic molecule, is also a chemoattractant and proinflammatory activator. *J Immunol* **174**, 5243–5248.
- Donlon, T.A., Krensky, A.M., and Clayberger, C. (1990). Localization of the human T lymphocyte activation gene 519 (D2S69E) to chromosome 2p12–q11. *Cytogenet Cell Genet* **53**, 230–231.
- Frodsham, A.J. (2005). Host genetics and the outcome of hepatitis B viral infection. *Transpl Immunol* **14**, 183–186.
- Guidotti, L.G., and Chisari, F.V. (2001). Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* **19**, 65–91.
- Guo, X., Zhang, Y., Li, J., Ma, J., Wei, Z., Tan, W., *et al.* (2011). Strong influence of human leukocyte antigen (HLA)-DP gene variants on development of persistent chronic hepatitis B virus carriers in the Han Chinese population. *Hepatology* **53**, 422–428.
- Hata, A., Zerboni, L., Sommer, M., Kaspar, A.A., Clayberger, C., Krensky, A.M., *et al.* (2001). Granulysin blocks replication of varicella-zoster virus and triggers apoptosis of infected cells. *Viral Immunol* **14**, 125–133.
- Janknecht, R., and Hunter, T. (1996). Versatile molecular glue. *Transcriptional control*. *Curr Biol* **6**, 951–954.
- Jongstra, J., Schall, T.J., Dyer, B.J., Clayberger, C., Jorgensen, J., Davis, M.M., *et al.* (1987). The isolation and sequence of a novel gene from a human functional T cell line. *J Exp Med* **165**, 601–614.
- Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., *et al.* (1994). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* **265**, 528–530.
- Kaspar, A.A., Okada, S., Kumar, J., Poulain, F.R., Drouvalakis, K.A., Kelekar, A., *et al.* (2001). A distinct pathway of cell-mediated apoptosis initiated by granulysin. *J Immunol* **167**, 350–356.
- Kida, Y., Kuwano, K., Zhang, Y., and Arai, S. (2001). Aholplasma laidlawii up-regulates granulysin gene expression via transcription factor activator protein-1 in a human monocytic cell line, THP-1. *Immunology* **104**, 324–332.
- Kida, Y., Shimizu, T., and Kuwano, K. (2002). Opposing roles of activator protein-1 and CCAAT/enhancer binding protein beta in the regulation of inducible granulysin gene expression in a human monocytic cell line, THP-1. *Immunology* **107**, 507–516.
- Kim, Y.J., Lee, H.S., Yoon, J.H., Kim, C.Y., Park, M.H., Kim, L.H., *et al.* (2003). Association of TNF-alpha promoter polymorphisms with the clearance of hepatitis B virus infection. *Hum Mol Genet* **12**, 2541–2546.
- Krensky, A.M. (2000). Granulysin: a novel antimicrobial peptide of cytolytic T lymphocytes and natural killer cells. *Biochem Pharmacol* **59**, 317–320.
- Lavanchy, D. (2004). Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* **11**, 97–107.
- Lee, W.M. (1997). Hepatitis B virus infection. *N Engl J Med* **337**, 1733–1745.
- Li, M., Yao, Y., and Gao, Y.Q. (2010). [Expression profile of immune effector molecules in natural killer cells in patients with chronic hepatitis B]. *Zhonghua Gan Zang Bing Za Zhi* **18**, 96–100.
- Litsov, V.I. (1975). [50th anniversary of the Borovoe Military Sanatorium]. *Voen Med Zh* **8**, 96.
- Lok, A.S., and McMahon, B.J. (2007). Chronic hepatitis B. *Hepatology* **45**, 507–539.
- Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* **370**, 650–652.
- Luoma, L.M., Deeb, T.M., Macintyre, G., and Cox, D.W. (2010). Functional analysis of mutations in the ATP loop of the Wilson disease copper transporter, ATP7B. *Hum Mutat* **31**, 569–577.
- Ma, J., Guo, X., Wu, X., Li, J., Zhu, X., Li, Z., *et al.* (2010). Association of NKG2D genetic polymorphism with susceptibility to chronic hepatitis B in a Han Chinese population. *J Med Virol* **82**, 1501–1507.
- Marchini, J., Howie, B., Myers, S., McVean, G., and Donnelly, P. (2007). A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat Genet* **39**, 906–913.
- Mink, S., Haenig, B., and Klemmner, K.H. (1997). Interaction and functional collaboration of p300 and C/EBPbeta. *Mol Cell Biol* **17**, 6609–6617.
- Morizane, S., Suzuki, D., Tsuji, K., Oono, T., and Iwatsuki, K. (2005). The role of CD4 and CD8 cytotoxic T lymphocytes in the formation of viral vesicles. *Br J Dermatol* **153**, 981–986.

- Park, N.H., Chung, Y.H., and Lee, H.S. (2010). Impacts of vaccination on hepatitis B viral infections in Korea over a 25-year period. *Intervirology* **53**, 20–28.
- Pena, S.V., Hanson, D.A., Carr, B.A., Goralski, T.J., and Krensky, A.M. (1997). Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. *J Immunol* **158**, 2680–2688.
- Ramji, D.P., and Foka, P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* **365**, 561–575.
- Robek, M.D., Wieland, S.F., and Chisari, F.V. (2002). Inhibition of hepatitis B virus replication by interferon requires proteasome activity. *J Virol* **76**, 3570–3574.
- Stefanovic, S., Stojkovic, L., Spuzic, I., Jancic, M., and Branicevic, B. (1977). [Vaccination against smallpox of patients with malignant hematopathy]. *Srp Arh Celok Lek* **105**, 145–148.
- Stenger, S., Hanson, D.A., Teitelbaum, R., Dewan, P., Niazi, K.R., Froelich, C.J., *et al.* (1998). An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* **282**, 121–125.
- Stenger, S., Rosat, J.P., Bloom, B.R., Krensky, A.M., and Modlin, R.L. (1999). Granulysin: a lethal weapon of cytolytic T cells. *Immunol Today* **20**, 390–394.
- Sun, Q., Burton, R.L., and Lucas, K.G. (2002). Cytokine production and cytolytic mechanism of CD4(+) cytotoxic T lymphocytes in *ex vivo* expanded therapeutic Epstein-Barr virus-specific T-cell cultures. *Blood* **99**, 3302–3309.
- Thimme, R., Wieland, S., Steiger, C., Ghayeb, J., Reimann, K.A., Purcell, R.H., *et al.* (2003). CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* **77**, 68–76.
- Thio, C.L., Mosbruger, T., Astemborski, J., Greer, S., Kirk, G.D., O'Brien, S.J., *et al.* (2005). Mannose binding lectin genotypes influence recovery from hepatitis B virus infection. *J Virol* **79**, 9192–9196.
- Thio, C.L., Mosbruger, T.L., Kaslow, R.A., Karp, C.L., Strathdee, S.A., Vlahov, D., *et al.* (2004). Cytotoxic T-lymphocyte antigen 4 gene and recovery from hepatitis B virus infection. *J Virol* **78**, 11258–11262.
- Thio, C.L., Thomas, D.L., Karacki, P., Gao, X., Marti, D., Kaslow, R.A., *et al.* (2003). Comprehensive analysis of class I and class II HLA antigens and chronic hepatitis B virus infection. *J Virol* **77**, 12083–12087.
- Yabe, T., McSherry, C., Bach, F.H., and Houchins, J.P. (1990). A cDNA clone expressed in natural killer and T cells that likely encodes a secreted protein. *J Exp Med* **172**, 1159–1163.
- Zhang, Z., Zhang, S., Zou, Z., Shi, J., Zhao, J., Fan, R., *et al.* (2010). Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. *Hepatology* **53**, 73–85.

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