S267F variant of sodium taurocholate cotransporting polypeptide and its clinical association in Korean patients with chronic hepatitis B

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ABSTRACT

S267F variant of sodium taurocholate cotransporting polypeptide and its clinical association in Korean patients with chronic hepatitis B

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Background: Sodium taurocholate cotransporting polypeptide (NTCP) was recently identified as a cellular receptor for hepatitis B virus (HBV). The substitution of serine residue at position 267 of NTCP with a phenylalanine residue (S267F) is an Asian-specific variation that hampers HBV entry *in vitro*. In this study, we aimed to evaluate the prevalence of S267F polymorphism in Korean patients with chronic hepatitis B (CHB) and its association with disease progression. We also investigated whether bile acids affect the entry of HBV *in vitro*.

Methods: The effect of S267F variant on HBV infection was assessed using mutagenesis, transfection, virus inoculation and ELISA. For HBV preparation, HepAD38 cell line was used. The presence of S267F polymorphism was assessed in 1,200 patients with CHB and 176 individuals seronegative for hepatitis B surface antigen (HBsAg). Genomic DNA was extracted from whole blood and the S267F polymorphism was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The association between S267F mutation and its clinical association was also

analyzed.

Results: *In vitro* investigation using HepG2-NTCP cell line revealed that the S267F variant of NTCP reduced HBV infection and appears to compete with ursodeoxycholic acid for the NTCP receptor. The frequency of the S267F variant (genotype CT or TT) in Korean CHB patients and HBsAg-seronegative individuals was 2.7% and 5.7% (P=0.031), respectively, and those who had S267F variant were less susceptible to HBV infection. The frequencies of the S267F variant in CHB, cirrhosis and hepatocellular carcinoma (HCC) patients were 3.3%, 0.9%, and 3.5%, respectively. S267F variant correlated significantly with a lower risk for cirrhosis (P=0.036), while there was no significant correlation with the risk for HCC (P=0.887). Among the 32 patients who had S267F variant (genotype CT), 10 had not received antiviral treatment; their median HBV DNA level was 2.0 $\log_{10}$ IU/mL. In contrast, the median HBV DNA level of the 477 antiviral treatment-naïve patients with wild-type NTCP (genotype CC) was 2.9 $\log_{10}$ IU/mL (P=0.179).

Conclusion: We confirmed S267F variant of NTCP reduced HBV infection *in vitro*. Also, the uptake of HBV and UDCA occurs competitively in interaction with NTCP. The S267F variant of NTCP is clinically associated with lower risk of HBV infection and cirrhosis development in Korean patients with CHB.

Key words: S267F variant, polymorphism, sodium taurocholate cotransporting polypeptide, hepatitis B

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I. INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a leading cause of cirrhosis and hepatocellular carcinoma (HCC).[1] Because progression of liver disease in patients with chronic hepatitis B (CHB) is fostered by active viral replication, a high level of HBV DNA, which indicates active virus replication, has been identified as an independent risk factor for development of cirrhosis and HCC.[2-4] Thus, eradication or permanent suppression of HBV could theoretically reduce the risk for liver disease progression.

HBV is an enveloped DNA virus with strict host-species and cell-type specificity.[5] A complex combination of environmental, pathogenic and host genetic factors play a role in determining both susceptibility to HBV and the course of the infection. Among these factors, cellular entry is vital for

infection of target organs by enveloped viruses. HBV entry into and replication in hepatocytes are prerequisites for its pathogenesis. The former is mediated by the cellular receptor for the virus.[6]

The viral membrane of the infectious HBV particle contains three envelope proteins: large (L, preS1+preS2+S), middle (M, preS2+S), and small (S only).[7] The preS domain contributes to the viral life cycle at several steps, including attachment to the hepatocyte and budding of the virus at the endoplasmic reticulum.[8] Particularly, the preS1 domain is associated with viral attachment to the receptor.[9] The S domain mediates HBV recruitment to the hepatocyte surface via heparin sulfate proteoglycans.[10,11] All three proteins have identical C-terminal S domains, which contain the hepatitis B surface antigen (HBsAg). HBV infection is characterized by the presence of noninfectious subviral particles. The L protein has a conserved 77-amino acid N-terminal sequence in the pre-S1 domain. Myristoylation of this conserved sequence is essential for infectivity.[12]

Recently, sodium taurocholate cotransporting polypeptide (NTCP), known previously as solute carrier family 10 member 1 (SLC10A1), was identified as a functional species-specific HBV receptor.[13,14] NTCP is specifically expressed at the basolateral membrane of hepatocytes and is encoded by the NTCP *(SLC10A1)* gene in humans. NTCP is involved in the essential reuptake of bile acids from the blood in the portal vein. NTCP is expressed on the hepatocyte surface and allowed for the bile acids to be resecretion into bile.[15]

Genetic variations may influence the expression levels and the function of proteins. Single-nucleotide polymorphisms (SNPs), some of which are

specific to certain ethnicities, have been identified in the NTCP gene.[16-19] The genetic variant I223T (c.668T>C) was identified in African-Americans at a frequency of 5.5%. S267F (c800C>T, p.Ser276Phe, and rs2296651) and I279T (c.836T>C) are Asian-specific SNPs, with allele frequencies of 7.5% and 0.5%, respectively. Patients with these variants also have lower bile acid transport activity. The S267F polymorphism is found in exon 4 of NTCP. Yan *et al*. conducted an *in vitro* investigation of the effect of the S267F polymorphism, which has been observed only in Asians, and at the highest frequency in East Asians.[13] They found that this variant resulted in loss of HBV receptor function. However, the clinical implications of this finding remain unknown. To date, no study of the effect of S267F variant on susceptibility to HBV infection in Korean CHB patients has been conducted.

This study aimed to investigate the frequency of S267F variant of NTCP in Korean CHB patients and the association between S267F mutation and the natural course of CHB.

II. MATERIALS AND METHODS

1. HBV Preparation

HepAD38 cells[20] were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco), penicillin, streptomycin (Welgene), and 1μg/ml doxycycline (Sigma-Aldrich). Medium was harvested every three or four days from HepAD38 cells 14-31 days post-induction of HBV by depletion of doxycycline. Stored medium was filtered through a 0.45-μm filter, precipitated with 40% (w/v) polyethylene glycol-8000 ([final]=6.2%, Sigma-Aldrich), mixed by end-over-end rotation overnight at 4°C. Viral precipitates were collected by centrifugation (4,500 rpm, 4°C, 1 h) and resuspended with fresh medium at ~1000-fold concentration. The HBV DNA was quantified by real time PCR.

2. Transient transfection

HepG2-NTCP cells were transfected with wild-type NTCP plasmid or S267F-containing NTCP plasmid using TransIT-X2 Dynamic Delivery System (Mirus). Cells were maintained for 48 h after transfection and then they were inoculated with HBV (genotype D)[20] in the presence of 4% PEG 8000 at 37°C for 24 h. Cells were maintained in the presence of 2.5% DMSO and culture supernates were collected every 3 days. A commercial enzyme linked immunosorbent assay (ELISA) kit was used to measure secreted HBeAg levels in the sample medium. (Wantai Pharm Inc.)

3. HBV infection in the presence of ursodeoxycholic acid

HepG2-NTCP cell line, which stably expresses NTCP, was kindly provided by Dr. Wang-Shick Ryu. HepG2-NTCP cells were maintained in high-glucose DMEM containing 10% FBS, penicillin and streptomycin (Welgene) at 37°C in a 5% CO_2 environment. HepG2-NTCP cells were seeded in 48-well plates at a density of 7.5×10^4 cells/well. HepG2-NTCP cells were infected with HBV in the presence of 4% PEG 8000 and ursodeoxycholic acid (UDCA) (0, 10, 30 and 90μM) at 37°C for 24 h. Cells were maintained in the presence of 2.5% DMSO and culture supernates were collected every 3 days. A commercial ELISA kit was used to measure secreted HBeAg levels in the sample medium (Wantai Pharm Inc.)

4. Patients

This study included 1,200 patients with CHB and 176 HBsAg-seronegative individuals. All participants were recruited at Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Of the 1,200 patients with CHB, 333 were diagnosed with liver cirrhosis and 318 were diagnosed with HCC. Patients infected with other hepatitis viruses (with the exception of hepatitis C virus), autoimmune disorders, and other non-HBV diseases were excluded. Written informed consent was obtained from the patients or a responsible family member. This study was approved by the independent Institutional Review Board of Severance Hospital and conformed to the ethical guidelines of the 1975 Helsinki Declaration.

5. DNA purification and genotyping of S267F polymorphism

Genomic DNA was extracted from whole blood using the MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 (TaKaRa) according to the manufacturer's instruction. The S267F polymorphism in NTCP was genotyped using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method. A 204-bp PCR fragment, including the S267F polymorphism in NTCP, was amplified using the following primer set: 5′-ATATGGCAATGAGGAGAAGC-3' and 5′-TTCCCTCTGAGTGTATGTGG-3′. PCR was performed in a 25-μL reaction containing 0.5 μL PfuTurbo DNA polymerase (2.5 U/μL), 2.5 μL 10x reaction buffer (Agilent), 50-100ng genomic DNA, 0.5 μL dNTP (10 mM), 0.5 μL (5 μM) of each primer, and up to 25 μL sterile double distilled water. The amplification was performed with the following conditions: first heated at 95°C for 30 s and then amplified for 35 cycles by denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s, extension at 68˚C for 30 s, and a final extension at 68°C for 5 min. The restriction fragments of S267F polymorphism in NTCP were obtained using *Hph*I (New England Biolabs) according to the manufacturer's instruction. Digested fragments were electrophoresed on 3% agarose gels, stained with ethidium bromide, and visualized with UV transilluminator. The genotypes of the polymorphism in the individuals were determined according to the digestion pattern: genotype CC with a fragment of 204 bp, genotype CT with three fragments of 204, 120, and 84 bp, and genotype TT with two fragments of 120 and 84 bp (**Fig. 1**).

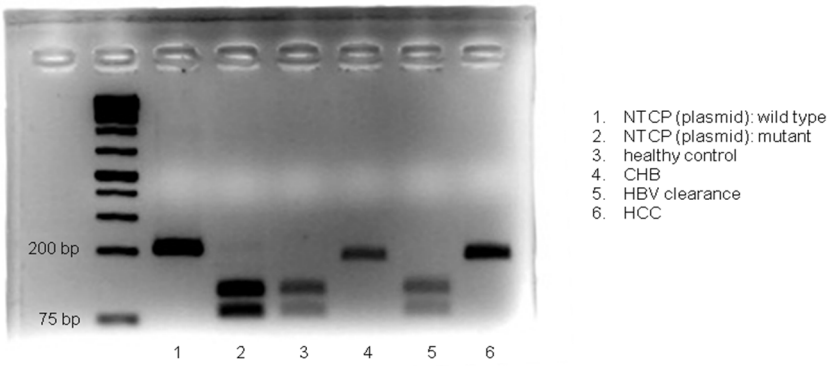

Figure 1. NTCP genotype determination based on restriction digestion pattern. Wild-type NTCP (genotype CC) appeared as one band, S267F variant (genotype TT) showed two bands upon digestion. NTCP, sodium taurocholate cotransporting polypeptide.

6. Clinical evaluation

At baseline, routine blood chemistry parameters, serum HBV DNA level, and other serologic viral markers were assessed. HBsAg, HBeAg, and anti-HBe were measured using ELISA (Abbott Laboratories, Chicago, IL). Serum HBV DNA levels were quantified using a commercially available real-time polymerase chain reaction (PCR) assay (COBAS AmpliPrep-COBAS TaqMan HBV test, Roche) with a linear detection range of 20–170,000,000 IU/mL. Serum alanine aminotransferase (ALT) levels were measured using standard laboratory procedures with the upper limit of normal set at 40 IU/L.

If histologic information was not available, clinically diagnosed liver cirrhosis was defined as follows: (1) platelet count <100,000/μL and ultrasonographic findings suggestive of cirrhosis, including a blunted, nodular liver edge accompanied by splenomegaly (>12 cm); or (2) esophageal or

gastric varices.[21] During follow-up, all patients underwent periodic surveillance with ultrasonography and laboratory workups, including determination of α-fetoprotein levels, at 3 or 6 month intervals.

7. Statistical analysis

Data are expressed as mean ± SD, median (range), or n (%), as appropriate. Differences among continuous variables were examined for statistical significance by Student's *t*-test (or Mann-Whitney test, if appropriate). Categorical variables were analyzed by chi-square test (or Fisher's exact test, if appropriate). The Cox proportional hazards model was used for multivariate analyses.

Hardy-Weinberg equilibrium (HWE) was tested using the Online Encyclopedia for Genetic Epidemiology HWE tool (OEGE).[22] Genotype association was analyzed with Chi-square (X^2) test.

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 20.0, Armonk, NY, USA) and SAS (version 9.2, SAS Inc., Cary, NC, USA). A value of $P < 0.05$ was considered to indicate statistical significance.

III. RESULTS

1. Transient transfection of NTCP plasmid

To confirm the role of S267F mutation in HBV infection, HepG2-NTCP cells were transfected with either wild-type NTCP or NTCP/S267F variant plasmid. We inoculated the transfected cells with HBV and measured secreted HBeAg as a reporter for HBV infection. Secreted HBeAg levels were about 2-fold lower in cells transfected with S267F-containing NTCP plasmid than those transfected with the wild-type NTCP plasmid (**Fig. 2**). This result indicates an association between S267F variant of NTCP and reduced HBV infection.

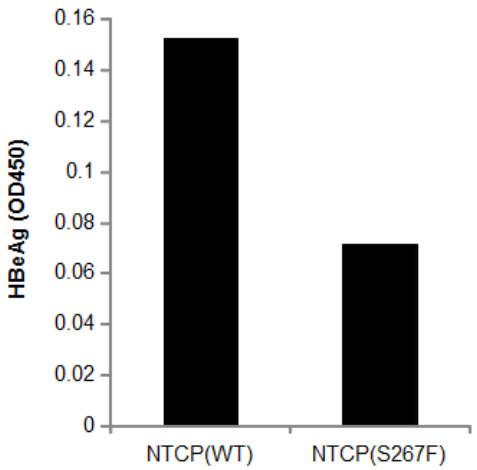

Figure 2. Comparison of HBV uptake levels between wild-type and S267F NTCP. The secreted HBeAg levels were markedly reduced in the cells transfected with S267F-containing NTCP plasmid. HBV, hepatitis B virus; HBeAg, hepatitis B e antigen.

2. Effects of ursodeoxycholic acid on hepatitis B virus infection

We investigated the changes in HBV entry upon UDCA treatment. UDCA (3α, 7β-dihydroxy-5β-cholanic acid), a dihydroxy bile acid, is one of the secondary bile acids.[23] We hypothesized that the uptake of UDCA and HBV entry would occur competitively because both processes employ the same NTCP protein. Therefore, we designed the experiment to determine the effect on HBV infectivity by varying the amount of UDCA. As the concentration of UDCA increases (from 0 to 90 μM), we observed a decrease in secreted HBeAg (**Fig. 3**). This result suggests that HBV and UDCA compete for interaction with NTCP.

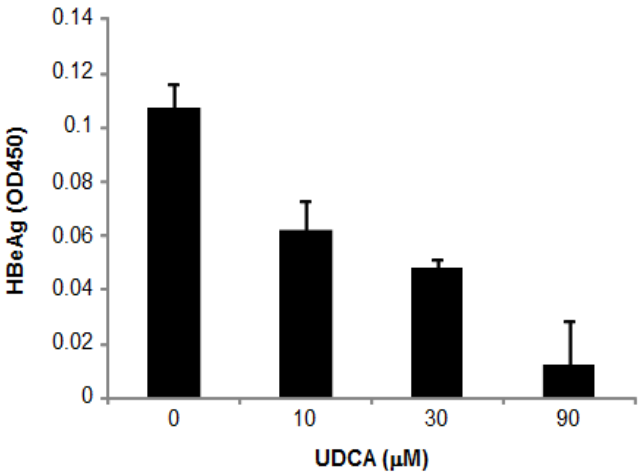

Figure 3. The changes in HBV entry in the presence of UDCA. Secreted HBeAg decreased when the amount of UCDA increased. HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; UDCA, ursodeoxycholic acid.

3. Baseline characteristics

The baseline characteristics of the 1,376 study subjects are summarized in **Table 1**. In total, we analyzed 1,200 patients with CHB and 176 HBsAg-seronegative individuals. For CHB patients, the mean age was 50.5 years, with more males than females (n = 803; 66.9%). Patients with CHB had lower platelet levels and higher AST and ALT levels than HBsAg-seronegative individuals. The mean HBV DNA level was 2.9 $\log_{10}$ IU/mL and 269 (22.4%) patients were positive for HBeAg. In total, 709 (59.1%) patients received antiviral treatment. In the CHB patients, the S267F polymorphism genotypes were distributed as follows: 1,168 (97.3%) patients were wild-type homozygote (CC) and 32 (2.7%) were heterozygote (CT).

Table 1. Baseline characteristics of the study population

Variable	CHB (n=1,200)	HBsAg negative individuals (n=176)	*P* value
Demographic			
Age, years	50.5 ± 11.7	47.8 ± 13.8	<0.001
Male gender	803 (66.9)	88 (50%)	<0.001
Laboratory			
Platelet (10^3/μL)	92.0 ± 91.2	117.9 ± 104.7	0.001
Serum albumin (g/dL)	3.5 ± 0.7	3.6 ± 0.5	0.134
AST (IU/L)	122.0 ± 368.9	48.6 ± 53.8	0.043
ALT (IU/L)	91.8 ± 169.5	36.8 ± 43.4	0.002
HBV-related			
HBV DNA, log $_{10}$IU/mL	2.9 ± 2.1		
HBeAg positivity	269 (22.4)		
Antiviral therapy	709 (59.1)		
Polymorphism-related			
S267F			0.031
CC	1,168 (97.3)	166 (94.3)	
CT	32 (2.7)	9 (5.1)	
TT	0 (0)	1 (0.6)	

Variables are expressed as mean ± SD (range) or n (%).

CHB, chronic hepatitis B; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBV, hepatitis B virus; HBeAg, HBV e antigen.

4. Frequency of S267F variant in controls and patients with CHB

Genotype distribution analysis showed that the frequency of the S267F was in Hardy-Weinberg equilibrium. No departure from the Hardy-Weinberg distribution was observed for this genotype (P=0.640) in CHB or HBs-seronegative individuals.

S267F polymorphism was associated with reduced infectivity in CHB patients compared with HBs-seronegative individuals (**Table 2**). The genotype distributions of S267F polymorphism differed significantly between Korean CHB patients and HBsAg-seronegative individuals. The frequency of genotype CT or TT in CHB patients was lower than HBs-seronegative individuals (2.7% vs. 5.7%). Also, the risk of HBV infection was significantly lower in patients with genotype CT than those with genotype CC (odds ratio [OR] 0.455, 95% confidence interval [CI] 0.220-0.942, P=0.034) .

Table 2. Association of S267F polymorphism with chronic HBV infection

	CHB	HBs-seronegative individuals	OR	95% CI	*P* value
S267F					
CC	1168 (97.3)	166 (94.3)	1.000		
CT	32 (2.7)	9 (5.1)	0.455	0.220-0.942	0.034
TT	0 (0)	1 (0.6)			

Variables are expressed as means ± SD (range) or n (%).

CHB, chronic hepatitis B; OR, odds ratio; 95% CI, 95% confidence interval.

5. Association of S267F polymorphism with disease course of chronic HBV infection

The population was further stratified for a subgroup analysis, distinguishing CHB patients without cirrhosis or HCC (n=549) from CHB patients with cirrhosis (n=333) and CHB patients with HCC (n=318) (**Table 3**). The S267F polymorphism was detected in 3.3% (n=18) of patients with CHB only, 0.9% (n=3) of patients with CHB and cirrhosis, and 3.5% (n=11) of patients with CHB and HCC. The frequency of genotype CT was higher in CHB only patients than in cirrhotic patients with CHB. As shown in Table 4, The presence of S267F variant correlated significantly with a lower risk of developing cirrhosis (OR 0.268, 95% CI 0.078-0.917, *P*=0.036). However, there was no significant difference in the risk of developing HCC in CHB patients (OR 0.946, 95% CI 0.441-2.029, *P*=0.887). Also, the S267F variant was associated with a decreased risk of developing HCC from cirrhosis (OR 0.254, 95% CI 0.070-0.918, *P*=0.037).

Table 3. Genotype of NTCP in patients with CHB, with or without either cirrhosis or HCC

S267F	CHB only (n=549)	CHB + LC (n=333)	CHB + HCC (n=318)
Genotype			
CC	531 (96.7)	330 (99.1)	307 (96.5)
CT	18 (3.3)	3 (0.9)	11 (3.5)

Data are expressed as n (%)

CHB, chronic hepatitis B; LC, liver cirrhosis, HCC, hepatocellular carcinoma.

Table 4. Association of S267F polymorphism with disease progression in patients with CHB infection

S267F	CHB vs. LC		CHB vs. HCC		LC vs. HCC	
	P	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)
Genotype						
CC	1.0		1.0		1.0	
CT	0.036	0.268 (0.078-0.917)	0.887	0.946 (0.441-2.029)	0.037	0.254 (0.070-0.918)

Data are expressed as n (%)

CHB, chronic hepatitis B; LC, liver cirrhosis, HCC, hepatocellular carcinoma; OR, odd ratio; 95% CI, confidence interval.

6. Correlation of S267F polymorphism with HBV DNA level in patients who did not receive antiviral therapy

The presence of S267F variant was associated with reduced HBV infection. Thus, we hypothesized that the S267F variant might decrease the spread of HBV-infected hepatocytes and result in low viral titers. HBV DNA levels of treatment-naïve patients with and without S267F variant were compared (**Fig. 4**). Among the 32 CT heterozygote patients, 10 (31.3%) were treatment-naïve, and these individuals had a median DNA level of 2.0 $\log_{10}$ IU/mL (range, 1.08-4.66 IU/mL). In comparison, the median DNA level of the 477 treatment-naïve CC homozygote patients was 2.9 $\log_{10}$ IU/mL (range, 1.30-8.10, P=0.179).

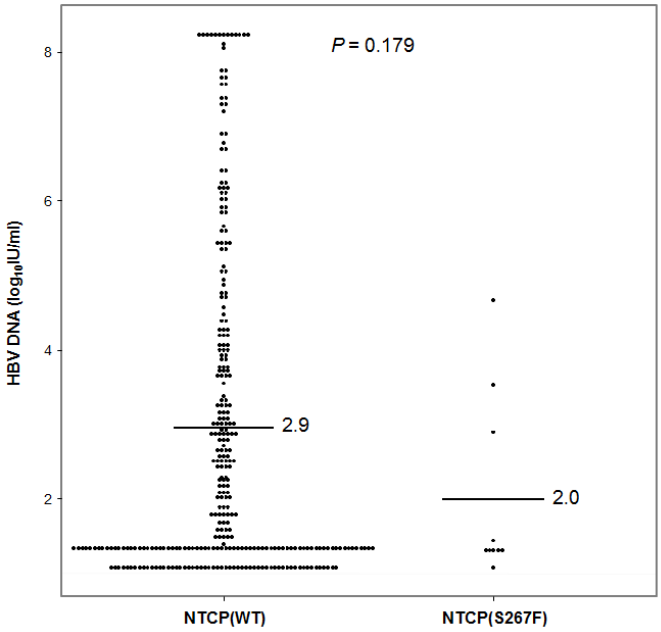

Figure 4. HBV DNA level in patients with and without S267F variant. The median HBV DNA level was higher in patients without S267F variant. HBV, hepatitis B virus.

IV. DISCUSSION

NTCP plays an important role in both the enterohepatic circulation of bile acids and in hepatocyte function.[24] Until recently, NTCP has been known as a bile acid transporter[18,19] and this protein also participates in transporting steroid hormones, drugs (e.g., cyclosporine), and peptide ligand. Human NTCP consists of 349 amino acids with nine predicted transmembrane domains.[25] To date, six SNPs have been identified in NTCP[19] and some of these SNPs are found in specific ethnic backgrounds. Among them, the S267F polymorphism in exon 4 of NTCP has been identified only in Eastern Asians.[6]

NTCP was recently proposed to be a functional cell surface receptor for HBV entry into hepatocytes.[13,26] The region between amino acids 157 and 165 is the most essential for HBV infection.[27] Amino acids 84-87 of NTCP are also considered important for HBV infection.[9] Interestingly, HBV entry and bile acid transport share common molecular determinants on NTCP. Thus, genetic variation of NTCP could have effects on not only the HBV entry, but also bile acids uptake. Firstly, we have been questioned how the uptake of bile acids and HBV would occur.

To confirm this question, an *in vitro* experiment was performed using UDCA. UDCA (3α, 7β-dihydroxy-5β-cholanic acid), is a dihydroxy, secondary bile acid. Its role in lowering ALT levels in chronic liver diseases has been reported.[28] In our study, HBV infection was inhibited in a dose-dependent manner when the cells were inoculated with HBV and UDCA. This result suggests that HBV and UDCA employed NTCP competitively. This opens up new possibilities for the management of HBV infection using NTCP

substrates. However, the concentration of UDCA in the experiment (0-90 μM) is different from the ones in real clinical settings. Standard doses of UDCA treatment is within the range of 12-15 mg/kg body weight.[29] In other words, the concentration of UDCA, when applied to patients, is relatively too high compared to when it is used in experiments. Thus, further studies are required regarding the influence of UDCA in the course of hepatitis B.

The frequency of Asian-specific S267F variant differs slightly according to ethnicity: 3.1-5.0% in Koreans, 7.4% in Chinese, 7.5% in Chinese-Americans, and 9.2% in Vietnamese.[6,17,19] In our study, the frequency of the S267F variant in HBsAg-seronegative individuals was 5.7%, consistent with the previous report. We also confirmed that HBV infection was inhibited in the presence of the S267F variant *in vitro*. We also sequenced the preS1 domain of HBV from the S267F variant-containing samples to see whether the presence of S267F variant exerted a selective pressure that is strong enough to drive viral evolution in the preS1 domain. However, we could not observe any sequence variation, especially in the essential residues for NTCP binding (data are not shown).

So far, three studies have been reported regarding the association between NTCP polymorphism and the clinical characteristics of CHB. We summarized the results of these studies including ours in Table 5.

Table 5. Summary of the previous studies on NTCP polymorphism

Authors	Li *et al.*[6]	Peng *et al.*[30]	Hu *et al.*[31]	This Study
Group	Chinese Han	Chinese Han	Taiwanese	Korean
Study population	n=244 (CHB patients) n=76 (HBV infection resolvers) n=113 (healthy controls)	n=1,899 (CHB patients) n=1,828 (healthy individuals)	n=3,801 (CHB patients) n=3,801 (HBsAg-seronegative controls)	n=1,200 (CHB patients) n=176 (HBsAg-seronegative controls)
S267F is associated with	Increased HBV infection	Decreased HBV infection	Decreased HBV infection	Decreased HBV infection
Clinical significance	No significant association between S267F and cirrhosis, HCC	Lower risk of ACLF	Lower risk of cirrhosis and HCC	Lower risk of cirrhosis

CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ACLF, acute-on-chronic liver failure.

All studies were performed for Asian patients. Taken together, patients with the S267F variant seem to be less susceptible to HBV infection. With the exception of the first study,[6] the S267F variant in NTCP is associated with reduced HBV infection and subsequent disease course, including acute-on-

chronic liver failure, cirrhosis and HCC. Only the first study[6] presents conflicting data about susceptibility. However, the clinical significance this variant with cirrhosis or HCC requires additional study because various factors in combination could affect disease progression.

Our results showed patients with the S267F variant were less susceptible to HBV infection. Patients with S267F variant also had a lower risk of developing cirrhosis. Cirrhosis develops as a result of inflammation and fibrosis in patients with CHB. Increased HBV DNA level is one of the most important risk factors for cirrhosis development.[2] Since S267F variant is associated with lower HBV DNA level, the chance of cirrhosis development could be reduced in patients with S267F variant. However, we did not find any significant, direct association between S267F variant and HCC development. Perhaps, carcinogenesis occurs from many different mechanisms including HBV infection, changes in the host immune system, and behavioral factors associated with HBV infection.[32]

This is the first study to investigate S267F variant of NTCP in Korean patients with CHB. Therefore, the S267F variant seems to associate with the susceptibility to, and chronicity, of HBV infection in Asian population. However, whether the level of HBV viremia is critical for interference with bile acid transport remains unclear. Treatment-naïve patients were analyzed separately to confirm the correlation between the HBV DNA level and S267F variant. We hypothesized that patients with S267F would have lower HBV DNA levels than those without. Although the median HBV DNA level was slightly lower with the patients who had S267F variant, the difference was not

statistically significant. Notably, this result is difficult to interpret because the number of treatment-naïve patients with S267F variant was small.

This study has some limitations. First, the study population was heterogeneous. Second, only the S267F variant of *SLC10A1* was genotyped. Because other factors might be involved in HBV entry, the role of NTCP could have been overestimated until additional analysis is conducted. Third, the sample size varied between the groups, and only a small number of patients harbored the S267F variant. However, the overall prevalence of S267F variant was consistent with previous reports (**Table 4**). Finally, we tried immunofluorescence to detect HBcAg from the infected cells but failed. We also used HBeAg ELISA, instead of HBsAg ELISA, to confirm the degree of HBV infection. Such method agrees with the fact that HBsAg is not a reliable marker of productive HBV infection, especially at early time points, as reported by Li *et al*.[33] Future validation would likely enhance our understanding of the role of NTCP.

As resistance to the existing antiviral agents increased, new therapeutic agents is urgently needed. To date, Myrcludex B is an antiviral candidate targeting NTCP,[34] and this protein could be considered as a new therapeutic target for curing chronic HBV infection.

V. CONCLUSION

The S267F variant of NTCP is associated with lower levels of HBV infection *in vitro*. In addition, UDCA treatment inhibited HBV infection in a dose-dependent manner.

The frequency of the S267F variant (genotype CT or TT) was 2.7% and 5.7% in Korean patients with CHB and HBsAg-seronegative individuals, respectively. Patients who had the S267F variant of NTCP were less susceptible to HBV infection. The frequency of S267F variant was 3.3% in CHB patients, 0.9% in cirrhosis patients, and 3.5% in HCC patients. The S267F variant correlated significantly with a lower risk of cirrhosis development. Further functional analysis of NTCP should increase our understanding of HBV biology.

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ABSTRACT (IN KOREAN)

국내 만성 B형간염 환자에서 B형간염 바이러스 세포 수용체인 NTCP의 S267F 변이와 임상적 연관성

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서론: Sodium taurocholate co-transporting polypeptide (NTCP)는 최근 B형간염 바이러스의 수용체로 밝혀졌다. NTCP 수용체의 267번 위치에 serine에서 phenylalanine로의 치환 (S267F 변이)은 아시아에서 특이적인 변이로 *in vitro* 실험에서 B형간염 바이러스의 감염을 방해하는 것으로 보고되었다. 본 연구에서는 국내 만성 B형간염 환자들에서 S267F 변이의 유병률을 살펴보고 만성 B형간염 질병 경과와의 연관성에 대해 알아보고자 한다. 또한, 담즙산이 B형간염 바이러스의 세포 내 감염에 영향을 주는지 *in vitro* 실험을 통해 확인하고자 한다.

방법: S267F 변이가 B형간염 감염에 미치는 영향을 보기 위해 mutagenesis, transfection, virus inoculation과 ELISA 방법을 이용하였다. B형간염 바이러스의 경우 HepAD38 세포주를 이용하여 virus induction, supernatant collection 및 concentration, real-time PCR을 통한 정량화 방법으로 준비하였다. 만성 B형간염 환자 1,200명과 B형간염 표면 항원 음성인 176명에 대하여 NTCP의 S267F 변이를 조사하였다. 혈액으로부터 genomic DNA를 추출하였고, 중합효소 연쇄반응-제한 효소 절편 길이 다형성 (PCR-RFLP)을 이용하여 S267F

변이 여부를 검사하였다. 또한, S267F 변이와 임상적 연관성에 대해서도 함께 분석하였다.

결과: HepG2-NTCP cell line을 이용한 in vitro 실험결과 NTCP 267F 변이가 B형간염 바이러스 감염에 음성적 효과 (negative effect)를 나타냈고, UDCA에 의해 용량 의존적으로 B형간염 바이러스 감염이 감소함을 확인하였다. NTCP의 267F 변이 (CT or TT 유전자형) 비율은 만성 B형간염 환자에서 2.7%, B형간염 표면 항원 음성인 군에서 5.7% 였고 (P=0.031), S267F 변이가 있는 경우 B형간염 감염에 덜 민감하다는 것을 확인하였다. S267F 변이의 비율은 만성 B형간염 환자에서 3.3%, 간경화에서 0.9%, 간암 환자에서 3.5% 였다. 통계적 분석 결과 S267F 변이가 있는 경우 간경화 발생의 위험이 낮아졌고 (P=0.036), 반면 간암 발생과는 통계적 유의성을 보이지 않았다 (P=0.887). S267F 변이가 있는 32명의 환자 중 10명의 환자가 이전에 B형간염 관련 치료를 받은 과거력이 없었으며 이들의 B형간염 바이러스 DNA 중앙값은 2.0 $\log_{10}$ IU/mL 이었다. 그러나 이전 항바이러스 치료력이 없고 동시에 S267F 변이가 없는 (CC 유전자형) 477명의 환자들의 B형간염 바이러스 DNA 중앙값은 2.9 $\log_{10}$ IU/mL (P=0.179)이었다.

결론: in vitro 실험을 통해 우리는 NTCP의 S267F 변이가 B형간염 바이러스의 감염을 감소시킴을 확인하였다. 또한 B형간염 바이러스와 UDCA의 세포 내 수용은 NTCP에 대하여 경쟁적으로 작용한다. NTCP의 267F 변이는 임상적으로 국내 만성 B형간염 환자들에서 B형간염 바이러스의 낮은 감염력 및 간경화 위험도와 관련이 있다.

핵심되는 말: rs2296652, 유전적 다형성, sodium tahrocholate cotransporting polypeptide, B형간염