의학석사 학위논문

Naturally occurring variants of hepatitis C virus NS5B protein in Korean patients with chronic Hepatitis C virus infection

한국 만성 C 형 간염 환자의 NS5B 단백의 자연발생 변이에 대한 연구

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김 동 원

A thesis of the Master’s degree

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Naturally occurring variants of hepatitis C virus NS5B protein in Korean patients with chronic Hepatitis C virus infection

by Dong Won Kim

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Abstract

Introduction: The nonstructural 5B (NS5B) protein of the hepatitis C virus (HCV) with RNA-dependent RNA polymerase (RdRp) activity plays a pivotal role in viral replication. Therefore, monitoring of its naturally occurring mutations is very important for the development of antiviral therapies and vaccines.

Methods: The mutations in the partial NS5B gene (492bp) from 166 quasispecies of 15 genotype-1b (GT) treatment-naïve Korean chronic patients were determined and mutation patterns and frequencies mainly focusing on the T cell epitope regions were evaluated.

Results: The mutation frequency within the CD8+ T cell epitopes was significantly higher than those outside the CD8+ T cell epitopes. Of note, the mutation frequency within predicted CD4+ T cell epitopes, a particular mutational hotspot in Korean patients was significantly higher than in patients from other areas, suggesting distinctive CD4+ T cell-mediated immune pressure against HCV infection in the Korean population. The mutation frequency in the NS5B region was positively correlated with patients with carrier-stage rather than progressive liver disease (chronic hepatitis, liver

cirrhosis, and hepatocellular carcinoma). Furthermore, the mutation frequency in four codons (Q309, A333, V338 and Q355) known to be related to the sustained virological response (SVR) and end-of treatment response (ETR) was also significantly higher in Korean patients than in patients from other areas.

Conclusions: In conclusion, a high level of mutation frequency in the HCV GT-1b NS5B region, particularly in the predicted CD4+ T cell epitopes, was found in Korean patients, suggesting the presence of distinctive CD4+ T cell pressure in the Korean population. This provides a likely explanation of why relatively high levels of SVR after a combined therapy of pegylated interferon (PEG-IFN) and ribavirin (RBV) in Korean chronic patients with GT-1b infections is observed.

Keywords: Hepatitis C virus (HCV), nonstructural 5B protein (NS5B), CD4+ T cell epitope, antiviral resistant mutations

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Introduction

According to the WHO, 3% of the global population is infected with hepatitis C virus (HCV), with 3–4 million people newly infected each year (1-4). Most HCV infections persist, with up to 80% of all cases leading to chronic hepatitis associated with liver fibrosis, cirrhosis, and hepatocellular carcinoma (5-7). Combinatorial treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV) provides good clinical efficacy in patients infected with genotypes 2 and 3 (GTs) but is less efficacious in patients infected with the most prevalent GT-1b, thereby emphasizing the urgent need for more effective specifically targeted antiviral therapies for GT-1b (8-11).

The HCV RNA-dependent RNA polymerase (RdRp) is an essential enzyme that lacks proofreading activity, thus leading to a population of distinctive but closely related viral variants, termed viral quasispecies, within an infected individual (12-14). Monitoring of the diversity of HCV quasispecies is important for the prediction of liver disease progression as well as HCV treatment outcome (15-18). Currently, studies regarding HCV quasispecies have mainly focused on structural genomic regions; therefore, relatively limited data are available regarding nonstructural regions. Recently, variations

in nonstructural 5B (NS5B) protein, particularly in specific codons, were reported to be positively related to sustained virological response (SVR) and end-of treatment response (ETR) of patients infected with GT-1b (15, 16).

It was also reported that the SVR rate in patients with HCV GT-1b treated with PEG-IFN plus RBV are higher in Asian patients as compared with Caucasians (10, 19). In particular, previous studies have shown that SVR rates in Korea patients infected with GT-1b range from 56% to 62% (20, 21). Recently, two SNPs, rs12979860 and rs8099917 of the IL28B gene showing the strongest association with treatment response, have been reported at a high frequency in Korean patients with the hepatitis C virus (HCV) GT-1b compared to the frequencies in other ethnic groups (22, 23). Although prior investigations can partly explain the high SVR rates in Korean patients, other mechanisms may also contribute to this effect. In the present study, to address this issue, we investigated the mutation frequencies and patterns in the partial NS5B from HCV GT-1b infected Korean patients, which are known to be related to the SVR rates, via quasispecies analysis.

Materials and Methods

1. Patients and HCV RNA Extraction

Serum samples were collected from a total of 73 treatment-naïve HCV-positive patients who visited Seoul National University Hospital in 2003. The clinical statuses of the HCV-positive patients were defined as carrier (C), chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC). General definitions of the carrier and chronic liver disease types are as follows: the diagnosis of a HCV carrier with normal transaminase values can be made in the presence of positive anti-HCV antibodies, of a positive HCV RNA by RT-PCR and of normal alanine aminotransferase (ALT) levels in at least three tests carried out at least two months apart over a period of six months (24); chronic hepatitis was defined as an elevation or fluctuation of serum ALT over 6 months without any evidence of any other chronic liver disease (25); liver cirrhosis was diagnosed as having clinically relevant portal hypertension (esophageal varices and/or ascites, splenomegaly with a platelet count of 100,000/mm3) (26) and ultrasonographic imaging features suggestive of liver cirrhosis (27); and HCC was diagnosed either histologically or radiologically based on the presence of a hypervascular liver

mass with serum alpha-fetoprotein (AFP) levels exceeding 400 ng/ml (28). HCV RNA was purified using the Viral Gene-Spin Viral DNA/RNA Kit (iNtRON Biotechnology Inc., Seongnam, Korea) according to the manufacturer's guideline. This work was approved by the institutional review board of Seoul National University Hospital (IRB No. C-1304-032-479). The experiment was mainly based on the viral RNA extracted from isolates; therefore, the research was done without informed consent and a waiver of informed consent was agreed upon by the IRB.

2. Quantitative PCR and cDNA synthesis

A quantitative PCR method was used to analyze viral RNA with an ABI7500 system (Perkin-Elmer Applied Biosystems, Warrington, UK). The primers were designed to amplify the NS2 region and the sequences were as follows: sense primer HCVF (5'-CGA CCA GTA CCA CCA TCC TT-3') and antisense primer HCVR (5'-AGC ACC TTA CCC AGG CCT AT-3'). Viral cDNA synthesis for Reverse-transcriptase (RT) PCR was done using the Maxime RT PreMix (iNtRON Biotechnology Inc., Seongnam, Korea) according to its own the protocol.

3. Nested PCR Amplification

The nested PCR method and primer pairs to amplify GT-1 to 4 are available in the literature (Table 1) (29). Briefly, as an example for GT-1b, the first round of amplification was carried out using the sense primer A1b (O/S) (accession no. M62321, positions 8113-8135, 5' – CTGACRACTAGCTGYG GTAAYAC - 3') and the antisense primer F1b (O/A) (positions 8678-8699, 5' - CCTGGAGAGTAACTRTGGAGTG - 3'). The first-round reaction was subjected to 30 cycles of amplification (30 s at 94℃, 30 s at 45℃ and 50 s at 68℃) followed by a 7 min of extension at 72℃. The second round of amplification was carried out using the sense primer B1b (I/S) (positions 8181-8205, 5' - GCTCCRGGACTGCACSATGCTCGTG - 3') and the antisense primer E1b (I/A) (positions 8654-8675, 5' – AATGCGCTRA GRCCATGGAGTC - 3') to amplify 495bp of the GT-1b NS5B region (Figure 1). The second-round reaction was subjected to 30 cycles of amplification (30 s at 93℃, 30 s at 55℃ and 1 min at 68℃) followed by a 7 min of extension at 72℃.

4. Cloning and Sequencing Analysis

PCR products of GT-1b were cloned using the TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, CA, USA). The NS5B regions were

sequenced using the M13 primer. For each subject, 10 to 12 subclones were sequenced (30, 31). Sequencing was conducted using the Applied Biosystems model 377 DNA automatic sequencer (Perkin-Elmer Applied Biosystems, Warrington, UK). If there were sequence variations between clones of a sample, a typical sequence of the variation site was determined as a major sequence. Nucleotides were aligned and their similarities were calculated using the multiple-alignment algorithm in Megalign (DNASTAR, Windows Version 3.12e). Mutations were determined with a combination of the consensus sequence of a total of 166 subclones and 10 GT-1b reference strains obtained from the LANL HCV database (http://hcv.lanl.gov) [accession numbers AB442219, AB691953, AF165047, D16435, D50485, D85516, D90208, M58335, S62220 and X61596] (32) . Because at aa 316 and 464, the two types of subclonal amino acids were conserved in each subject, both amino acids were considered to be a consensus sequence (33, 34). For a further comparison of the analyzed sequences, 45 HCV GT-1b sequences from other countries (Japan: 15, China: 12, Taiwan: 3, Germany: 7 and Switzerland: 8) were also retrieved from the LANL HCV database and relevant nucleotide positions were compared with consensus sequences of 15 subjects.

5. Phylogenetic analysis

HCV GTs were confirmed by a phylogenetic analysis based on 12 reference strains representing each of the GTs of 1-6 obtained from GenBank [accession numbers AF009606 (1a), AF064490 (5a), AY232745 (2b), D63821 (3a), D90208 (1b), DQ480515 (6a), JX961069 (2a), M58335 (1b), M67463 (1a), S62220 (1b), X61596 (1b) and Y11604 (4a)]. Phylogenetic trees were inferred using the neighbor-joining method (35). Neighbor-joining was carried out using MEGA version 4.0.2 (36). The resultant neighbor-joining tree and topology were evaluated by bootstrap analyses based on 1,000 re-samplings.

6. Prediction of novel CD4+ epitopes

15-mer peptides containing an association between a particular HLA class II molecule and the sequenced NS5B with binding capacity < 500mM were screened for the presence of the relevant HLA-binding motif (37).

7. Statistical analyses

The results were expressed as percentages, means ± SD, or as medians (range). The differences between the categorical variables were analyzed using Fisher's exact test or a Chi-square test. For continuous variables, the Student's t-test was used when the data showed a normal distribution, or the

Mann-Whitney U test was used when the data was not normally distributed. The level of significance of each test was adjusted for multiple tests via Bonferroni correction. A p-value of < 0.05 (two-tailed) was considered to be statistically significant.

Table 1. Primers used in PCR targeting the NS5B region of HCV.

Primer	Sequence (5' → 3')	Nucleotide position[a]	Specific genotype
A1b (O/S)	CTGACRACTAGCTGYGGTAAYAC	8113-8135	1
F1b (O/A)	CCTGGAGAGTAACTRTGGAGTG	8678–8699	1
B1b (I/S)	GCTCCRGGACTGCACSATGCTCGTG	8181–8205	1
E1b (I/A)	AATGCGCTRAGRCCATGGAGTC	8654–8675	1/4
2HCV-OS	GTGTTCACCACYAGCATGGGGA	8110–8131	2
2HCV-OA	WSAGTTCGTGGGGAGWGTATGT	8687–8708	2
2HCV-IS	TGGTRTGTGGMGACGACYTGGT	8201–8222	2
2HCV-IA	GCCCGTGTAGCCTTTCAATTAT	8644–8665	2
A3a (O/S)	ACAATCACTTGTTACATCAAGGCC	8134–8157	3a
F3a (O/A)	TCTACTGGAGAGTAACTGTGGA	8681–8703	3a
B3a (I/S)	GGAACCCGGACTTTCTTGTC	8186–8205	3a
E3a (I/A)	CCATGGAGTCTTTCAATGATTG	8642–8663	3a
4HCV-OS	ACCACCAGCTTYGGRAACAC	8116–8135	4
4HCV-OA	TTCGTGTGGAGAGTATCCRTGCA	8681–8704	4
4HCV-IS	CTGAGAGACTGCACSATGYTGGT	8182–8204	4

a. Amino acid positions correspond to the reference sequence M62321.

(A)

(B)

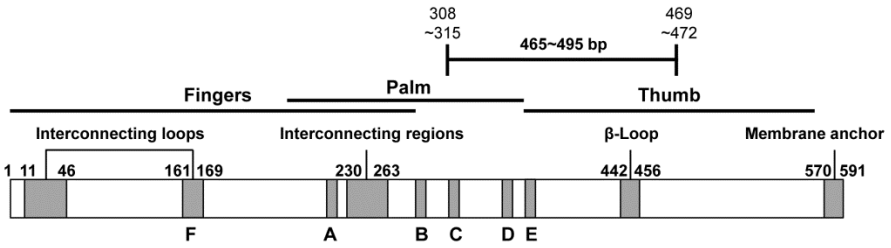

Figure 1. Schematic figure of amplified region of HCV NS5B. (A) The genomic structure of HCV. (B) Primer binding sites and the size of amplicon obtained in the nested PCR. Alphabets indicate conserved regions of the NS5B.

Results

1. Nested PCR-based distribution of GTs

Among 73 treatment-naïve samples which were subject to cDNA synthesis, 23 (31.5%) were amplified. Similar to the serological prevalence, GT-1b (15 patients, 65.2%) and 2 (6 patients, 26.1%) were dominant in the Koreans. GT-3a and 4 were amplified in one subject each (4.3%) (Table 2). Clinical details of the GT-1b patients for whom the sequences were amplified are presented in Table 3.

Table 2. Distribution of amplified subjects by PCR targeting NS5B sequences.

Genotypes	No. of subjects NS5B were amplified (%)
1	15 (65.2)
2	6 (26.1)
3	1 (4.3)
4	1 (4.3)
Overall	23 (31.5)
Not amplified	50 (68.5)
Total	73 (100)

Table 3. Clinical features of 15 Korean patients in this study.

Clinical factors	15 subjects (%)
Age in years, mean ± SD	63.9 ± 9.8
Male (%)	7 (47.7)
Liver disease (no.) C:CH:LC:HCC	6:1:6:2
ALT (IU/L), mean ± SD	60.6 ± 36.5
History of antiviral therapy	0

2. Phylogenetic analysis of GT-1b and its characteristics

A phylogenetic analysis based on the 492bp GT-1b sequenced NS5B region of randomly selected subclones showed distinctive sequence variation between each subject (Figure 2). All 15 subjects belong to GT-1b. 12 subjects with N316/E464 and three subjects with C316/Q464 were phylogenetically segregated. The C316/Q464 group has significantly lower Cp values [C316/Q464 (31.69 ± 0.92) vs. N316/E464 (32.28 ± 1.05), p = 0.033] (Table 4) and exclusively showed advanced liver disease (CH + LC + HCC) (100%) compared to the other group (48.1%, p = 0.001) at the subclonal level (Table 5). This finding indicates a positive correlation between viral replication and the clinical severity of liver disease. The nucleotide sequence of 166 patients will be available in GenBank nucleotide sequence databases with the following accession numbers: KF422017-KF422027.

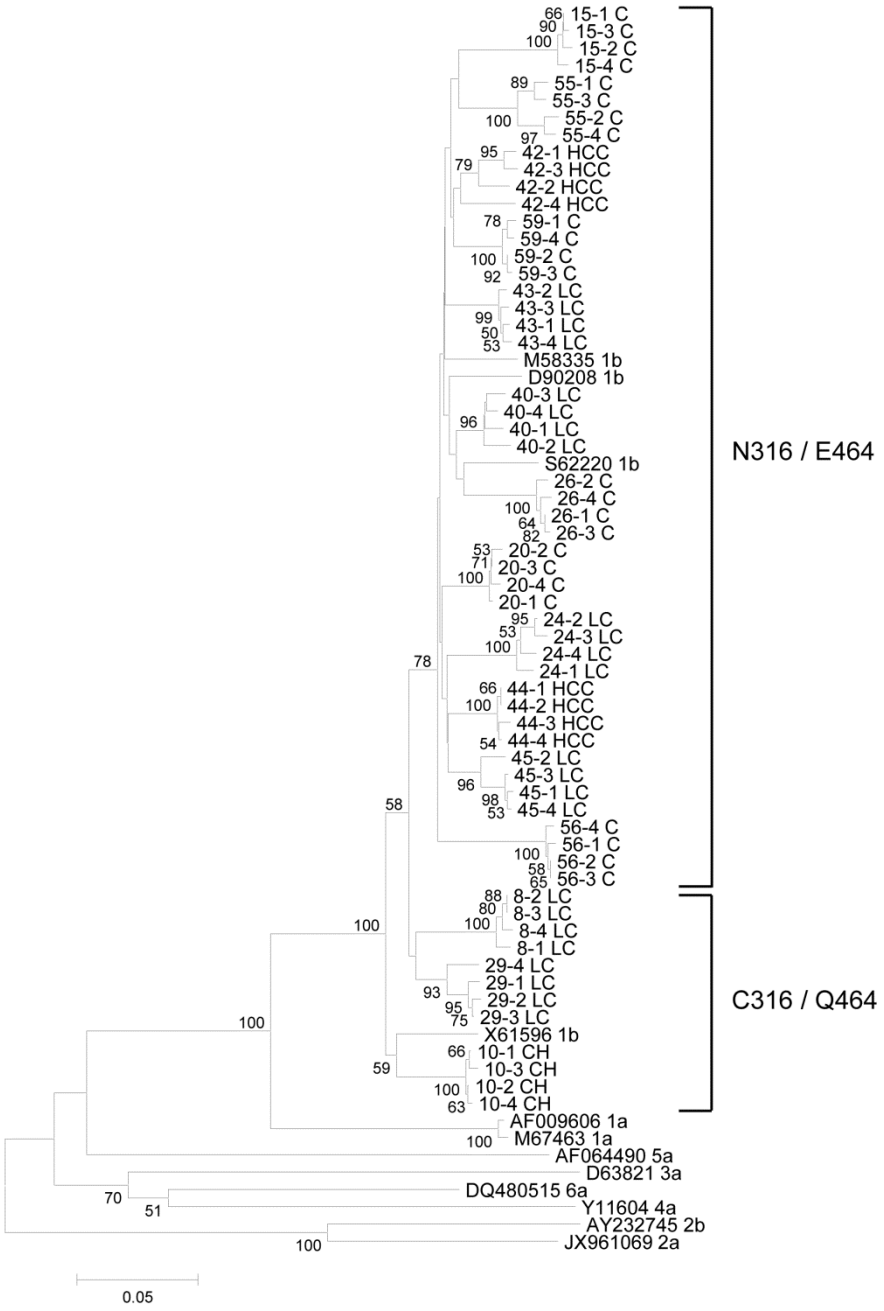

Figure 2. Phylogenic tree of 492bp of the genotype 1b NS5B region.

Table 4. Comparison of Cp values between types at the major codons including antiviral resistance and SVR related in the sequenced NS5B region.

Amino acid variants	Cp values of WT /major type, mean ± SD (No of patients, %)	Cp values with Mutation /minor type, mean ± SD (No of patients, %)	*P*-value
C316N	31.69 ± 0.92 (3, 20.0)	32.28 ± 1.05 (12, 80.0)	0.033
A333E/V	33.12 ± 1.2 (3, 20.0)	32.1 ± 0.5 (12, 80.0)	0.184
S335N	32.8 ± 1.1 (7, 46.7)	33.1 ± 1.4 (8, 53.3)	0.669
V338A	32.9 ±1.3 (6, 40.0)	1.2 ± 0.5 (9, 60.0)	0.953
P353L	33.3 ± 11.2 (7, 46.7)	32.6 ± 1.2 (8, 53.3)	0.307
Q355K/R	33.4 ± 0.9 (3, 20.0)	31.3 ± 0.5 (12, 80.0)	0.003
E440D/G/K	32.9 ± 1.3 (7, 46.7)	33.0 ± 1.2 (8, 53.3)	0.935
C451H/T/Y	33.0 ± 1.4 (6, 40.0)	33.0 ± 0.5 (9, 60.0)	0.978
E464Q	32.28 ± 1.05 (12, 80.0)	31.69 ± 0.92 (3, 20.0)	0.033

a. C316/Q464 and N316/E464 were found in an ex clusive manner.

Table 5. Mutation frequency at the major codons including antiviral resistance and SVR-related codons in the sequenced NS5B region.

Amino acid variants	No. of variants (n = 166)	Rate (%)	No. of patient	Mutation type[a]	Note (Reference)
Q309R	96	57.8	15	Diverse	SVR and ETR 27%, NR: 9% in Japan (15)
C316N[b]	133	80.1	12	Conserved	63.3% with C316N/Y in patients treated with IFN / RBV in Japan (15)
A333E/V	16	9.6	3	Conserved (E) Diverse (V)	SVR and ETR 23%, NR: 9% in Japan (15)
S335N	63	38.0	7	Diverse	29.6% with S335A/N in patients treated with IFN / RBV in Japan (15)
V338A	46	27.7	6	Diverse	SVR and ETR 18%, NR: 5% in Japan (15)
P353L	57	34.3	7	Diverse	17.3% with S335A/N in patients treated with IFN / RBV in Japan (15)
Q355K/R	34	20.5	3	Conserved	SVR and ETR 25%, NR: 5% in Japan (15)
E440D/G/K	40	24.1	7	Conserved (D) Diverse (G/K)	Immunosuppressant resistant mutation (38)
C451H/T/Y	46	27.7	6	Conserved (T/Y) Diverse (H)	The frequency of 2 AA are almost same (16)
E464Q[b]	33	19.9	3	Conserved	The frequency of 2 AA are almost same (16)

a. 'Diverse' indicates the mutation type of the coexistence with the wild type in a quasispecies distribution of a subject. Otherwise, 'Conserved' indicates the presence of only mutation types alone without the wild type in a quasispecies distribution of a subject.

b. C316/Q464 and N316/E464 were found in subclones with exclusive manner. Total 33/33 (100%) C316/Q464 subclones (100%) were found in advanced liver disease [$p < 0.001$, compared to N316/E464 64/133 (48.1)].

3. Distribution of mutations in the sequenced NS5B region

The distribution of the mutations from the sequenced GT-1b NS5B region aa 164 is shown in Figure 3. There are six known CD8+ T cell epitopes (Table 6), and the mutation frequencies inside the CD8+ T cell epitope regions (2.9%) are significantly higher than those outside the epitope regions (2.3%, $p = 0.001$) (Table 7). The mutation frequencies inside the predicted CD4+ T cell epitopes (4.8%) are significantly higher than those outside the CD4+ T cell epitope (1.4%) and are even higher than that inside the known CD8+ T cell epitopes ($p < 0.001$). We designated the region including the aa 333-355 section of the CD4+ T cell epitopes as a mutational hotspot where an extraordinary high mutation frequency (6.7%) was observed. Of note, the region is predicted to have high binding affinity for the various MHC class II HLA types prevalent in Koreans, raising the possibility that there may be distinctive MHC class II restricted immune pressure against HCV GT-1b in the mutational hotspot (Table 8).

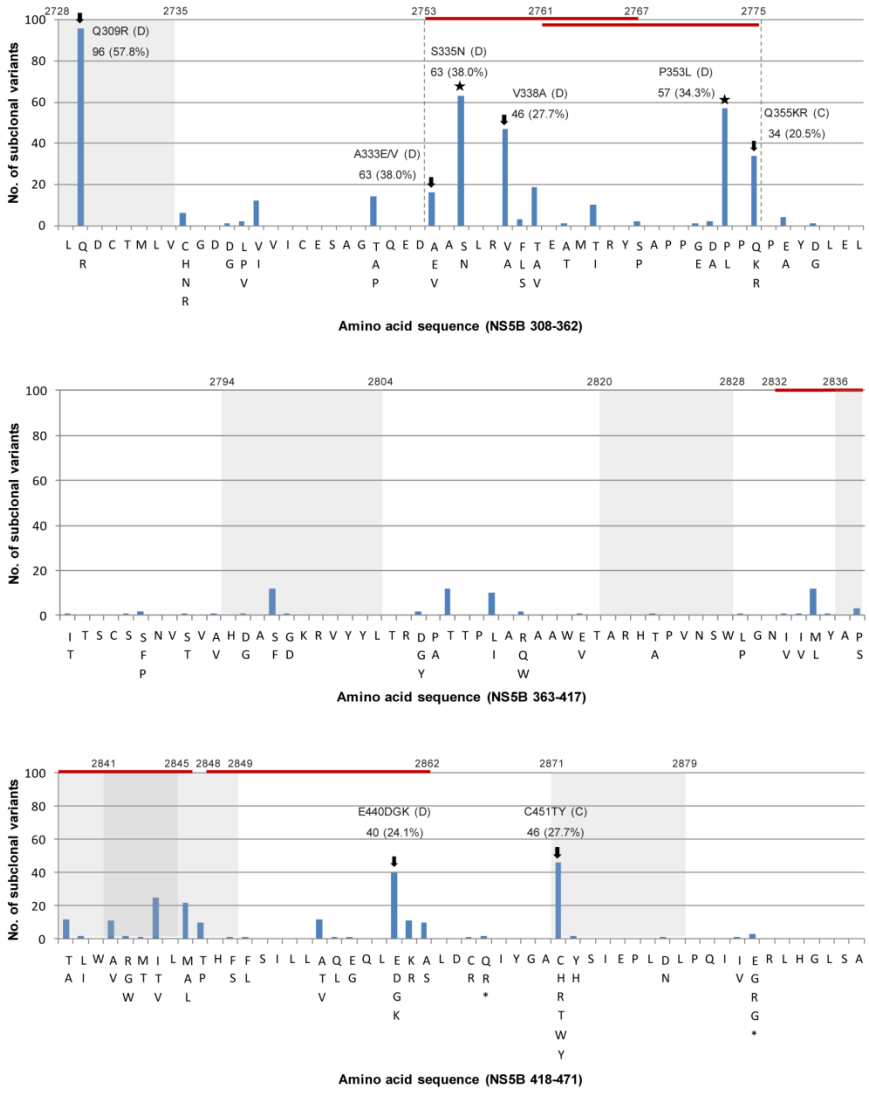

Figure 3. Distribution and frequencies of the amino acid mutations in the NS5B AA 308-471 regions. The blue-shaded regions are the known MHC class I restricted regions and the red-lined regions are regions expected to be Korean-specific CD4+ epitope-binding regions. The region between the dotted lines is a mutational hotspot. The arrow indicates amino acid

substitution related to IFN/RBV and other agents in HCV and the asterisks denote the novel mutations found in this study. The letters C and D indicate ‘conserved’ and ‘diverse’ in the subclones, respectively.

Table 6. Comparison of amino acid sequences, dominant HLA allele, nonsynonymous/synonymous (d_N/d_S) ratio between six regions of Known CD8+ T cell epitopes in the NS5B region.

NS5B region	AA sequence	HLA allele (Reference)	HLA alleles in Koreans[a] (Frequencies in Koreans, %)	d_N/d_S (ratio)	AA with high mutation frequency (No. of variants, %)
308 - 315	LQDCTMLV	A02:01 (38)	A02:01 (16.5) A02:06 (9.5)	96 / 47 (2.04)	Q309R (96, 57.8)
374 - 384	HDASGKRVYYL	B38 (32)	B38:02 (0.9) A02:01 (16.5) A02:06 (9.5)	14 / 214 (0.07)	-
400 – 408	ARHTPVNSW	B27 (40)	None	1 / 102 (0.01)	-
416 – 425	APTLWARMIL	B07 (32)	None	78 / 297 (0.26)	-
421 - 429	ARMILMTHF	B27 (40,41)	B27:05 (3.6)	72 / 191 (0.38)	-
451 - 459	CYSIEPLDL	A24:02 (39)	A24:02 (22.9)	49 / 94 (0.52)	C451H/R/T/W (46, 27.7)

a. Binding capacity < 500mM were selected.

Table 7. Comparison of mutation rates between the CD4+ and CD8+ T cell epitope regions.

Regions (n = 27,224)	No. of mutations / No. of codons	Mutation rate (%)	*P*-value
Outside known CD8+ epitopes	425 / 18,758	2.3	-
Inside known CD8+ epitopes	249 / 8,466	2.9	0.001[a]
Outside predicted CD4+ epitopes	250 / 18,426	1.4	-
Predicted CD4+ epitopes	424 / 8,798	4.8	<0.001[b]
Mutational hotspot_1	254 / 3,818	6.7	<0.001[b]

a. P-values were determined by a comparison of mutation rates of inside and outside of the CD8+ T cell epitopes.

b. P-values (<0.001) were obtained by a comparison of the mutation rates of outside of the CD4+ T cell epitopes as well as inside the CD8+ T cell epitopes.

Table 8. Relationships of predicted CD4+ T cell epitopes of the NS5B with MHC class II HLA types prevalent in Korean population.

NS5B	AA sequence	HLA allele[a] [%, binding level (mM)]
333 – 347	AASLRVFTEAMTRYS	DRB1*15:01 (8.0%, 82.9)
		DRB1*09:01 (10.4%, 110.5)
		DRB1*04:05 (8.5%, 134,6)
		DRB1*01:01 (7.4%, 58.9)
		DRB1*07:01 (7.3%, 101.6)
		DRB1*11:01 (3.2%, 89.5)
341 – 355	EAMTRYSAPPGDPPQ	DRB1*09:01 (10.4%, 331.4)
		DRB1*15:01 (8.0%, 339.4)
412 – 426	IIMYAPTLWARMILM	DRB1*09:01 (10.4%, 68.4)
		DRB1*15:01 (8.0%, 37.9)
		DRB1*01:01 (7.4%, 32.1)
428 – 442	HFFSILLAQEQLEKA	DRB1*09:01 (10.4%, 65.8)
		DRB1*04:05 (8.5%, 16.8)
		DRB1*11:01 (3.2%, 133.1)
		DRB1*03:01 (2.2%, 59.9)

a. Binding capacity < 500mM were selected.

4. Comparison of d_S and d_N according to the NS5B region

The distinctive CD4+ T cell-mediated immune pressure was examined by comparing d_N to d_S. The d_N/d_S ratio inside the known CD8+ T cell epitopes (0.29) was slightly higher than that of the outside (0.21) region with d_N frequencies of 2.9% and 2.3%, respectively. The d_N/d_S ratio inside the predicted CD4+ T cell epitopes (0.49) was much higher than that outside (0.13) with d_N frequencies of 4.8% and 1.4%, respectively, although d_S frequency outside the predicted CD4+ T cell epitopes was higher at a statistically significant level. The odds ratio of the d_N inside and outside predicted CD4+ T cell epitopes was 3.55. In the mutational hotspot, d_N frequencies (11.1%) were found higher than d_S frequencies (4.8%), resulting in and elevated d_N/d_S ratio (1.4). This suggests there are strong MHC class II restricted immune pressures against HCV NS5B in Korean chronic patients (Table 9).

Table 9. Frequencies of d_N and d_S according to the NS5B region.

Regions	Total	Inside	Outside	Odds ratio
Known CD8+	Codons	8,466	18,758	-
(51 AA)	d_N (%)	249 (2.9)	425 (2.3)	1.30[a]
	d_S (%)	868 (10.3)	1,992 (10.6)	0.97
	d_N/d_S ratio	0.29	0.21	1.34
Known CD4+	Codons	8,798	18,426	-
(51 AA)	d_N (%)	424 (4.8)	250 (1.4)	3.55[b]
	d_S (%)	863 (9.8)	1,997 (10.8)	0.91
	d_N/d_S ratio	0.49	0.13	3.92
Mutational hotspot	Codons	3,818	23,406	-
(23 AA)	d_N (%)	254 (6.7)	420 (1.8)	3.71[b]
	d_S (%)	182 (4.8)	2,678 (11.4)	0.42[b]
	d_N/d_S ratio	1.4	0.13	8.90

a. $p = 0.001$, b. $p < 0.001$, c. $p = 0.01$

5. Comparisons of d_S and d_N in the NS5B region between Korean patients and patients from other countries

To examine whether there is distinctive immune pressure against HCV NS5B at the CD4+ T cell level in Koreans, we compared d_S and d_N in the NS5B region between 15 Korean patients and 45 patients from other countries (Japan: 15, China: 12, Taiwan: 3, Germany: 7 and Switzerland: 8). In the Koreans subjects, we used the consensus sequences of NS5B from more than 10 subclones of patients. Regarding the patients of other countries, we used retrieved sequences from the LANL HCV database. In the NS5B region, the d_N/d_S ratio for the Korean subjects (0.23) was nearly identical to that of those from other countries. The d_N frequency (3.6) in the known CD8+ T cell epitopes from other countries was higher than that of Korean patients (3.1), but the difference was not statistically significant ($p = 0.572$). However, the d_N frequency (4.5%) in the predicted CD4+ T cell epitope regions in the Korean patients was significantly higher than that of those from other countries (2.1%) ($p = 0.001$). The d_N/d_S ratio in the predicted CD4+ T cell epitope regions were higher in the Koreans (0.52) by more than twofold compared to those of the patients from other areas (0.24). In particularly, the difference in the d_N frequency between the Koreans (6.4%) and the patients from other countries (3.1%) were more pronounced in the mutational hotspot.

Collectively, these results suggest the presence of distinctive CD4+ T cell mediated immune pressure against HCV NS5B in Koreans (Table 10).

Table 10. Comparison of the frequencies of d_N and d_S of the NS5B regions between 15 Korean patients consensus sequence and patients from other countries.

Regions	Total	Korea (n = 15)	Worldwide (n = 45)	Odds ratio
Total	Codons	2,460	7,380	-
(164 AA)	d_N (%)	57 (2.3)	161 (2.2)	1.06
	d_S (%)	245 (10.0)	712 (9.6)	1.03
	d_N/d_S ratio	0.23	0.23	1.03
Known CD8+	Codons	765	2,295	-
(51 AA)	d_N (%)	24 (3.1)	83 (3.6)	0.87
	d_S (%)	79 (10.3)	223 (9.7)	1.06
	d_N/d_S ratio	0.30	0.37	0.82
Predicted CD4+	Codons	795	2,385	-
(53 AA)	d_N (%)	36 (4.5)	51 (2.1)	2.12[a]
	d_S (%)	69 (8.7)	213 (8.9)	0.97
	d_N/d_S ratio	0.52	0.24	2.18
Mutational hotspot	Codons	345	1,035	-
(23 AA)	d_N (%)	22 (6.4)	32 (3.1)	2.06[b]
	d_S (%)	16 (4.6)	53 (5.1)	0.91
	d_N d_S ratio	1.38	0.60	2.28

a. p = 0.001, b. p = 0.01

6. Correlation between NS5B mutations and the severity of liver disease

The overall frequency of the mutation frequency of the entire NS5B region in carrier (C) (2.8%) was significantly higher than in the comparison group, patients with chronic hepatitis (CH), those with liver cirrhosis (LC) and those with hepatocellular carcinoma (HCC) (2.2%) ($p = 0.002$). The mutation frequency in known CD8+ T cell epitopes was also significantly higher in C than in the comparison group [C (3.4%) vs. CH + LC + HCC (2.6%), $p = 0.05$]. This tendency was also found in the predicted CD4+ T cell epitopes [C (5.7%) vs. CH + LC + HCC (4.2%), $p = 0.001$] and in the mutational hotspot [C (7.7%) vs. CH + LC + HCC (5.9%), $p = 0.004$] with an increased frequency of mutations at a statistically significant level. This shows that increases in the mutation rate in the NS5B region are negatively related to the progression of liver disease in hepatitis C chronic patients (Table 11).

Table 11. Comparison of the mutation rates in NS5B regions according to the clinical status of liver disease.

Regions (n = 27,224)	Carrier (%) / No. of codons	CH+LC+HCC (%) / No. of codons	*P*-value
Total	320 (2.8) / 11,316	354 (2.2) / 15,908	0.002[a]
Known CD8+	119 (3.4) / 3,519	130 (2.6) / 4,947	0.05
Predicted CD4+	208 (5.7) / 3,657	216 (4.2) / 5,141	0.001[a]
Mutational hotspot	122 (7.7) / 1,587	132 (5.9) / 2,231	0.004[a]

a. Statistically significant after a Bonferroni post hoc analysis ($p < 0.05$).

7. Mutation frequency in codons related to SVR and ETR in Korean patients

Mutations at 309, 333, 338 and 355 codons are reported to be related to SVR and ETR groups as compared to non-responders (NR) (15). Interestingly, a very high mutation rate in four SVR-related codons was found in Korean treatment-naïve patients, with an average mutation frequency of 28.9% (192/664) in the quasispecies distributions. Of note, the average mutation frequency (31.7%) in four codons as calculated from 15 Korean patients was significantly higher than any of the other regions, including that from Japan (Table 12).

A quasispecies analysis showed a total of 10 mutations including SVR or antiviral resistance in the sequenced NS5B region. These can be divided into two distinct groups. One is the diverse (D) type which coexists with other quasispecies members in a patient and the other is made up of conserved (C) types which exist alone without a quasispecies counterpart in a patient (Figure 3, Table 5). The coexistence of diverse quasispecies at a specific codon may be indirect evidence of an important target for immune pressure or/and viral fitness. Notably, the coexistence of Q and R at codon 309 located in one of the CD8+ T cell epitopes regions (aa 308 and 315) was found in all 15 Korean subjects via a quasispecies distribution analysis (Table 13), which may be due

to the distinct CD8+ T cell immune pressure against a region between aa 308 and 315 among Koreans. In addition, there were other D type mutations: A333V, S335N, V338A, P353L, E440G/K and C451H. On the other hand, there were only three C types of mutations (C316N, Q355K/R and E464Q). Interestingly, in all the three C-type mutations, significantly different Cp values between two counterparts in the respective mutation type were found (Table 4).

Table 12. Comparison of mutation rates in four SVR-related codons (309, 333, 338 and 355) in the NS5B region between 15 Korean consensus sequence and patients from other countries.

Geological region (n = 15)	No. of mutations (n = 60)	Mutation rate (%)	*P*-value[a]
Korea	19	31.7	-
Japan	7	11.5	0.014
East Asia[b]	4	6.7	0.001[c]
Europe[d]	8	13.3	0.028

a. P-values were determined by a comparison with the mutation rate of 15 Korean consensus sequences.

b. China = 12, Taiwan = 3

c. Statistically significant after a Bonferroni post hoc analysis ($p < 0.05$).

d. German = 7, Switzerland = 8

Table 13. Quasispecies distribution at the codon 309 in 15 Korean patients.

Subjects	Subclones with Q309	Subclones with Q309	Q309 / R309 Ratio	Sum
8 (LC)	4	8	0.50	12
10 (CH)	4	6	0.67	10
15 (C)	6	6	1.00	12
20 (C)	5	7	0.71	12
24 (LC)	2	9	0.22	11
26 (C)	5	6	0.83	11
29 (LC)	5	6	0.83	11
40 (LC)	5	5	1.00	10
42 (HCC)	3	9	0.33	12
43 (LC)	4	7	0.57	11
44 (HCC)	5	5	1.00	10
45 (LC)	6	4	1.50	10
55 (C)	3	9	0.33	12
56 (C)	6	4	1.50	10
59 (C)	7	5	1.40	12
Total	70	96	0.73	166

Discussion

The presence of distinct HLA types among an ethnic group could lead to distinct MHC class I or II restricted immune pressures within its population (32, 40-42). Therefore, the frequency and patterns of escape variants against structural and nonstructural HCV proteins reflect the background HLA types among an ethnic group (43, 44). The aim of the present study is to investigate the background mutation frequency and patterns of HCV NS5B, reported to be related to a high SVR, from treatment-naive Korean patients chronically infected with GT-1b in as effort to explain the high SVR in Korean patients. The significant findings of this study are as follows.

First, the entire mutation frequency in the sequenced NS5B region was positively correlated with carriers with a normal ALT level but not with patients showing disease progression (chronic hepatitis, cirrhosis and HCC) [C (2.8%) vs. CH + LC + HCC (2.2%), p = 0.002]. Furthermore, similar mutation frequencies were also noted within both the CD4+ (p = 0.001) and CD8+ T cell epitope regions (p = 0.05) (Table 11). This suggests that the accumulation of multiple mutations in NS5B may be induced by vigorous and multi-specific immune pressure in the HCV-acute infection phase and may

lead to the functional abnormality of HCV RdRp activity, resulting in the attenuation of HCV pathogenic potentials. This strongly supports the previous results which showed that mutations in NS5B were related to the high SVR and EVR of GT-1b chronically infected patients (15).

Second, a pronounced d_N frequency in the predicted CD4+ T cell epitopes in the NS5B region [Korean (4.5%) vs. those of patients from other countries (2.1%), p = 0.001], particularly in the mutational hotspot [Korean (6.4%) vs. other countries (3.1%), p = 0.01], was found in Korean patients, and not in patients from other areas (Table 10). This suggests that there may be distinct intrahepatic MHC class II restricted immune pressure at least against HCV NS5B among the Korean population. Broadly directed virus-specific immune pressure at the CD4+ T cell level was recently reported to play a very pivotal role in spontaneous resolution at the very early phase of HCV-acute infection (45). Furthermore, the presence of the multi-specific CD4+ T cell response against HCV can aid not only the induction of a vigorous antiviral CD8+ T cell response but also antibody production for the inhibition of the spread of virus (46). Particularly, because three codons (A333, V338, and Q355) of the four reported to be related to the high SVR are located in the mutational hotspot, an acquisition of the mutations within this region induced by the distinctive Korean immune pressure at the CD4+ T cell level may contribute

to the high SVR found in GT-1b infected Korean patients. In fact, the prediction of the MHC class II HLA allele showed that a region of the CD4+ T cell epitope from the NS5B, covering aa 333 to 347, one of two predicted epitopes comprising the mutational hotspot, have high binding affinity for most HLA DRB1 alleles prevalent in Korean populations (47). In addition, HLA DQB1 03:01 and 03:02, prevalent at higher than 10% frequencies in Koreans, also are noted to be associated with viral clearance (48-50). Our previous study also showed that there are distinct mutation patterns and a very high mutation frequency of the CD4+ T cell epitopes of the HBV preC/Core region in Korean chronic patients, strongly supporting the hypothesis of this study (51).

Third, the frequency of d_N within the CD8+ T cell epitope region of NS5B was significantly higher than those outside the CD8+ T cell epitope region [inside CD8+ (2.9%) vs. outside (2.3%), $p = 0.001$], suggesting the presence of immune pressure at the CD8+ T cell level against HCV NS5B among Korean patients, as shown in patients from other areas (Table 9) (32, 41, 42, 52). However, pronounced differences in the mutation frequency between six regions of CD8+ T cell epitopes were found. Two of the six CD8+ T cell epitopes (308 to 315 aa and 451 to 459) with high binding affinity to two HLA allele types, HLA-A02:01 and HLA-A24:02 prevalent in Koreans,

showed a higher d_N frequency compared to other epitopes [308-315: 96 (7.2%) and 451-459: 49 (3.3%)], suggesting the presence of distinct MHC class I restricted immune pressure in Korean patients (47). Particularly, it is noteworthy that the extraordinary high d_N/d_S ratio (2.04) found in a region of the CD8+ T cell epitope covering codons 308 to 315, was mainly due to the presence of frequent mutations in codon 309, one of four codons related to SVR rates (Table 6, Figure 3). The mutation type, Q309R, is known to be frequently mutated in NS5B particularly in Asian patients. However, even compared to Japanese patients, also an Asian country like Korea, the strikingly high mutation frequency of Q309R was observed in only the Korean patients (15, 16). All of the 15 patients harbored this mutation in their quasispecies distribution and the more than half (96/166, 57.8 %) of the entire quasispecies from 15 patients had the mutation type R309 (Table 5). Interestingly, co-existence of both mutated and wild types, not exclusive of the existence of one type alone, was found in all 15 patients, suggesting the advantage of the coexistence of two variants in a patient over the exclusive existence of either type alone in an escape of host immune surveillance or viral fitness (Table 13). Therefore, the high frequency of Q309R mutation in Korean patients may be induced by CD8+ T cell immune pressure which may in part provide a likely explanation for the high SVR rates in Korean.

Finally, it is well known that mutations in NS5B can affect the HCV replication capacity. We found a total of three types of mutations (C316N, Q355K/R, and E464Q) which had a significant effect on HCV replication (Cp value: C316N, E464Q $p = 0.014$, Q355K/R $p = 0.001$) (Table 12). Interestingly, our quasispecies analysis showed that two polymorphisms in aa 316, C316 and N316, were strongly related to two polymorphisms in codon 464, Q464 and E464, respectively, in an exclusive manner (Figure 1). The type with both C316 and Q464 signatures showed a significantly higher HCV replication capacity and was more related to patients with advanced liver disease compared to the type with both N316 and E464 signatures. The exclusive combination of the SNPs of two codons may be due to the structural constraint of NS5B. Furthermore, the coexistence of both types (C316/Q464 and N316/E464) was not found in any patients, suggesting that these two types may be from completely different resources and not a different quasispecies version induced by immune pressure from a patient. Our data showing phylogenetic segregation between the two types also supports the above hypothesis.

In conclusion, our data showed that the distinct MHC class II restricted immune pressure against HCV NS5B in Korean patients led to a pronounced high mutation frequency and to distinct mutation patterns in HCV NS5B of

Korean patients. This finding provides a novel insight into the high SVR and ETR rates during the treatment of GT-1b infected Korean patients.

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초 록

서론: C형간염 바이러스 (Hepatitis C virus, HCV) nonstructural 5B (NS5B) 부분은 바이러스의 증식에 중요한 역할을 담당하는 RNA-의존성 RNA 폴리머라제 (RNA-dependent RNA polymerase) 기능을 담당하는 것으로 알려져 있는데 이러한 NS5B 부분의 변이를 조사하는 것은 항바이러스 치료 및 백신 개발에 중요하다.

방법: 항바이러스제 치료 기록이 없는 (treatment-naïve) 환자로부터 얻어진 혈청을 정량적으로 HCV 감염을 확인한 후 viral RNA로부터 역전사 중합효소연쇄반응법으로 cDNA를 합성하여 nested PCR 방법으로 NS5B 부분의 DNA를 획득하였다. 획득된 15개의 유전자형 1b 증폭 산물을 TOPO TA cloning으로 각 시료별로 10-12개의 subclone을 확보하여 492bp의 총 166개 subclone으로부터 간질환의 임상 진단과 T 세포 에피톱 (epitope)을 중심으로 변이 빈도 및 패턴이 조사되었다.

결과: N316/E464와 계통학적으로 분리되는 C316/Q464 3개 검체는 통계적으로 유의하게 낮은 Cp value를 보였으며 (31.69 ± 0.92 vs. 32.28 ± 1.05, p = 0.016) 이는 C316/Q464 검체가 HCV의 복제능과 연관이 있음을 보여준다. 간질환의 정도와 변이 빈도를 비교한 결과 HCV

보균자에서 심각한 간질환인 만성 C형간염, 간경변 그리고 간질환보다 통계적으로 유의하게 높은 변이 빈도가 확인되었다 (2.8% vs. 2.2, p = 0.002). 기존에 알려진 CD8+ T 세포 에피톱 부분은 에피톱 바깥 부분과 비교할 때 통계적으로 유의하게 높은 변이 빈도가 확인되었으며 (2.9% vs. 2.3%, p = 0.001), 예측된 CD4+ T 세포 에피톱 및 이에 속하는 mutational hotspot 부분은 예측된 CD4+ T 세포 에피톱에 속하지 않는 부분 및 CD8+ T 세포 에피톱 부분에 비하여도 통계적으로 유의하게 높은 변이 빈도가 확인되었다 (p < 0.001). 이는 CD4+ T 세포 매개 면역 작용이 간질환의 발전을 억제하는 것으로 추측된다. 국내 및 해외의 전체 NS5B 부분의 변이 빈도는 큰 차이가 없었으며 (국내 2.3% vs. 해외 2.2%) CD8+ T 세포 에피톱 부분의 변이 빈도는 오히려 국내에서 낮았지만 (국내 3.1% vs. 해외 3.6%), 예측된 CD4+ T 세포 에피톱 (국내 4.5 vs. 해외 2.1%, p = 0.001)과 mutational hotspot (국내 6.7 vs. 해외 3.1%, p = 0.01)에서 통계적으로 유의하게 높은 국내 변이 빈도가 확인되었다. 이는 국내 인구에 HCV에 대한 CD4+ T 세포 매개 면역작용이 있음을 제시한다. 또한 지속 바이러스반응 (sustained virological response, SVR)과 치료 완료 후 바이러스 반응 (end-of treatment response, ETR) 진단에서 높은 빈도의 변이가 생성되는 것으로 알려진 네 개 아미노산 (Q309, A333, V338 그리고 Q355)의 변이 빈도는 국내 treatment-naïve 환자에게서 다른 지역에 비하여 통계적으로 유의하게 높게 확인되었는데 (한국 31.7% vs. 일본 11.5%, 동아시아 6.7% 그리고 유럽 13.3%, p < 0.028), 이러한 네

개의 아미노산 중 세 개가 예측된 CD4+ T 세포 에피톱에 속하였다.

결론: 국내 유전자형 1b C형간염 환자의 NS5B 부분, 특히 예측된 CD4+ T 세포 에피톱에서 높은 빈도의 변이가 발견되는 것은 국내 인구에 특이 CD4+ T 세포 매개 면역작용이 있음을 제시한다. 이러한 결과는 국내 만성 C형간염 환자에 대한 인터페론 알파와 리바비린 병합요법의 높은 SVR 비율에 대한 근거로 제시될 수 있다.

주요어: C형간염 바이러스, viral quasispecies, CD4+ T 세포 에피톱, 항바이러스제 내성변이
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