

## Basic Study

**Naturally occurring mutations in the reverse transcriptase region of hepatitis B virus polymerase from treatment-naïve Korean patients infected with genotype C2**

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**Abstract****AIM**

To report naturally occurring mutations in the reverse transcriptase region (RT) of hepatitis B virus (HBV) polymerase from treatment naïve Korean chronic patients infected with genotype C2.

**METHODS**

Here, full-length HBV reverse transcriptase RT sequences were amplified and sequenced from 131 treatment naïve Korean patients chronically infected with hepatitis B genotype C2. The patients had two distinct clinical statuses: 59 patients with chronic hepatitis (CH) and 72 patients with hepatocellular carcinoma (HCC). The deduced amino acids (AAs) at

42 previously reported potential nucleos(t)ide analog resistance (NAr) mutation positions in the RT region were analyzed.

### RESULTS

Potential NAr mutations involving 24 positions were found in 79 of the 131 patients (60.3%). Notably, AA substitutions at 2 positions (rt184 and rt204) involved in primary drug resistance and at 2 positions (rt80 and rt180) that functioned as secondary/compensatory mutations were detected in 10 patients (1 CH patient and 9 HCC patients) and 7 patients (1 CH and 6 HCC patients), respectively. The overall mutation frequencies in the HCC patients (3.17%, 96/3024 mutations) were significantly higher than the frequencies in the CH patients (2.09%, 52/2478 mutations) ( $P = 0.003$ ). In addition, a total of 3 NAr positions, rt80, rt139 and rt204 were found to be significantly related to HCC from treatment naïve Korean patients.

### CONCLUSION

Our data showed that naturally occurring NAr mutations in South Korea might contribute to liver disease progression (particularly HCC generation) in chronic patients with genotype C2 infections.

**Key words:** Hepatitis B virus; Polymerase; Reverse transcriptase; Potential nucleos(t)ide analog resistance; Chronic hepatitis; Hepatocellular carcinoma

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**Core tip:** To date, naturally occurring mutations in hepatitis B virus (HBV) reverse transcriptase region (RT) in genotype C2-infected patients have rarely been introduced in terms of clinical severity. So, this study characterized the AA substitutions at the aforementioned 42 potential NAr mutation positions in HBV RT sequences from a cohort of 131 Korean treatment-naïve CHB patients with genotype C2 infections. Notably, AA substitutions at positions involved in primary (rt184 and rt204) or secondary drug resistance (rt80 and rt180) were detected in 10 patients [1 CH patient and 9 hepatocellular carcinoma (HCC) patients] and 7 patients (1 CH and 6 HCC patients), respectively. The overall mutation frequencies in the HCC patients (3.17%, 96/3024 mutations) were significantly higher than the frequencies in the CH patients (2.09%, 52/2478 mutations), suggesting that naturally occurring NAr mutations in South Korea might contribute to liver disease progression (particularly HCC generation) in chronic patients with genotype C2 infections. In addition, a total of 3 NAr positions, rt80, rt139 and rt204 were found to be significantly related to HCC from treatment naïve Korean patients.

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## INTRODUCTION

Despite the availability of an effective vaccine, more than 240 million people are chronic carriers of the virus<sup>[1]</sup>. The annual number of deaths caused by hepatitis B virus (HBV)-related diseases, including cirrhosis and hepatocellular carcinoma (HCC), is estimated to be approximately 786000 worldwide<sup>[2]</sup>. HBV infection is endemic in South Korea; based on the Korean National Health and Nutrition Survey of 2011, the prevalence of hepatitis B virus surface antigen (HBsAg) positivity was 3.4% among men and 2.6% among women<sup>[3]</sup>. There is increasing evidence that specific HBV genotypes may play significant roles in the development of different disease profiles during chronic hepatitis B (CH) infection<sup>[4,5]</sup>. Notably, an extraordinary prevalence of genotype C2, which is more virulent than genotype B<sup>[4]</sup>, has been reported in South Korea<sup>[6-8]</sup>. Furthermore, the high prevalence of basal core promoter (BCP) double mutations and the presence of a distinct immune response against HBV proteins in the Korean population can lead to the generation of unique HBV variants that are rarely encountered in other areas, resulting in distinct clinical manifestations in Korean chronic patients<sup>[9]</sup>. Indeed, several unique types of HBV mutations related to the progression of liver disease (particularly HCC) that are rarely, if ever, encountered in other areas have been found in South Korea<sup>[10-27]</sup>.

HBV has an incomplete double-stranded DNA genome that is approximately 3.2 kb in length and contains 4 overlapping open reading frames (ORFs) encoding the polymerase (P), core (C), surface antigen (S), and X protein<sup>[28]</sup>. The HBV reverse transcriptase (RT) lacks proofreading ability, which can lead to HBV mutations that occur at a 10-fold higher frequency compared to other DNA viruses<sup>[29]</sup>. The high rate of mutations in the HBV genome compromises antiviral therapy with nucleos(t)ide analogues (NAs), leading to the generation of drug-resistant viral strains and disease. Although antiviral therapy using NAs is an effective control measure<sup>[30]</sup>, obstacles remain, including the limited types of NAs available and the inevitable emergence of antiviral resistance conferred by viral mutations during long-term treatment<sup>[31]</sup>. Thus, elucidating the mechanisms underlying the evolutionary basis of the drug resistance mutations is important for their prevention and control. Drug resistant mutations in RT have been extensively explored during antiviral therapy using NAs, including lamivudine (LMV)<sup>[32]</sup>, adefovir (ADV)<sup>[33]</sup>, entecavir (ETV)<sup>[34]</sup>, telbivudine (LdT)<sup>[35]</sup> and tenofovir (TNF).

Recently, RT mutations from treatment-naïve Chinese patients has been reported *via* analysis of 42 RT positions that were previously reported to be NAr<sup>s</sup><sup>[36]</sup>. These mutations could be divided into 4 categories [*i.e.*, primary drug resistance mutation (Category 1), secondary/compensatory mutation (Category 2), putative NAr mutation (Category 3) and pretreatment mutation (Category 4)].

To date, naturally occurring mutations in HBV RT in genotype C2-infected patients have rarely been introduced in terms of clinical severity. Therefore, this study characterized the AA substitutions at the aforementioned 42 potential NAr mutation positions in HBV RT sequences from a cohort of 131 Korean treatment-naïve CHB patients with genotype C2 infections. The clinical characteristics and significance of these identified NAr mutations were also investigated in the present study.

## MATERIALS AND METHODS

### Patients

Serum samples were collected from 135 chronic hepatitis B patients who visited Konkuk University Hospital and met the inclusion criteria of hepatitis B surface antigen (HBsAg) positivity and HBV DNA positivity and were LMV, ADV, ETV, LdT and TNF treatment-naïve. All patients had negative tests for hepatitis C virus, human immunodeficiency virus and markers for coexisting autoimmune liver disease. Among 135 patients, the 131 patients proved to be infected with genotype C2, which showed two clinical statuses: chronic hepatitis (CH, 59 patients) and hepatocellular carcinoma (HCC, 72 patients), were used in the NAr mutation analysis. This study was approved by the Institutional Review Boards (IRB) of Seoul National University Hospital (IRB-1605-065-761) and Konkuk University Hospital (KUH-1010544). The experiments were primarily based on extracted virion DNA from isolates; hence, the study did not require informed consent and the waiver of informed consent was agreed upon by the IRBs.

### HBV DNA extraction and PCR amplification

HBV DNA was extracted from the secured 200  $\mu$ L of serum samples using the QIAamp DNA Blood Mini Kit (QIAGEN Inc, Hilden, Germany). To analyze the mutation patterns and the frequencies of mutations in the RT region, a nested PCR based sequencing protocol was used as previously described<sup>[37]</sup>. The first-round PCR was performed using the sense primer Pol-RT-F1 (5'-CAGCCTACTCCATCTCTCCACCTCTAAG-3') and the antisense primer Pol-RT-R1 (5'-GCTCCAGACCGGCTGCGAGC-3') to yield a 1375-bp amplicon between positions 3157 nt and 1316 nt of the HBV genome. The second-round PCR was performed using the sense primer Pol-RT-F2 (5'-CCTCAGGCCATGCAGTGGA-3') and the antisense primer Pol-RT-R2 (5'-GTATGGA TCGGCAGAGGAGC-3') to yield a 1291-bp amplicon

between positions 3196 nt and 1271 nt of the HBV genome. The PCR was initiated in a 20  $\mu$ L PCR mixture containing 1.5 mmol/L MgCl<sub>2</sub>, 250  $\mu$ mol/L dNTPs, and 1.0 U of the ProFi Taq DNA polymerase (Bioneer). For both rounds, the protocol was as follows: 95 °C for 10 min, followed by 15 cycles at 94 °C (15 s), 55 °C (15 s) and 68 °C (3 min). A final extension step was performed at 72 °C for 5 min. The second-round PCR protocol used 2  $\mu$ L of the product from the first-round PCR and was identical to the conditions described above except that 30 cycles were performed. The PCR products were analyzed by electrophoresis on 1.0% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

### HBV genotyping

For genotyping, a phylogenetic analysis based on entire sequences of the RT region (1032 bp) was performed for PCR-positive 135 HBV strains. The 1032-bp RT sequences of the 135 HBV strains were compared with the sequences of eight reference strains representing each of the genotypes (A-F including the C2 strains) obtained from GenBank [accession numbers M57663 (A), AB100695 (B), AB074755 (C1), AY247032 (C2), AY641559 (C2), X02496 (D), AB106564 (E), and X75663 (F)]. Phylogenetic trees were inferred using the neighbor-joining method in MEGA version 7.0.14<sup>[38]</sup>. A mutation was defined through comparisons with the consensus sequence of the HBV strains in our cohort and the eight reference strains. The RT sequences of 131 patients with HBV genotype C2 infections were registered at GenBank [CH patients (GenBank Nos: KX264864-KX264922) and HCC patients (GenBank Nos: KX264792-KX264863)]

### Mutation analysis and definitions

Generally, the mutation definition and analysis were performed as previously described. Briefly, 42 potential NAr-relevant AA positions in RT were analyzed for AA mutations and concomitant HBsAg mutations. The AA mutations were identified by comparing HBV RT sequences with the genotype C-matched consensus sequence generated based on the HBV sequences obtained in this study and the reference sequences reported in previous studies<sup>[39]</sup>. A mutation type referred to the replacement of the consensus AA of genotype C with a novel AA.

### Statistical analyses

The results were expressed as means  $\pm$  SD, percentages. Differences between categorical variables were analyzed using Fisher's exact test or the  $\chi^2$  test. For continuous variables, Student's *t*-test was used when the data showed a normal distribution, and the Mann-Whitney *U* test was used when the data were not normally distributed. The level of significance of each test was adjusted for multiple tests *via* Bonferroni correction. The *P* value < 0.05 (two-tailed) was considered statistically significant. To adjust *P* values for

**Table 1 Comparison of clinical data between chronic hepatitis and hepatocellular carcinoma patients**

Clinical factors	CH (n = 59)	HCC (n = 72)	Total (n = 131)	P value
Age, yr, mean ± SD	38.9 ± 11.1	52.3 ± 9.7	45.7 ± 12.3	< 0.001
Gender, male	47.40%	79.10%	64.80%	< 0.001
HBeAg negative	35.50%	54.10%	45.80%	0.04%
ALT (IU/L), mean ± SD	94.5 ± 105.6	74.2 ± 85.1	106.8 ± 191.2	NS
AST (IU/L), mean ± SD	70.4 ± 92.0	127.1 ± 139.8	113.2 ± 141.0	< 0.001
HBV DNA	6.5 ± 2.0	5.3 ± 1.1	6.53 ± 1.7	< 0.001
HBsAg	3.7 ± 0.6	3.3 ± 0.7	3.43 ± 0.6	< 0.001

HbsAg: Hepatitis B virus surface antigen.

**Table 2 Correlation between the frequency of potential nucleos(t)ide analog resistance mutation and clinical features**

No. of mutations	CH/HCC	HBeAg (positive/negative)	ALT (IU/L)	AST (IU/L)	HBV DNA	HBsAg
0	27/25	29/23	108.51 ± 105.8	122.92 ± 106.8	6.50 ± 6.5	3.97 ± 3.8
1	18/16	21/13	101.61 ± 108.7	71.42 ± 126.1 <sup>a</sup>	6.91 ± 6.4	3.95 ± 3.8
2	9/19	15/13	81.10 ± 113.8	113.75 ± 113.0	6.65 ± 6.1	3.65 ± 3.9 <sup>a</sup>
3	4/7	3/8	211.45 ± 97.3	205.18 ± 104.8 <sup>a</sup>	6.84 ± 6.5	3.84 ± 3.8
4	1/4	2/3	56.60 ± 108.8	72.00 ± 114.8	5.85 ± 6.5	3.68 ± 3.8
5	0/1	1/0	24.23	39.05	7.49	2.83
≥ 1 (n = 79)	32/47	42/37	105.81 ± 216.19	106.86 ± 131.51	5.11 ± 1.55	3.37 ± 0.77
Total (n = 131)	59/72	71/60	107.21 ± 191.16	113.2 ± 141.06	4.98 ± 1.51	3.43 ± 0.73

The significant values were shown in boldface and marked with asterisk (<sup>a</sup>*P* < 0.05). HbsAg: Hepatitis B virus surface antigen; HCC: Hepatocellular carcinoma.

multiple testing and control the false discover rate for multiple testing was used<sup>[40]</sup>. We appreciate statistical consultation from the Medical Research Collaborating Center at the Seoul National University Hospital and the Seoul National University College of Medicine.

## RESULTS

### **Genotype distribution and clinical features of treatment-naïve patients**

The 131 patients were proved to be infected with genotype C2 by phylogenetic analysis based on 1032bp RT sequences and were used for the NAr mutation analysis. The clinical features of our cohort are summarized in Table 1. The patient cohort consisted of CH (45%, 59 patients) and HCC patients (55%, 72 patients) and included 85 males (64.8%) and 46 females (35.1%) with a median age of 45 years (range 22-73 years). The main characteristics of the CH and HCC patients were compared. The HCC patients were significantly older (*P* < 0.001), included a significantly higher number of male patients (*P* < 0.001), have a lower number of HBeAg-positive patients (*P* = 0.037) and had significantly lower HBV DNA (*P* < 0.001) and secreted HBsAg levels (*P* < 0.001) compared to the CH patients.

### **Correlation between the frequency of potential NAr mutation and clinical features**

Using sequence analysis of the full-length HBV RT from 131 patients, we deduced the amino acids (AAs) at 42 previously reported potential NA resistance (NAr)

mutation positions in the RT region<sup>[36]</sup>. The correlation between the frequency of potential NAr mutation in 131 patients and clinical features were summarized in Table 2. The analysis indicated that potential NAr mutations were present in 79 of the 131 patients (60.3 %). Of these, 32 (40.5%) and 47 patients (59.5%) belonged to the CH and HCC patient groups, respectively and 42 (53.2%) and 37 patients (46.8%) were HBeAg-positive and negative, respectively. Of the 79 patients with NAr mutations, 34 patients (43.03%) had a single mutation and 45 patients (56.96%) had multiple mutations, including 28 patients (35.44%) with double mutations, 11 patients (13.92%) with triple mutations, 5 patients (6.3%) with a quadruple mutation, and 1 patient (1.26%) with a quintuple mutation. No significant difference in clinical factors was observed between the patients with potential NAr mutations (79 patients) and those without mutations (52 patients) (Table 2).

### **Characterization of potential NAr mutation from treatment naïve patients**

All AA substitutions detectable in the 79 patients with NAr mutations at the 42 positions previously reported to be potential NAr mutations that could be grouped into 4 categories<sup>[36]</sup>, and a total of 24 NAr mutation sites were detectable among the 79 patients. Two dominant mutation sites rt128 in Category 3 and rt224 in Category 4 were present in 16 patients (20.25%, CH: 11 and HCC: 5) and 16 patients (20.25%, CH: 4, HCC: 12), respectively (Table 3). The AA substitutions at 2 positions (rt184 and rt204) in Category 1 consisting of 8 primary NAr mutation positions (rt169, rt181, rt184,

**Table 3** Characterization of potential 42 NAr mutation from treatment naïve Korean patients of genotype C2 infections

Category	Mutation	Drug resistance	CH	HCC	P value
Primary drug resistance	T184A/C/F	ETV	1	-	0.021
	M204I/V	LMV, ETV, TNF	-	9	
Mutation number (%) / no. of patients number			1/472 (0.21%)	9/576 (1.56%)	
Secondary mutation	L80I	LMV	1	9 patients	NS
	L180M	LMV, ETV, LdT	1	2	
Mutation number (%) / no. of patients number			1/177 (0.56%)	7/216 (3.24%)	
Putative NAr mutation	S53N	LMV	1	6	N.S
	L82M/V	LMV	-	1	
	V84M/I	ADV	1	-	
	H126C/Y/Q	ADV	5	6	
	I128I/N/A	LMV	11	5	
	R/W153Q	LMV	2	-	
	V191I/D	LMV, ADV	2	3	
	V207I	LMV	-	1	
	S213T	ADV	-	3	
	Q215P/S/H	LMV, ADV	-	2	
	L217R	ADV	1	-	
	F221Y	ADV	3	9	
	L229G/V/W	LMV	-	2	
	P237H	ADV	-	2	
	N238D/S/T	ADV	3	6	
Mutation number (%) / no. of patients number			29/1475 (1.96%)	41/1800 (2.27%)	
Pre-treatment mutation	T38A		23	31	NS
	Y124H	Found	9	5	
	D134E/N/C	Before	4	6	
	N139K/H	Therapy	4	8	
	I224V		-	8	
Mutation number (%) / no. of patients number			4/351 (1.14%)	12/432 (2.78%)	
Total Mutation number (%) / no. of patients (%)			52/2478 (2.09%) 32 patients (54.2)	96/3024 (3.17%) 47 patients (65.2)	0.003
			148/5502 (2.68%) 79 (60.3)		

ETV: Entecavir; LMV: Lamivudine; TNF: Tenofovir; ADV: Adefovir; CH: Chronic hepatitis; HCC: Hepatocellular carcinoma.

rt194, rt202, rt204, rt236 and rt250) were detected in 10 patients (1 CH patient and 9 HCC patients). The mutation frequency in primary drug resistance Category was significantly higher in the HCC patients than in the CH patients ( $P = 0.021$ ). The AA substitutions at 2 positions (rt80 and rt180) in the second Category, which included 3 secondary/compensatory NAr mutation positions (rt80, rt173 and rt180), were detected in 7 patients (1 CH patient and 6 HCC patients), indicating that the mutation frequency in Category 2 tended to be higher in the HCC patients than in the CH patients ( $P = 0.109$ ). Potential NAr mutations in Categories 3 and 4 were found in 54 patients (23 CH patients and 31 HCC patients) and 48 patients (17 CH patients and 31 HCC patients), respectively, but had no significant difference in the variant frequencies in Categories 3 and 4 were found between the CH and HCC patients (Table 3).

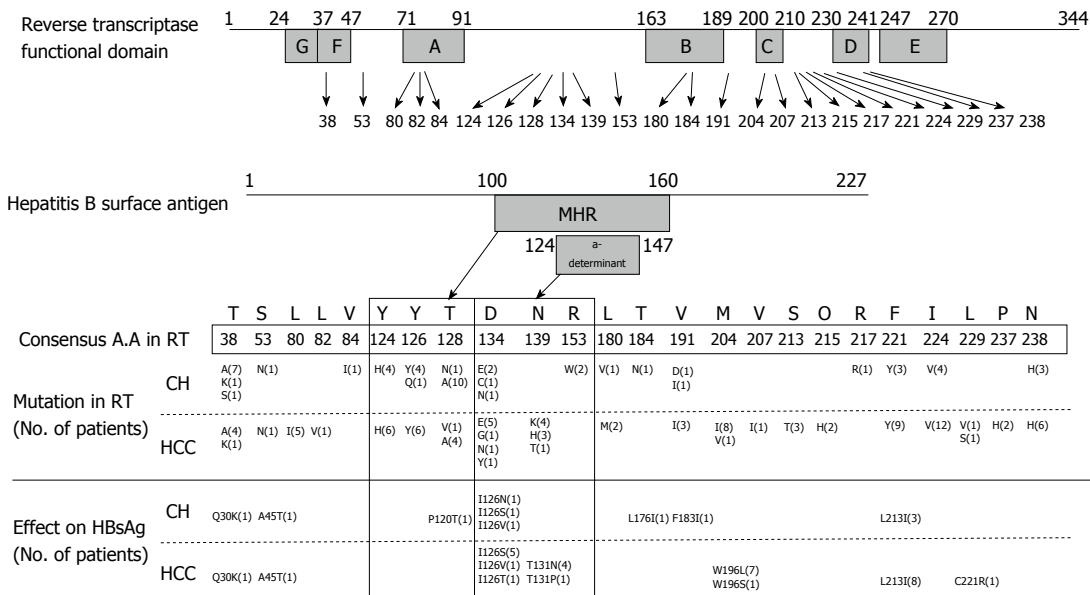
#### Mutation rates of potential NAr mutation in terms of clinical stages

Mutation rates of 79 patients with NAr mutations were compared in terms of clinical liver disease stages. The mean values of potential NAr mutation rates at the 42

positions of 131 our cohort was 2.68% (148/5502) (Table 3). Overall, mutation frequencies in the HCC patients (3.17%, 96/3024), were significantly higher than in those of CH patients (2.09%, 52/2478) ( $P = 0.003$ ) (Table 3).

#### Mutation distribution and frequency in different RT sections

HBV RT region consists of 7 functional domains (G, F, A, B, C, D and E) and 6 interdomains (G-F, F-A, A-B, B-C, C-D and D-E) connecting domains (Figure 1)<sup>[41]</sup>. Our mutation distribution analyses revealed that of 11 domains, mutations within A-B interdomain were the most frequently found in our cohort (58.22%, 46/79 patients). The mutations in this region were detected in all 6 reported sites (6/6, 100%), rt124 (10 patients), rt126 (11 patients), rt128 (16 patients), rt134 (12 patients), rt139 (8 patients) and rt153 (2 patients) (Figure 1 and Table 4). The mutation frequency of A-B interdomain (7.50%, 59/786) was also higher than those of the domain (1.07%,  $P = 0.008$ ) and non A-B interdomain (3.16%) (Table 4), in line with the previous report that potential NAr positions within this region



**Figure 1** Mutations identified in reverse transcriptase region and the overlapped hepatitis B virus surface antigen. The identified NAr mutations in this study in the hepatitis B virus (HBV) reverse transcriptase (RT) sequence (1-344 aa) and overlapped HBsAg (1-227 aa) were shown. RT consists of 6 functional domains, G (24-36 aa), F (37-47 aa), A (71-91 aa), B (163-189 aa), C (200-210 aa), D (230-241 aa) and E (247-270 aa). HBsAg contains MHR region (100-160 aa) including “a” determinant region (124-147 aa). Lower box indicated 24 identified NAr mutations in this study and comparison of mutation types between CH and hepatocellular carcinoma patients. CH: Chronic hepatitis; HCC: Hepatocellular carcinoma.

**Table 4** Mutation site distributions and mutation rate in different sections of hepatitis B virus RT and overlapped hepatitis B surface antigen regions

	Region (amino acid)	No. of mutation site	Mutation frequency	P value
Reverse transcriptase	Domain (22)	9 (40.9)	1.07%	0.008
	A-B interdomain (6)	6 (100)	7.50%	-
	Non A-B interdomain (14)	10 (71.4)	3.16%	NS
	Total (42)	25 (59.5)	2.68%	-
HBsAg	A-determinant (3)	2 (66.6)	3.81%	
	Non A-determinant (37)	8 (21.6)	0.55%	< 0.001
	Total (40)	10 (28.5)	0.80%	-

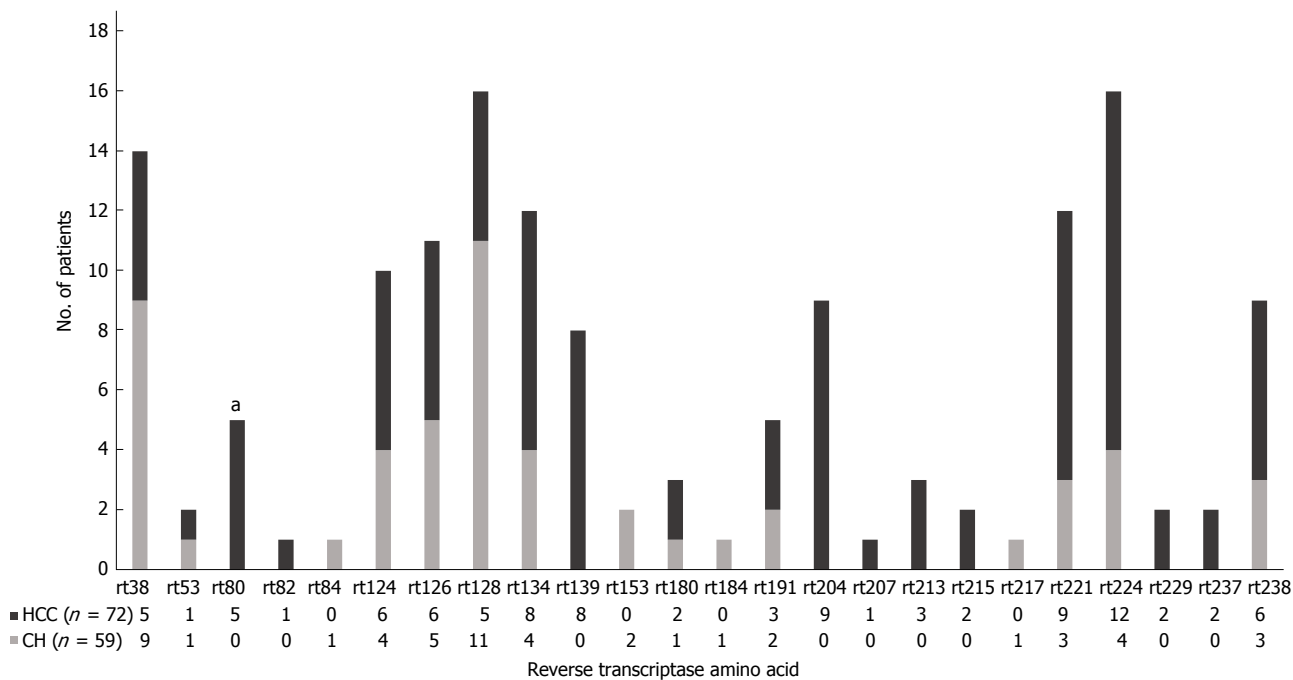
Statistics were calculated between Domain/Non A-B interdomain and A-B interdomain in reverse transcriptase region. In category 2, the statistical significant showed between A-determinant and Non-A-determinant in HBsAg region.

might be hotspots of naturally occurring mutation in this treatment-naïve population<sup>[36]</sup>. The RT include the complete HBsAg region<sup>[42]</sup>. In this study, 35 out of 42 mutated positions in RT were within the corresponding region of HBsAg positions (except mutations at rt236, rt237, rt238, rt242, rt245, rt256 and rt250). Our data showed that the AA mutations at 10 of 42 NAr positions were accompanied by 15 types of AA changes of HBsAg in 32.06% (42/131) patients. It should be noted that the 15 AA mutations at 3 NAr positions, rt134, rt139 and rt153 were found in the “a” determinant region of s126,s131 at HBsAg in 11.45% (15/131) patients (Figure 1) and these region had significantly higher mutation frequency, compared to non-“a” determinant (3.81%: 0.55%,  $P < 0.001$ , Table 4).

**Identification of NAr and overlapped HBsAg mutations related to HCC**

Of detected 24 mutated positions, a total of 3 NAr

positions, rt80, rt139 and rt204 were found to be significantly related to HCC from treatment naïve Korean patients (Figure 2 and Table 5). Of these, two, rt139 and rt204 except rt80 led to simultaneous HCC related mutations in overlapped HBsAg, s131 and s196. In this study, 2 mutation type of M204I (8 patients) and M204V (1 patient) at rt204 in Category 1, leading to YMDD motif mutations were found from 9 HCC patients but not from CH patients ( $P = 0.004$ ), which also led to the simultaneous W196L (7 patients) and W196S (1 patient) HBsAg mutations in 8 HCC patients. The only one type mutation, L80I at rt80 in Category 2 were also found only in HCC patients (5 patients), but not in CH patients ( $P = 0.036$ ). The third mutation types, N139K (4 patients), N139H (3 patients) and N139T (1 patient) at the rt139 in the Category 4 mutations were found from 8 HCC patients, but not in CH patients ( $P = 0.008$ ), which also led to the simultaneous T131N (4 patients) and T131P (1 patients) HBsAg mutations in 5



**Figure 2 Comparison of nucleos(t)ide analog resistance variants frequency between chronic hepatitis and hepatocellular carcinoma patients.** Of identified 24 mutations, mutation frequency at the 3 sites, rt80, rt139 and rt204 was significantly higher in hepatocellular carcinoma patients than in CH patients. <sup>a</sup>P < 0.05. CH: Chronic hepatitis; HCC: Hepatocellular carcinoma.

**Table 5 Frequency and patterns of 3 types of NAr Mutations related to hepatocellular carcinoma**

Mutations	No. of patients		Nucleotide sequences	Codons in RT genes (patients)	Codons in HBsAg genes (patients)	P value
	CH	HCC				
rtL80I	0	5	GGCTAT→GGATAT	CTA(L)→ATA(I) (5)	TAT(Y)→TAT(Y) (5)	0.036
rtN139K/T/H (sT131N/P)	0	8	GGAACC→GGA AAC →GGCACC →GGACCC	AAC(N)→AAA(K) (4) →CAC(H) (3) →ACC(T) (1)	ACC(T)→AAC(N) (4) →ACC(T) (3) →CCC(P) (1)	0.008
rtM204I/V (sW196L/S/W)	0	9	ATATGG→ATATTG →ATATCG →ATGIGG	ATG(M)→ATT(I) (7) →ATC(I) (1) →GTG(V) (1)	TGG(W)→TTG(L) (7) →TCG(S) (1) →TGG(W) (1)	0.004

The point mutation bases of the three truncations are shown in bold and italic. H: Histidine; I: Isoleucine; K: Lysine; L: Leucine; M: Methionine; N: Asparagine; P: Proline; S: Serine; T: Threonine; V: Valine; W: Tryptophan; Y: Tyrosine; CH: Chronic hepatitis; HCC: Hepatocellular carcinoma.

HCC patients (Table 5).

## DISCUSSION

Naturally occurring RT mutations associated with HBV drug resistance have been reported from treatment naïve chronic patients from several countries<sup>[43-46]</sup>. In South Korea, higher mutation rates and unique mutation patterns related to clinical implications in several HBV ORFs (the HBsAg, preS, X, and preC/C regions) compared to other countries have been reported to date<sup>[10-15]</sup>. Furthermore, higher relapse rates after antiviral therapy in Korean chronic patients have also been reported<sup>[47]</sup>. However, there have been no reports regarding potential NAr mutations from Korean treatment naïve patients to date. In this study, we analyzed potential NAr mutations from 131 Korean treatment naïve patients with genotype C2 infections using direct sequencing protocols.

There are three notable findings in our study. First, our data demonstrated that the prevalence of patients with potential NAr mutations was 60.3% (79/131) (Table 3), which was almost two times higher than the prevalence of these mutations [30.73% (59/192)] in a treatment naïve Chinese cohort<sup>[36]</sup> using the same direct sequencing protocols applied for the detection of potential NAr mutations in the treatment naïve Korean cohort. The difference in the mutation rates between the 2 cohorts was more pronounced with the Korean and Chinese cohorts [2.68% (148/5502) (Table 3) and 0.94% (76/8064)<sup>[36]</sup>, respectively]. In particular, we found primary NA mutations at Category 1 positions from 10 patients (7.8%) [rt184 related to ETV resistance (1 patient) and rt204 related to LMV resistance (9 patients)] and the so called YMDD mutation or secondary/compensatory mutations at Category 2 positions [rt80 and rt180 from 7 patients (5.3 %)] (Table 3). These findings were in contrast to

**Table 6** Comparison of clinical features between patients with or without L80I

Clinical factors	Wild type (n = 126)	L80I (n = 5)	Total (n = 131)	P value
Age, yr, mean $\pm$ SD	45.8 $\pm$ 12.2	57.2 $\pm$ 8.1	45.7 $\pm$ 12.3	0.043
Gender, Male	63.50%	100%	64.80%	NS
HBeAg negative	45.20%	60.00%	45.80%	NS
ALT (IU/L), mean $\pm$ SD	84.0 $\pm$ 96.8	68.6 $\pm$ 19.7	100.8 $\pm$ 191.2	NS
AST (IU/L), mean $\pm$ SD	100.9 $\pm$ 125.3	118.6 $\pm$ 65.0	113.2 $\pm$ 141.0	NS
HBV DNA	5.8 $\pm$ 1.7	6.7 $\pm$ 0.2	6.5 $\pm$ 1.7	< 0.001
HBsAg	3.4 $\pm$ 0.65	3.5 $\pm$ 0.32	3.4 $\pm$ 0.6	NS
CH: HCC, HCC (%)	59/67 (53.9)	0/5 (100)	59/72 (54.9)	0.036

HBV: Hepatitis B virus; CH: Chronic hepatitis; HCC: Hepatocellular carcinoma.

the two previous reports of Chinese cohorts<sup>[36,46]</sup> which showed that any mutations were not found within both regions. This findings may partially provide a likely explanation for why relapse after antiviral therapy is so prevalent in Korean patients<sup>[48]</sup> and also suggest that these patients should be treated with newer NAs, such as tenofovir (TDF), which is very potent and has a high genetic barrier to antiviral resistance.

Second, potential NAr mutations in our cohort were distributed in a non-random manner, as was shown in other studies<sup>[36]</sup>. The potential NAr mutations were found more frequently in the A-B interdomain overlapped with the HBsAg MHR region than in domain regions (7.50% vs 1.07%,  $P = 0.008$ ) (Table 4), which was in line with the previous report<sup>[36]</sup> that potential NAr positions within this region might be hotspots of naturally occurring mutations in this treatment naïve population. Notably, significantly higher mutation frequencies were found in 2 overlapped "a" determinant positions (3.81%, 15/393) compared to non-"a" determinant region (0.55%, 27/4847,  $P < 0.001$ ) (Table 4). These findings suggest that host immune pressure against B cells could contribute to the generation of potential NAr mutations<sup>[36]</sup>.

Third, our data showed there was significant difference in overall frequency of potential NAr mutations between CH patients (2.09%) and HCC patients (3.17%,  $P = 0.003$ , Table 3). In particular, mutations at the 3 NAr positions (rt80, rt139, and rt204) seemed to be the most pronounced contributors to hepatocarcinogenesis in the Korean Cohort [CH (0%, 0/59) vs HCC (30.6%, 22/72),  $P < 0.001$ ]. Of these, the YMDD-motif mutation at rt204 was reported to naturally occur in chronic HBV patients without antiviral treatment, such as lamivudine therapy, by several studies<sup>[49,50]</sup>. The other HCC-related mutation (rtL80I) was first introduced as a mutation associated with LMV resistance<sup>[51]</sup>. These authors found that these mutants were associated with increased viral loads accompanied by an elevation in serum aminotransferase activity and exacerbation of liver disease in every case. In line with the previous report<sup>[51,52]</sup>, our data indicated that L80I might have contributed to clinical deterioration<sup>[52]</sup>. Notably, our findings that L80I was combined with the rtM204I/V mutations in all 5 patients (data not shown) and L80I was also significantly related to

increased HBV replication (Table 6) suggested that this mutation might play a role in compensating for the defective replication of rtM204I/V. Thus, our finding regarding relationships of exacerbation of liver disease with rtL80I and rtM204I/V in treatment naïve patients may be primarily attributed to the co-selection of these two mutation types. These results suggest that potential NAr mutations may contribute to hepatocarcinogenesis, possibly *via* increases in HBV replication fitness or evasion of B cell immune responses against HBsAg.

In conclusion, our data showed that potential NAr mutations, including the classical antiviral resistance mutations, were very prevalent in treatment naïve Korean patients compared to populations from other countries. Naturally occurring potential NAr mutations may contribute to liver disease progression (particularly HCC generation) in Korean chronic patients with genotype C2 infections and provide a likely explanation for why patients with advanced liver disease are difficult to treat with NAs. Additionally, we identified 3 HCC-related NAr mutations (L80I, N139K/T/H and M204I/V).

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## COMMENTS

### Background

Naturally occurring reverse transcriptase mutations associated with hepatitis B virus (HBV) drug resistance have been reported from treatment naïve chronic patients from several countries. However, there have been no reports regarding potential nucleos(t)ide analog resistance (NAr) mutations from Korean treatment naïve patients to date.

### Research frontiers

Here, they found naturally occurring potential NAr mutations may contribute to liver disease progression in Korean chronic patients with genotype C2 infections.

### Hotspots or important area

Notably, the authors identified 3 hepatocellular carcinoma (HCC)-related NAr



mutations (L80I, N139K/T/H and M204I/V).

### Applications

The three HCC-related NAr mutations (L80I, N139K/T/H and M204I/V) found in this study could be applied as markers of molecular detection method for the HCC of HBV infected chronic patients in the future.

### Peer-review

The authors showed that potential NAr mutations, including the classical antiviral resistance mutations, were very prevalent in treatment naïve Korean patients and naturally occurring potential NAr mutations may contribute to liver disease progression in Korean chronic patients with genotype C2 infections. In addition, authors identified 3 HCC-related NAr mutations (L80I, N139K/T/H and M204I/V).

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