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Heterogeneous HBV mutants coexist in Korean hepatitis B patients

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Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; anti-HBe, antibody to HBeAg; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; ALT, alanine aminotransferase; AST, aspartate aminotransferase

Abstract

Although many hepatitis B virus (HBV) mutants have been found in all open reading frames since the precore defective mutant was initially reported, systematic investigations of diverse HBV mutant populations in hepatitis B patients have not been performed. Therefore, we examined whether heterogeneous mutant populations simultaneously exist in Korean hepatitis B patients. In order to detect hepatitis B virus mutants, we amplified a conserved core region and a surface antigen region of HBV DNA by PCR from sera of 27 Korean chronic hepatitis B patients, and then performed single strand confor-mational polymorphism analysis followed by DNA sequencing analysis. The results showed that heterogeneous HBV mutants in both regions were present in a single as well as in various hepatitis B patients. Sequence analysis revealed a defective interfering particle with missense mutation in the core region. We also found that two subtypes of *adr* and *adw* coexisted in a single patient. In addition, a point mutation causing a stop codon in the surface antigen region was observed. We are further analyzing the clinical implications of HBV mutants to identify their roles in the pathogenesis of chronic hepatic disorders induced by HBV.

Keywords: HBV DNA, HBV mutant, PCR-SSCP

Introduction

Hepatitis B virus (HBV) is a small circular, partially double-stranded DNA virus (3.2 kb) and requires reverse transcription of pregenomic RNA during replication (Summers and Mason, 1982). This reverse transcription accounts for the greater genetic variability of HBV genome in comparison with other DNA viruses (Will *et al*., 1987).

Since PCR was used for detection of HBV DNA, many HBV mutants have been reported. The first identified precore defective mutant was found in the patient whose HBeAg was seroconverted to negative (Carman *et al*., 1989). Thereafter, many HBV mutants in surface antigen, core and polymerase regions as well as precore region have been observed in persistently infected hosts (Blum *et al*., 1991; Ackrill *et al*., 1993; Carman *et al*., 1993; Hasegawa *et al*., 1994; Miska *et al*., 1994; Wallace *et al*., 1994). One of the well characterized mutants is precore mutant which does not encode e antigen because of the nonsense mutation created in the upstream of the start codon of core antigen (Ulrich *et al*., 1990). Thus, patients infected by these precore mutants showed negative results in HBeAg test providing incorrect informations about the progression of hepatitis in hepatitis B patients because seroconversion of HBeAg (+) to HBeAg (-)/anti-HBe (+) was interpreted as an indicator of a favorable prognosis (Fiordalisi *et al*., 1990; Nordenfelt *et al*., 1995).

Several HBV variants with mutations in determinant sites of surface antigen region were identified from patients in whom the HBV vaccine had failed to provide protection (Karthigesu *et al*., 1994; Hawkins *et al*., 1996; Chiou *et al*., 1997). Precore/core and presurface HBV mutants were shown to emerge spontaneously during the natural course of chronic infection, during interferon therapy or in immunosupressed patients (Blum, 1993). The importance of HBV mutants in pathogenicity, immunity, progression, and responsiveness to treatment has emerged as an area for intensive study. However, systematic investigations of diverse HBV mutant populations in hepatitis B patients have not been performed. Instead, most studies were focused on specific mutant types. Therefore, we systematically attempted to investigate HBV mutants in chronic hepatitis B patients in Korea. In order to do this, we amplified a conserved core region as well as surface

antigen region of HBV DNA by PCR, performed SSCP analysis, and then analyzed DNA sequence. The results show that the heterogeneous HBV mutant populations exist simultaneously in Korean chronic hepatitis B patients. We are investigating the potential clinical implications of mutant forms of HBV DNA.

Materials and Methods

Patient serum

Serum specimen were obtained from patients at Kyung Hee University Hospital of Oriental Medicine. All patients were HBsAg (+). The informed consent was obtained from each patient. Serological tests for HBsAg/anti-HBs, HBeAg/anti-HBe and anti-HBc were performed with radioimmunoassays using the commercially available kits (Abbott Laboratories).

Oligonucleotide primers

Primers for PCR amplification were designed in the conservative core regions to avoid mismatch as follows. The forward primer of set 1, 5'-TTCTTAGGGGCATGGACA TTGA-3', begins at map position 1776 and its backward primer, 5'-AATTCCCTGGATGCTGGGTC-3', begins at map position 2025. The forward primer of set 2, 5'-CATTGAC CCGTATAAAGAATT-3', begins at map position 1779 and its backward primer, 5'-TCCCTGGATGCTGGGTCTTCCAA A-3', begins at map position 2022. To amplify various regions in the whole HBV genomic DNA, 15 oligonucleotide primer sets were designed as shown in Table 1.

PCR amplification of HBV DNA extracted from virion in serum

HBV DNA was extracted using NaOH method as described by Kaneko *et al*. (1990). PCR amplification of HBV DNA was performed as described by Keum *et al*. (1997). To avoid contamination from floating amplicon in the air, PCR mixture containing 1 × buffer, $MgCl_2$, dNTP and primers were prepared and then 8-methoxypsoralen was added to the reaction mixture to give a final concentration of 10 μg/ml. After this PCR mixture was exposed to UV light for 10 min at room temperature, *Taq* polymerase and template were added. One picogram of pGEM-HBV vector DNA was subjected to PCR as a positive control.

Table 1. The primer sets used for the amplification of whole HBV genome.

I. S site (pre S1, pre S2, S) 2722-3215, 1-709			
+ (→) 5'-		- (←) 5'-	
S 2657	2657 agagaaactacacgcagcgc	S 2950	2950 accccaaaagaccgccgtgt
S 2905	2905 gagacttcgggccagggttc	S 3110	3110 gcttggtggaatgttgtgga
S 3066	3066 tcatcctcaggccatgcagt	S 40	40 tgtgctctccatgttcggtg
S 20	20 caccgaacatggagagcaca	S 260	260 gcagacacatccagcgatag
S 241	241 ctatcgctggatgtgtctgc	S 460	460 gtccgaaggttttgtacacc
S 408	408 ctgctcaaggaacctctatg	S 650	650 tgttgtacagacttggcccc
S 631	631 ggggccaagtctgtacaaca	S 906	1906 tgtaaaaggggcagcaaagc
II. Ia site 710-1247			
Ia 887	887 gctttgctgccccttttaca	Ia 1260	1260 cccgagcagccatgggaagg
III. X site 1248-1712			
X 1131	1131 tctgccgatccatactgcgg	X 1520	1520 cttaggcaagacctggtggg
XP 1481	1481 catggagaccaccgtgaacg	X 1776	1776 atgccccaaagccacccaag
IV. C site (pre C, C) 1688-2326			
Pre C 1643	1643 tgtactaggaggctgtaggc	C 1960	1960 ccaacacaggatagcttgcc
C 1941	1941 ggcaagctatcctgtgttgg	C 2180	2190 ataggggcatttggtggtct
C Pol 2171	2171 agaccaccaaatgcccctat	C 2340	2340 gagtccaagggatactaaca
V. Ib site 2327-2721			
Ib 2321	2321 tgttagtatcccttggactc	Ib 2530	2530 ggatagaacctagcaggcat
Ib 2511	2511 atgcctgctaggttctatcc	Ib 2722	2722 tgctgtagctcttgttccca

Nonisotopic DNA/PCR-SSCP analysis

Nonisotopic PCR-SSCP analysis was performed as previously described by Chi *et al.* (1994). Briefly, the extracted DNA from sera of 25 chronic hepatitis B patients was subjected to PCR for amplification of the conserved core region of HBV DNA using oligonucleotide primers described above, and then 20 μl of PCR products were mixed with 7 μl of alkaline solution and 15 μl of denaturing loading buffer. Total volume was adjusted to 45 μl by adding H_2O. After heating at 95˚C for 5 min, each sample was rapidly loaded onto an 8% or 10% nondenaturing polyacrylamide gel in 0.5× TBE buffer. The upper chamber was pre-cooled at 4˚C with buffered ice before the samples were loaded. Each sample was applied to two gels with and without 5% glycerol, and the gels were run at 18-20˚C, and 6-8˚C. Therefore, each PCR product was applied to gel electrophoresis under four different conditions. Following a 3-5 h run at 450 volts, the gel was stained with ethidium bromide for 5 min and destained with H_2O and photographed under ultraviolet light.

Subcloning and sequencing of HBV PCR products

The amplified region of HBV DNA from a single patient showing multiple migration shifts on SSCP analysis were ligated into the TA cloning vector (Invitrogen). The resulting plasmids were then used to transform *Escherichia coli* cells INV F'(Invitrogen). The multiple colonies were selected and the inserted HBV DNA sequences were amplified using the HBV DNA region-specific primers. After confirmation of migration shifts of these cloned PCR products using SSCP analysis, the representative clones were subjected to sequencing analysis. We used the same protocol for generating single-stranded templates for subsequent sequencing as the previously reported (Saunders *et al*., 1992). All specimens were sequenced in both directions to confirm the findings.

Results and Discussion

SSCP analysis of core region of HBV DNA in chronic hepatitis B patients

It is believed that certain amino acid changes in core protein may abolish the effectiveness of the T- and B-cell responses in controlling HBV infection, thereby allowing recurrent viraemia (Chisari and Ferrari, 1995). Although the presence of quasi-species was reported in an anti-HBe (+) patient infected with precore mutant virus (Alexopoulou *et al*., 1997), the presence of a mixture of core variants in a serum and the extent of their variation in chronic HBV infection has not been fully investigated in Korean hepatitis B patients. Therefore, we attempted to find HBV core mutants by PCR after amplification of HBV DNA in sera of 25 chronic hepatitis B patients (Table 2). PCR products amplified from 25 patients showed a whole variety of DNA bands after single strand conformational polymorphism (SSCP) (Figure 1). Multiple bands shown in each lane suggest that heterogeneous HBV mutants are present in a single patient. The difference in band intensities further suggests that certain types of mutants prevail dominantly over the other types. To confirm our SSCP findings that multiple mutants coexist in a single patient, we transformed *Escherichia coli* with HBV DNA amplified from patient 3 in Table 2 (lane 3 in Figure 1). After reamplification of HBV DNA isolated from 40 transformed colonies by the same primer set, we performed SSCP again. The results of this second SSCP revealed that at least 7 different mutant types were indeed present in this patient (Figure 2). The second PCR-SSCP analysis revealed that some mutants were actually more frequently found. These findings further suggest that certain mutant types prevail dominantly.

Table 2. Serologic and laboratory data of 27 Korean chronic hepatitis B patients at the time of study. Seventeen patients are HBeAg (+) and 10 patients are anti-HBe (+).

Patient number	Age/Sex	HBsAg	HBeAg/ anti-HBe	ALT/AST (IU/ l)	HBV strain subtype
1	40/M	+	-/+	46/37	*adr*
2	35/M	+	-/+	47/40	*adr*
3	35/M	+	-/+	109/46	*adr*
4	32/M	+	-/+	24/20	*adr*
5	41/M	+	-/+	38/25	*adr*
6	36/M	+	-/+	51/32	*adr*
7	39/F	+	-/+	99/63	*adr*
8	31/F	+	-/+	22/19	*adr*
9	36/M	+	-/+	51/32	*adr*
10	28/M	+	+/-	103/54	*adr*
11	38/M	+	+/-	43/28	*adr*
12	42/M	+	+/-	21/19	*adr*
13	44/M	+	+/-	88/40	*adr*
14	41/M	+	+/-	47/42	*adr*
15	38/M	+	+/-	45/24	*adr*
16	32/M	+	+/-	112/44	*adr*
17	23/F	+	+/-	269/184	*adr*
18	41/M	+	+/-	114/71	*adr*
19	45/F	+	+/-	148/59	*adr*
20	34/F	+	+/-	139/80	*adr*
21	43/M	+	+/-	85/60	*adr*
22	29/F	+	+/-	46/50	*adr*
23	48/F	+	+/-	98/79	*adr*
24	45/M	+	+/-	35/53	*adr*
25	44/F	+	+/-	21/26	*adr*
26	40/F	+	-/+	145/88	*adr*
27	53/M	+	+/-	61/83	*adr*

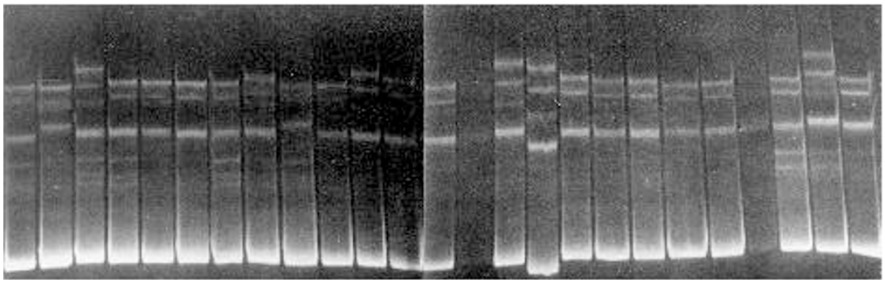

Figure 1. The SSCP patterns of HBV DNA of a core region amplified by PCR from 25 hepatitis B patients. The number represents each chronic hepatitis B patient listed in Table 2 (patient 1 to 25). The extracted DNA from serum of 25 chronic hepatitis B patients was subjected to PCR for amplification of the conserved core region of HBV DNA. For SSCP analysis, 20 μl of PCR products were denatured with 7 μl of alkaline solution and 15 μl of denaturing loading buffer. After heating at 95˚C for 5 min, each sample was subjected to gel electrophoresis by 8% nondenaturing polyacrylamide gel and SSCP analysis was performed as described in 'Materials and Methods'.

Sequence analysis of HBV DNA core region

To further characterize HBV mutants found in a single chronic hepatitis B patient with anti-HBe (+) (patient 3 in Table 2), we determined DNA sequences (Figure 3). These results confirmed our SSCP findings that many HBV mutants coexist in a single patient's serum. At present, we do not know how these multiple mutants were generated. HBV mutants may arise due to selective pressure during infection. or several HBV mutants may simultaneously co-infect a patient. The well characterized HBV mutant with mutation in the precore region (nucleotide 1896) which is unable to express HBeAg was not found in our patient. However, among HBV mutants which were confirmed by DNA sequence analysis, we found one single base-pair deletion mutant (Figure 4). Actually, this deletion mutant was shown in SSCP where double stranded DNA band of this mutant migrated faster than those in other lanes (lane 5 in Figure 2). This deletion mutant is interesting because deletion site is located in the open reading frame of core region, thus encoding a completely different or nonfunctional protein. This mutant is defined by a defective interfering (DI) particle (Huang *et al*., 1987). At present, there is no information of whether this DI particle with single base pair deletion is able to replicate at the expense of wild type HBV like other DI particles found in almost every other virus groups (Okamoto *et al*., 1993). Also, there is no information of whether this DI particle interrupts host immune respon-ses against HBV. Since some of HBV mutants have been reported to escape immune responses (Koziol *et al*., 1976; Swenson *et al*., 1983), the further examinations of HBV mutants including DI particles will provide us with the clues about the immune escape phenomena of HBV occurring in chronic hepatitis B patients.

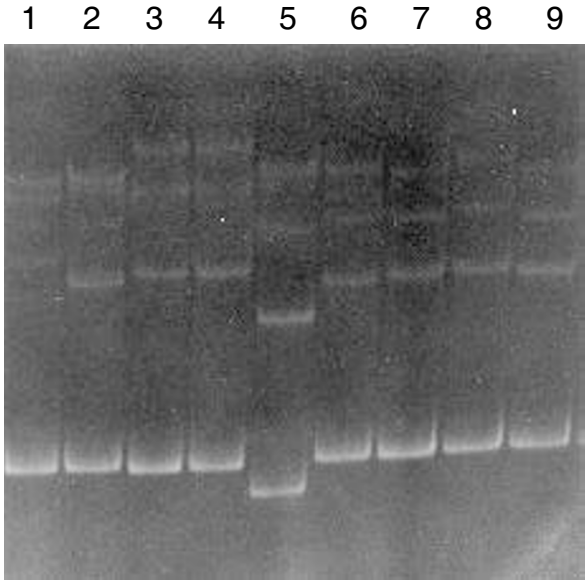

Figure 2. The SSCP patterns of HBV DNA of a core region reamplified by PCR from a single patient. The PCR products amplified from a single patient (patient 3 in Table 2) were ligated into pGEM-T vector, which was transformed into *E. coli*. HBV DNA was reamplified from 40 colonies, and then screened by SSCP analysis. In this result, the SSCP patterns of HBV DNA isolated from only 9 colonies are demonstrated.

SSCP analysis of whole HBV genomic area in chronic hepatitis B patients

Since we found that heterogeneous mutant populations in core region coexisted in a single or multiple hepatitis B patients, we next determined whether HBV mutants were found in whole HBV genomic area. To do these analyses, we amplified whole HBV genomic DNA from two hepatitis B patients (patient 26, 27 in Table 2) using 15 different primer sets (Table 1) and performed SSCP. The results clearly showed that HBV mutants arose in whole region of HBV genome (Figure 5). However, susceptibility to mutation seems to be different depending

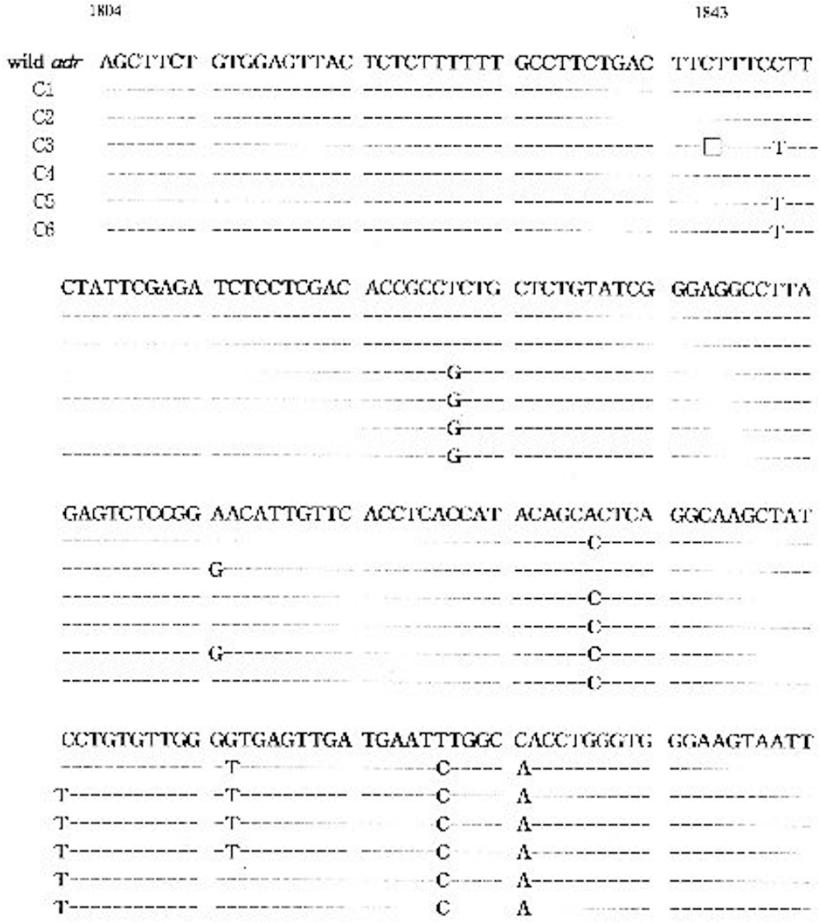

Figure 3. Comparison of nucleotide sequences of precore and core regions of wild *adr* type and mutants isolated from a single patient. Plasmids containing HBV PCR products amplified from a single patient (patient 3 in Table 2) were isolated from each of 6 bacterial colonies (C1-C6), and their DNA sequences were lined up with that of wild *adr* type. The mutated nucleotides are demonstrated in letters. The empty square (□) represents a deleted nucleotide found in DI particle.

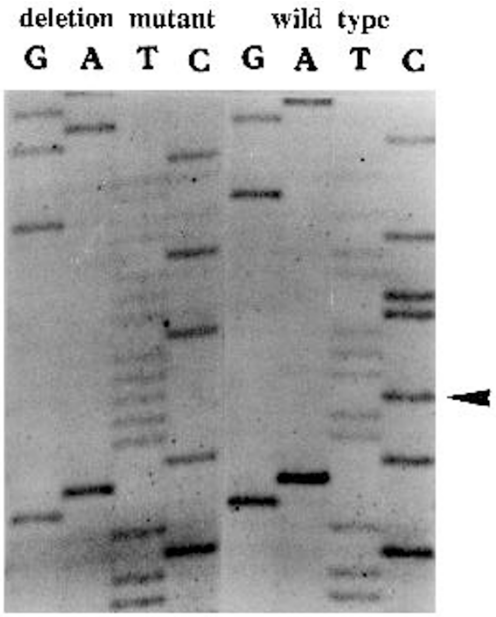

Figure 4. The nucleotide sequence of defective interfering (DI) particle. This DI particle (left panel) does not contain cytosine residue which is indicated by an arrow in a wild *adr* type.

on the genomic area because some lanes (S6, S7, X1, X2 and C2) have more bands than the other lanes. The result also shows different DNA band patterns for each patient, suggesting that the different HBV mutants exist in two patients

Sequence analysis of surface antigen region

HBsAg carries a number of antigenic determinants, which are classified into two categories. One is a common '*a*' determinant and the other is subtypic mutually-exclusive '*d*' and '*y*' or '*w*' and '*r*' determinant. As a result, the four major subtypes are created, i.e., *adw, adr, ayw* and *ayr* (Ohnuma *et al*., 1990). To study the HBV mutants in surface antigen region, we analyzed HBV DNA sequence of Korean hepatitis B patients. After PCR-SSCP analysis, *E. coli* was transformed with HBV DNA samples of two patients in Figure 5 (patient 26, 27 in Table 2), and then eight colonies were sequenced within the S gene. The first three colonies (colony S1-S3) are from patient 26 with anti-HBe (+) and the other five clones (clone S4-S8) are from patient 27 with HBeAg (+). In Figure 6, DNA sequence of HBV mutants found in these patients was compared with wild-type HBV subtype adr. An Arg-145 variant (Moriyama *et al*., 1991; Fuji *et al*., 1992), a well

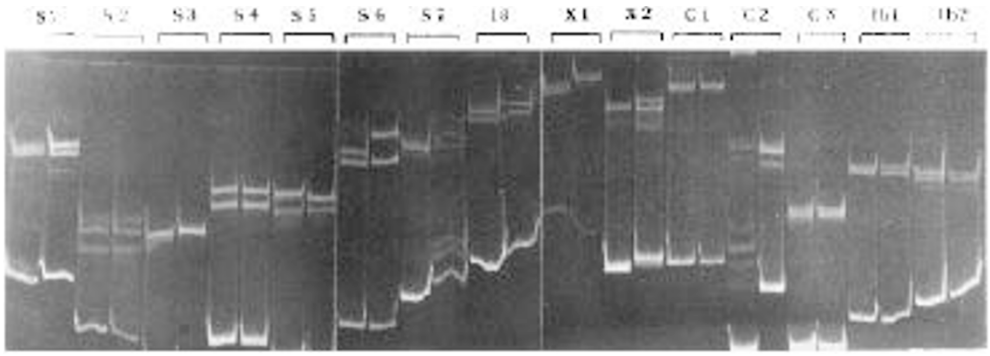

Figure 5. The SSCP patterns of whole genomic area of HBV DNA amplified from 2 hepatitis B patients. The two patients are listed in Table 2 (patient 26, 27). DNA fragments covering the entire HBV genome were separately amplified by various primer sets listed in Table 1, and then SSCP analysis of these PCR products was performed as 'Materials and Methods'.

```
                 455                 466                                     495
wild adrTCGGAC GGAAACTGCA CTTGTATTCC CATCCCATCA TCCTGGGCTT
S1       ------- ---------- ---------- ---------- ----------
S2       ------- ---------- ---------- ---------- ----------
S3       ------- ---------- ---------- ---------- ----------
S4       ------- ------T----- ---------- ---------- ----------
S5       ------- ---------- ---------- ---------- ----------
S6       ------- ------T----- ---------- ---------- ----------
S7       ------- ------T----- ---------- ---------- ----------
S8       ------- ------T----- ---------- ---------- ------A---
 TCGCAAGATT CCTATGGGAG TGGGCCTCAG TCCGTTTCTC CTGGCTCAGT
S1---------- ---------- -------T   G ----------
S2---------- ---------- -------T-- ---------- ----------
S3---------- ---------- ---------- -G ---------- ----------
S4---------- ---------- ---------- -G-------- ----------
S5-------A---- ---------- ---------- -A ---------- ----------
S6-------A---- ---------- ---------- ---------- ----------
S7-------A---- ---------- ---------- ---------- ----------
S8---------- -----A ---------- ---------- ----------
 TTACTAGTGC CATGGTATTG GGGGCCAAGT CTGTACAACA GGGCTTTCCC
S1---------A-- ---------- ---------- ---------- ----------
S2---------- ---------- ---------- ---------- ----------
S3---------- ---------- ---------- ---------- ----------
S4---------G-- ---------- ---------- ---------- ----------
S5---------A-- ---------- ---------- ---------- ----------
S6---------- ---------- ---------- ---------- ----------
S7---------- ---------- ---------- ---------- ----------
S8---------- ---------- ---------- ---------- ----------
 CCACTGTTT
S1----------
S2----------
S3----------
S4----------
S5----------
S6----------
S7----------
S8----------
```

Figure 6. Comparison of nucleotide sequences of S region of wild *adr* type and mutants isolated from two patients. Plasmids containing HBV PCR products amplified from two patients were isolated (patient 26, 27 in Table 2). The first three clones (S1-S3) were from patient 26 and the second five clones (S4-S8) were from patient 27. Their DNA sequences were lined up with those of wild *adr* type, and the mutated nucleotides are demonstrated in letters.

characterized immune escape mutant, which shows altered antigenicity due to mutation in '*a*' determinant (amino acids 124-147) was not present in our two patients. However, we found a point mutation leading to stop codon (TGG to TAG) at nucleotide 495 (codon 156) in colony S8. The nonsense mutation at this position of surface region (patient 27) was not previously reported. In addition, two serological subtypes were observed in this patient. The nucleotide 507 (codon 160) was either G (Arg-160; *adw* type) in clones S4, S8 or A (Lys-160; *adr* type) in clones S5-S7. At present, we do not know how two different sub-types coexist in this patient. It is possible that an HBV mutant with G to A point mutation at nucleotide 507 occurred in this patient resulting in

Lys-160 and subtypic change from *adr* to *adw*; such a mutant may have survived its predecessor (*adr*) owing to immune pressure exerted by antibodies to the '*r*' determinant. Another possibility is that this patient was infected with two HBV strains of different subtypes (*adr* and *adw*). Okamoto *et al.* reported that a single amino acid substitution by site-directed mutagenesis at codon 160 was responsible for the *r/w* allelic determinants in the S gene product (Okamoto *et al*., 1987).

In conclusion, our results show that the diverse hetero-geneous HBV mutants including DI particles exist in Korean chronic hepatitis B patients. At present, it is difficult to establish a certain relationship between a given mutation and its biological effect since most individuals were infected by more than one variant. Further studies on these diverse mutant populations will help us to ultimately understand this relationship and possibly the nature of hepatic disorder caused by HBV infection.

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