Comparison of genetic and derived amino acid changes in full-length hepatitis B virus genome between the patients with self-limited acute hepatitis B and those with acute exacerbation (AE) in chronic hepatitis B (CHB) during HBeAg seroconversion

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Abstract

The aim of study was to compare the genetic differences in full-length HBV DNA between the patients with acute hepatitis B (AHB) and those with acute exacerbation (AE) in chronic hepatitis B (CHB) during HBeAg seroconversion. The sequence was determined from serial sera of AHB (n=3) and CHB (n=3) at presentation and after HBeAg seroconversion. All patients have been infected by subgenotype C2 of HBV. There was no marked genetic diversity in full-length genome of HBV between AHB and CHB during AE (CHB vs. AHB (%): 1.33 ± 0.49 vs. 1.03 ± 0.25) and after HBeAg seroconversion (CHB vs. AHB (%): 1.40 ± 0.61 vs. 1.35 ± 0.35). However, In C and Sgene, the genetic diversity was higher in patients with CHB than in those with AHB at AE (CHB vs. AHB (%): C gene, 1.23 ± 0.23 vs. 0.40 ± 0.17; S gene, 1.03± 0.55 vs. 0.57 ± 0.38), but it didn't show remarkable differences after HBeAg seroconversion (CHB vs. AHB (%): C gene, 1.23 ± 0.31 vs. 1.20 ± 0.71; S gene, 0.97 ± 0.61 vs. 0.75 ± 0.35). Interestingly, the substitutions in these genes were likely to occur on putative HLA class I/II restricted epitopes, and the changes of gene in core and surface epitope-related codon were higher in CHB than AHB. In AHB, genetic change of C gene was higher after HBeAg seroconversion than in acute status (HBeAg (-) vs. acute status (%): C gene, 1.20 ± 0.71 vs. 0.40 ± 0.17). In contrast, there were no remarkable differences in CHB before or after HBeAg seroconversion. The different pattern of genetic variation that related to immune escape between AHB and CHB might be associated with the different clinical consequences after HBeAg seroconversion. (J Med Life Sci 2009;6:342-350)

Key Words : Hepatitis B virus, Acute hepatitis B, Chronic hepatitis B, Acute exacerbation, HBeAg seroconversion

INTRODUCTION

Hepatitis B virus (HBV) is one of the major causes of various liver disease including acute hepatitis B (AHB), chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) in endemic areas[1]. In adults, over 90% of AHB resolves with rare reactivation[2-4]. In contrast, during the chronic HBV infection, acute exacerbation(AE) of CHB, defined as defined as elevation of serum ALT levels to more than 10 times upper limit of normal and more than twice the baseline value, and following spontaneous HBeAg seroconversion, defined as HBeAg negative, anti-HBe positive, and normal ALT[5] frequently occurred and accompanied with increasing genetic mutations[6-9]. In addition, reactivation of hepatitis frequentlyobserved in patients with CHB[10-11].

The mechanism of AE during HBeAg seroconversion was not clear yet and had been suggested tobe associated with the imbalance between host factor (immune system) and viral factor (HBV genotype and genetic variants in HBV DNA)[12-13].

Because HBV polymerase lacksproofreading function, mutations in HBV genome naturally occurred during viral replication throughout the whole HBV genome[14-15].

As a non-cytopathic pathogen, the clearance of HBV was mostly dependent on virus specific T-cell response[16-17]. Recently, different frequency of virus-specific $CD4^+$ helper- and $CD8^+$ cytotoxic-Tlymphocyte (CTL) had been found in AHB and CHB and assumed to be associated with the various clinical courses of HBV infection[18, 19, 20, 21]. Moreover, residue substitution in the epitopes recognized by virus-specific CTL, mutations in HBV genes, such as precore/basal core promoter (PC/BCP) relating to viral

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replication or in genes encoding the protein (core and surface protein) that mainly affected by the immune system had also been reported to relate to the persistence of chronic HBV infection[22-24].

The purpose of present study was to compare the genetic and derived amino acid changes in full-length genome of HBV before and after HBeAg seroconversion from the patients with AHB or CHB.

PATIENTS AND METHODS

Patients

Consecutive sixpatients who were diagnosed as self-limited acute hepatitis B (AHB n=3) and chronic hepatitis B (CHB n=3) with AE followed by HBeAg seroconversion were selected. Their serial serum samples were collected at clinical presentation of hepatitis and after spontaneous HBeAg seroconversion. The clinical data of these 6 patients are presented in Table 1.

To evaluate the genetic diversity, the individual HBV DNA sequence was compared with the consensus HBV DNA sequence (subgenotype C2) deduced from eight of HBV DNA registered in GenBank using Molecular Evolutionary Genetics Analysis (MEGA4) software (http://www.megasoftware.net) (GenBank No.: AY641558; AY641560; AY641561; AF286594; DQ683578; X01587; AY123041; D000630).

Patients were excluded if they had any of the followings: history of receiving anti-virus therapy or immunosuppressive therapy before collecting serum sample, concomitant hepatitis C or D virus infection, and historyof heavy alcohol drinking. Ethics committee approved this study and patients gave written informed consent.

Serologic testing

Hepatitis B surface antigen (HBsAg), anti-HBs, HBeAg, and anti-HBe were analyzed using commercial enzyme immunoassay kits (Abbott, North Chicago, IL, USA). Serum levels of HBV DNA were measured using the Digene Hybrid Capture assay (detection limit, 0.5 pg/mL) (Digene Corporation, Gaithersburg, MD, USA). At the time ofdata analysis, serum HBV DNA levels were measured in the appropriate samples of stored serum using Cobas Amplicor HBV Monitor kits (detection limit, 60 IU/mL) (Roche Molecular Systems, Pleasanton, CA, USA).

Amplification and sequencing analysis of the full-length of HBV DNA

HBV nucleic acids were extracted from 200 ㎕ of serum that had been stored at −80 ℃ using a High Pure Viral Nucleic Acid Kit (Roche, Penzberg, Germany). The full-length of the HBV genome was amplified with two overlapping fragments by nested polymerase chain reaction (PCR). The primers introduced by Gunther et al., Takahashi et al. and Sugauchi et al. were modified and used for amplification of the full-length of HBV DNA and sequencing PCR (Table2)[25-27]. In brief, the long fragment about 3.2kb long was amplified with the primer pair, P1/P2, and the short fragment referred to the nick site including P1 and P2 primer regions was amplified with primer pair S1/S2. The second PCR were carried out with four primer pairs that overlapped and covered the whole length of the long fragment and short fragment. Briefly, the first round PCR was carried out in a tube containing 50 ㎕, which was composed of the following components: 0.2 ㎛M concentration of each of the external primer, 0.2mM concentration of each of the four dNTP, 25 ㎕ of 2×PCR buffer (Takara LA taq with GC buffer, Japan) and 10 ㎕ of solution extracted from serum. The first round PCR was programmed to the first incubation of the samples at 94℃ for 5min, followed by 40 cycles at 94℃ for 1 min, at 60℃ for min and then at 72℃ for 4 min, with a 10 minutes extension step at 72℃. The

Table 1. The basic clinical characteristics of the study patients

			At clinical presentation			HBeAg seroconversion		
Patients	Sex/Age	Diagnosis	ALT (IU/L)	HBV DNA (pg/ml)	HBeAg /anti-HBe	ALT (IU/L)	HBV DNA (pg/ml)	HBeAg /anti-HBe
1	F/43	AHB	646	<0.5	−/+	55	ND	−/+
2	F/26	AHB	1689	0.9	+/−	76	ND	−/+
3	F/41	AHB	5185	NA	+/−	15	ND	−/+
4	F/29	CHB	159 (534)	0.9	+/−	27	ND	−/+
5	M/37	CHB	430	59	+/−	20 (88)	ND (147)	−/+ (−/+)
6	F/61	CHB	126 (472)	0.8	−/+	24	ND	−/+

Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B; ALT, Alanine aminotransferase; ND, not detected.
(), Data in the parentheses showed the biochemical and virological data in reactivated status (Patient 5).

second PCR was programmed same to the first PCR except annealing temperature (52℃ or 60℃) and the amounts of templates (5 ㎕of the first PCR products). Five micro liters of the second round PCR products were analyzed by electrophoresis in a 1% Agarose gel stained with ethidium bromide and visualized with an ultraviolet transIluminator. The size of PCR products was estimated according to the migration pattern of a 1 Kb DNA ladder (Promega Co. USA). Then, the second PCR products were purified from 1% of agarose gel using QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany).

The purified PCR product were used as template for

Table 2. Primer pairs for the full-length of HBV DNA amplification

First PCR (Full-length genome)	
Long fragment	
P1 (forward: nt 1821-1841)	5' - TTT TCA CCT CTG CCT AAT CA-3'
P2 (reverse: nt1 823-1806)	5' - AAA AGT TGC ATG GTG CTG G-3'
Short fragment	
T711 (forward: nt 1255-1274)	5' - CCT CTG CCG ATC CAT ACT GC-3'
HC24 (reverse: nt 2048-2072)	5' -CCT GAG TGC TGT ATG GTG AGG-3'
Second PCR	
P1 (forward: nt 1821-1841)	5' - TTT TCA CCT CTG CCT AAT CA-3'
T731 (reverse: nt 2911-2930)	5' - TGA TCG GGA AAG AAT CCC AG-3'
PS8 (forward: nt 2816-2835)	5' - GTC ACC ATA TTC TTG GGA AC-3'
#S2-2 (reverse: nt 668-687)	5' - GGC ACT AGT AAA CTG AGC CA-3'
#S2-1 (forward: nt 455-474)	5' - CAA GGT ATG TTG CCC GTT TG-3'
T716 (reverse: nt 1576-1595)	5-GGT GAA GCG AAG TGC ACA CG-3'
T713 (forward: nt 1421-1440)	5' -TTG TYT ACG TCC CGT CGG CG-3'
T717 (reverse: nt 1872-1892)	5' -GCC ACC CAA GGC ACA GCT TGG-3'
Sequencing PCR	
P1 (nt 1821-1841)	5' - TTT TCA CCT CTG CCT AAT CA-3'
HC11 (nt 2191-2210)	5' - CAG ACA ACT ATT GTG GTT TC-3'
T726: (nt'2457-2476)	5' - CCT TGG ACT CAT AAG GTG GG-3'
PS8 (nt 2816-2835)	5' - GTC ACC ATA TTC TTG GGA AC-3'
T732 (nt 3075-3094)	5' - GTG GAG CCC TCA GGC TCA GG-3'
#S1-1 (nt 192-211)	5' - TCG TGT TAC AGG CGG GGT TT-3'
#S2-1 (nt 455-474)	5' - CAA GGT ATG TTG CCC GTT TG-3'
T707 (nt 637-656)	5' - CCT ATG GGA GTG GGC CTC AG-3'
HB4F (nt 970-992)	5' - CCTATTGATTGGAAAGTATGTCA-3'
T711 (nt 1255-1274)	5' - CCT CTG CCG ATC CAT ACT GC-3'
T713 (nt 1421-1440)	5' -TTG TYT ACG TCC CGT CGG CG-3'

Table 3. Comparison of genetic diversity of HBV DNA between AHB and CHB

	Full Genome		Core gene		Surface gene	
	AHB	CHB	AHB	CHB	AHB	CHB
At clinical presentation	1.0	1.1	0.5	1.5	0.3	0.7
	0.8	1.0	0.5	1.1	0.4	1.1
	1.3	1.9	0.2	1.7	1.0	1.8
Mean ± SD	1.03±0.25	1.33±0.49	0.40±0.17	1.23±0.23	0.57±0.38	1.03±0.55
After HBeAg seroconversion	1.1	1.0	0.7	1.5	0.5	0.3
	1.6	1.1	1.7	1.3	1.0	1.1
	NA	2.1	NA	0.9	NA	1.5
Mean ± SD	1.35±0.35	1.40±0.61	1.20±0.71	1.23±0.31	0.75±0.35	0.97±0.61

Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B; ALT, Alanine aminotransferase; NA, not available

sequencing PCR, which was carried out with the 11 primers using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, USA) by an automatic sequencing machine-ABI PRISM 3100 Genetic Analyzer. (HITACHI, Tokyo, Japan) All necessary precautions to prevent cross-contamination were performed, and negative controls were included in each assay.

Phylogenetic analysis

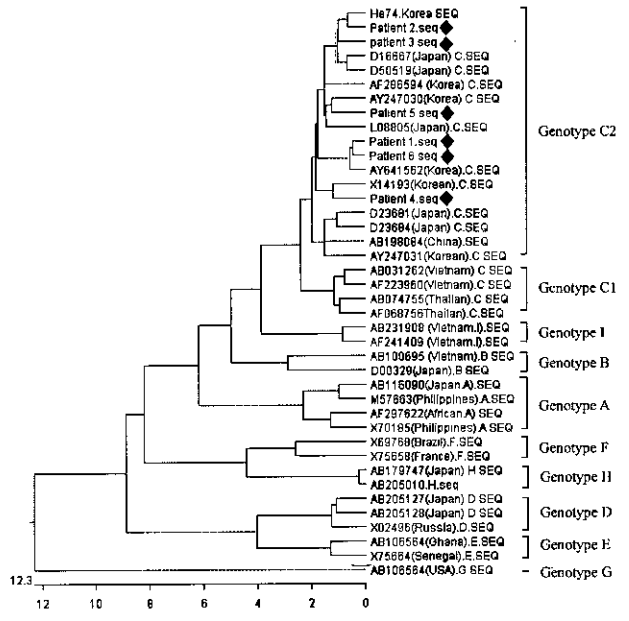
The genotype of the HBV was determined by phylogenetic analysis comparing with 34 reference strains from GenBank. Thealignment of the full-length sequencing was performed by cluster method and the phylogenetic tree was constructed by neighbor joining method[28].

RESULTS

HBV DNA from all the six patients was extracted at clinical presentation and after HBeAg seroconversion except patient 3. In patient 3, its nucleotide sequence was determined only in clinical presentation because failure of HBV DNA amplification by PCR at the time of HBeAg seroconversion. All patients have been infected with genotype C2 and subtype adr of HBV.

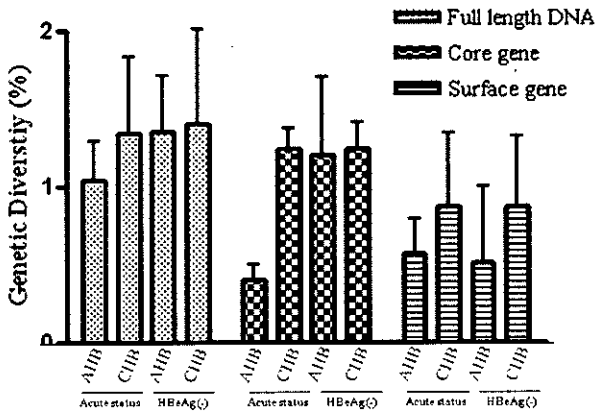
To evaluate the genetic diversity between AHB and CHB, we compared the full-length genome, core (C) gene and surface (S) gene of HBV according to clinical status. Because of lacking the serum sample before the onset of acute manifestation in AHB and CHB, we compared the genes with consensus HBV DNA sequences, which were deduced from eight HBV DNAs (Genotype C2) registered in GenBank. As results, the average genetic diversity of full-length HBV DNA was slightly higher in CHB than that in AHB at acute status (CHB vs. AHB (%): 1.33 ± 0.49 vs. 1.03 ± 0.25), and there were no remarkable differences after HBeAg seroconversion (CHB vs. AHB (%): 1.40 ± 0.61 vs. 1.35 ± 0.35) (Table3 and Fig. 2). In contrast, in C and S gene, the average genetic diversity was markedly higher in patients with CHB than in those with AHB at presentation (CHB vs. AHB (%): Cgene, 1.23 ± 0.23 vs. 0.40 ± 0.17 S gene, 1.03 ± 0.55 vs. 0.57 ± 0.38). But it didn't show marked genetic distances after HBeAg seroconversion (CHB vs. AHB (%): core gene, 1.23 ± 0.31 vs. 1.20 ± 0.71 surface gene, 0.97 ± 0.61 vs. 0.75 ± 0.35) (Fig. 1). In AHB, average genetic diversity of full-length HBV DNA was slightly higher in sera after HBeAg seroconversion than in acute status (HBeAg (-) vs. acute status (%): 1.35 ± 0.35 vs. 1.03 ± 0.25). Especially, in C gene, the genetic diversity after HBeAg seroconversion was about 3 times higher than that in acute status (HBeAg (-) vs. acute status (%): C gene, 1.20 ± 0.71 vs. 0.40±0.17). In S gene, it was also obtained similar result (HBeAg (-) vs. acute status (%): S

Figure 1. Determinant of HBV genotype by phylogenetic analysis



HBV genotype was determined by phylogenetic analysis comparing with 34 reference strains from GenBank. The study subjects were labeled with.

Figure 2. Genetic diversity between AHB and CHB during HBeAg seroconversion



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gene, 0.75 ± 0.35 vs. 0.57 ± 0.38), but not much like that in C gene (Fig. 2). In contrast, in CHB, there were no remarkable genetic differences in full-length HBV DNA, core and S gene between acute status and after HBeAg seroconversion (Fig. 2).

A total of 20 and 39 of nucleotide changes in core gene as well as 47 and 96 of nucleotide changes in surface gene were detected in AHB and CHB, respectively. Among them, 50% (10/20), 56.4% (22/39), 42.5% (20/47) and 45.8% (44/96) were nonsynonymous mutations in these regions, respectively. Interestingly, the nonsynonymous mutations detected in CHB were seemed to more frequent on putative human leukocyte antigen (HLA)class I/II restricted epitope-related codon than that in AHB (CHB vs. AHB: C gene, 68.2% (15/22) vs. 30% (3/10); S gene, 34% (15/44) vs. 14% (3/20) (Fig. 3).

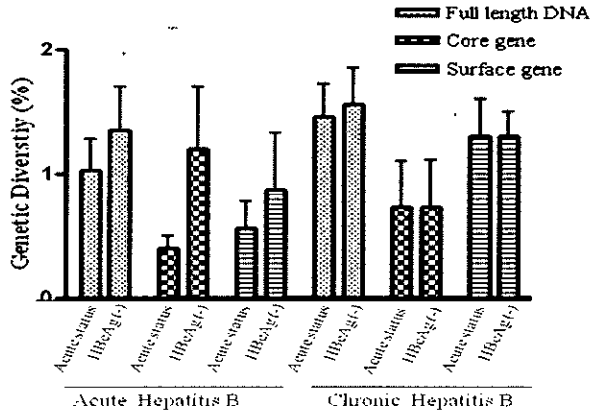
In the study, we also analyzed the genetic diversity in a reactivated patient (Patient 5). In full-length HBV DNA and surface gene, the genetic diversity was not much different during HBeAg seroconversion and reactivation. In Cgene, the genetic diversity was slightly higher after HBeAg seroconversion (1.1 %) and lower after reactivation (1.3 %) than in acute status (0.9 %) (Fig. 4).

The basal core promoter/precore (BCP/PC) mutations were broadly detected in study patients and didn't show marked distinction in AHB and CHB (Fig. 5). The A1762T/G1764A double mutations in BCP region, which produce K130M and V131I amino acid substitutions in HBx protein, were found in the Patient 2 and 5. The G1764A single mutation was detected in two AHB patients (Patient 2 and 3) and in one CHB (Patient 5). However, alternatively C1766T/T1768A mutation was determined in these three patients. The PC mutation (G1896A), which makes a stop codon at codon 28 to eliminate the HBe protein production, was observed in the Patient 6.

DISCUSSION

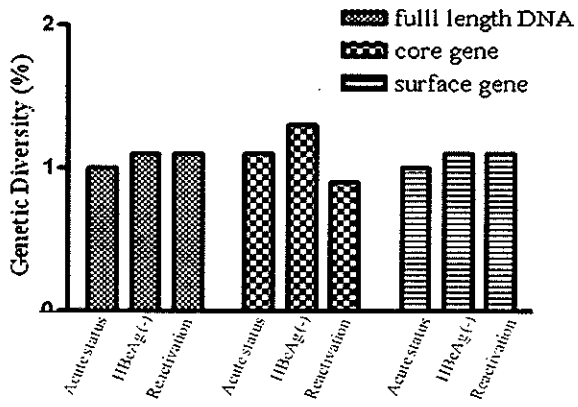
In the study, the genetic diversity of full-length HBV DNA was not much differentbetween CHB and AHB regardless of clinical status while comparing with consensus HBV DNA. However, there existed less genetic variants in AHB compared with CHB in core and surface gene. In core gene, the geneticvariants were more frequent in acute status of CHB than AHB, but not much different after HBeAg seroconversion. Especially, these variants tended to center on the genes coding core- or surface-epitope and were more frequent in CHB than that of AHB regardless of clinical status. This finding suggested that genetic mutations on these epitope-related codons might be mostly selected, not randomly, during the viral clearance under the immunopressure. Because the changes of amino acid on epitope decreased the binding capacity with HLA complex or recognition by T cell receptor, HBV with this kind of

Figure 3. Genetic diversity between acute status and HBeAg seroconversion



The individual HBV DNA sequence was compared with the consensus HBV DNA sequence (genotype C2) deduced from eight of HBV DNA registered in GenBank using Molecular Evolutionary Genetics Analysis (MEGA4) software (http://www.megasoftware.net) (GenBank No.: AY641558; AY641560; AY641561; AF286594; DQ683578; X01587; AY123041; D000630). Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B

Figure 4. Comparison of intra-genetic diversity in a reactivated patient (Patient 5)



The HBV DNA sequence of Patient 5was compared with the consensus HBV DNA sequence (genotype C2) deduced from eight of HBV DNA registered in GenBank using Molecular Evolutionary Genetics Analysis (MEGA4) software (http://www.megasoftware.net) (GenBank No.: AY641558; AY641560; AY641561; AF286594; DQ683578; X01587; AY123041; D000630). Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B

changes, in viral aspect, might escape from the immune system to survive[29]. Interestingly, different pattern of genetic changes existed between AHB and CHB during HBeAg seroconversion. In AHB, the frequency of genetic or amino acid substitution was higher in sera after HBeAg seroconversion than in acute status. Particularly, this difference was remarkable in core gene. On the contrary, in CHB, there were no remarkable differences in full-length HBV DNA, core and surface gene before or after HBeAg seroconversion and still maintained the high frequency of genetic variants. Core protein was known as the main target of immune system and the mutations in core epitope-related codon were frequently detected during immune tolerance phase around the time of HBeAg clearance[22, 30-32]. Previously, Whalley SA et al., reported that the most variants in AHB appeared transiently and were rapidly replaced by wild-type sequences in hepatitis B resolvers that achieved clearance of serum HBsAg[18]. This phenomenon might be related to the vigorous, efficient and short period of immune response, compared with CHB, on virus in AHB[18, 19, 33]. Therefore, this different immune response might result in different pattern of genetic changes and it might be one of the mechanisms of the frequent reactivation after spontaneous HBeAg seroconversion in CHB

Figure 5. Residue substitutions in core and surface protein.

Residue substitutions were compared with the consensus amino acid sequence (genotype C2) deduced from eight of HBVs registered in GenBank (GenBank No.: AY641558; AY641560; AY641561; AF286594; DQ683578; X01587; AY123041; D000630). Abbreviation: AHB, Acute Hepatitis B;

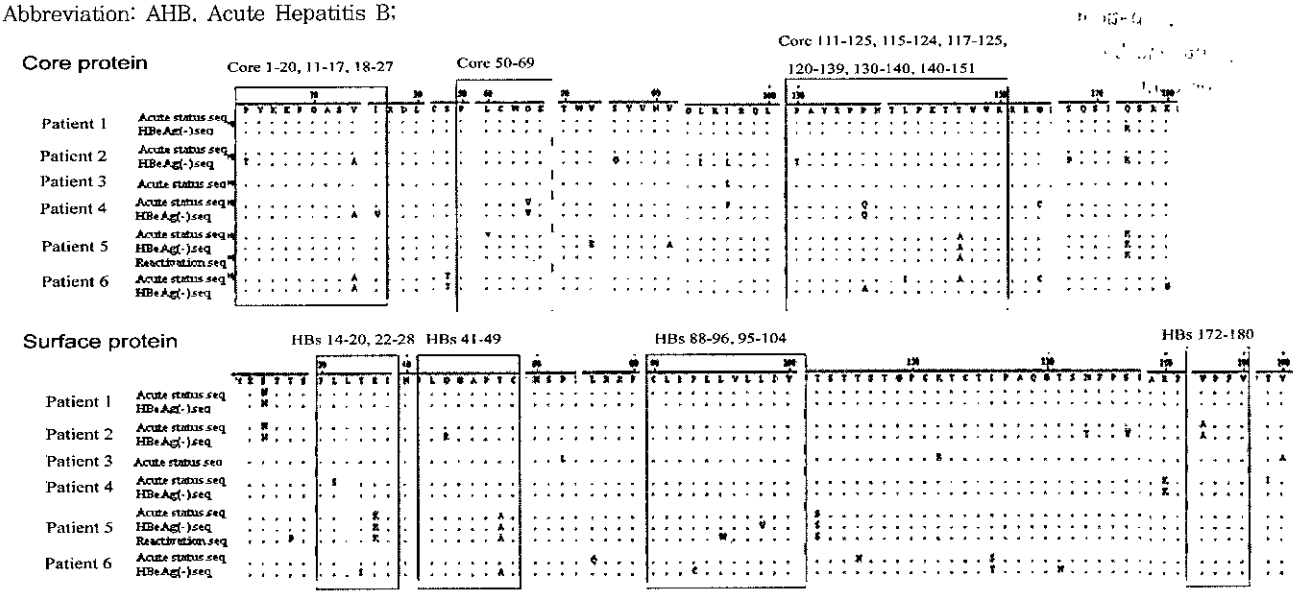
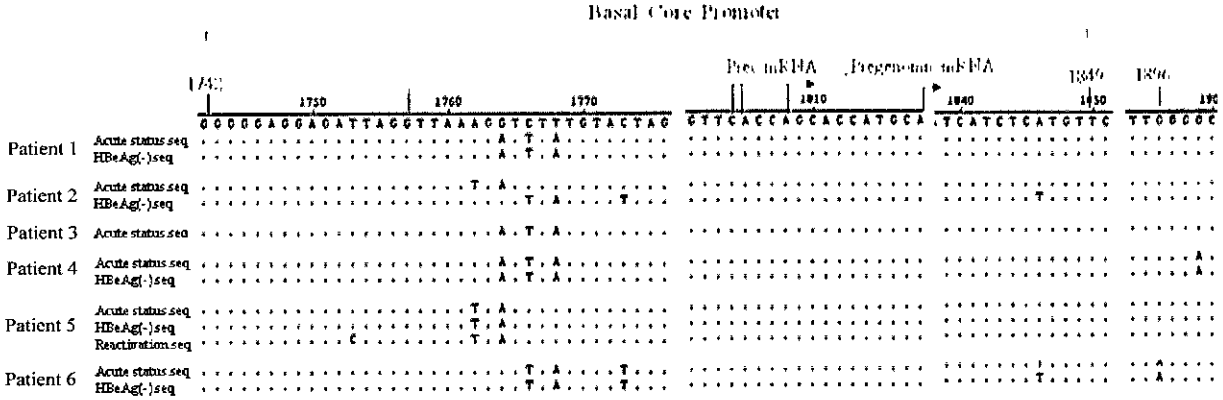


Figure 6. Nucleotide mutations in basal core promoter and precore region

Nucleotide sequences in basal core promoter were compared with the consensus HBV DNA sequence (genotype C2) deduced from eight of HBV DNA registered in GenBank (GenBank No.: AY641558; AY641560; AY641561; AF286594; DQ683578; X01587; AY123041; D000630). Abbreviation: AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B



that already possessed high frequency of genetic mutations before AE, although AE during CHB had similar clinical course to AHB that could lead to spontaneous HBeAg seroconversion. This speculation also might be explained by the result from a CHB patient (Patient 5), who reactivated with similar genetic diversity to the other two clinical statuses (Figure 5).Although, the genetic changes slightly decreased after reactivation, it still had higher genetic diversity than that in acute status of AHB.

Out of the mutations in core and surface gene, the BCP (A1762T/G1764A) mutation had been supposed to associate with severity and progression of liver disease and was detected in up to 90% of CHB infected with genotype C of HBV[27, 34]. In In vitrostudy, this double mutation increased viral replication and reduced HBeAg production[35-37]. In the present study, BCP mutation was broadly detected throughout the patients and seemed not to be related to different outcome between AHB and CHB.

In the present study, there were some limitations. First, lack of the HLA genotyping, we could not exactly explain the detected mutations in epitope-related codon, most of which were HLA-A2 restricted epitope, were result of the immune selection. However, considering the HLA-A2 haplotypic allele is one of the major HLA allele in Korea, it could be indirectly explained that such mutations in epitope might be due to the immune selection[38, 39]. Second, owing to the small size of samples and PCR based direct sequencing method, the statistics analysis of genetic diversity and the proportion of mutations between wild type and mutant type had not been available in this study.

Overall, the different consequences in AHB and CHB during HBeAg seroconversion might be associated with different pattern of genetic variations in HBV DNA that related to immune escape or viral replication.

Nucleotide changes were located within core or surface gene, in which resides several important immunogenic epitopes. Only a few HLA classI-restrited T-cell epitopes for HBV have been identified until now, and most are HLA-A2 restricted[40].

However, such phenomenon may be a result that coordinated by multiple mutations in epitope region to escape from immune system for survive or in some specific region that influencing the viral transcription and replication.

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