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Comparison of Full Genome Sequences Between Two Hepatitis B Virus Strains With or Without preC Mutation (A1896) from a Single Korean Hepatocellular Carcinoma Patient

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Abstract This report describes the full-length sequences of 2 HBV clones from a hepatocellular carcinoma (HCC) patient, one with preC mutation (1896A) and the other without preC mutation. The high level of discrepancy in mutation frequency between these 2 strains was observed in the Core (C) region among 4 ORFs. These data support previous results that Korean HBV strains, belonging to genotype C2, are prone to mutations. It is possible that the mutations (BCP and preC mutations) associated with the HBeAg defective production might contribute to the diversity of mutations related to HBV persistence, playing an important role in hepatocarcinogenesis in this patient.

Keywords: Hepatitis B virus (HBV), hepatocellular carcinoma (HCC), preC mutation, quasispecies, full-length genome sequence

Chronic Hepatitis B virus (HBV) infection affects more than 350 million people, some of whom develop severe liver diseases, which include cirrhosis and hepatocellular carcinoma (HCC) [13]. Moreover, Korea is an endemic area of HBV infection, and based on the Korean National Health and Nutrition Survey of 1998, the prevalence of HBsAg was 5.1% in men and 4.1% in women [9].12

HBV has a quasispecies distribution in infected patients owing to an error-prone HBV reverse transcriptase lacking 3'-5' proofreading capacity [2, 4]. Because the quasispecies distribution of HBV facilitates the selection of variants bearing survival advantage against host immune response, which could contribute to the persistence of viral replication, it should be monitored to ensure the adequate management of chronic infections.

Genotype C has a higher disease-inducing capacity, is more prone to mutations than genotype B, and is known to infect almost all Korean patients [7, 9, 18, 20]. The higher prevalence of double mutations in the basal core promoter (BCP) region, which is closely related to the reduction of HBeAg, an immune tolergen, or major hydrophilic region (MHR) variants is also found in more Korean chronic patients than patients of other countries [19, 22]. Therefore, it seems likely that these characteristic features of Korean chronic patients might contribute to the construction of a HBV quasispecies composed of diverse variants.

Although Korea is a hepatitis B virus (HBV) endemic area [9], relatively few full-length genome sequences are available [8, 9, 18]. In particular, to our knowledge, comparative studies based on the complete genome sequences between HBV strains with different polymorphisms in preC mutation from patients have not yet been performed.

To understand the molecular dynamics between 2 strains with or without preC mutation within a HCC patient, comparisons of the nucleotide and amino acid sequences between these 2 strains are presented, and their characteristic mutation patterns are evaluated in the present study.

A serum sample was collected from a HBeAg negative patient who visited Cheju National University Hospital, Jeju, Korea, in 2004. The patient, designated by code name JH4, was diagnosed with HCC either histologically or radiologically. Clinical data are shown in Table 1.

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Table 1. Clinical characteristics of an HCC patient, JH4, the subject of this study.

Samples	Sex	Age (years)	ALT (1 U/l)	HBeAg	Anti-HBe	HBV DNA (pg/ml)	Diagnosis
JH4	M	52	52	-	+	70	Hepatocellular carcinoma

DNA was extracted using phenol/chloroform/isoamylalcohol (50:49:1) from 200 ml of serum. To amplify the full-length genome of HBV, the nested Expand High Fidelity PCR System (Roche, Penzberg, Germany) was used. The HBV sequences of this patient were amplified using a two-step full-length genome amplification method as previously described [7, 8].

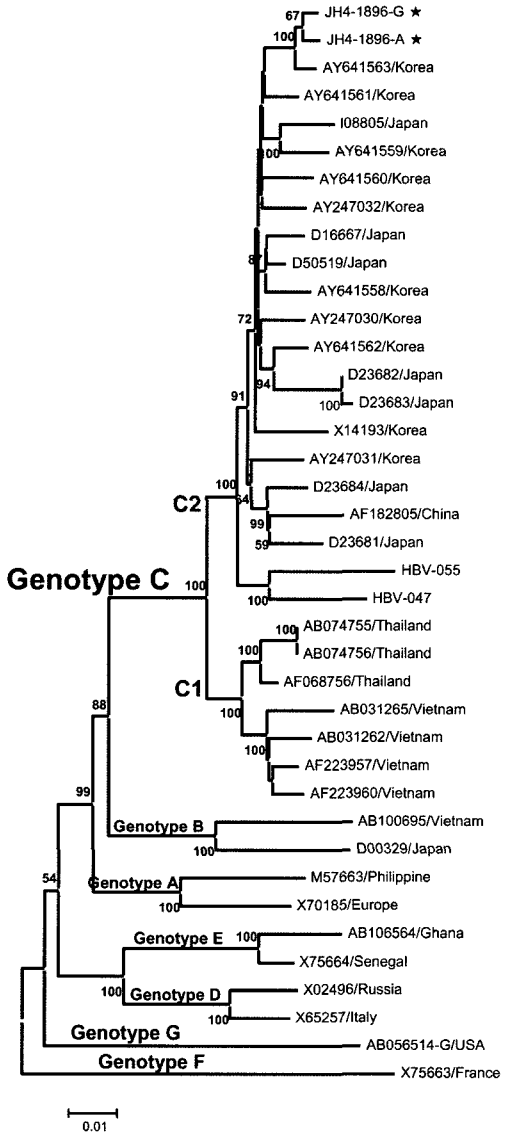

Fig. 1. A phylogenetic tree based on the entire genome sequences of 37 reference HBV strains and of the 2 HBV strains sequenced in this study.

Genetic distances were estimated by using the Kimura two-parameter matrix and the phylogenetic tree was constructed by the neighbor-joining method. Stars (★) indicate HBV strains sequenced in this study; Country origins of all the reference HBV strains are given. Percentages at nodes represent levels of bootstrap support from 1,000 resampled datasets. Bootstrap values of less than 50% are not shown. The bar indicates 1% estimated sequence divergence.

PCR amplicons were purified using a QIAEXII gel extraction kit (QIAgen, Hilden, Germany), and cloned into a TOPO TA cloning vector (Invitrogen, California). The HBV DNA insert was sequenced with vector- and HBV-specific primers as previously described [7].

Based on full nucleotide sequences, phylogenetic analysis was performed on the 2 HBV clones, JH4-1896-G and JH4-1896-A, and results were compared with the sequences of 37 reference clones retrieved from GenBank. Phylogenetic trees were inferred using the neighbor-joining method [3, 6, 11, 21], which was carried out using MEGA version 2.1 [10]. A phylogenetic tree showed that the 2 strains belonged to genotype C2, which predominates in Far East Asia (Japan, Korea, and China), and that they form a closely related branch (Fig. 1).

The most discernible feature observed between these 2 HBV strains is the polymorphism of nucleotide position 1896 in the preC region (G or A), which is known to be associated with interruption of HBeAg production. One is with preC mutation, designated as a JH4-1896-A strain (GenBank No. EF137803) not producing HBeAg, but the other is without preC mutation, designated as a JH4-1896-G strain (GenBank No. EF137802), which can produce HBeAg (Table 2).

All two HBV strains from the HCC patient, JH4, had the same nucleotide lengths of 3,200 bp, with deletion of 15 bp in preS1 and in the overlapping P region. These nucleotide deletions led to 5 and 6 amino acid deletions in the P and preS1 regions, respectively. However, since these deletions affect the start codon in the overlapping preS1 region and the next start codon was located at the 12th amino acid position, both strains contained 11 amino acids deleted large S antigens (389 amino acids), characteristic of genotype D strain. Moreover, both strains showed premature termination in the 665th codon of P protein, and they had P proteins with length of 659 amino acids, much shorter than that of wild strain with a length of 843 amino acids (Table 2, Table 3). Although the catalytic domain of the reverse transcriptase domain of truncated P protein was intact, the RNase H domain was completely deleted. Ribonuclease H activity encoded by the RNase H domain is essential for cleavage of the RNA in the RNA-DNA hybrid during reverse transcription [14]. The possibility that the loss of this activity might be compensated for by the intact P protein of different quasispecies HBV strains in this patient

Table 2. Amino acid substitutions deduced from full genome sequences, and mutation patterns of the BCP region of 2 HBV strains from an HCC patient, JH4.

Samples	Polymerase	PreS1	PreS2	Surface	X protein	BCP		Precore		Core
						1762 A	1764 G		1896 G	
JH4-1	E17A, G32D, E39D, V69I, Q85H, T100I, 182 Del 186, E258K, A272S, S335F, H401R, V612A, L613Q, Q648R, 665 Stop	1 Del 6, 77 Seop	P36S	P62L, L88P, I152T, L209W	T82A, I127T, K130M, V130I	A to T	G to A	Clear	G	E117G
JH4-4	E17A, G32D, V69I, Q85H, 182 Del 186, A272S, H401R, L613Q, Q648R, 665 Stop	1 Del 6	Clear	V180A, L209W, V224A	T82A, I127T, K130M, V130I	A to T	G to A	28 Stop	G to A	P5T, Y38H, L60V, V149I, G153S, Q177K

(JH4) could not be excluded. However, the exact mechanisms in compensation of the lost activity of the truncated P protein remain for future study.

Both strains have X (154 amino acids) and C (183 amino acids) proteins of normal length. Both strains contained double mutations at nucleotides 1762 (A→T) and 1764 (G→A) in the basal core promoter (BCP) region, which simultaneously led to two amino acid substitutions at amino acid positions 130 (K→M) and 131 (V→I) in the overlapping X region. Besides these two mutations related to the BCP double mutations, the other mutation at the amino acid position 127 (I→T) known to be associated with progressive forms of liver disease was also found in the X region of both strains (Table 2).

Generally, some discrepancies between 2 HBV strains in mutation patterns and frequencies of almost all regions except for the X region were observed. In particular, discrepancy in the mutation frequency of the C region was the most clearly shown. For example, whereas only one amino acid change was observed in the JH4-1896-G strain, six amino acid changes were observed in the JH4-1896-A strain. The amino acid differences between 2 HBV strains were observed in 8 (4.4%) among 183 codons in C proteins (Table 2).

HBeAg is known to be a strong immune tolergen, which could suppress the host immune response against HBV antigens [11]. HBV strain showing the complete loss of HBeAg might be expected to be more prone to mutations against host immune response than one producing HBeAg. In fact, comparison of mutation patterns between the 2 HBV strains of the present study showed that amino acid sequence divergence between the 2 HBV strains was higher in HBsAg [2.2% (5/226 amino acids)] and HBcAg [4.4% (8/183)], eliciting a strong host immune response, than in functional proteins, P [0.8% (5/659 amino acids)] and X [0% (0/154 amino acids)] proteins, strongly supporting the above notion. In particular, the fact that the highest level of divergence between both strains was observed in the C region having common amino acid sequences with HBeAg suggested that HBeAg might suppress the host immune response against HBeAg more specifically rather than suppress the whole host immune response against HBV antigens in a nonspecific manner.

In addition to preC mutation, some mutation patterns suspected as a factor associated with hepatocelluar carcinogenesis of this patient (JH4) were observed in the present study. First, BCP double mutations, which are known to be associated with the severance of clinical outcome [1, 5], were observed in both strains. This result was also in accordance with the previous result that BCP double mutations are frequent in Korean HBV isolates, irrespective of HBe seroconversion and disease severity [21]. Second, the deletion of preS1 start codon leading to the N terminally 11 amino acids deleted large S protein characteristic of genotype D strain was observed in both HBV strains. The deletion type of preS1 start codon had also been introduced in 2 among 3 Korean HCC patients in the previous report [18], but had been rarely detected in genotype C strains of other countries. Third, the three mutations (I127T, K130M, and V131I) observed in the carboxy terminal region of the X protein known to be associated with transactivating function [16, 17] were also observed in both strains.

In conclusion, our data support previous results that Korean HBV strains, belonging to genotype C2 extraordinarily, are prone to mutations and have several characteristic mutations. It could be that the mutations

Table 3. Comparison of amino acid length of encoded proteins between 2 HBV strains from an HCC patient, JH4.

	Polymerase	Large surface Ag	Middle surface Ag	Small surface Ag	X Ag	Core Ag	HBeAg
Genotype C	843	400	281	226	154	183	P
JH4-1896-G	659	339	281	226	154	183	P
JH4-1896-A	659	339	281	226	154	183	N

(BCP and preC mutations) associated with the HBeAg defective production might contribute to the diversity of mutations related to HBV persistence, playing an important role in hepatocarcinogenesis in this patient.

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REFERENCES

1. Baptista, M., A. Kramvis, and M. C. Kew. 1999. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. *Hepatology* **29:** 946–953.
2. Cane, P. A., D. Mutimer, D. Ratcliffe, P. Cook, G. Beards, E. Elias, and D. Pillay. 1999. Analysis of hepatitis B virus quasispecies changes during emergence and reversion of lamivudine resistance in liver transplantation. *Antivir. Ther.* **4:** 7–14.
3. Cho, S. J., K. M. Cho, E. C. Shin, W. J. Lim, S. Y. Hong, B. R. Choi, J. M. Kang, S. M. Lee, Y. H. Kim, H. Kim, and H. D. Yun. 2006. 16S rDNA analysis of bacterial diversity in three fractions of cow rumen. *J. Microbiol. Biotechnol.* **16:** 92–101.
4. Gunther, S., L. Fischer, I. Pult, M. Sterneck, and H. Will. 1999. Naturally occurring variants of hepatitis B virus. *Adv. Virus Res.* **52:** 25–137.
5. Kao, J. H., P. J. Chen, M. Y. Lai, and D. S. Chen. 2003. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology* **124:** 327–334.
6. Kim, B. J., Y. H. Koh, J. S. Chun, C. J. Kim, S. H. Lee, M. J. Cho, J. W. Hyun, K. H. Lee, C. Y. Cha, and Y. H. Kook. 2003. Differentiation of actinomycete genera based on partial *rpoB* gene sequences. *J. Microbiol. Biotechnol.* **13:** 846–852.
7. Kim, H., Y. M. Jee, B. C. Song, J. W. Hyun, H. S. Mun, H. J. Kim, E. J. Oh, J. H. Yoon, Y. J. Kim, H. S. Lee, E. S. Hwang, C. Y. Cha, Y. H. Kook, and B. J. Kim. 2007. Analysis of Hepatitis B virus quasispecies distribution in a Korean chronic patient based on the full genome sequences. *J. Med. Virol.* **79:** 212–219.
8. Kim, H., Y. M. Jee, B. C. Song, J. W. Shin, S. H. Yang, H. S. Mun, H. J. Kim, E. J. Oh, J. H. Yoon, Y. J. Kim, H. S. Lee, E. S. Hwang, C. Y. Cha, Y. H. Kook, and B. J. Kim. 2007. Molecular epidemiology of hepatitis B virus (HBV) genotypes and serotypes in patients with chronic HBV infection in Korea. *Intervirology* **50:** 52–57.
9. Kim, S. Y., H. S. Kang, and Y. S. Kim. 2000. Phylogenetic analysis of hepatitis B virus genome isolated from Korean patient serum. *J. Microbiol. Biotechnol.* **10:** 823–828.
10. Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. *MEGA2: Molecular Evolutionary Genetics Analysis* software, Arizona State University, Tempe, Arizona, U.S.A.
11. Kwak, J., D. H. Lee, Y. D. Park, S. B. Kim, J. S. Maeng, H. W. Oh, H. Y. Park, and K. S. Bae. 2006. Polyphasic assignment of a highly proteolytic bacterium isolated from a spider to *Serratia proteamaculans*. *J. Microbiol. Biotechnol.* **16:** 1537–1543.
12. Lee, D. H., J. H. Kim, J. J. Nam, H. R. Kim, and H. R. Shin. 2002. Epidemiological findings of hepatitis B infection based on 1998 National Health and Nutrition Survey in Korea. *J. Korean Med. Sci.* **17:** 457–462.
13. Lee, W. M. 1997. Hepatitis B virus infection. *N. Engl. J. Med.* **337:** 1733–1745.
14. Locarnini, S., J. McMillan, and A. Bartholomeusz. 2003. The hepatitis B virus and common mutants. *Semin Liver Dis.* **23:** 5–20.
15. Odgerel, Z., K. B. Nho, J. Y. Moon, S. H. Kee, K. S. Park, K. J. Song, L. J. Baek, J. E. Yeon, K. S. Byun, C. H. Lee, and J. W. Song. 2003. Complete genome sequence and phylogenetic analysis of hepatitis B virus (HBV) isolates from patients with chronic HBV infection in Korea. *J. Med. Virol.* **71:** 499–503.
16. Pumpens, P., E. Grens, and M. Nassal. 2002. Molecular epidemiology and immunology of hepatitis B virus infection - an update. *Intervirology* **45:** 218–232.
17. Rossner, M. T. 1992. Review: Hepatitis B virus X-gene product: A promiscuous transcriptional activator. *J. Med. Virol.* **36:** 101–117.
18. Song, B. C., H. Kim, S. H. Kim, C. Y. Cha, Y. H. Kook, and B. J. Kim. 2005. Comparison of full length sequences of hepatitis B virus isolates in hepatocellular carcinoma patients and asymptomatic carriers of Korea. *J. Med. Virol.* **75:** 13–19.
19. Song, B. C., S. H. Kim, H. Kim, Y. H. Ying, H. J. Kim, Y. J. Kim, J. H. Yoon, H. S. Lee, C. Y. Cha, Y. H. Kook, and B. J. Kim. 2005. Prevalence of naturally occurring surface antigen variants of hepatitis B virus in Korean patients infected chronically. *J. Med. Virol.* **76:** 194–202.
20. Usuda, S., H. Okamoto, H. Iwanari, K. Baba, F. Tsuda, Y. Miyakawa, and M. Mayumi. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J. Virol. Methods* **80:** 97–112.
21. Yeo, S. H., H. J. Kim, J. H. Yoon, H. S. Kim, Y. I. Hwang, and S. C. Lee. 2006. Characterization of antioxidant-producing *Alteromonas macleodii* HJ-14 isolated from seawater. *J. Microbiol. Biotechnol.* **16:** 731–739.
22. Yoo, B. C., J. W. Park, H. J. Kim, D. H. Lee, Y. J. Cha, and S. M. Park. 2003. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. *J. Hepatol.* **38:** 98–103.