

# Occult HBV among Anti-HBc Alone: Mutation Analysis of an HBV Surface Gene and Pre-S Gene

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**Purpose:** The aim of this study is to investigate the molecular characteristics of occult hepatitis B virus (HBV) infection in 'anti-HBc alone' subjects.

**Materials and Methods:** Twenty-four patients with 'anti-HBc alone' and 20 control patients diagnosed with HBV were analyzed regarding S and pre-S gene mutations. All specimens were analyzed for HBs Ag, anti-HBc, and anti-HBs. For specimens with an anti-HBc alone, quantitative analysis of HBV DNA, as well as sequencing and mutation analysis of S and pre-S genes, were performed.

**Results:** A total 24 were analyzed for the S gene, and 14 were analyzed for the pre-S gene through sequencing. A total of 20 control patients were analyzed for S and pre-S gene simultaneously. Nineteen point mutations of the major hydrophilic region were found in six of 24 patients. Among them, three mutations, S114T, P127S/T, M133T, were detected in common. Only one mutation was found in five subjects of the control group; this mutation was not found in the occult HBV infection group, however. Pre-S mutations were detected in 10 patients, and mutations of site aa58-aa100 were detected in 9 patients. A mutation on D114E was simultaneously detected. Although five mutations from the control group were found at the same location (aa58-aa100), no mutations of occult HBV infection were detected.

**Conclusion:** The prevalence of occult HBV infection is not low among 'anti-HBc alone' subjects. Variable mutations in the S gene and pre-S gene were associated with the occurrence of occult HBV infection. Further larger scale studies are required to determine the significance of newly detected mutations.

**Key Words:** Occult HBV infection, HBV S gene mutation, HBV pre-S gene mutation

## INTRODUCTION

Hepatitis B virus (HBV) infection causes chronic B viral hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), which are all associated with considerable mortality.<sup>1</sup> HBV infection is usually diagnosed by detecting serum HBs Ag and anti-HBc. Chronic hepatitis B can be diagnosed by persistent detection of HBs Ag with simultaneously detection of HBV

DNA. In some cases of HBV infection, a state of detectable HBV DNA without HBs Ag is considered occult HBV infection. Occult HBV infection is defined by the presence of HBV DNA in liver tissue (and in some cases also in serum) from HBs Ag negative individuals.<sup>2</sup> The mechanism or clinical significance of occult HBV infection has not been revealed, although its incidence is high in endemic regions.<sup>2</sup> Concurrent infection with hepatitis C virus (HCV) and other risk factors, such as alcohol consumption, are associated with occult HBV infection progressing to chronic liver diseases.<sup>3</sup> Transmission of HBV through transfusion or transplant is a risk that must be closely monitored.<sup>4</sup>

The detection of anti-HBc alone without HBs Ag and anti-HBs has been previously reported.<sup>2,5</sup> In such cases, the status of HBV infection is difficult to determine. Positive anti-HBc can be seen in various circumstances, such as acute infection, chronic HBV infection, and recovery from a past HBV infection. In particular, 'anti-HBc alone' can be seen when the anti-HBs titer is substantially decreased during a long period after HBV

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infection,<sup>5</sup> occult HBV infection,<sup>6</sup> coinfection with human immunodeficiency virus (HIV) or HCV,<sup>7</sup> the 'window period' of an acute HBV infection,<sup>4</sup> and in false positive cases.<sup>8</sup> In highly endemic regions, including Korea, determining the clinical significance of anti-HBc alone has proven difficult.<sup>3</sup>

The surface (S) gene encodes a 226aa protein, named the major antigen (small HBs Ag). The major hydrophilic region (MHR) of the HBs Ag binding site is located at aa100–aa160, and cysteine residues form two major large loops at aa107–aa138 and aa139–aa147. A binding site of aa124–aa147 that determines the antigen binding ability of HBs Ag is called the 'a' determinant. In particular, aa139–aa147 is a highly conserved region showing high affinity for HBs Ag. The pre-S2 gene encodes a 55aa protein, which together with the previous major antigen, forms the medium antigen. The pre-S1 gene encodes a 119aa protein, which together with the medium antigen, forms the large antigen.<sup>9</sup> Medium-HBs Ag (pre-S2) and large-HBs Ag (pre-S1) are very important for virus clearance, because they are more immunologic and appear earlier in the course of infection than the small-HBs Ag.<sup>2</sup> Especially, aa58 to aa100 of pre-S1 is speculated to be recognized by antibodies involved in viral clearance.<sup>9</sup> Several studies suggest that a specific mutation type in the HBV pre-S/S region in occult HBV infections may be responsible for altering HBs Ag antigenicity or secretion capacity. This may lead to vaccine escape infections and the emergence of highly virulent variants in certain populations.<sup>10</sup>

The mechanism leading to occult HBV infection remains unknown. There are several suggested mechanisms to explain occult HBV infection.<sup>11–13</sup> First is genetic mutation of the HBV S gene, which causes HBs Ag to be non-detectable. There are reports on non-detectable HBs when mutation occurs at the 'a' determinant.<sup>14,15</sup> The second mechanism explains occult HBV infection through suppressed viral replication, in which viral copy numbers exist below the detection limit. This mechanism is particularly likely, because occult HBV infection can be reactivated during immune-suppressed conditions.<sup>16</sup> The third mechanism is that various mutations in genomic regulatory elements may negatively affect viral replication.<sup>17</sup> The last suggested mechanism is that there are mutations affecting post-transcriptional mechanisms regulating HBs Ag expression and secretion.<sup>18</sup>

Several studies have demonstrated the prevalence of occult HBV infection in 'anti-HBc alone' and their relationship, but not many have explored the molecular genetic characteristics of occult HBV infection. In this study, the molecular genetic traits of occult HBV infection among 'anti-HBc alone' subjects were analyzed through sequencing study of the S gene and pre-S gene.

## MATERIALS AND METHODS

### Specimens

Among patients referred for laboratory analysis of viral hepatitis with known results of HBs Ag, anti-HBc, and anti-HBs, anti-HBc alone was found in 1060 patients. Of these, 42 patients (3.96%) were HBV DNA positive. The study included 24 individuals with available remaining specimen aliquots. Twenty patients with an initial diagnosis of chronic HBV infection, with both positive HBs Ag and HBV DNA, during the same period were selected as a control group. The S and pre-S gene sequencing analysis was compared between groups. Clinical data were obtained through retrospective review of electronic medical records.

This study was approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong (KHNMC MD IRB 2012-004).

### Serologic analysis

All specimens were analyzed with a Roche Modular Analytics E170 (Roche Diagnostics, Inc., Mannheim, Germany) to measure HBs Ag, anti-HBc, and anti-HBs. Specimens with 'anti-HBc alone' were stored at -70°C for further HBV DNA analysis.

### HBV DNA test

For 1060 specimens with anti-HBc alone, the COBAS AmpliPrep/COBAS TaqMan HBV test (Roche Diagnostics GmbH, Mannheim, Germany) was used for quantitative analysis of HBV DNA. COBAS TaqMan HBV test is a real-time PCR method using COBAS TaqMan, with a detection sensitivity of 4–12 IU/mL (20–60 genome copies/mL). The primers used in this test were HBV-104UB and HBV-104D, which respectively amplify 145 nucleotides (genotype A–F and H) and 181 nucleotides (genotype G) within the highly conserved pre-Core/Core region of the HBV genome. Quality control was performed for each batch with high and low positive controls, as well as a negative control, as included with the test kit.

### Detection of HBV surface and pre-S gene mutations

Primers used for S and pre-S gene mutation analysis by nested PCR and sequencing analyses are shown in Table 1. The PCR methods used for amplification and sequencing analysis of S and pre-S genes were conducted as follows. First, PCR was performed using primers PS1 and PS2 under the following protocol: 5 minutes at 95°C, 60 seconds denaturation at 95°C, 60 seconds annealing at 51.5°C, 60 seconds extension at 72°C for 40 cycles, followed by 10 minutes extension at 72°C. After the first amplification, 5 µL of PCR product was obtained, and primers PS3 and PS4 were used under the same conditions for another 30 cycles as mentioned above. Amplification and sequencing analysis of the S gene were conducted as follows. First, PCR was performed using primers S1 and S2 under the following protocol: 5 minutes at 95°C, 60 seconds denaturation at 95°C,

**Table 1.** Primer Sequences Used for Polymerase Chain Reaction of the Surface Gene and Pre-S Gene

Primer no.	Sequence (5'–3')	Nucleotide position	Reference no.
Surface gene			19
S1	CTC GTG TTA CAG GCG GGG TTT TTC	191–214	
S2	CAT CAT CCA TAT AGC TGA AAG CCA AAC A	748–721	
S3	TTG TTG ACA AGA ATC CTC ACA ATA CC	215–240	
S4	GCC CTA CGA ACC ACT GAA CAA ATG G	710–686	
Pre-S gene			20
PS1	AAA ATT AAT TAT CCT GCT AGG	2627–2648	
PS2	GAG AAG TCC ACC ACG AGT C	269–251	
PS3	TTT ACA ACT CTG TGG AAG GC	2747–2767	
PS4	GAG TCT AGA CTC TGT GGT ATT GTG	255–232	

60 seconds annealing at 51.5°C, 60 seconds extension at 72°C for 40 cycles, followed by 10 minutes extension at 72°C. After the first amplification, a PCR product amount of 5 µL was obtained. Primers S3 and S4 were used under the same conditions for another 30 cycles as mentioned above. An ABI PRISM 3730XL Analyzer (Applied Biosystems, Foster City, CA, USA) was used for sequence analysis of the product, and obtained sequences were aligned and analyzed using the SEQUENCHER™ version 4.9 (Gene Code Co., Ann Arbor, MI, USA) program. Sequences were aligned with full-length HBV genotype C sequences previously obtained from an asymptomatic carrier (GeneBank accession number X04615).

## RESULTS

### Characteristics of patients

HBV DNA positivity was found in 42 of 1060 specimens (3.96%) that were 'anti-HBc alone'. The demographic characteristics and laboratory results of 42 patients with occult HBV infection and positive HBV DNA were reviewed. Twenty-six males and 16 females were included. Mean age was 64.4 years (range 39–87), including 16 patients from 40–60 years, 20 patients from 61–80 years, and 6 patients over 81 years. The mean aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels of patients were 28.1±16.4/23.8±11.3 IU/mL, both within normal limits. Among 42 patients, 31 patients (73.8%) had less than 20 IU/mL of HBV DNA. The mean HBV DNA quantity of the other 11 patients was 73.3±69.22 IU/mL.

Among 42 specimens, 24 were analyzed for S gene and 14 were analyzed for pre-S gene through sequencing. The remaining specimens had insufficient DNA for sequencing analysis. The clinical courses of patients are shown in Table 2. Besides one HBV carrier (patient #20), no other patients had a prior history or symptoms of HBV infection. The HBV carrier was HBs Ag positive at 2011, found during routine health examination, although he was negative during this study. Hepatitis B immune globulin or anti-viral agents were not used. One patient (patient #3) was simultaneously positive for HCV and was later confirmed to have HCV by recombinant immunoblotting

assay. There was no prior history of diagnosis or treatment for HBV.

The control patients included 11 men and nine women. Chronic HBC infection was found in 15 patients, HBV carrier state (negative for HBeAg, normal range of ALT, and below 200 IU of HBV DNA) was found in four patients, and acute HBV infection was found in one patient. The mean age of control patients was 43.95 (range 31–60), and the mean HBV DNA quantity was  $2.5 \times 10^7 \pm 5.6 \times 10^7$  IU/mL.

### Analysis of HBV S gene

Mutations of the MHR region were found in six of 24 patients with occult HBV infection, as shown in Table 2 and Fig. 1. A total of 11 mutations were found in six of 24 patients. Patients #7 and 14 showed single mutations of S132F and M133T in a determinant, respectively. Another four patients had multiple mutations. Patients #12 and 21 showed the same mutations of Y100S, M103I, P105R, G112K, T113A, S117G, and P127T. Patient #2 showed eight mutations of Q101R, L104S, P105N, S114T, I126M, P127S, M133T, and D144E. Patient #10 showed 11 mutations of Q101K, S114T, P120T, T123A, I126N, P127T, T113N, M133T, G145A, S154P, and P160K. Among them, S114T (patients #2 and 10), P127S/T (patients #2, 12, and 21), and M133T (patients #2, 10, and 14) were commonly found. Patient #3 was co-infected with HCV and patient #20 was co-infected with chronic HBV and did not show mutation of the S gene.

Only I126T was found in five subjects of the control group (C4, C8, C15, C16, and C20), although this mutation was not found in the occult HBV infection group. Other mutations detected in occult HBV infections were not observed in the control group.

### Analysis of HBV pre-S gene

Only 14 specimens were analyzed pre-S gene mutation due to insufficient volume. Mutations of the pre-S1 region were detected in nine of 14 patients (Table 2). Mutations at aa58–aa100 of pre-S1, known as the antibody recognition region, were detected in nine patients. G83S was detected in five patients (patients #14 and 18–21) who all simultaneously showed muta-

**Table 2.** Description of Patients with Detectable HBV-DNA and Mutations of Major Hydrophilic Region (MHR) in HBV S Gene, Pre-S1 Gene, and Pre-S2 Gene

No. case	Sex/age (yrs)	Clinical diagnosis	AST/ALT (IU/mL)	Total bilirubin	HBV DNA quantity (IU/mL)	HCV	HIV	MHR mutation	Pre-S1 mutation	Pre-S2 mutation
1	M/49	Routine health screening	18/19	0.6	<2.0×10	N	N	None	*	*
2	F/40	HIVD operation	15/18	0.8	<2.0×10	NT	N	Q101R, L104S, P105N, S114T, I126M, P127S, † M133T, † D144E†	*	*
3	F/62	Subdural hematoma, DM	44/37	0.7	<2.0×10	P	N	None	*	*
4	M/87	CHF c pulmonary edema	21/11	0.4	9.01×10	N	N	None	None	None
5	M/58	Osteoarthritis, GB polyp	22/20	0.5	<2.0×10	N	N	None	*	*
6	M/61	Bell's palsy, DM, HTN	24/29	0.8	<2.0×10	N	N	None	M46H, T86A	A11T, G19D, I42T
7	M/85	Lung ca., pneumonia	116/34	5.5	<2.0×10	N	N	S132F	W4R, P65S	N33H
8	M/63	Colon ca., DM, HTN	16/15	0.3	<2.0×10	N	N	None	*	*
9	M/67	BPH	17/15	0.4	<2.0×10	N	N	None	None	F22L
10	F/78	DM, HTN, stroke	27/34	1	2.56×10 <sup>2</sup>	N	N	Q101K, S114T, P120T, T123A, I126N, P127S, † T131N, M133T, † G145A, † S154P	*	*
11	M/59	BPH, HTN	50/35	0.6	5.70×10	NT	N	None	F34L, L82H	D14N, R16K, G30E, P54Q
12	F/63	Aneurysm, HTN	23/21	0.3	2.56×10	NT	NT	Y100S, M103I, P105R, G112K, T113A, S117G, P127T†	None	None
13	M/58	Routine health screening	40/50	1	3.18×10	N	NT	None	*	*
14	F/60	Spondylolisthesis	22/15	0.8	<2.0×10	N	N	M133T†	G83S, D114E, H116R	None
15	F/69	Fracture of humerus	29/26	1.2	<2.0×10	N	N	None	*	*
16	M/41	Routine health screening	25/18	1	<2.0×10	N	N	None	*	*
17	M/45	Routine health screening	32/22	0.9	<2.0×10	N	N	None	*	*
18	M/87	Ischemic heart disease, DM, HTN	36/22	0.3	6.27×10	N	N	None	G83S, D114E	None
19	M/71	Atherosclerosis of arteries of extremities	31/38	0.6	<2.0×10	N	N	None	G83S, D114E	None
20	M/67	HTN, chronic HBV carrier	28/26	1.1	<2.0×10	N	N	None	G83S, D114E	None
21	F/87	DM, HTN	19/17	0.5	3.17×10	N	N	Y100S, M103I, P105R, G112K, T113A, S117G, P127T†	G83S, D114E	None
22	M/73	Allergic dermatitis	25/18	0.6	6.27×10	NT	NT	None	I84T	P54T
23	M/71	HTN	35/47	1.1	<2.0×10	N	N	None	None	F22L
24	F/65	Amnesia, HTN	26/14	NT	<2.0×10	N	N	None	None	None

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HIVD, herniated intervertebral disc; DM, diabetes mellitus; CHF, chronic heart failure; GB, gallbladder; HTN, hypertension; BPH, benign prostate hypertrophy; N, negative; P, positive; NT, not tested.  
 \*Sequencing was not performed due to insufficient specimens, †Mutations shared by two or more occult HBV infection strains.

	101	111	121	131	141	151
Reference (X04615)	<b>Y QGMLPVCPLL</b>	<b>PGTSTTSTGP</b>	<b>CRCTCTIPAQG</b>	<b>TSMFPSCCCT</b>	<b>KPSDGNCTCI</b>	<b>PIPSSWAFAR</b>
Patient #1	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #2	<b>R - - SN - - - -</b>	<b>- - - T - - - -</b>	- - - - -	<b>- - - MS - - - -</b>	<b>- - - L - - - -</b>	<b>- - - E - - - -</b>
Patient #3	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #4	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #5	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #6	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #7	- - - - -	- - - - -	- - - - -	- - - - -	<b>- - - F - - - -</b>	- - - - -
Patient #8	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #9	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #10	<b>K - - - - -</b>	<b>- - - T - - - -</b>	<b>- - - T - - - -</b>	<b>- - - A - - - NT -</b>	<b>- - - N - T - - - -</b>	<b>- - - A - - - -</b>
Patient #11	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #12 <b>S</b>	<b>- - I - R - - - -</b>	<b>- KA - - - G - - -</b>	- - - - -	- - - - -	<b>- - - T - - - -</b>	- - - - -
Patient #13	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #14	- - - - -	- - - - -	- - - - -	- - - - -	<b>- - - T - - - -</b>	- - - - -
Patient #15	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #16	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #17	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #18	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #19	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #20	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #21 <b>S</b>	<b>- - I - R - - - -</b>	<b>- KA - - - G - - -</b>	- - - - -	- - - - -	<b>- - - T - - - -</b>	- - - - -
Patient #22	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #23	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Patient #24	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -

**Fig. 1.** Amino acid sequences of major hydrophilic region (100–160) including ‘a’ determinant (124–147, shadow) in hepatitis B virus (HBV) S gene of 24 patients with occult HBV infection.

tion on D114E. Five mutations from the control group were found at the same location (aa58–aa100), including A60V in nine patients (C2, C3, C12, C13, and C16–C20), L74I in C2, I84T in C1, L85V in C2, and A95V in C3. Besides I84T found in C1, no mutations of occult HBV infection were detected.

Ten point mutations were found on the pre-S2 gene in six patients (Table 2). A11T, D14N, R16K, G19D, F22L, G30E, N33H, I42T, and P54Q/T were found; these mutations were not reported previously. Among these, F22L (patients #9 and 23) and P54T (patient #22) were detected in the control group (C1, C8, C9, and C10). T49I was seen in three controls (C4, C6, and C7), but was not observed in the occult HBV infection group.

## DISCUSSION

The prevalence of occult HBV infection varies by method of detection (ELISA, PCR, or real-time PCR), primers used for PCR, study population, and with different endemic areas.<sup>21</sup> In this study, the prevalence of occult HBV infections was 3.96% (42/1060), somewhat higher than a prevalence of 1.7% reported from the same institute in 2010.<sup>3</sup> This difference in prevalence is likely explained by differences in the population studies, as the population in this study was larger and more diverse

than that of the previous study.

In our study, the average age of subjects with occult HBV infection was 64.6 years, and the subjects had normal ALT levels, undetectable HBeAg, and very low viral loads. There were more males in the occult HBV infection group (n=42; ratio of males to females, 2:1). A previous report indicated that the viral loads are significantly lower among patients with occult HBV infection than asymptomatic carriers or patients with chronic hepatitis.<sup>21,22</sup> This phenomenon may explain the first cause of occult HBV infection, suppressed viral replication in patients with occult HBV infection, although the reason for this is not clear. Detection of HBV DNA in liver tissue is a definitive diagnosis of occult HBV infection; thus, very low or even undetectable levels of HBV DNA may be relevant results.<sup>11</sup>

Several amino acid substitutions were detected in the MHR from aa100 to aa160 in subjects with occult HBV infection and ‘anti-HBc alone’. According to a study by Svicher, et al.,<sup>23</sup> Y100S, found in patients #12 and 21, resulted in decreased antigen-antibody reactivity of HBs Ag, because other variants are incorporated in a step-by-step manner. This Y100S mutation appears to be the route towards occult states.<sup>23</sup> A high frequency of mutations at aa119–aa123 has been reported to have a role in the occurrence mechanism of occult HBV infection. A mutation at aa120–aa123 (P120T and T123A) found in patient #10 was deemed essential for the antigenicity of HBs Ag in a previ-

ous report.<sup>24</sup> Amino acid substitution of the 'a' determinant (aa124–aa147) causes conformational changes that affect binding of the neutralizing antibody, resulting in a false negative HBs Ag test.<sup>25</sup> Mutations of the 'a' determinant abolish the two loop structure of the 'a' determinant and produce changes in hydrophilicity, electrical charge, or acidity of the loop. A total of 11 mutations in the 'a' determinant were found in six of 24 patients. The 'a' determinant mutations found in this study were I126M/N, P127S/T, T131N, S132F, M133T, F134L/T, D144E, and G145A. The P127S mutation found in four (patients #2, 10, 12, and 21) patients and G145A found in one patient (patient #10) were previously reported to abolish the two loop structure of the 'a' determinant, resulting in altered hydrophilicity, electrical charge, or acidity of the loops.<sup>9</sup>

In particular, the mutations of aa144–aa148 were common escape mutants that interfered with HBs Ag detection and/or evasion of vaccine-induced neutralizing antibodies.<sup>21</sup> Only D144E and G145A were detected from patients #2 and 10, respectively, while other known mutations were not found. P127T, found in four of six patients with mutations, appears to be a meaningful mutation contributing to the occurrence of occult HBV infection. A study of untreated Korean patients with chronic hepatitis B showed that S protein mutations occur spontaneously.<sup>26</sup> Among the spontaneously occurring mutations, T123A and T131N were found in patient #10, but not in other patients.

The newly detected mutations in this study were Q101K (patients #2 and 10), M103I (patients #12 and 21), L104S (patient #2), P105N/R (patients #2, 12, and 21), G112K (patients #12 and 21), T113A (patients #12 and 21), S132F (patient #7), S154P (patient #10), and P160K (patient #10). However, these mutations were concurrently detected with other previously reported mutations, except for S132F. Therefore, further analyses are required to determine whether these mutations were actually associated with occult HBV infection or were results of natural polymorphism. S132F, found in patient #7 alone, seems to be associated with occult HBV infection of the host patient. Compared to previous results in patients with chronic HBV infection,<sup>26</sup> mutations of MHR were observed at lower prevalence. In contrast to a previous study in which subjects had chronic HBV infection with severe liver disease or HCC, the subjects of this study were first found to exhibit anti-HBc alone in a health medical examination or preoperative test by accident. Thus, further study as to whether these variants influence disease severity or course may be warranted.

A previous study suggested that mutations in the vicinity of the pre-S2/S splice donor site were common in occult HBV infections.<sup>13</sup> A more recent report also described pre-S1 deletions in the start codon and pre-S2 deletions.<sup>10</sup> The 14 patients whose pre-S region was studied all had no pre-S1 and pre-S2 deletions. In the same study, S17A, P32L, W43L/R, H51P/R, and I84T/M of the pre-S1 region and W3R/Stop and S5A of the pre-S2 region were found in occult HBV infections.<sup>10</sup> In our

study, I84T was found in patient #22 and was also seen in a control patient, suggesting its origin as a natural polymorphism. The other mutations of F34L, N46H, P65S, T86A, L82H, G83S, and D114E seen in this study are mutations of previously unreported regions. Particularly, F34L and N46H were located in the hepatocyte binding site of the pre-S1. The corresponding region of pre-S1 (especially from aa58 to aa100) is speculated to be recognized by antibodies involved in the viral clearance process.<sup>9</sup> We found mutations of P65S, T86A, L82H, and G83S in this site, detecting G83S in 5 patients. Mutations of A11T, D14N, R16K, G19D, F22L, G30E, I42T, and P54Q/T were found in pre-S2. Other than F22L and P54T also found in the control group, these pre-S2 mutations were previously not reported or mentioned.

Further analyses of HBV strain and determining genotype were impossible due to an insufficient amount of specimens in this study. Based on sequences of the S gene and comparing to BLAST search, their strain appears to be genotype C, which is the most prevalent in Eastern Asia. Mutation studies of occult HBV infection in genotype C are very rare, and the clinical significance thereof has rarely been discussed in the literature. We detected novel mutations that were not previously reported. However, we were unable to draw any conclusions as to what role these mutations play in occult HBV infection. Further large-scale studies are required to determine the significance of these mutations and differences in this specific group with mutations.

There are several limitations in this study. Further evaluations, such as additional test, family history of liver disease, etc., were impossible because this study was a retrospective study. Also, additional evaluations were not done due to limited sample volume. The viral load below the confirmatory assay detection limit and inability to study full-length HBV genome were also limitations of this study. The possibility of a false positive real-time PCR result could not be ruled out. Finally, patients with anti-HBc alone were not tested for the presence of HBV DNA in liver tissue. Thus, the cases enrolled in this study may underestimate the prevalence of occult HBV infection in subjects positive for anti-HBc alone, since liver HBV DNA was not assayed.

Despite meaningful detection of several new mutations, a limited number of study samples provide insufficient evidence to prove that occurrence of occult HBV infection is related to these newly found mutations. The retrospective nature of this study resulted in difficulty obtaining specimens. Therefore, analysis was not available for all mutations of every patient with occult HBV infection enrolled in this study. While there are not many studies on mutation of the HBV genotype C mutation, this study provides meaningful insight into this subject.

In conclusion, variable mutations in the MHR of the S gene were associated with the occurrence of occult HBV infection, although S gene mutations did not show higher genetic diver-

sity in this study. Mutations of the S and pre-S genes were frequently observed in subjects with occult HBV infection. Nevertheless, further large-scale studies are required to determine the significance of these newly detected mutations.

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