Clinical significance of hepatitis B virus-DNA in hepatocellular carcinoma negative for hepatitis B virus surface antigen

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Abstract. It is relatively rare for hepatocellular carcinoma (HCC) to develop in patients that are serologically negative for hepatitis B virus surface antigen (HBsAg) or anti-hepatitis C virus antibody (HCV-Ab). In addition, hepatitis B virus (HBV) is sometimes detected and associated with hepatocarcinogenesis in HCC cases without HBsAg and HCV-Ab (NBNC-HCC). In this study we focused on the characteristics of resected NBNC-HCC, and occult HBV infection in resected liver tissues was also examined in these cases. A total of 32 cases (26 males and 6 females, median age 65.7±13.9 years) that underwent liver resection were enrolled in this study. Clinical data from 19 cases with NBNC-HCC were compared with those from 13 cases of HCC related to hepatitis viruses (HV-HCC). Subsequently, occult HBV infection was assessed by the detection of HBV-DNA from extracted liver tissue in NBNC-HCC. Mutation and variation were also examined by the PCR-direct sequencing method in the occult HBV cases. The average diameter of NBNC-HCC was significantly larger than that of HV-HCC. In addition, the activity and fibrosis scores of the surrounding liver tissues were significantly higher in HV-HCC. Among 19 cases of NBNC-HCC, HBV-DNA was detected in 7. Four out of the 7 cases were detected in the Pre-S/S region. The insertion of four amino acids in the α-loop region was detected in 1 case. No significant difference between the occult HBV cases and the others was found in NBNC-HCC. All cases were classified into genotype C on phylogenetic analysis. HBV-DNA was frequently detected in the liver tissues of NBNC-HCC. Thus, our data revealed that HBV may be associated with hepatocarcinogenesis in cases of occult HBV infection.

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Introduction

Hepatocellular carcinoma (HCC) can be a fatal, and chronic infection caused by hepatitis B virus (HBV) and/or hepatitis C virus (HCV) is usually related to hepatocarcinogenesis. Globally, HBV carriers are estimated to total 3 million, and approximately 1 million people suffer from HBV-related HCC (1). HBV is an oncogenic virus which integrates with the genome of hepatocytes. The quantity of HBV-DNA is strongly associated with the generation of HCC (2,3).

In general, the diagnosis of HBV infection is based on the detection of hepatitis B surface antigen (HBsAg) in the serum. However, it was recently reported that HBV-DNA is periodically detected in the serum or liver and is recognized as occult HBV infection (4,5). In particular, HBcAb positivity is thought to be a latent risk factor for hepatocarcinogenesis, since the covalently closed circular DNA (cccDNA) of HBV is responsible for the persistent HBV infection of hepatocytes in these cases (6,7).

In order to examine the clinical characteristics of HCC negative for HBsAg and the anti-hepatitis C virus antibody (HCV-Ab) (NBNC-HCC) and HBV-DNA in relation to hepatocarcinogenesis in NBNC-HCC, 32 cases of resected HCC were analyzed.

Materials and methods

Sample collection. Thirty-two cases of HCC (26 males and 6 females, median age 65.7 years) that underwent surgical resection in Kobe University and related hospitals were enrolled in this study. Clinical characteristics including age, gender, biochemical data and surrounding liver tissues were examined. Histological features were graded according to the New Inuyama Classification (8).

Analysis of occult HBV infection. To examine occult HBV infection in HBsAg-negative cases, DNA was extracted in 12 cases from surrounding liver tissues using the QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan). The detection of HBV-DNA was carried out by employing PCR analysis using specific primers (9,10). PCR products were directly sequenced using an ABI PRISM® 3100 *Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and nucleotide align-

Table I. Clinical characteristics of NBNC-HCC and hepatitis virus-related HCC.

	NBNC-HCC	HV-HCC	P-value
Cases (no.)	19	13	
Age (years)	68.70±8.27	61.40±14.50	0.0794
Tumor size (mm)	74.70±36.20	48.80±29.60	0.0415[a]
Activity	0.50±0.514	1.090±0.302	0.0019[a]
Fibrosis	1.53±1.81	3.000±0.444	0.0061[a]
PLT (x10^4/μl)	20.40±9.80	15.90±8.01	0.1849
ALT (IU/l)	41.40±28.20	46.50±26.50	0.6151
AFP (ng/ml)	1,302±3,988	13,508±24,412	0.0984
PIVKA-II (mAU/ml)	16,672±36,142	923±1,250	0.0738

NBNC-HCC, HCC without HBsAg and HCV-Ab; HV-HCC, hepatitis virus-related HCC; PLT, platelets; ALT, alanine transaminases; AFP, α-fetoproteins; PIVKA-II, prothrombin induced by vitamin K absence or antagonist-II. Data are the mean±SD. [a]p<0.05.

Table II. Clinical data of NBNC-HCC between occult HBV-infected cases and others.

	Occult HBV in NBNC-HCC	Non-occult HBV in NBNC-HCC	P-value
Cases (no.)	7	12	
Age (years)	65.7±11.4	70.10±6.45	0.2925
Tumor size (mm)	84.7±46.3	70.2±10.2	0.4335
Activity	0.20±0.22	0.615±0.137	0.1284
Fibrosis	1.170±0.672	1.540±0.457	0.6530
PLT (x10^4/μl)	24.40±3.91	18.50±2.68	0.2341
ALT (IU/l)	38.5±33.0	42.8±27.2	0.7690
ICG-R 15% (%)	11.30±1.62	17.80±2.01	0.0199[a]

PLT, platelets; ALT, alanine transaminases; ICG, indocyanine green test. Data are the mean±SD. [a]p<0.05.

ment using 20 reference strains was performed with Clustal X software. The HBV genotype was determined by phylogenetic analysis using the Molecular Evolutionary Genetics Analysis 4 (MEGA4) software program (available at http://www.megasoftware.net) (11).

Statistical analysis. Statistical analysis was carried out using JMP7 software (SAS Institute Japan, Co., Ltd.). P<0.05 was considered significant.

Results

Clinical data. The etiology of the HCC cases is illustrated in Fig. 1. Seven cases (22%) were positive for HBsAg, 6 (19%) were positive for HCV-Ab and 19 (59%) were negative for both HBsAg and HCV-Ab. Clinical data in the hepatitis virus-related HCC (HV-HCC) cases were compared with those in NBNC-HCC (Table I). The activity and fibrosis scores of the background liver tissues in HV-HCC were higher than those in NBNC-HCC. The average diameter of the cancer in HV-HCC was smaller than that in NBNC-HCC.

Occult HBV infection in NBNC-HCC. To examine occult HBV infection, HBV-DNA was assessed by PCR analysis from extracted liver tissue in NBNC-HCC. The total characteristics of occult HBV were clinically compared with those of non-occult HBV cases (Table II). No significant difference, excluding the indocyanine green test, was detected between the two groups.

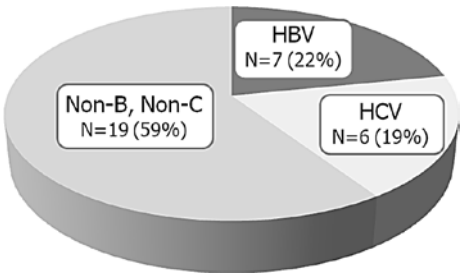

Figure 1. Serological background of surgically operated HCC. Seven cases (22%) were positive for HBsAg, 6 (19%) were positive for HCV-Ab and 19 (59%) were negative for both HBsAg and HCV-Ab.

HBV-DNA in the pre-S/S region was amplified and detected in 4 out of 7 cases (57%); the X region was detected in 4 cases (57%), and the core promoter/pre-core region was detected in 3 cases (43%). Mutation of codon 38 was detected in 1 case (12,13). C1653T in the enhancer box α-region was

Table III. Mutation/variation of occult HBV infection.

Case no.	HBsAg	Pre-S/S	X region		BCP region
			C1653T	T1753V	A1762T/G1764A
2	ND	ND	T	T	T/A
3	ND	ND	T	T	T/A
5	+	+	T	T	T/A
6	+	4-AA insertion	ND	ND	ND
11	-	+	ND	ND	ND
12	ND	+	ND	ND	ND
16	+	ND	C	T	ND

BCP, basic core promoter; ND, not determined; AA, amino acid.

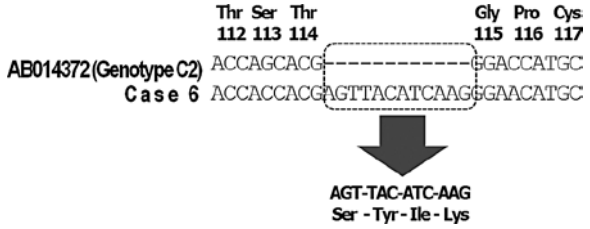

Figure 2. Alignment of the S genome in NBNC-HCC. A 12-nucleotide insertion (4-amino acid insertion) was detected in case no. 6. The nucleotide sequence was aligned and compared with the reference genotype C strain from Japan.

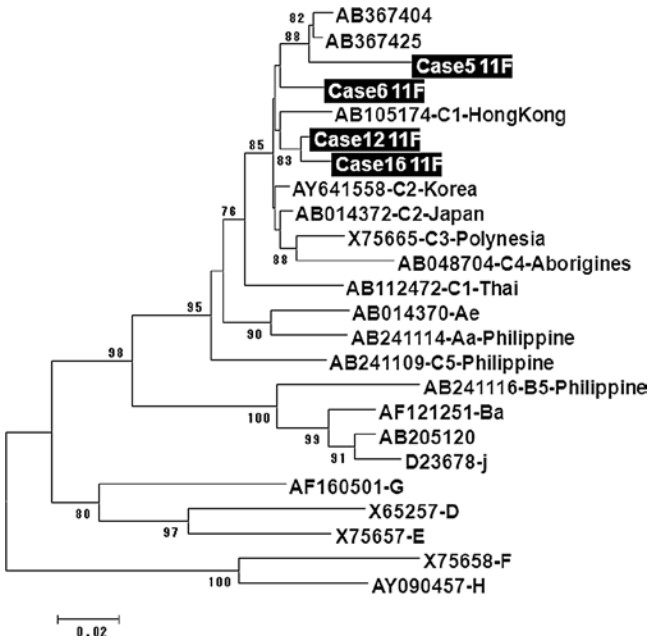

Figure 3. Phylogenetic analysis of HBV-DNA in NBNC-HCC. The HBV genotype was determined by phylogenetic analysis based on the Pre-S/S region. The nucleotide sequence was amplified and compared with 20 reference sequences. All 4 cases were grouped into genotype C.

found in 3 out of 4 cases, and A1762T/G1764A in the basic core promoter region was detected in all 4 cases (Table III). A 12-nucleotide insertion (4-amino acid insertion) in the S region was noted in 1 case (Fig. 2). Phylogenetic analysis showed that all 4 cases were grouped into genotype C (Fig. 3).

HBcAb was serologically examined in 12 out of 19 NBNC-HCC cases. Five out of 12 cases (42%) were positive for HBcAb, which were thought to have been previously infected and resolved naturally.

Discussion

Liver resection for HCC is a potentially curative treatment. However, it has been reported that resection is possible in only 30-35% of the HCC cases evaluated for surgical therapy due to co-existing cirrhosis or the multicentric generation of HCC (14). NBNC-HCC is estimated to comprise 10% of HCC in Japanese patients. In this study, 19 out of 32 resected HCC cases (56%) were diagnosed as NBNC-HCC. It has been suggested that cases of hepatic fibrosis of HCC with hepatitis viral infection are usually more severe than those of NBNC-HCC. In this study, HCC of more than 5 cm in diameter was present in 13 out of 19 cases (68%) of NBNC-HCC. In addition, the average diameter of NBNC-HCC was larger than that of HV-HCC. It is thought that HV-HCC is diagnosed at an early stage, since patients with chronic infection of hepatitis virus are regularly examined. It was recently reported that the prevalence of non-alcoholic steatohepatitis is increasing among NBNC-HCC cases (15). In this study, 5 cases (26%) showed impaired glucose tolerance, and 3 cases (16%) were obese. Thus our findings indicated that abnormal glucose tolerance was associated with hepatocarcinogenesis in this study.

Occult HBV infection is diagnosed when HBsAg in serum is negative and HBV-DNA in serum or liver is positive, regardless of the positivity for anti-HBc. It is still controversial whether or not occult HBV infection is associated with liver damage. Althought it was reported that occult HBV infection does not progress to severe liver disease (16), another study showed that occult HBV infection caused liver disease progression in cases of chronic infection of HCV (17,18). In this study, the activity and fibrosis scores in the HCC cases with occult HBV infection were lower than those in HV-HCC. Thus, our data indicated that occult HBV infection is not related to the progression of liver disease. As for hepatocarcinogenesis, it is also controversial whether or not occult HBV infection

is associated with HCC. Although it was reported that occult HBV infection is an independent risk factor for carcinogenesis in patients with HCV (19,20), another study showed that occult HBV infection was not related to hepatocarcinogenesis (21).

Several studies have demonstrated that the basic core promoter region in the HBV genome is associated with hepatocarcinogenesis (22,23). In Japan, it was reported that C1653T and T1753V are associated with HCC (24,25). Although T1753V was detected in only 1 case, C1653T and A1762T/G1764A were relatively common in this study. It was also reported that a mutation of the X region is associated with HCC (12,13), but the codon 38 mutation was detected in only 1 case and no mutation was found in codon 31 in the present study. It is probable that the mutation/variation in occult HBV infection in relation to HCC is different from that in HBsAg-positive cases. A recent study showed that virological factors of HBV related to HCC are different between occult HBV-infected and HBsAg-positive patients, and G1721A, M1I and Q2K in the pre-S2 gene might be useful viral markers for HCC in occult HBV carriers (24).

In this study, a 12-nucleotide insertion (4-amino acid insertion) between nt 114 and 115 in the S region was found in 1 case (Fig. 2). We considered that the α-loop region in the S region was important for the conformation of the S antigen, and amino acid substitutions at 122, 123, 126, 141 and 145 were found in the vaccine-escaping mutant. It was possible that the insertion caused the conformational change to the S antigen, and this resulted in the serological HBsAg negativity in this case. It has also been reported that several insertions in the HBx and Pre-S2 regions are associated with HCC (25,26), but there were no such mutations identified in this study.

In the present study, phylogenetic analysis revealed that all cases of occult HBV infection were grouped into genotype C. Since HBV genotype C is the most prevalent in Japanese patients, it is reasonable that occult HBV in Japan is derived from genotype C.

In conclusion, HBV-DNA was frequently found in the NBNC-HCC tissues. No significant difference was noted between occult HBV and other forms of infection among the NBNC-HCC cases. Therefore, further investigation is necessary to assess whether occult HBV infection is related to hepatocarcinogenesis.

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