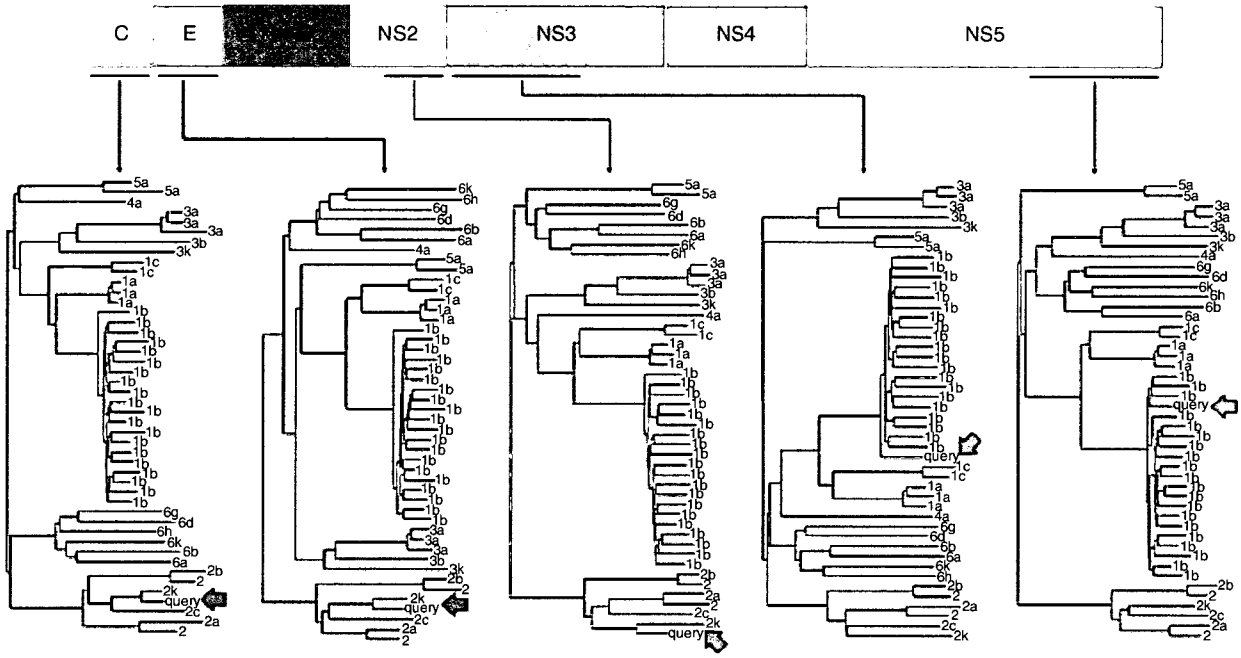

Figure 6 Result of genome typing of the recombinant hepatitis C virus genome: Horizontal lines below the locus map show the regions that were used as five queries. Each phylogenetic tree, which is estimated by genome typing service, shows the relation among the query (block arrows) and fragments whose genome types are well defined.

components and viewers can be shared among the master databases, making it possible to simplify the overall system structure.

Users may need multiple-step procedures in their data analyses. For example, genotyping requires mapping to the genome, the extraction of reference sequences, multiple alignment, genetic distance and phylogenetic tree estimation, and sometimes bootstrap resampling. The genotyping service in the HVDB is designed to eliminate this laborious process. It provides users with an easy-to-use analysis pipeline to estimate a genotype from a sequence fragment specified as a query.

Usage flow

Users can retrieve and analyze data in various ways as shown in Figure 7. Users who want data of the specific locus or region can retrieve them by selecting a region in "map viewer". Users who want data of the specific genotype or phylogenetic cluster can retrieve them by selecting a node in "tree viewer". The retrieved data can also be downloaded as GenBank or FASTA format and then be used by other software beyond the HVDB.

When users do their own analyses of the retrieved data, the data are compiled into a private division by selecting a corresponding option in the viewers. When users want to analyze their own data with published ones in the HVDB, they may upload their data and then merge the data into a private division retrieved from the HVDB as described above. Once private divisions are made, they can be phylogenetically analyzed by various algorithms and parameters, and the result prepared in the HVDB can be viewed by viewers.

DISCUSSION

RECENTLY, TWO HCV sequence databases have been opened to the public; one in the USA[23] and another in France.[24] The US database was constructed by collecting HCV sequences from INSDC and adding some information about patients to them. The French database is made up of HCV sequences of INSDC, the SRS (Sequence Retrieval System) keyword query tool, and sequence analysis tools. While neither database includes data of hepatitis virus other than HCV, the HVDB includes data of HBV and HEV, as well as HCV. The other property of the HVDB is that it has the precomputed "map information" and phylogenetic trees, which the US and French databases do not. The

Table 2 Number of entries that are contained in each master database. Each number shows the number of entries that cover whole area of the corresponding locus

Virus	Total entries	Locus	Nucleic acids	Amino acids
HCV	44 709	Whole genome	178	
		C	901	865
		E1	1456	1377
		NS1/E2	376	359
		E2	414	394
		p7	462	390
		NS2	370	351
		NS3	314	305
		NS4	284	275
		NS4a	545	472
		NS4b	284	275
		NS5	178	173
		NS5a	812	797
		NS5b	180	175
HBV	11 895	Whole genome	1009	
		P	1015	793
		X	1239	954
		C	1736	1444
		pre-C	1534	860
		S	2662	2488
		pre-S1	1033	635
		pre-S2	1287	893
HEV	1 611	Whole genome	77	
		ORF1	77	76
		ORF2	89	82
		ORF3	87	84

HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus, ORF, open reading frame.

information enables users to obtain data of the specific locus or genotype very easily. As a functional property, the HVDB provides the genotyping service. Although the US database has a similar function, users have to process all the steps of analyses manually. On the other hand, the genotyping service of the HVDB can process the analyses automatically once users set their queries and parameters.

Enhancement plan

There are several other hepatitis-related viruses; the major ones are hepatitis A virus (HAV) and hepatitis D virus (HDV). To enrich HVDB, we are now preparing the HAV and HDV master databases in the same manner as HCV (linear genome) or HBV (circular genome).

So far the genotyping service can only be used for HCV sequences, since the strict classifications of other viruses are still arguable. We will add the service of other viruses once a consensus of classification of each virus is reached.

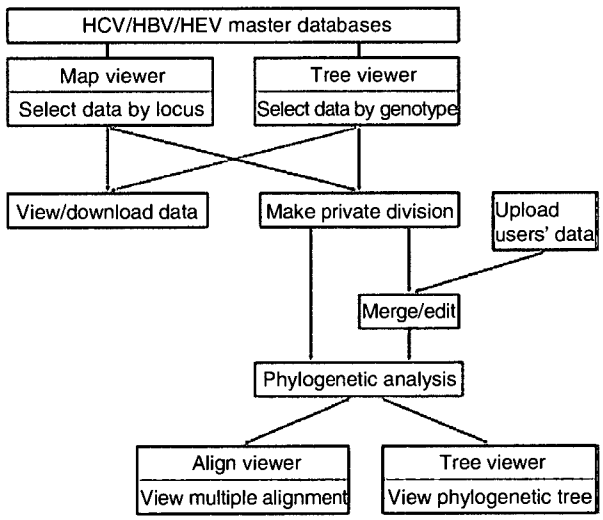

Figure 7 Flow chart of the hepatitis virus database usage. Dashed arrows show steps of data retrieval, and the solid arrows show steps of data analyses. HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

In the field of vaccine and drug development, it is highly important to refer to the tertiary structure of a target protein. Therefore, we are now designing a tool to handle the tertiary structure data of virus proteins as a new feature. At present, some structural data of the NS5b (RNA polymerase) region of the HCV protein is available through the Protein Data Bank (PDB). We will make a library of the putative structure of the NS5b region by using PDB entries as templates and homology modeling as an algorithm. The structure data will be linked to the relevant sequence and annotated data, and they will be viewable on the WWW using a 3-D structure viewer.

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Classification of Hepatitis B Virus Genotype B into 2 Major Types Based on Characterization of a Novel Subgenotype in Arctic Indigenous Populations

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Hepatitis B virus genotype B (HBV/B) has been classified into 5 subgenotypes. Except for Bj/B1 in Japan, the subgenotypes (Ba/B2–B5) have undergone recombination with HBV/C in the core promoter/precore/core genomic region. Phylogenetic analyses of complete sequences show that the Arctic strains belong to a novel subgenotype (HBV/B6) without the recombination, analogous to what is seen with Bj/B1. Comparison of 50 HBV/B6 carriers from the Arctic versus 50 Bj and 50 Ba age- and sex-matched carriers from Asia revealed that clinical characteristics of HBV/B6 carriers were similar to those of Bj/B1 carriers in Japan. The results suggest that HBV/B may be classified into nonrecombinant (Bj/B1 and B6) and recombinant (Ba/B2–B5) types.

Hepatitis B virus (HBV) infection affects >350 million people and is one of the major causes of acute and chronic liver diseases in the world. The majority of acute HBV infections are self-limiting, whereas chronic HBV infection can cause chronic hepatitis (CH), liver cirrhosis (LC), or hepatocellular carcinoma (HCC). Eight major genotypes (and their subgenotypes) of HBV have been classified by molecular evolutionary analyses and have been designated "A"–"H" [1, 2].

HBV genotype B (HBV/B) can be divided into 2 major geographically distinct groups, provisionally designated "Bj" ("j" for "Japan") and "Ba" ("a" for "Asia") [3, 4]. Our previous molecular evolutionary analyses have shown that HBV/Ba includes, first, 3 subgenotypes B2–B4 [3, 5] and, later, a fourth, B5 [4]. Distinguishing themselves from the HBV/Bj/B1 subgenotype, the HBV/Ba/B2–B5 subgenotypes demonstrate evidence of intergenotypic recombination with HBV/C, in a genome part corresponding to the core promoter/precore/core (Cp/preC/C) genomic region [5]. Clinically, HBV/Ba infection has been found, in many Asian countries, such as Taiwan, to be associated with a higher risk of development of HCC in HBV carriers [6]. By contrast, in Japan, HCC has less commonly been associated with subgenotype Bj [7]. In addition, the prevalence of hepatitis B e antigen (HBeAg) in persons infected with HBV/Bj has been found to be lower than that in persons infected with HBV/C or HBV/Ba [7]. With regard to the geographical distribution of HBV/Bj without recombination, studies to determine whether this subtype occurs outside Japan have not been conducted.

Studies performed in several Arctic regions and countries, including Alaska, Canada, and Greenland, have indicated that HBV is also endemic in their indigenous populations. High rates of HCC have been found in Yupik Eskimos and Inuit, in both Alaska and Greenland [8]. A recent molecular epidemiological study of HBV in indigenous Alaskans has identified HBV/B in Yupik Eskimos in southwestern Alaska [9]. However, thus far there are no reports describing HBV/B-associated HCC in indigenous Alaskans. Two independent groups have identified HBV/B in hepatitis B surface antigen (HBsAg)–positive Inuits from Baker Lake, Canada, and from Greenland and have reported a low prevalence of HBeAg and low levels of HBV DNA in these HBV carriers [10]. Assuming that it is possible that HBV/B was carried from Asia to the Arctic during the time of human migration, we sought, in the present study, to determine whether the HBV/B strains found in the Arctic were similar to those endemic to Japan. Furthermore, we sought to compare clinical and virological features of the HBV/B strains circulating in the Arctic and Asia.

Materials and methods. During 2005, 31 HBV/B-infected carriers were enrolled in Alaska, 11 HBV/B carriers were en-

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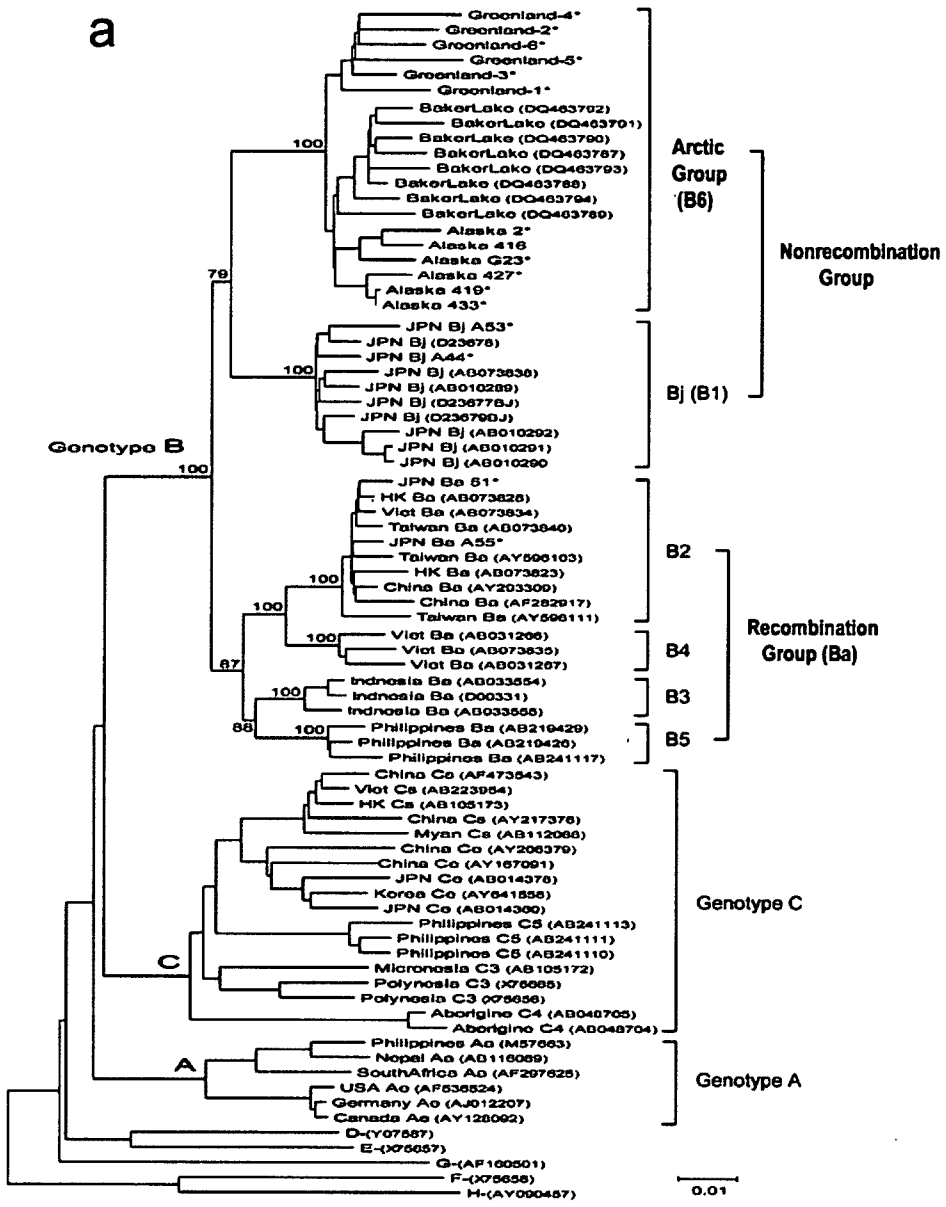

Figure 1. *a*, Phylogenetic tree based on complete genome sequences. The following 62 representative sequences were retrieved from databases: 8 HBV/Bj (B1) strains, 8 original HBV/Ba (B2) strains, 3 HBV/B strains from Indonesia (HBV/B3), 3 HBV/B strains from Vietnam (HBV/B4), 3 HBV/B strains from Philippines (HBV/B5), 8 HBV/B strains from Baker Lake, Canada, 5 HBV/Cs (C1) strains, 5 HBV/Ce (C2) strains, 3 HBV/C3 strains, 2 HBV/C4 strains, 3 HBV/C5 strains, and 11 HBV strains representative of the other 6 genotypes (i.e., Aa/A1, Ae/A2, and D–H). The present study found 16 strains, which are indicated by an asterisk (*): 6 strains in Alaska, 6 strains in Greenland, 2 HBV/Bj (B1) strains in Japan, and 2 HBV/Ba (B2) strains in Japan. Each representative strain from the database is identified with accession numbers, followed by the subgenotype and the country of origin (Hong Kong [HK], Japan [JPN], and Vietnam [Viet]). The genetic distance was estimated with the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/). The numbers in the tree indicate bootstrap reliability by the interior branch test. The length of each horizontal bar on the left indicates the number of nucleotide substitutions per site. *b*, Phylogenetic tree for all HBV strains shown in panel *a*, reconstructed by use of recombinant sequences, in a part of the core-promoter/precore/core genomic region (nt 1740–2443), of all HBV/B (B1-B6) strains. Reference sequences from the databases are identified as outlined in panel *a*.

rolled at Sisimiut in western Greenland, and 8 HBV/B carriers were enrolled at Baker Lake, Nunavut (Canada). All samples from Alaska and Baker Lake were obtained through a serosurvey for HBV and were from participants who had an average of 20 years of follow-up. The samples from Greenland were from a serum bank created during a large population serosurvey in 1998 in Sisimiut in western Greenland. All participants were members of the indigenous populations in their respective regions. The

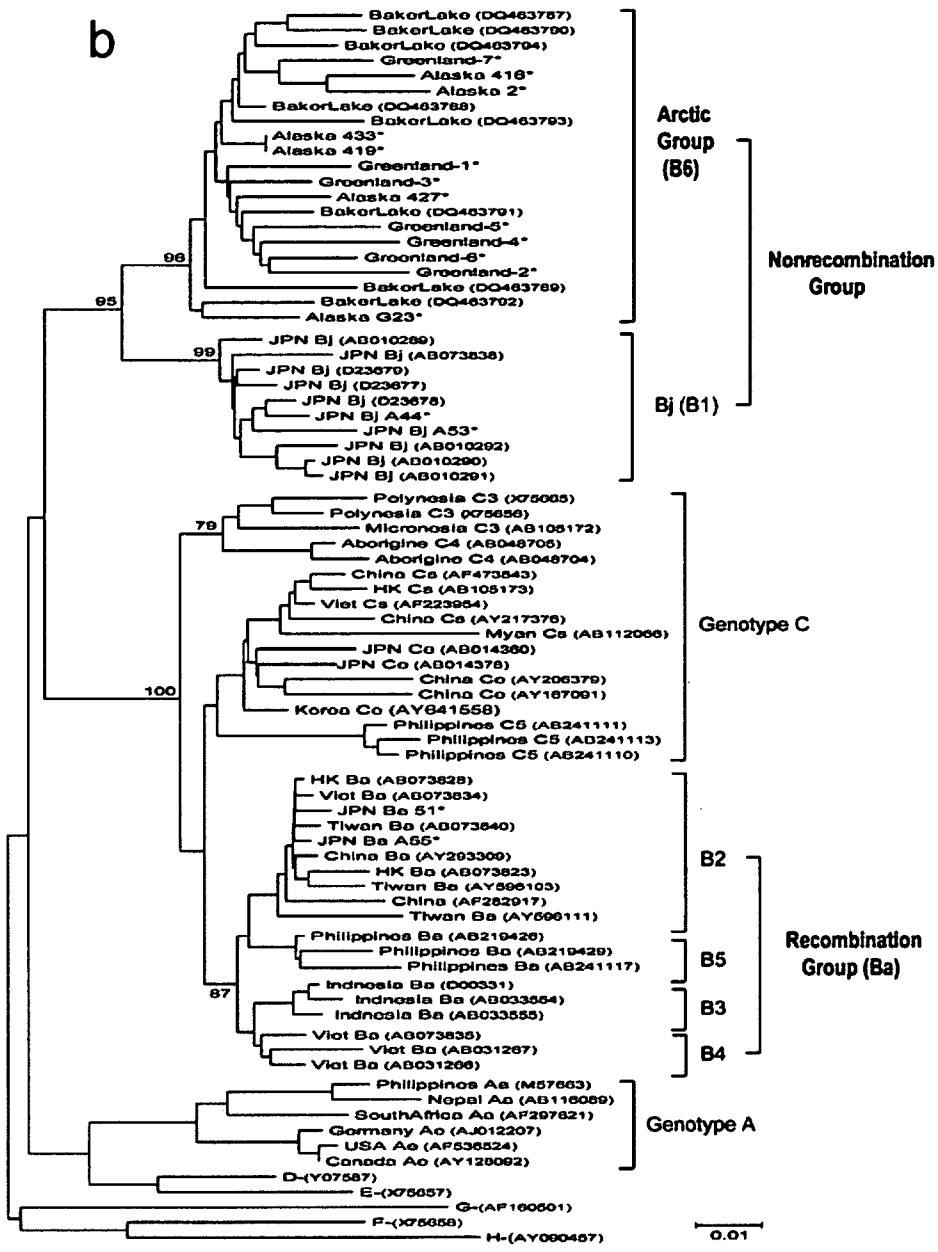

Figure 1. *(Continued)*

participants were classified into 3 clinical groups: (1) asymptomatic carriers, (2) patients with chronic liver disease, and (3) patients with LC or HCC, all of whom were diagnosed on the basis of clinical findings and diagnostic imaging. None of the participants was coinfected with either hepatitis C virus or human immunodeficiency virus. No patients had received antiviral treatment. The study protocol was approved by the ethics committees of the participating institutions, in accordance with the 1975 Declaration of Helsinki, and informed consent was obtained from each participant before they underwent any study-related procedures.

The complete sequences of HBV/B, as well as clinical data on the 8 carriers in the indigenous population of Baker Lake, have been reported elsewhere [10]. A total of 16 complete genomes (12 B6, 2 Bj/B1, and 2 Ba/B2) and 30 partial HBV genes bearing the enhancer II (EnhII)/Cp/preC/C genomic regions (nt 1611–2009) were amplified by polymerase chain reaction (PCR) using several primers sets [3]. The sequences reported herein have been deposited in the GenBank/DDBJ/EMBL databases, under the following accession numbers: Alaska433 (AB287314), Alaska419 (AB287315), Alaska427 (AB287316), Alaska416 (AB287317), Alaska2 (AB287318), AlaskaG23 (AB287319),

Greenland-1 (AB287320), Greenland-2 (AB287321), Greenland-3 (AB287322), Greenland-4 (AB287323), Greenland-5 (AB287324), Greenland-6 (AB287325), JPN Bj A44 (AB287326), JPN Bj A53 (AB287327), JPN Ba 51 (AB287328), and JPN Ba A55 (AB287329).

Available clinical and serological data on participants persistently infected with HBV/Bj and HBV/Ba (50 participants for each) were included in the study. The control specimens represent a randomized selection from samples previously investigated in our laboratory, to provide samples from individuals who are age and sex matched to the 50 HBV/B carriers from the Arctic. All HBV/Bj and 10 HBV/Ba samples were obtained from hepatitis B carriers living in Japan [11]; the remaining 40 HBV/Ba carriers were from Hong Kong and China [5, 12].

Statistical differences were evaluated by the Mann-Whitney nonparametric test, Fisher's exact probability test, χ^2 test with Yates's correction, and Student's t test, as appropriate. $P < .05$ was considered to be statistically significant.

Results and discussion. A total of 42 HBV/B strains, including 31 from Alaska and 11 from Greenland, were successfully amplified in their EnhII/Cp/preC/C genomic regions, spanning 398 bp, and were compared with genotype B strains previously deposited in GenBank/DDBJ and with other selected references for other genotypes. All of these 42 Arctic HBV/B strains formed a common phylogenetic cluster along with the 8 strains from Baker Lake, with a 100% bootstrap index (data not shown). The complete genome sequences were obtained from 12 of the 42 HBV/B strains (6 of the 31 Alaskan strains and 6 of the 11 Greenland strains). As shown in figure 1*a*, a phylogenetic analysis of the complete genome sequences from the samples obtained from the Arctic and of those from Japan and Asia revealed 6 distinct clusters within HBV/B that were supported by a 100% bootstrap resampling index; 5 of these clusters were previously designated as "Bj/B1," "Ba/B2," "Ba/B3," "Ba/B4" and "Ba/B5," according to recent proposals regarding HBV nomenclature [2], and the sixth (unclassified) cluster consisted of the 6 HBV/B strains from Alaska, 6 HBV/B strains from Greenland, and 8 HBV/B strains from Baker Lake. This sixth cluster was tentatively designated "B6" and was closely related to HBV/Bj/B1, having bootstrap values exceeding 75%, and had a branch more distant from the ancestral point, suggesting that the group has diverged from the other subgenotypes B (i.e., B1–B5) and has evolved independently.

The most significant virological feature of the HBV/Ba/B2-B5 strains is the presence of recombination with genotype C in part of the Cp/preC/C genomic regions, whereas HBV/Bj/B1 strains have no evidence of this recombination [3]. To test for the presence of the possible intergenotypic recombination, bootscan analyses were performed with the complete genome sequences. No evidence of recombination was observed in any of the B6 strains, in contrast to what was observed for the HBV/Ba/B2-B5 strains (data not shown). The phylogenetic tree for all of the HBV strains is shown in figure 1 and was reconstructed by use of the partial genomic region corresponding to the Cp/preC/C regions (nt 1740–2443) (figure 1*b*). In this region, HBV/B6 strains were phylogenetically very similar to HBV/Bj strains. A clear separation of HBV/B6 and HBV/Bj/B1 strains from the HBV/Ba/B2-B5 strains, which grouped with HBV/C strains, was observed in the tree, therefore suggesting that all of the HBV/B strains may be classified into 2 major forms: "nonrecombinant" and "recombinant." This is the first report of a novel HBV/B subgenotype without recombination that has been discovered outside Japan.

According to previous reports, HBV/Ba is associated with development of HCC in persons of younger age [6], in striking contrast with HBV/Bj in Japan. The prevalence of HBeAg has previously been demonstrated to be significantly lower in carriers of HBV/Bj than in carriers of HBV/Ba [5]. To clarify the clinical characteristics of HBV/B6, we performed an age- and sex-matched control study to examine clinical and virological differences between the 50 HBV/B6 carriers and the control subjects (consisting of 50 HBV/Bj carriers and 50 HBV/Ba carriers) [5, 11, 12]. Table 1 summarizes comparative data on HBeAg status, levels of HBV DNA, levels of alanine transaminase (ALT), and clinical findings. In the present study, the level of HBV DNA was defined as high when it was >5 log IU/mL, because this value is known to be predictive for disease progression. The prevalence of HBeAg was significantly lower in carriers of HBV/B6 or HBV/Bj than in carriers of HBV/Ba ($P = .0026$ and $P = .0143$, respectively), whereas the levels of HBV DNA and of ALT were significantly higher in carriers of HBV/Ba than in carriers of HBV/B6 or HBV/Bj ($P < .001$). Seroconversion, at an older age, from positivity for HBeAg to the presence of antibodies against HBeAg; high levels of DNA; and high levels of ALT may be related to the risk of more-severe liver disease [13]. Indeed, when we compared the clinical findings for the 3 HBV groups (HBV/B6 vs. Bj vs. Ba), in whom the mean age was 48 years, the proportion of asymptomatic carriers was significantly higher in the HBV/B6 group than in the HBV/Ba group ($P = .0001$), whereas patients with LC and/or HCC were more prevalent in the HBV/Ba group than in the HBV/B6 group or the HBV/Bj group ($P < .0001$ and $P = .0214$, respectively). These results support previous reports that HBV/Ba infection may be a risk factor for the development of HCC in Taiwanese HBV/Ba carriers <50 years of age [6]. In contrast, HBV/Bj was rarely found in Japanese patients with HCC, and HBV/Bj-associated HCC was observed mainly in elderly persons (mean age, 67 years) [5, 14].

In the participants infected with HBV/B6, we looked for mutations, particularly the basal core-promoter mutation (T1762/A1764), which previously had been found to be associated with HCC [15]. It has been reported that, in Japan, this mutation is found less frequently in HBV/Bj/B1 strains (13%) than in HBV/Ba strains (33%) [5]. In Taiwan and Hong Kong, the frequency of the T1762/A1764 mutation in HBV/Ba carriers has

Table 1. Results of age- and sex-matched case-control study of clinical differences between HBV/Bj, HBV/Ba, and HBV/B6.

Feature	HBV/B6 (n = 50)	HBV/Bj (n = 50)	HBV/Ba (n = 50)	P
Male sex	33 (66)	34 (68)	36 (72)	Matched
Age, mean ± SD, years	48.1 ± 19.6	48.1 ± 16.9	47.9 ± 13.1	Matched
Hepatitis B e antigen	6 (12)	8 (16)	20 (40)[a]	<.02
DNA >5 log copies/mL	9 (18%)	18 (36)	36 (72)[b]	<.001
Alanine transaminase	40.3 ± 36.3	43.1 ± 33.4	94.0 ± 94.1[c]	<.001
Clinical state				
Asymptomatic	35 (61)[d]	22 (44)	15 (30)	<.02
Chronic hepatitis	15 (30)	24 (50)	21 (42)	NS
Liver cirrhosis/hepatocellular carcinoma	0	4 (8)	14 (28)[e]	<.03

NOTE. Data are no. (%) of participants, unless otherwise indicated. HBeAg, hepatitis B e antigen; NS, no significant difference.

[a] For B6 vs. Ba, P = .0026; for Bj vs. Ba, P = .0143.
[b] For B6 vs. Ba, P < .0001; for Bj vs. Ba, P = .0006.
[c] For B6 vs. Ba, P = .0006; for Bj vs. Ba, P = .0005.
[d] For B6 vs. Ba, P = .0001; for B6 vs. Bj, P = .0154.
[e] For B6 vs. Ba, P < .0001; for Bj vs. Ba, P = .0214.

been reported to be 29% and 46%, respectively [15]. The present study has revealed that the prevalence of the T1762/A1764 mutation is quite low (4.0%) in HBV/B6 carriers, occurring at a frequency similar to that in age- and sex-matched HBV/Bj/B1 carriers.

The precore stop mutation (A1896) that inhibits translation of the HBeAg precursor and induces an HBeAg-negative phenotype has been reported more frequently in HBV/Bj/B1 carriers than in HBV/Ba carriers [5]. In the present study, the prevalence of the A1896 mutation was very high (88%) in HBV/B6 carriers. Thus, within the Cp/preC regions, the virological features of HBV/B6 strains were found to be similar to those of HBV/Bj/B1 strains, providing further evidence of the relatedness of these strains.

In the phylogenetic analyses, HBV/B6 was found to have 3 independent clusters, each of which was associated with 1 of the 3 groups of infected indigenous participants from the 3 geographical regions—Yupik Eskimos from Alaska in the United States, Inuit from Baker Lake in Canada, and Inuit from Sisimiut in western Greenland—but they all were found to belong to the same cluster, with significant bootstrap values. The majority of individuals living in each of these regions are members of its indigenous population. These native peoples of the Arctic share many genetic characteristics with Asians and, like the Japanese people, are believed to have originated from peoples of Mongolian ancestry. Historically, the first Eskimos migrated from Siberia to Alaska via the Bering Land Bridge ~10,000 years ago, and 1000 years ago the forefathers of present-day Inuit spread eastward from northern Alaska to inhabit Arctic Canada and Greenland. These results suggest that the original HBV/Bj/B1 strain in Japan might be an ancestor of the novel HBV/B6 strain in the Arctic and that it might have been carried by indigenous groups when they migrated to North America and Greenland.

In conclusion, we have found a novel subgenotype, "B6," of HBV/B in indigenous Arctic populations with chronic liver disease. Our results demonstrate that clinical and virological characteristics of the HBV/B6 strain are similar to those of the HBV/Bj/B1 strain but different from those of the Ba/B2-B5 strains. We believe that the presence of the intergenotypic recombination affecting basal core-promoter and precore/core genes is a key element in these differences. Thus, we recommend that HBV/B be considered to be a genotype present in 2 major forms: a nonrecombinant type (Bj/B1 and B6) and a recombinant type (Ba/B2-B5). Further prospective studies and case-control studies in the Arctic, with larger cohorts of patients infected with HBV/B6, should be designed to further evaluate the clinical manifestations of HBV/B6 infection.

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Case Report

Japanese case of hepatitis B virus genotypes C/D hybrid

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Hepatitis B virus (HBV) is classified into eight genotypes based on complete genome sequence. Each genotype is related to geographic distribution and race. In Japan, most of the genotypes are B and C. In the present study, we report the first Japanese strain of HBV having a recombination between genotypes C and D. A 30-year-old woman was admitted to Kobe Medical Center because of liver dysfunction. She was diagnosed with spontaneous reactivation of chronic hepatitis B. She had no history of blood transfusion and her parents were negative for HBV. The phylogenetic analysis based on the complete genome sequences revealed that this strain was classified into genotype C, whereas the analysis based on S gene sequence showed that this strain was genotype D. By using a SimPlot program, this strain was confirmed as a recombinant strain between genotypes C and D. Compared with previous recombinant strains in China, the breakpoint was the same and the difference was only 0.8% of the complete genome sequence. It was unclear whether or not this strain was transmitted from China, but the recombinant strains and intergenotypes of HBV have already existed in Japan.

Key words: genotype, hepatitis B virus, recombination

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection affects more than 350 million people worldwide. HBV has been classified into eight genotypes, A–H, based on the nucleotide difference of more than 8% of the entire genome.[1] The HBV genotypes have distinct geographic distributions.[2] In Japan, the most common is genotype C, and genotype B is the second most common. Genotype D from Japan is quite rare and the clinical characterization is still unclear.[3]

Recently, on the basis of molecular evolutionary analyses using complete genomic sequences, several strains having recombination were reported. In particular, subgenotype Ba, which was determined as a recombinant strain between genotype C and genotype B, is common in South-east Asia.[4] Here, we report the first Japanese case of chronic hepatitis B with a recombination between genotypes C and D.

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CASE REPORT

Patient

A 30-YEAR-OLD Japanese woman was identified with liver dysfunction and admitted to Kobe Medical Center in April 2006. In early April 2006, she found skin eruptions in her entire body and consulted a dermatologist. Allergic dermatitis was suspected and she was treated with anti-allergy drugs. The symptoms soon improved but, at the same time, liver dysfunction was accidentally discovered and she consulted our Division of Internal Medicine. She had no fever, abdominal pain nor gastrointestinal symptoms. Her general condition was good and laboratory data were as follows: platelet count $17.9 \times 10^4/\mu L$, prothrombin time 88.5%,

aspartate aminotransferase (AST) 219 IU/L, alanine aminotransferase (ALT) 415 IU/L, γ-glutamyltranspeptidase (γ-GTP) 26 IU/L, hepatitis B surface antigen (HBsAg) 93.2 cutt-off index positive, hepatitis Be antigen (HBeAg) > 99% positive, antibody to hepatitis B core antigen (anti-HBc) 94.4% positive. Antibody to hepatitis Be antigen (anti-HBe) and anti-immunoglobulin (Ig)M-HBc antibody were negative. She was born in Japan and had no past history including blood transfusion. She had no history of traveling abroad nor receiving alternative medicine such as Chinese acupuncture. Her parents were not HBV carriers and she had no sexual intercourse other than with Japanese men. She was suspected of spontaneous reactivation of chronic hepatitis B, and treated by resting. However, the liver enzymes were gradually elevated and AST reached 799 IU/L and ALT 1384 IU/L. At the same time, the prothrombin time was gradually prolonged and it was thought that she might have subacute fulminant hepatitis. She rejected any blood transfusion for religious reasons, so antiviral therapy was started and the transaminase level improved. Liver biopsy was not done during her admission.

DNA extraction and direct sequencing

The patient's DNA was extracted from 100 μL serum using a DNA extractor kit (DNA Blood Mini Kit; QIAGEN, Hilden, Germany). To access the complete nucleotide sequences, polymerase chain reaction (PCR) amplifying two overlapping amplicons of the HBV genome was carried out.[5] Amplified fragments were directly sequenced by dideoxy sequencing using Taq Dye Deoxy Terminator cycle sequencing kit with 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA, USA).

SimPlot analysis

Recombination was examined by the SimPlot program (available at http://sray.med.som.jhmi.edu/Raysoft/SimPlot) and bootscanning analysis[6,7] (Fig. 1). The following complete genomes (represented by their accession numbers) were used in SimPlot and phylogenetic analysis: genotype A, *X70185*; genotype B, *D23678*; genotype C, *AY217376, AB105174, AY641558, AB014372, X75665, AB048704, AB241112*; genotype D, *X65257, AY721612, AB210820, AB188242*; genotype E, *X75657*; genotype F, *X75663*; genotype G, *AF160501*; genotype H, *AY090457*. This strain was also compared with previous C/D recombinant strains (*AY817509, AY800249, AY57948, AY57947*). The recombination breakpoint of the Japanese strain was estimated at nt 10 and 799 (Fig. 1).

Phylogenetic trees

The complete genome sequences were compared with those of the 21 reference sequences retrieved from the DDBJ/EMBL/GenBank database. The subtypes of the strains used for comparison were obtained from published articles.[2,8] Sequences were aligned using CLUSTAL X software. Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Fig. 2a).[6] To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. These analyses were carried out using the Molecular Evolutionary Genetics Analysis (MECA) software program (available at http://www.megasoftware.net).[7] This analysis was also carried out based on the region at nt 10 and 799 (Fig. 2b), and the region at nt 800 and 9 (Fig. 2c). Although the phylogenetic analysis based on the complete genome sequences revealed that this strain was classified into genotype C, the analysis based on the region at nt 10 and 799 showed that this strain was classified into genotype D.

Nucleotide differences between the present case and reference strains

Divergences of the present strain with genotype C; *AB014372*, C/D recombinant strain; *AY800249, AY057947*, and genotype D; *AB210820* were also examined. Percentage divergences of complete genome, *Pre-S/S* gene, *X* gene, precore/core (*Pre-C/C*) and *polymerase* genes are summarized in Table 1. Based on the complete genome, this strain had 99.2% identity with the Chinese strain, *AY800249*, although the identity was 96.6% with the Tibet strain, *AY057947*. The percentage divergence of this strain from genotype D in the *Pre-S/S* gene (1.5%) was lower than that from genotype C (5.6%), whereas the difference from genotype D in the *Pre-C/C* gene (9.5%) was higher than that from genotype C (0.9%).

DISCUSSION

THE HBV GENOTYPES have a distinct geographic distribution.[2] While genotype C is common in Australia and Asian countries including Japan, genotype D spreads worldwide, but predominates in the Mediterranean area and North-west Asia. As for genotype C, subgenotype C1 was common in Japan, Korea, and China; C2 in China, South-East Asia, and Bangladesh; C3 in

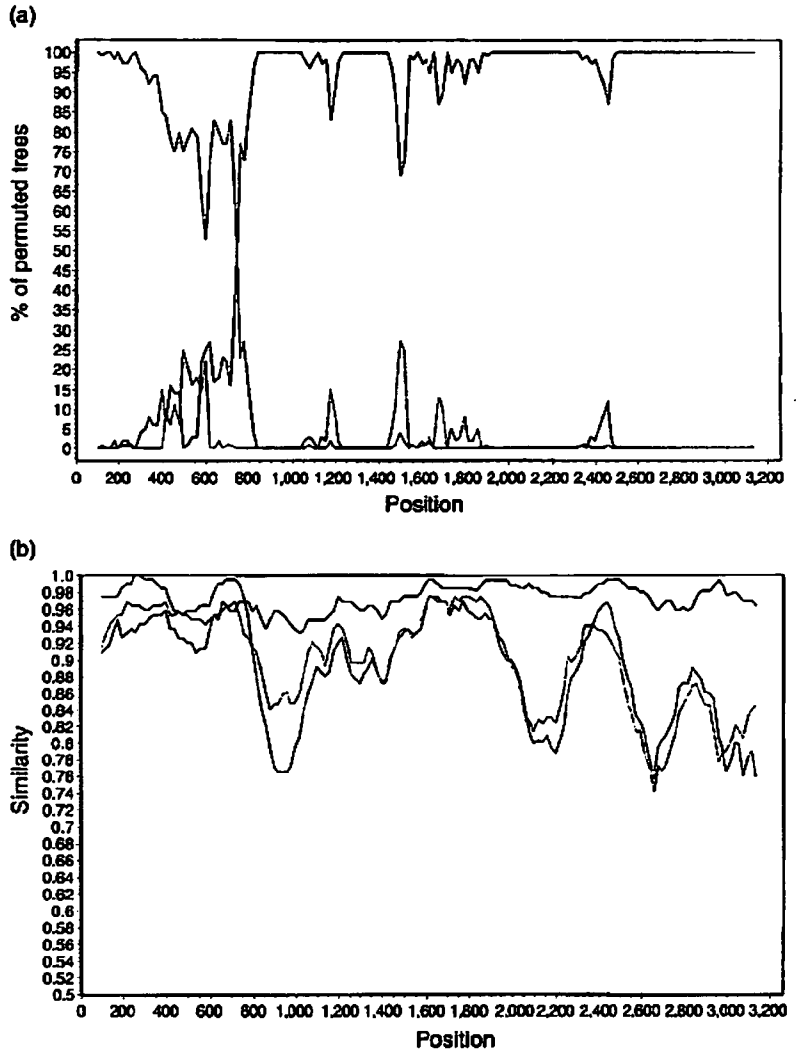

Figure 1 (a) The recombination breakpoint was determined by the SimPlot program. This strain was compared with three reference strains of genotype C (*AB014372*), genotype D (*AB210820*), and outgroup genotype E (*X75657*). (b) Nucleotide similarity comparison of genotypes C and D using SimPlot. (—), C; (—), D; (—), E.

Table 1 Percentage divergences of nucleotide sequences between the present case and reference strains

GeneBank Accession no.	Genotype (Country)	Nucleotide divergences				
		Complete genome	*Pre-S/S* gene	*X* gene	*Pre-C/C* gene	*Pol* gene
AB014372	C (Japan)	3.6	5.6	2.2	0.9	6.5
AY800249	C/D (China)	0.8	1.5	1.1	0.2	0.4
AY057947	C/D (Tibet)	3.4	4.1	3.2	0.2	6.5
AB210820	D (Japan)	9.1	1.5	4.7	9.5	4.7

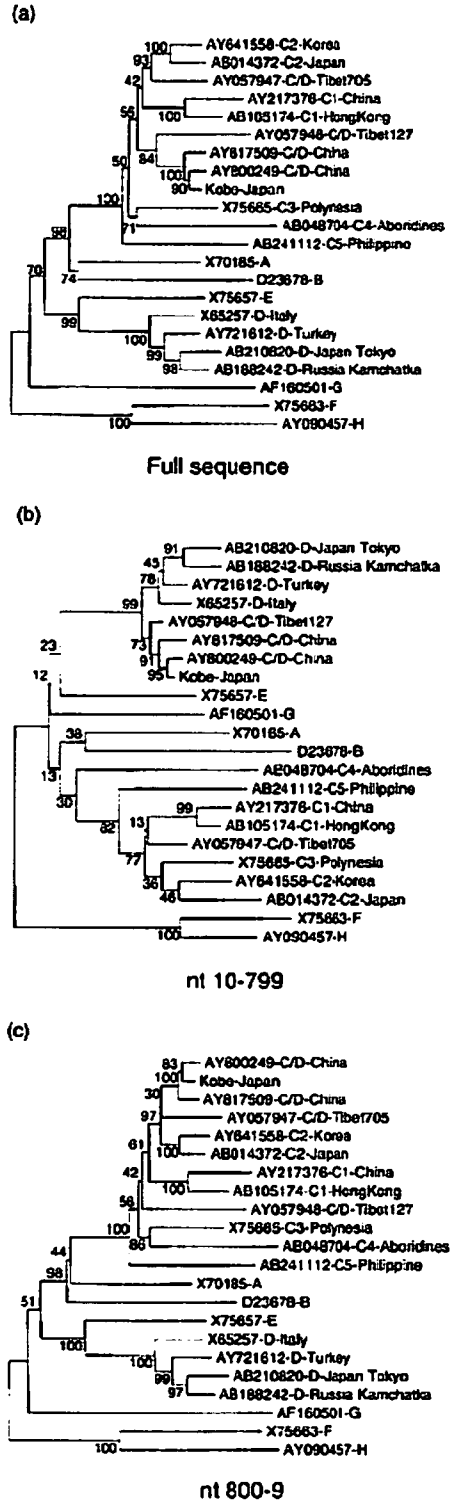

Figure 2 Phylogenetic analysis of the hepatitis B virus strain isolated from Kobe compared with 21 reference strains. The regions included in this analysis were: (a) the complete genome, (b) nt 10–799, and (c) nt 800–9.

Oceania comprising strains specifying adrq-; and C4 specifying ayw3 was encountered in Aborigines from Australia.[7] Genomic recombination between different genotypes of HBV was first described in 1991, and is increasingly reported because of technical progression.[9,10] Several recombinant strains formed between A and D,[11,12] B and C,[11,13,14] C and C,[15] and C and D[16,17] have been reported.

So far, recombinant strains of genotype C/D were reported from China and Tibet, and it is understood that they are the cross-sectional regions of both genotypes. But genotype D in Japan is quite rare and only a few strains were reported in Tokyo and Ehime.[18,19] Based on the analysis of mitochondrial DNA polymorphism, most mainland Japanese are derived from the continental gene flow after the Yayoi Age.[20] But, recently, HBV genotypes from other countries are increasing and, in particular, genotype A is increasing in acute hepatitis B.[21] It has been unclear whether this strain was transmitted from China or originally generated in Japan, but intergenotype recombinants might result from coinfection with different genotypes as recently reported.[22]

The present patient's parents were negative for HBsAg and they have not been checked for anti-HBc antibody. But this strain has no escape mutation in the S region, and was thought not to be transmitted from the parents. Although this patient had no relationship with Chinese individuals, the complete genome revealed that this strain had high similarity with Chinese recombinants (C/D hybrid strains), showing 99.2% similarity of nucleotides. This strain, as well as previous strains from China and Tibet, belonged to genotype C with the recombinant fragments which were genotype D at nt 10–799.[17] It was reported that many recombinant strains have breakpoints in the pre-S1/S2 region, but the recombinant fragments at nt 10–799 were detected only in the recombinant C/D strains.

Many studies have reported differences in the clinical course of HBV infection among different subgenotypes. It was reported from Japan and Taiwan that HBV/C induces a more severe course of disease compared to HBV/B in chronically infected patients.[3,23] Previous reports showed that genomic recombination affected the clinical outcome; subgenotype Ba in Taiwan, which

is a hybrid between genotype C and genotype B, was associated with developing hepatocellular carcinoma in young adults.[24] Meanwhile, it was reported in India that HBV/D was associated with more severe disease than HBV/A, but the clinical features of A/D hybrid strains are unknown. There is a report comparing HBV/C and HBV/D,[25] but no report comparing C/D hybrid and HBV/C or HBV/D. Further cohort studies should be conducted to elucidate the clinical manifestation of the C/D hybrid.

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Journal of Gastroenterology

A weak association between occult HBV infection and non-B non-C hepatocellular carcinoma in Japan

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Background. In Japan, approximately 10% of hepatocellular carcinoma (HCC) patients are negative for both hepatitis B surface antigen (HBsAg) and antibodies to hepatitis C virus (anti-HCV), i.e., they constitute the so-called category of non-B non-C (NBNC) HCC. Little is known about the characteristics of NBNC-HCC. ***Methods.*** Potential risk factors for carcinogenesis (including occult HBV infection [HBsAg is negative but HBV DNA is positive by polymerase chain reaction (PCR)], obesity, and diabetes) were assessed in 233 HCC patients grouped according to hepatitis virus serological status (152 with HCV-HCC, 36 with HBV-HCC, and 45 with NBNC-HCC). ***Results.*** The prevalence of patients with obesity or diabetes was significantly higher in the NBNC-HCC group than in the HBV-HCC group. The same trend was observed even when patients with massive alcohol intake were excluded from the analysis. Only 8 patients (18%) in the NBNC-HCC group had detectable serum HBV DNA, and this was at very low levels (HBV/Ce/C2 and HBV/D were determined in 7 and 1 patients, respectively). In the NBNC-HCC group, the determined nucleotide sequences of the enhancer II/core promoter/precore/core region did not contain any HCC-associated mutations, whereas 25 of 30 patients in the HBV-HCC group carried strains with C1653T, T1753V, and/or A1762T/G1764A mutations. ***Conclusions.*** A weak association between occult HBV infection and HCC development was observed in the NBNC patients. This study indicates that nonalcoholic steato-hepatitis should be further investigated to assess its contribution to HCC development in this category of patients.

Key words: non-B non-C hepatocellular carcinoma, obesity, diabetes, occult HBV

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, with a particularly high incidence in Japan, Southeast Asia, and southern Europe. Documentation of the geographical variation in the incidence of HCC has led to the clear identification of several of the most common risk factors. These include chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) or exposure to aflatoxin.[1] In a previous study from Japan, in most of the patients diagnosed with HCC there was evidence of viral etiology. However, there were also approximately 10% of patients without detectable HBV surface antigen (HBsAg) or antibodies to HCV (anti-HCV) and the disease was defined as non-B non-C (NBNC) HCC.[2]

A number of recent studies have also indicated the increasing significance of such factors as nonalcoholic steato-hepatitis (NASH),[3–5] excessive alcohol intake,[6,7] and occult HBV infection[8,9] in the development of HCC. Occult HBV infection is defined by serologically undetectable HBsAg despite the presence of HBV DNA in serum or liver[8,10,11] Interestingly, a previous report indicated that HBV DNA was integrated into the human genome in NBNC-HCC tissues, suggesting that occult HBV infection might play a role in hepatocarcinogenesis in patients with so-called NBNC.[12] However, another report has demonstrated that levels of HBV-DNA in patients with occult HBV infection are very low, and covalently closed circular HBV-DNA cannot be detected,[13] which makes the role of occult HBV infection in the development of HCC unclear.

In the present study, we compared possible risk factors (such as occult HBV infection, obesity, and diabetes) in patients with NBNC-HCC with these risk factors in patients with HBV- or HCV-related HCC. Furthermore, we investigated the contribution of occult HBV infection to the development of HCC by examining not only the prevalence of occult HBV infection but also

Table 1. Characteristics of patients with non-B non-C HCC, HBsAg-positive HCC, and anti-HCV-positive HCC

	Viral markers (HBsAg/anti-HCV)			
	NBNC-HCC (−/−)	HBV-HCC (+/−)	HCV-HCC (−/+)	Total
Number of patients	45	36	152	233
Sex (male:female)	28:17	31:5	107:45	166:67
Age (years)	65.8 ± 9.0	52.1 ± 10.8*	65.8 ± 7.6	63.7 ± 9.7
History of BTF	4 (9%)**	9 (25%)	55 (36%)	68 (29%)
History of operation	14 (31%)***	14 (39%)	79 (52%)	107 (46%)
Alcohol (>80 g/day)	14 (31%)	10 (28%)	48 (32%)	72 (31%)
Family history of liver disease	3 (7%)	14 (39%)*	9 (6%)	26 (11%)
Obesity (BMI > 25 kg/m^2)	15 (33%)****	5 (14%)	33 (22%)	53 (23%)
Diabetes	17 (38%)	2 (6%)*	39 (26%)	58 (25%)
HBV markers	22 (49%)	36 (100%)*	78 (51%)	136 (58%)
Anti-HBc	18 (40%)	36 (100%)	70/145 (48%)	124/226 (55%)
Anti-HBs	6 (13%)	1 (3%)	22/148 (15%)	29/229 (13%)
HBV DNA	8 (18%)	30/30 (100%)*	8/89 (9%)	46/127 (36%)
Anti-HCV	0 (0%)	0 (0%)	152 (100%)*****	152 (65%)
ALT (U/l)	42.0 ± 35.0**	93.9 ± 91.9	81.9 ± 55.5	76.0 ± 61.7
ALP (U/l)	304.5 ± 146.2	372.8 ± 274.0	296.4 ± 133.8	310.3 ± 168.5
γ-GTP (U/l)	104.4 ± 92.1	88.1 ± 108.3	96.6 ± 85.2	96.2 ± 90.0
Platelets (×10^4/μl)	14.6 ± 8.8	11.7 ± 7.3	10.9 ± 5.6	11.7 ± 6.7
AFP (ng/ml)	9047 ± 33539	2458 ± 7815	1452 ± 7915	3102 ± 16603
Number of patients positive for AFP	20 (44%)**	27 (75%)	106 (70%)	153 (66%)
Tumor number (single:multiple)	30:15	22:14	102:50	154:79
Tumor diameter (mm)	44.0 ± 26.6***	33.9 ± 21.3	28.8 ± 15.7	32.6 ± 20.1
Tumor histology (well:moderate:poorly)	7:21:3	5:14:3	55:69:7	67:104:13

* $P < 0.001$, HBV-HCC vs NBNC-HCC or HCV-HCC; ** $P < 0.05$, NBNC-HCC vs HBV-HCC or HCV-HCC; *** $P < 0.05$, NBNC-HCC vs HCV-HCC; **** $P < 0.05$, NBNC-HCC vs HBV-HCC; ***** $P < 0.0001$, HCV-HCC vs NBNC-HCC or HBV-HCC
Values for age, ALT, ALP, γ-GTP, platelets, AFP, and tumor diameter are expressed as means ± SD

HBV DNA levels in serum, HBV genotypes, and HCC-associated mutations, such as basal core promoter (BCP) double mutations.[14]

Patients and methods

Patients

Serum specimens were collected from 233 patients with HCC during the period between January 2000 and December 2004. Clinical data of each patient were retrospectively retrieved from the clinical record archives. Of the patients, 166 (71%) were male, and the overall mean age was 63.7 ± 9.7 years. The patients were categorized into three groups according to the status of viral hepatitis serological markers: (1) the HBV-HCC group, including 36 patients positive for HBsAg and negative for anti-HCV, (2) the HCV-HCC group, including 152 patients negative for HBsAg and positive for anti-HCV, and (3) the NBNC-HCC group, including 45 patients who were negative for both HBsAg and anti-HCV (Table 1). Patients with both HBsAg and anti-HCV were excluded from the current study. The diagnosis of HCC was established on the basis of histological findings in biopsy specimens or on the basis of radiological findings using ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and/or angiography, as well as clinical biochemical investigations (alpha-fetoprotein [AFP] and/or serum protein induced by vitamin K absence, termed PIVKA-II ["protein induced by vitamin K absence or antagonist II"]).

Methods

The following factors were compared in the above three groups; sex, age, history of blood transfusion, operation, excessive intake of alcohol, body mass index (BMI), presence of diabetes, various laboratory findings, HBV markers (HBsAg, antibody to HBV core [anti-HBc], antibody to HBs [anti-HBs] and HBV DNA [detection limit: 100 copies/ml]), HCV markers (anti-HCV), and characteristics of HCC (tumor numbers, tumor diameter, and histological differentiation of the tumors).

In addition, to clarify the association of occult HBV infection with the development of HCC, several factors were compared in subgroups of patients in the NBNC-HCC group with and without occult HBV infection.

Alcohol intake exceeding 80 g/day was defined as an excessive. BMI exceeding 25 kg/m^2 was used to define obesity. The normal cutoff level of AFP was set at 20 ng/ml and AFP was defined as positive when it exceeded this value.

Viral serological markers
HBsAg, anti-HBs, and anti-HCV in sera were determined by commercial enzyme immunoassay (Axcysm, Abbott Japan, Tokyo, Japan; or Lumipulse forte, Fujirebio, Tokyo, Japan), according to the manufacturer's instructions. Anti-HBc was examined by radioimmunoassay (Dinabot, Tokyo, Japan) or chemiluminescent enzyme immunoassay (CLEIA; Fujirebio). HBV core-related antigen (HBcrAg), which correlates with HBV DNA in serum, was measured in serum, using a CLEIA described previously (detection limit, 1.0 kU/ml).[15]

Sequencing of HBV genome
To investigate HBV genotypes and specific mutations that could have been associated with HCC if it had developed as a result of occult HBV infection, serum samples (100 μl) from patients in the NBNC-HCC group were submitted to nucleic acid extraction using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and polymerase chain reaction (PCR)-based amplification of HBV genome parts bearing the enhancer II/core promoter/precore/core or small S regions, as described previously.[16] Amplified HBV DNA fragments were sequenced directly using the ABI Prism Big Dye version 3.0 kit (Applied Biosystems, Foster city, CA, USA) on an AMI 3100 DNA automated sequencer (Applied Biosystems). All sequences were analyzed in both forward and reverse directions. Similarly, serum samples were also examined from patients in the HBV and HCV groups.

Quantification of serum HBV DNA
HBV DNA part spanning the S gene (Int 427-606) were amplified by real-time detection polymerase chain reaction (RTD-PCR) according to a previously described protocol,[17] with slight modification.[18] The detection limit of this study was 100 copies/ml.

Molecular evolutionary analysis of HBV
Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and were aligned by CLUSTAL X (free software available at ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalIX) and genetic distances were estimated by the six-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/). Based on these values, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the midpoint rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis
Data were analyzed where applicable by Fisher's exact test, or the Mann-Whitney *U*-test or Student's *t*-test implemented with STATA software version 8.0 (StataCorp LP, College Station, TX, USA). Differences were considered to be statistically significant if the *P* value was equal to or less than 0.05.

Results

Characteristics of non-B non-C HCC patients

The mean age of patients in the HBV-HCC group was 52.1 ± 10.8 years, and this was significantly lower than that of the other groups ($P < 0.001$). The NBNC-HCC group was characterized by a low proportion of patients with a history of blood transfusion (9%) and a lower mean level of serum alanine aminotransferase (ALT; 42.0 ± 35.0 U/l) as compared to the other groups ($P < 0.05$); however, the platelet concentration was slightly higher than that in the other groups. The prevalence of AFP-positive patients (44%) was significantly lower in the NBNC-HCC group as compared to prevalences in the other groups ($P < 0.05$); however, the mean AFP value (9047 ng/ml) and the mean tumor diameter (44.0 mm) tended to be higher in this group (Table 1). The prevalence of auto-antibodies (anti-nuclear antibody) in the NBNC-HCC group was low (4/37; 11%).

The prevalence of patients with obesity or diabetes was significantly higher in the NBNC-HCC group than in the HBV-HCC group ($P < 0.05$ and $P < 0.001$, respectively), but it was equal to that in the HCV-HCC group (Table 1). Even if the patients with massive alcohol intake were excluded from these three HCC groups, the prevalence of patients with obesity or diabetes was still significantly higher in the NBNC-HCC group (obesity, 11/31 [35%]; diabetes, 12/31 [39%]) than in the HBV-HCC group (3/26 [12%] and 2/26 [8%], respectively; $P < 0.05$ and $P < 0.01$, respectively), but the prevalence was equal to that in the HCV-HCC group (22/104 [21%] and 25/104 [24%], respectively). Comparison of HBV markers such as anti-HBc, anti-HBs, and HBV-DNA in serum showed no significant difference between the NBNC-HCC group and the HCV-HCC group (NBNC-HCC group, 49% vs HCV-HCC group, 51%). In the NBNC-HCC group, eight patients (18%) were positive for HBV DNA (occult HBV infection).

Significance of occult HBV infection

When the characteristics of patients in the NBNC-HCC group were compared between the subgroups of patients with and without occult HBV infection, the rate of obesity was significantly higher in the patients without occult HBV infection, while the prevalence of anti-HBc and/or anti-HBs tended to be higher in the patients with occult HBV infection (Table 2). No significant differences were found in the mean age, history of blood transfusion, alcohol intake, presence of diabetes, vari-

Table 2. Characteristics of non-B non-C HCC patients with and without occult HBV infection

	Occult HBV (+) ($n = 8$)	Occult HBV (–) ($n = 37$)	P value
Mean age (years)	64.1 ± 6.8	66.2 ± 9.4	NS
Sex (male:female)	5:3	23:14	NS
History of BTF	1 (13%)	3 (8%)	NS
Family history of liver disease	0 (0%)	3 (8%)	NS
History of operation	3 (38%)	11 (30%)	NS
Alcohol (>20 g/day)	1 (13%)	13 (35%)	NS
Obesity (BMI > 25 kg/m^2)	0 (0%)	15 (41%)	0.0274
Diabetes	2 (25%)	15 (41%)	NS
Autoantibody (anti-nuclear antibody)	0/4 (0%)	4/33 (12%)	NS
HBV serological markers			
Anti-HBc	4/8 (50%)	15/37 (41%)	NS
Anti-HBs	2/8 (25%)	5/37 (14%)	NS
Anti-HBc and/or Anti-HBs	4/8 (50%)	15/37 (41%)	NS
ALT (U/l)	35.5 ± 16.9	43.5 ± 37.8	NS
ALP (U/l)	387.8 ± 226.9	286.4 ± 122.3	NS
γ-GTP (U/l)	124.4 ± 38.5	100.0 ± 100.2	NS
Platelets (/μl)	10.4 ± 4.8	15.5 ± 9.2	NS
Number of patients positive for AFP	3 (38%)	17 (46%)	NS
AFP (ng/ml)	701 ± 1821	10852 ± 36816	NS
Tumor number (single:multiple)	6:2	24:13	NS
Tumor diameter (mm)	31.5 ± 13.2	46.6 ± 28.4	NS
Tumor histology (well:moderate:poorly)	2:4:2	5:17:1	NS

Values for age, ALT, ALP, γ-GTP, platelets, AFP, and tumor diameter are expressed as means ± SD

ous laboratory findings, or characteristics of HCC (Table 2).

We should note that the levels of HBV DNA were very low in the eight patients with occult HBV infection (mean, 1701 copies/ml; range, 140–5200 copies/ml; Table 3). Anti-HBc was positive in four patients (50%), but there was no association with the levels of HBV-DNA in serum. In spite of the low levels of HBV DNA, HBcrAg was detected in two of these eight patients.

Genetic characteristics of HBV strains in different HCC groups

Genotyping of HBV in the eight patients in the NBNC-HCC group with occult HBV infection indicated that seven had HBV/Ce (C2) and one had HBV/D infection (Fig. 1). Nucleotide sequences in the enhancer II/core promoter/precore/core region were available in only three of the eight HBV DNA positive-patients in the NBNC-HCC group, who had proven HBV/Ce infection (Fig. 2). The prevalence of HBV BCP mutations that have previously been described in association with HCC development, such as C1653T, T1753V (not T), and A1762T/G1764A, was compared in the HBV and the NBNC with occult HBV infection groups, as summarized in Table 4; 25 of 30 patients (83%) in the HBV group had one of these mutations, and this percentage was significantly higher than that in the NBNC group ($P = 0.01$). The pre-S sequence was available for only one strain obtained from 1 patient in the NBNC-HCC group; however no pre-S deletion was found in this patient. In the HCV-HCC group, 8 patients (9%; 8/89) were positive for HBV DNA, and HBV/C was determined in all of them by the sequencing of a short region in the S gene. Nucleotide sequences in the enhancer II/core promoter/precore/core region were not available for these strains.

Discussion

The role of occult HBV infection in the development of HCC remains unclear, and a few studies have been published with contradictory conclusions.[8,9,13] In our present study, 45 HCC patients were negative for both HBsAg and anti-HCV, with evidence of occult HBV infection found in 18% of the 45. Although the prevalence of occult HBV was slightly higher than that in a previous study, which reported that HBV DNA was detected in 12 (6.9%) of 175 HBsAg-negative and anti-HBc-positive blood units from a general population,[19] the levels of HBV DNA in our NBNC-HCC patients were quite low, consistent with previous reports demonstrating that the HBV DNA level in patients with occult HBV infection was significantly lower than that in HBsAg-positive carriers.[20] Interestingly, HBcrAg, which has a tentative association with covalently closed circular DNA in liver (manuscript submitted for publication), was detected in two of our NBNC-HCC patients who had relatively high HBV DNA levels (3900

Table 3. Clinical data of the eight patients in the non-B non-C HCC group infected with occult HBV

No.	Age (years)	Sex	Alcohol	Obesity	Diabetes	Anti-HBc	Anti-HBs	HBcr Ag (kU/ml)	HBV DNA S	HBV DNA X ~ core	HBV DNA (copies/ml)	Genotype	ALT (U/l)	Platelets ($\times 10^4/\mu l$)	AFP (ng/ml)	Tumor size (mm)
1	71	M	–	–	+	–	–	0.0	+	–	670	Ce	60	18.2	5200	50
2	59	F	–	–	–	–	–	0.1	+	+	1400	Ce	57	5.9	7.8	22
3	54	M	–	–	–	+	–	0.0	+	–	420	Ce	47	5	11	18
4	65	M	–	–	+	–	–	0.0	+	–	780	D	20	7.2	330	20
5	58	F	–	–	–	+	+	0.0	+	–	140	Ce	33	8	18	50
6	64	F	–	–	–	–	–	3.1	+	+	5200	Ce	24	16.1	32.8	25
7	68	M	–	–	–	+	–	0.0	+	–	1100	Ce	17	11	3.6	27
8	74	M	–	–	–	+	+	1.3	+	+	3900	Ce	26	11.7	6.7	40
Mean	64.1										1701		35.5	10.4	701	31.5

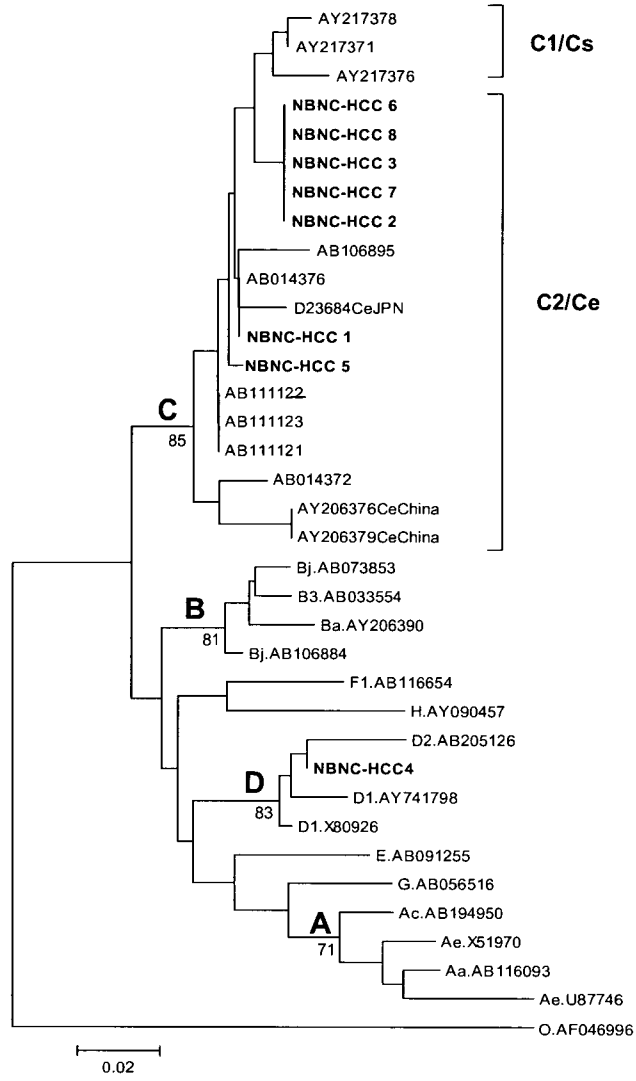

Fig. 1. Neighbor-joining phylogenetic tree constructed from partial S region. The seven hepatitis *B* virus (HBV)/*Ce* and one HBV/*D* strains were compared with representative sequences, including 4 HBV/*A*, 4 HBV/*B*, 12 HBV/*C*, 3 HBV/*D* strains, and reference strains of other genotypes. Strains from this study are shown in *bold*. *NBNC-HCC*, non-B non-C hepatocellular carcinoma

and 5200 copies/ml, respectively), suggesting the possibility that only a few of the NBNC-HCC patients had viral replication in the liver.

In general, occult HBV infection was prevalent in patients with evidence of HBV infection in the past (anti-HBc and/or anti-HBs antibodies), suggesting that it can follow a typical chronic hepatitis B infection,[21] or that it can be a result of exposure and apparent clearance of an acute hepatitis B infection.[22,23] However, a low prevalence of occult HBV infection was found even in HBV-endemic areas such as Taiwan (about 13%),[24]

Table 4. Genotypes and gene mutations in the non-B non-C (NBNC) HCC with occult HBV infection group compared with the HBV-HCC group

	Genotypes	C1653T	T1753V	A1762T/G1764A	One of these mutations
HBV	Bj:Ce:D = 2:27:1	14/30 (46.7%)	9/30 (30%)	24/30 (80%)	25/30 (83.3%)
NBNC	Ce:D = 7:1	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)

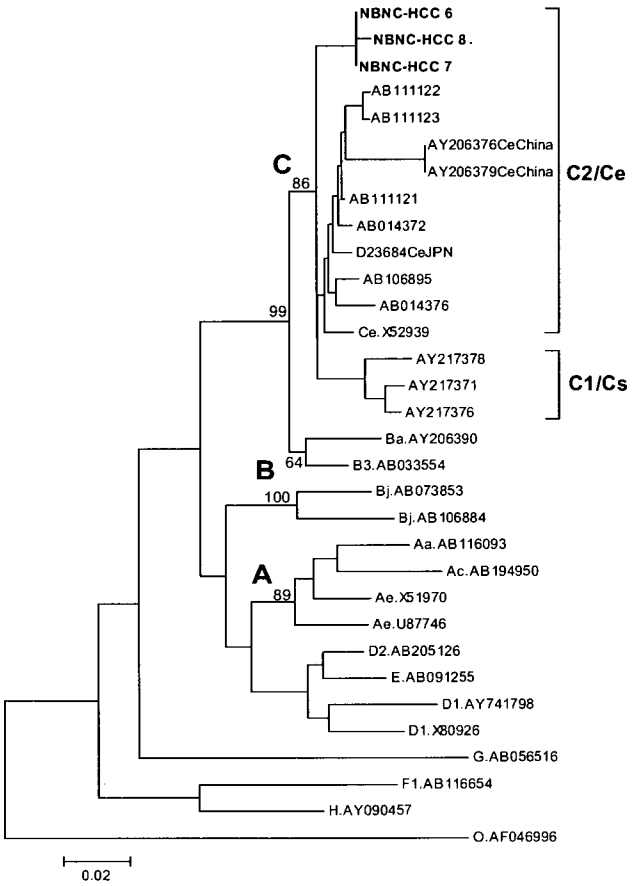

Fig. 2. Neighbor-joining phylogenetic tree constructed from precore and core region. The three HBV/*Ce* strains from the patients with non-B non-C hepatocellular carcinoma (HCC) were compared with representative sequences, including 8 HBV/*A*, 4 HBV/*B*, 13 HBV/*C* strains, and reference strains of other genotypes. Strains from this study are shown in *bold*

suggesting that the potential risk of hepatocarcinogenesis from occult HBV infection might be low.

On the other hand, some previous studies in patients with occult HBV infection have concluded that HBV could contribute to the development of HCC by integration into the human genome;[12,25] however, no population-based study of NBNC patients has been done to evaluate the association of occult HBV infection with the risk of HCC development.

Previous reports on HBV-related HCC cohorts showed that the specific core promoter double mutations T1762/A1764 could be associated with HCC development,[14,26] and our recent case-control study of age- and sex-matched groups of patients has confirmed that, in addition to the T1762/A1764 double mutation, the T1653 mutation in the box alpha increased the risk of HCC in anti-HBe-positive-patients with HBV genotype C.[27] In the present study, available nucleotide sequences from HBV DNA positive-patients in the NBNC-HCC group did not contain specific mutations in the enhancer II/core promoter/precore/core region such as C1653T, T1753V (not T), A1762T/G1764A (Fig. 3), suggesting that these HBV strains with low viral levels in serum and no specific mutations would not play a significant role in hepatocarcinogenesis. Additionally, it was reported that the prevalence of core promoter T1802C/T1803G mutants was significantly higher in patients with occult HBV infection with chronic hepatitis C than in those with HBsAg, and it was suggested that these mutations may be associated with the regulation of transcription,[28] but the T1802C/T1803G mutations were not found in patients with occult HBV infection in this study (Fig. 3).

It is reported that NASH is a liver disease characterized by the histological features of steatohepatitis in the absence of significant alcohol consumption.[29] The diagnosis requires a confirmation by liver biopsy; however, it has been shown that many patients with NASH had obesity, hyperlipemia, and insulin resistance, features which are associated with the metabolic syndrome.[30,31] In fact, particularly in males, the number of patients with metabolic syndrome has been increasing in Japan according to the latest National Health and Nutrition survey carried out in 2004 by the Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/houdou/2006/05/h0508-1a.html). Although no liver biopsy data to confirm the presence of NASH was available in our present study, the higher incidence of obesity and diabetes in our NBNC-HCC group may have been associated with a higher prevalence of this disorder as compared to that in the other groups. Further studies are needed to assess the role of NASH in hepatocarcinogenesis in NBNC patients.

In conclusion, a weak association between occult HBV infection and HCC development was observed in the NBNC-HCC group in this study. On the other hand, as the prevalence of obesity and diabetes increases in Japan, it may be important to assess the contribution of NASH to end-stage liver disease (liver cirrhosis [LC]