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Hepatitis B Virus DNA Integration in Hepatocellular Carcinoma After Interferon-Induced Disappearance of Hepatitis C Virus

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OBJECTIVES: Hepatocellular carcinoma (HCC) has been reported in patients in whom hepatitis C virus (HCV) was eliminated by interferon (IFN) therapy. We examined the pathogenesis of HCC in patients with sustained viral response.

METHODS: Operable HCC developed in 7 of 342 patients cured of HCV infection by IFN monotherapy. No patient abused alcohol or had diabetes mellitus or obesity. Resected specimens of HCC were histologically evaluated. DNA extracted from HCC was examined by polymerase chain reaction (PCR) to locate hepatitis B virus (HBV) DNA. HBV integration sites in human genome were identified by cassette-ligation-mediated PCR.

RESULTS: HBV DNA was not amplified in serum samples from any of the seven patients with HCC and was found in liver in four patients. In the latter four patients, HBV DNA was integrated into the human genome of HCC. In two of these patients, covalently closed circular HBV (cccHBV) was also detected. The patients with HBV DNA integration were free of HCV for more than 3 yr. In two of the three patients without HBV DNA integration, the surrounding liver showed cirrhosis. The liver of HCC with HBV DNA integration had not progressed to cirrhosis. Three of the four tumors with HBV integration had one integration site each, located at chromosomes 11q12, 11q22-23, and 22q11, respectively. The other tumor had two integration sites, situated at chromosomes 11q13 and 14q32. At chromosome 11q12, HBV DNA was integrated into protein-coding genome, the function of which remains unclear.

CONCLUSION: Integrated HBV DNA may play a role in hepatocarcinogenesis after the clearance of HCV by IFN treatment.

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INTRODUCTION

Interferon (IFN) has potent antiviral activity against hepatitis C virus (HCV). Previous studies have shown that IFN can reduce the incidence of hepatocellular carcinoma (HCC) in patients with HCV infection (1–3). After complete eradication of HCV by IFN therapy, HCC was thought to rarely occur (4). Recent studies have shown that HCC develops in 2.5–4.2% of such patients (5–7). These patients may have had advanced liver fibrosis at the time of HCV eradication, and subclinical tumors might have already existed in the liver at the end of IFN therapy (8). In some patients, however, HCC might develop from liver without fibrosis several years after the eradication of HCV by IFN. The etiology of such cases of HCC remains obscure. New regimens combining IFN with antiviral drugs can improve the rate of HCV clearance (9, 10). The risk of HCC might increase in patients with chronic hepatitis who have complete responses to IFN therapy. It is important to delineate the features of HCC occurring after elimination of HCV. Occult hepatitis B virus (HBV) infection is defined as the detection of HBV DNA in the serum or liver of patients without hepatitis B surface antigen (HBsAg) (11). In patients with chronic hepatitis C, occult HBV coinfection may exacerbate liver disease (12). Occult HBV infection is present in a substantial proportion of patients with HCV infection and has a pro-oncogenic effect (13). In the present study, we examined resected liver specimens to evaluate the role of occult HBV infection in the development of cancer after the clearance of HCV by IFN treatment. We also describe the clinical course of such patients with HCC.

METHODS

Patients

At our department, 1,286 patients with chronic hepatitis C without cirrhosis and without HBsAg received IFN

Table 1. Clinical Characteristics of Seven Patients

Case	Gender	Age at Operation	Anti-HBS/ Anti-HBC	Anti-HCV/ HCV-RNA	HCV Genotype Before IFN	Alcohol Intake	Body Mass Index (kg/m^2)	Period from End of IFN to Diagnosis for HCC	HBV DNA Integration	Stage of Fibrosis
1	M	65	−/−	+/−	2a	none	23.1	13 mon	−	2
2	M	59	+/+	+/−	1b	rare	23.7	45 mon	+	2
3	M	65	+/+	+/−	2a	rare	23.7	19 mon	−	4
4	M	61	+/+	+/−	2a	none	23.6	20 mon	−	4
5	M	59	−/−	+/−	2b	rare	23.4	41 mon	+	1
6	M	66	+/+	+/−	2a	none	18.1	80 mon	+	2
7	M	60	−/−	+/−	2a	none	21.5	103 mon	+	1

monotherapy for 24 wk from 1992 through 2002. In 342 patients, serum HCV RNA disappeared, and alanine aminotransferase activity (ALT) was within the normal range for 6 months after the end of IFN therapy. We are now monitoring 144 of these patients every half year. HCC was diagnosed in seven patients, four of whom were regularly monitored (cases 1, 4, 6, and 7). Seven patients underwent hepatectomy at our hospital. Their clinical characteristics are described in Table 1. No patient had alcohol abuse, drug usage, or diabetes mellitus. All patients had a body mass index of less than 25 kg/m^2. The surrounding liver tissue was pathologically classified according to the criteria proposed by Desmet *et al.* (14).

Detection of HBV DNA in Serum and Liver

DNA was extracted from 100 μL of serum or 10 μg of liver tissue by means of proteinase K digestion followed by phenol/chloroform extraction, as described previously (15). HBV DNA in serum or in liver was amplified with specific primers for HBX, HBS, and HBC (sequences of the primers shown in Table 2). Amplification was done in a thermal cycler for 35 cycles: 95°C for 30 s, 55°C for 60 s, and 72°C for 60 s in 40 μL of a reaction buffer containing 30 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 mM, polymerase chain reaction (PCR) buffer, and 2.5 units of Gold Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). With 2 μL of the first PCR product, a second PCR was done. To examine covalently closed circular HBV (cccHBV) in liver, extracted DNA was amplified with primers P23, 24, 25, and p26 (Table 2). The amplification procedure and primers have been described previously (16).

Detection of Integrated HBV DNA in Human Genome

We used cassette-ligation-mediated PCR to detect HBV DNA integrated into the human genome as described previously (15). Briefly, 10 μg of DNA was digested with *Eco*RI, *Hin*dIII, or *Pst*I and ligated to double-stranded DNA cassettes with compatible ends. The cassette-ligated DNA fragments were used as a template for nested PCR with the cassette- and HBV-specific primers. One microliter of the DNA solution was amplified in 40 μL of a reaction buffer containing 10 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 mM, PCR buffer, and 2.5 units of LATaq polymerase. The amplifications were carried out in a thermal cycler for 33 cycles (45 s at 94°C, 2 min at 55°C, 2 min at 72°C), followed by final extension for 10 min at 72°C. With 1 μL of the first PCR product, a second PCR was done. Table 2 shows the sequences of the primers used. The amplified cassette-ligated DNA fragments were subcloned and sequenced with a DNA sequencing system (377A, Applied Biosystems, Tokyo). To identify the integrated site of the host genome, we used the GenomeNet (http://www.genome.ad.jp) to compare the sequences adjacent to the integrated HBV DNA with the human sequence.

Statistical Analysis

Ages, intervals, and tumor sizes in the two groups were compared by Student's *t*-test.

RESULTS

Pathological Findings of the Resected Liver

The seven liver tumors were diagnosed as four poorly differentiated HCC and three moderately differentiated HCC (Table 3). The surrounding liver tissues were diagnosed as chronic hepatitis. The stage of liver fibrosis was IV in two specimens, II in three specimens, and I in two specimens. The activity grade was II in four specimens and I in three specimens. There was no evidence of fat deposits in any of the specimens.

HBV DNA in Serum

HBV DNA was not detected in serum of any of the seven patients with HCC.

HBV DNA in Liver

We detected HBV DNA in five of the seven HCC and three of five noncancerous liver samples (Fig. 1). In detail, HBX was detected in three of the seven tumors and one specimen of noncancerous liver tissue. HBC was detected in four tumors and three liver tissues. HBS was detected in two tumors and three liver tissues. Covalently cccHBV was detected in case two (both HCC and noncancerous liver) and in case four (only liver tissue).

Table 2. Oligonucleotides Used as PCR Primers

Sense Primers			Antisense Primers		
Name	Nucleotide Sequence	Position	Name	Nucleotide Sequence	Position
HBX					
HB-1	5′ CTCTCTCGGAAATACACCTC	220–1239	B-2	5′ GTAACTCCACAGAAGCTCCA	1818–1799
HB-3	5′ TGCCAACTGGATCCTGCGCGGGACGTCCTT	264–1293	B-4	5′ GGCTTGAACAGTAGGACATG	1742–1723
HBS					
HB-5	5′ AAGACCTGCACGATTCCT	391–408	B-6	5′ TAGAGGTAAAAAGGGACTC	672–654
HB-7	5′ TTCGCAAGATTCCTATGGG	99–517	B-8	5′ GCCCCCAATACCACATCA	634–617
HBC					
HB-9	5′ AACTTTTTCACCTCTGCCT	690–1708	B-10	5′ GCTTGCCTGAGTGCTGT	1945–1929
HB-11	5′ ACTGTTCAAGCCTCCAAGC	1731–1749	B-12	5′ AAGGAAAGAAGTCAGAAGGC	1848–1829
cccHBV					
P23	5′ CTGAATCCCGCGGACGACCC	1443–1462	P24	5′ ACCCAAGGCACAGCTTGGAGG	1891–1871
P25	5′ GTCTGTGCCTTCTCATCTGCC	1553–1573	P26	5′ AGATGATTAGGCAGAGGTGAAAAA	1846–1823
Cassette-ligation					
X-5	5′ ACTCTACCGTCCCCTTCTTCATGTGCCGTT	1351–1380	C-1	5′ GTACATATTGTCGTTAGAACGCGTAATACGACTCA	
X-6	5′ CTCTTTACGCGGTCTTTTTGTCTGTGCCTTC	1404–1434	C-2	5′ CGTTAGAACGCGTAATACGACTCACTATAGGGAGA	

HBV DNA Integrated in Human Genome

Our results provide evidence that HBV DNA was integrated into human genome in four of the seven patients with HCV infection in whom HCC developed after complete responses to IFN therapy (cases 2, 5, 6, and 7). HBV DNA was integrated into chromosome 11q23 in case 2, chromosome 22q11.23-12 in case 5, chromosome 11q12 in case 6, and chromosomes 11q13 and 14q32 in case 7. In case 6, HBV DNA was integrated into protein-coding sequences, hypothetical LOC387771 protein, the function of which remains unclear. HCC developed more than 3 yr after clearance of HCV in the patients with HBV DNA integration (Table 4). The interval from HCV eradication to the diagnosis of cancer was significantly longer in HCC with HBV DNA integration than in HCC without it. In HCC without HBV DNA integration, noncancerous liver tissue showed cirrhosis. In HCC with HBV DNA integration, the fibrosis stage of liver tissue was 1 or 2.

Clinical Courses of the Seven Patients

In four patients, in whom more than 3 yr had elapsed since the clearance of HCV RNA, HBV DNA was integrated into the human genome of HCC. In two of the three patients without HBV DNA integration, the surrounding liver showed cirrhosis. In contrast, the surrounding liver of HCC with HBV DNA integration did not progress to cirrhosis. Four patients are alive as of this writing. Tumor recurrence has not been detected in two of these patients (Fig. 2). The other three patients have died: two died of tumor progression and one of a myocardial infarction at operation. There was no correlation between clinical outcome after surgical treatment and HBV DNA integration.

DISCUSSION

In the present study, HCC developed in 4 (2.8%) of 144 patients who were regularly followed up after complete eradication of HCV by IFN monotherapy. Previous studies showed that HCC developed in 8 (2.2) of 363 patients, 6 (4.2%) of 142 patients, and 27 (2.3%) of 1,197 patients with sustained virus responses to IFN (5–7). Our findings are consistent with these results. The interval from the end of IFN therapy to the detection of HCC varied in these reports. In patients with a short interval, HCC most likely existed before the eradication of HCV by IFN. Makiyama *et al.* described the relation between tumor doubling time and the interval to the detection of HCC (7). They estimated that more than 6 yr were required for a single tumor cell to proliferate into a tumor measuring 1 cm in diameter. To our knowledge, 11 cases (including 2 in the present study) in which more than 60 months elapsed from the end of IFN therapy to the detection of HCC have been documented (5–7, 17–19). The longest interval between the end of treatment and diagnosis was 103 months, recorded in a patient in our study. HCC with long intervals between therapy and detection developed in noncirrhotic liver, usually not present in HCC with continuous HCV

Table 3. Histological Findings and the Existence Status of HBV DNA in Patients with HCC

Case	Tumor Size (mm)	Tumor Histology	Resected Liver (Stage)	Resected Liver (Grade)	HBV DNA In Serum	HBV DNA In Liver	cccHBV	HBV DNA Integration
1	19 × 18	Poorly	2	1	−	−	−	−
2	60 × 45	Poorly	2	1	−	+	+	chr. 11q22-23
3	50 × 45	Moderate	4	2	−	−	−	−
4	21×19	Poorly	4	2	−	−	−	−
5	30 × 22	Moderate	1	2	−	+	+	chr. 22q11
6	16 × 18	Moderate	2	2	−	+	−	chr. 11q12
7	40 × 30	Poorly	1	1	−	+	−	chr. 11q13 and 14q32

infection, suggesting that other etiologies are responsible for HCC developing a considerable time after the elimination of HCV. Available evidence thus indicates that factors other than chronic HCV infection play a role in the development of HCC detected after the eradication of HCV. This explanation seems more plausible rather than assuming that a tumor present before the start of IFN therapy grew for more than 5 yr without being detected by imaging studies, performed at regular intervals.

Few studies have examined the status of HCC in patients without HBsAg and antibodies to HCV antigen (anti-HCV), so called non-B, non-C patients. Patients with exposure to aflatoxin B1, alcohol addiction, diabetes mellitus, primary biliary cirrhosis, and steatohepatitis are considered at high risk for HCC (20–24). However, none of the patients in our study had these conditions. A recent review proposes that occult HBV is a carcinogenic factor, particularly in the absence of other risk factors for HCC (25). Pollicino *et al.* suggested that occult HBV infection is an independent factor for carcinogenesis in patients with chronic hepatitis C (13). In the present study, HBV DNA was found in four of seven cases of HCC, and cccHBV, virus-growing form, was also detected in two cases. HBV DNA was not detected in the serum of any patient. Small amounts of HBV, detected only in liver, are unlikely to induce hepatic injury or cause inflammation leading to carcinogenesis. In contrast, hepatocytes with HBV DNA integrated into the genome may be transformed, independently of the amount of HBV present. In our patients with occult HBV infection, HBV DNA was integrated into the human genome of HCC. There was a protein coding sequence near the HBV DNA integration site in one of the four HCC with HBV DNA integration. In this case, HBV DNA appeared to directly affect protein expression near the integration site. Integrated HBV DNA most likely caused instability of the human genome, without directly activating or disrupting protein function. A recent European study has reported that HCC was not detected for 5 yr in patients who had sustained virological responses to IFN monotherapy (26). The relation between HBV and HCC may be affected by patient demographics. Henry *et al.* reported that in an area with a high prevalence of HBV infection, occult HBV infection is common among patients with cryptogenic liver cirrhosis (27). To our knowledge, the annual incidence of HCC is higher in Japanese patients with HCV than that in countries in which

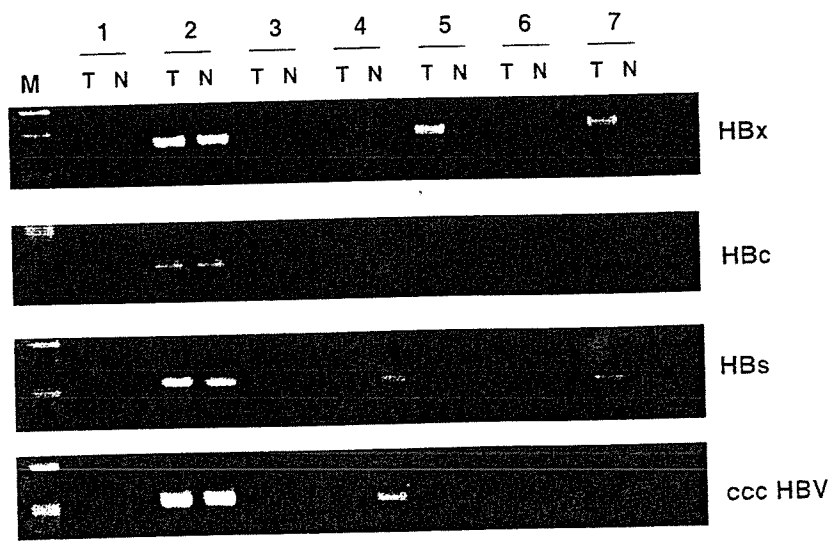

Figure 1. Amplification of HBV DNA and covalently cccHBV in resected liver. M. Marker (*pBR322/AluI*); cccHBV, covalently closed circular HBV.

Table 4. Relations Between Clinical Findings and HBV DNA Integration in HCC

	HCC with HBV DNA Integration (n = 4)		HCC Without HBV DNA Integration (n = 3)		*p*
Age	61 ± 3		64 ± 2		0.295
Interval (month)	67 ± 30		17 ± 4		0.0364*
Tumor size (mm)	32 ± 15		29 ± 16		0.7688
Tumor histology	Moderately D. HCC	2	Moderately D. HCC	1	
	Poorly D. HCC	2	Poorly D. HCC	2	
Fibrosis of resected liver	Stage 1	2	Stage 1	0	
	Stage 2	2	Stage 2	1	
	Stage 3	0	Stage 3	0	
	Stage 4	0	Stage 4	2	

Results are shown as means ± SD.
*Statistically significant.

HBV infection is rare. Occult HBV coinfection or past HBV infection in patients with HCV may partially account for the different incidences of HCC. We have previously reported that HBV DNA integration induces hepatocarcinogenesis in Japanese patients with HCV (28). HBV DNA integration was detected in HCC obtained from the patients with HCV, irrespective of the response to IFN therapy. A history of HCV infection thus seems to increase the risk of HCC in patients with HBV DNA integration.

In conclusion, our findings provide compelling evidence that occult HBV, especially integrated HBV, plays an important role in the development of HCC in patients with HCV eliminated by IFN therapy. Our results confirm the hypothesis that the elimination of HCV by IFN is not the endpoint of therapy for liver disease.

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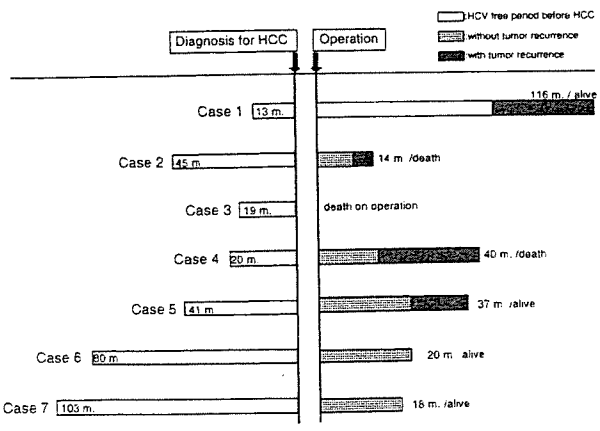

Figure 2. Clinical courses of seven patients with HCC who remained HCV free after IFN treatment. White bar indicates the period from the end of IFN treatment to the diagnosis of HCC. HCV RNA was not detected in any patient after treatment. Small dotted bar shows tumor-free period after liver resection. Meshed bar shows period with tumor recurrence. Two patients died of their tumors, and one died of a myocardial infarction at operation.

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Interferon reduces somatic mutation of mitochondrial DNA in liver tissues from chronic viral hepatitis patients

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SUMMARY. We recently reported that the genetic instability resulting in the high rate of mitochondrial DNA (mtDNA) mutation in noncancerous liver tissue is consistent with the multicentric hepatocarcinogenesis detected clinically. Interferon (IFN) has been reported to reduce hepatocarcinogenesis in individuals with hepatitis virus infection. Liver biopsy specimens were obtained from 26 patients with chronic hepatitis C virus (HCV) infection before and after IFN therapy (total dose: 252 million units). The mean (±SD) age of the study population was 45 ± 9 years and 13 (50%) were male [mode of acquisition: blood transfusion (27%), unknown (73%); viral load: 5.2 ± 1.1 k copies/mL; duration of infection: 17 ± 9 years (65%), unknown (35%); genotype: I (4%), II (80%), III (8%), IV (8%); alcohol intake: positive (31%), negative (69%)]. DNA samples were extracted from the specimens and subjected to direct sequencing. The mtDNA mutation frequency in the D-loop was increased in liver specimens from individuals with HCV infection compared with 21 controls (2.5 *vs* 0.6, $P < 0.001$). IFN therapy decreased the mtDNA mutation (mean difference = 0.7, $P < 0.001$) and the decreased number of mtDNA mutations was positively correlated with suppression of the total histological activity index score (mean difference = 1.3, $P < 0.01$). These results clearly indicate that the mutational rate of mtDNA is strongly associated with IFN therapy. Thus, analysis of mtDNA could provide a new criterion for the therapeutic evaluation of the effect of IFN, and may be useful for the prediction of risk of carcinogenesis.

Keywords: chronic hepatitis, hepatocarcinogenesis, interferon, mitochondrial DNA, oxidative stress.

INTRODUCTION

Persistent infection with hepatitis C virus (HCV) can ultimately result in the development of hepatocellular carcinoma (HCC). Thus, unlike other types of cancer, HCC is usually preceded by chronic inflammation for 20–40 years, although development of HCC can be rapid, particularly in individuals with viral hepatitis and high plasma levels of alanine aminotransferase [1]. Chronic inflammation induced by hepatitis viruses thus plays an important role in hepatocarcinogenesis. The extent of oxidative stress is increased in the liver of individuals infected with hepatitis viruses [2,3], and reactive oxygen species are important inducers of DNA mutations [4].

Mutations accumulate to a greater extent in mitochondrial DNA (mtDNA) than in nuclear DNA, predominantly because mitochondria generate substantial amounts of reactive oxygen species, while they lack histone-like nucleoproteins [5]. Because, expression of the entire mitochondrial genome is necessary for the maintenance of mitochondrial functions, including electron transport, small changes in the mtDNA sequence can result in profound impairment of such functions, thereby enhancing generation of free radicals, which in turn accelerates the rate of DNA mutation.

We have previously reported that the frequency of mtDNA mutations is markedly increased in both noncancerous and cancerous liver specimens from individuals with HCC [6], and showed that the frequency of mtDNA mutations in HCC tissue was greater than that previously described for other types of cancers [7–9]. Furthermore, the extent of accumulated mtDNA mutations in HCC tissues showed a positive correlation with the degree of malignancy. The genetic instability that results in the high rate of mtDNA mutation in noncancerous liver tissues is consistent with the multicentric hepatocarcinogenesis that is detected clinically.

It is suggested that the treatment of hepatitis patients with interferon (IFN) reduces the incidence of HCC [10,11]. Normalization of the plasma levels of alanine aminotransferase by IFN treatment reduces the rate of hepatocarcinogenesis, even if the hepatitis virus is not eliminated [12]. Thus, we

Abbreviations: HAI, histological activity index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; mtDNA, mitochondrial DNA.

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hypothesized that mtDNA in the liver might be affected by hepatitis virus infection and IFN therapy. The present work, demonstrates that HCV infection increases mtDNA mutation in the liver, so the treatment of HCV-infected patients with IFN significantly decreases the frequency of mtDNA mutation.

MATERIALS AND METHODS

Tissue specimens

We analysed liver mtDNA in 26 patients with chronic HCV infection who had received IFN-β (a total dose of 252 million units/patient) for 2 months. The mean (±SD) age of the study population was 45 ± 9 years and 13 (50%) were male [mode of acquisition: blood transfusion (27%), unknown (73%); viral load: 5.2 ± 1.1 k copies/mL; duration of infection: 18 ± 9 years (65%), unknown (35%); genotype: I (4%), II (80%), III (8%), IV (8%); alcohol intake: positive (31%), negative (69%)]. The liver specimens were obtained from these patients by needle biopsy before and after IFN therapy. The histological activity index (HAI) score of the liver specimens was evaluated by Knodell's scoring system. Twenty-one control liver samples were obtained from individuals (age: 57 ± 12 years; male/female: 60/40%) without viral infection, but with liver disease other than HCC (including hepatolithiasis and colorectal carcinoma with liver metastasis).

This study was performed in accordance with the Helsinki Declaration of 1975 (1983 revision) and was approved by the Ethics Committee of Osaka City University Medical School and Osaka City Medical Center. Informed consent was obtained from each patient.

PCR amplification

Freshly obtained liver specimens were frozen, micro dissected using a cryostat, and digested with proteinase K (0.1 mg/mL) in the presence of 1% sodium dodecyl sulphate (SDS). DNA was extracted using phenol–chloroform and ethanol precipitation, as described previously [6]. Each DNA sample (50 ng) was amplified by the polymerase chain reaction (PCR) with the use of overlapping sets of primers to screen the entire mitochondrial genome. PCR (initial incubation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min) was performed in a final volume of 50 μL, using a GeneAmp PCR system 9600 (Perkin–Elmer, CA, USA).

The generation of large PCR products excluded the possibility of nuclear pseudogenes complicating the analysis [13]. In addition, the primers were selected to avoid such co-amplification by analysis of cell lines devoid of mtDNA.

Sequence analysis

Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, CA, USA). The entire mtDNA sequence was examined for one control subject and for one chronic hepatitis type C patient, and the sequence of the displacement (D)-loop (nucleotides 100–600) was examined for the remaining 20 control subjects and 25 individuals with chronic HCV infection. All mutations were confirmed by repeated analysis of DNA extracted from the specimens.

Statistics

The Student's *t*-test was used to analyse differences in the number of mtDNA mutations between the groups of controls and chronic hepatitis. The significance of differences between median values was evaluated by the Paired *t*-test in the number of mtDNA mutations and the changes of HAI score before and after IFN treatment. A P value of <0.05 was considered significant.

RESULTS

The entire mitochondrial genomes of liver specimens obtained from an individual with chronic hepatitis type C before, just after, and 3 years after IFN treatment, and of one control liver specimen were amplified by PCR and sequenced manually. The mtDNA mutations detected are indicated in Fig. 1. Compared with a mtDNA sequence deposited in GenBank (accession no.J01415), the mtDNA sequence obtained from the liver specimen of the control subject contained three single-base variants, all of which were located in the D-loop. The mtDNA sequences obtained from the chronic hepatitis type C specimen before IFN therapy contained 24 mutations, only half of which were detected in the mtDNA from specimens obtained just after and 3 years after IFN treatment. Consistent with previous observations [6–9], most of the mutations detected in this present study were homoplasmic.

The greatest frequency of mtDNA mutations was apparent in the D-loop, especially in the region between nucleotides 100 and 600. All mtDNA samples contained a G → A transition at nucleotide 263, a T → C transition at nucleotide 489, and a C insertion between nucleotides 311 and 312. We then compared the number of mutations in the D-loop region (between nucleotides 100 and 600), excluding the three mutations (variants) common to all samples, among control liver specimens and tissues from individuals with chronic hepatitis type C obtained before and after IFN treatment (Figs 2 & 3). The individuals with hepatitis type C virus had much more mtDNA mutations in D-loop than control (2.8 *vs* 0.5, $P < 0.01$). The mean difference in the number of mtDNA mutations was 0.7 for specimens obtained before and just after IFN therapy ($P < 0.001$).

The decreased number of mtDNA mutations in D-loop (ΔmtDNA mutation) was positively correlated with the

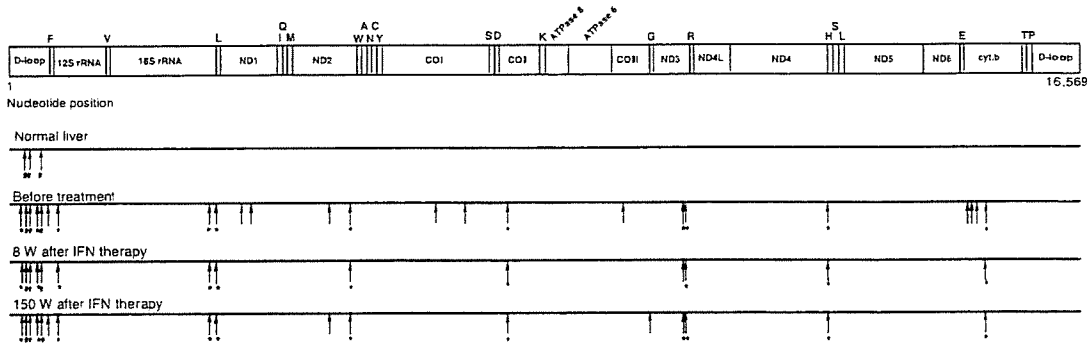

Fig. 1 Location of mtDNA mutations in specimens of control liver and liver tissues from a patient with chronic hepatitis C virus (HCV) infection. Arrows indicate the positions of mutations in mtDNA from liver specimens obtained from a control subject and from chronic HCV infection patients before and after interferon (IFN) treatment. The three common mutations (#) and the identical sites of mutations in the liver specimens before and after IFN therapy (*) are indicated. ND1, NADH (reduced nicotinamide adenine dinucleotide) dehydrogenase 1; COI, cytochrome c oxidase I; ATPase 8, ATP synthase 8; Cyt b, cytochrome b; F, V, L, I, Q, M, W, A, N, C, Y, S, D, K, G, R, H, E, T and P, tRNAs for phenylalanine, valine, leucine, isoleucine, glutamine, methionine, tryptophan, alanine, asparagine, cysteine, tyrosine, serine, aspartate, lysine, glycine, arginine, histidine, glutamate, threonine, and proline, respectively.

decrease (mean difference = 1.3, $P < 0.01$) in the total HAI score (?Activity), as shown in Fig. 4.

The variation of the mtDNA mutations were a C deletion at nucleotide 164, a G insertion between 184 and 185, a G → A transition at 207, a C insertion between 303 and 304, a C → T transition at 317, 320 and 530, and a deletion of GA at 514–515.

DISCUSSION

The present work, shows that IFN treatment markedly decreases the number of liver mtDNA mutations in individuals with chronic HCV infection, and that this change is correlated with a concomitant decrease in total HAI score.

Although each hepatocyte contains hundreds of mitochondria and each mitochondrion contains one to 10 genomes [14], most of the mutations identified in mtDNA were homoplasmic. Tumour cell mitochondria have been reported to proliferate preferentially when tumour cells are fused with normal cells [7,15], suggesting the presence of a mechanism that stimulates the replication of tumour-associated mutant mtDNA molecules. The D-loop region of mtDNA is important in the replication and expression of mitochondrial genome, because it contains both a leading-strand replication origin and transcriptional promoter regions [16]. Thus, mutations within the D-loop might affect the rate of DNA replication by modulating its interaction with trans-acting factors.

Mitochondria that undergo replication appear to acquire DNA damage more readily, resulting in an accumulation of mutations, compared with those maintained under resting conditions. Our data suggest that mtDNA mutations

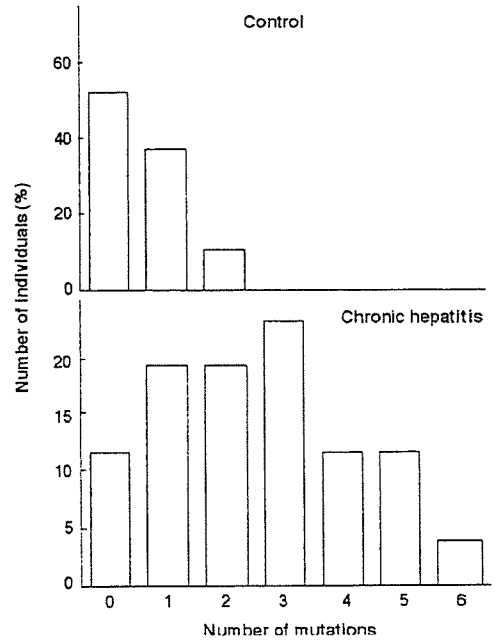

Fig. 2 The number of mtDNA mutations in the D-loop from control livers and liver specimens from patients with chronic hepatitis C virus (HCV) infection. The number of mutations in the D-loop (nucleotides 100–600), with the exception of the three mutations common to all subjects, was compared among the control liver specimens ($n = 21$) and liver tissue obtained from chronic HCV infection patients before interferon therapy ($n = 26$).

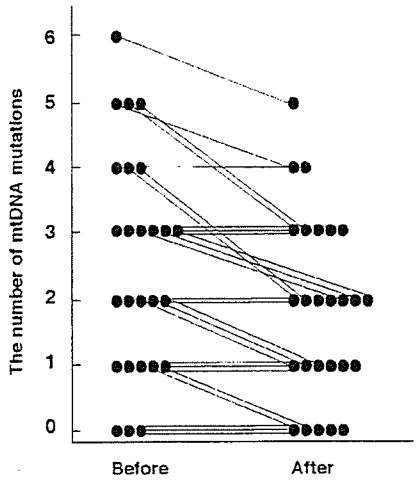

Fig. 3 Changes in the number of mtDNA mutations in the D-loop from liver specimens from patients with chronic hepatitis C virus (HCV) infection before and just after interferon (IFN) therapy. The number of mutations in the D-loop (nucleotides 100–600), with the exception of the three mutations common to all subjects, was compared among the liver tissue obtained from chronic HCV infection patients before and just after IFN therapy (n = 26).

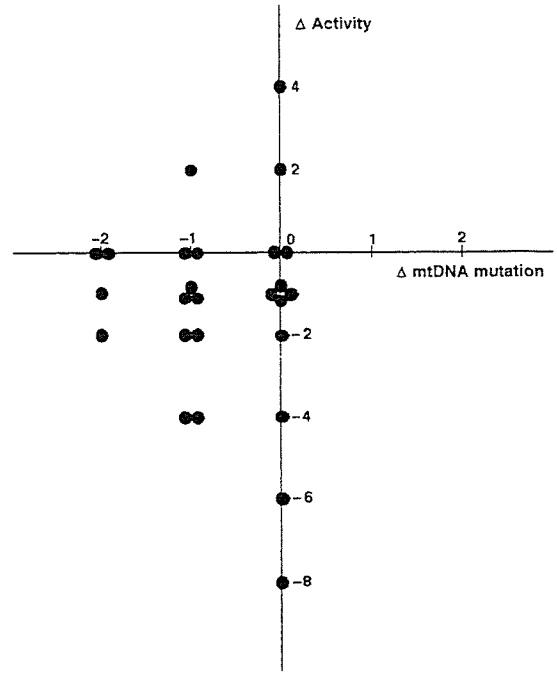

Fig. 4 The relationship between changes in histological activity index (HAI) score and the number of mtDNA mutations. Changes in the number of mtDNA mutations in the D-loop were compared with the changes in HAI score (ΔActivity) before and after interferon (IFN) treatment for each subject (n = 26). (?mtDNA mutation) = (the number of mtDNA mutations after IFN therapy) − (the number of mtDNA mutations before IFN therapy); and (?Activity) = (HAI score after IFN therapy) − (HAI score before IFN therapy).

accumulate during the neoplastic transformation of hepatocytes. In mtDNA harbouring certain mutations might generate abnormal RNAs or proteins, the latter of which may promote leakage of electrons from the mitochondrial electron transport chain. The amounts of endogenously produced reactive oxygen species, such as superoxide and related free radicals, might thus be increased in cells with mutant mtDNA, and the resulting oxidative modification of DNA may contribute to the early stages of hepatocarcinogenesis.

Although activated leucocytes infiltrate into inflammatory liver tissue, the number of mitochondria in infiltrated inflammatory cells is significantly smaller than in hepatocytes. Thus, the contribution of contaminating inflammatory cells to the increased mtDNA mutation frequency observed in HCC tissues is likely to be negligible. Furthermore, mtDNA preparations from liver tissues in individuals with chronic HCV infection were compared with those from paired blood samples. No mutations were found in the blood samples, except for the three common mutations in the D-loop.

Despite our attempts to detect deletions in mtDNA with the use of multiple primers for PCR analysis, no deletion was found in liver tissues from individuals with chronic HCV infection, suggesting that hepatocytes with large mtDNA deletions might be eliminated, while cells and/or mitochondria without such deletions preferentially undergo proliferation.

The high frequency of mtDNA mutations in the liver of patients with chronic hepatitis suggests that hepatocytes in such patients continuously undergo malignant transformation during inflammation induced by HCV infection. The observation that most of the mutations detected were homoplasmic in nature indicates that the mutated mtDNA had become dominant in the liver. Given the clonal nature and large number of mtDNA copies, the unusually high rate of mtDNA mutation in the liver of patients with chronic HCV infection indicates genomic instability, which is likely to contribute to hepatocarcinogenesis. Therefore, analysis of mutations in mtDNA could provide a new criterion for the therapeutic evaluation of the effect of IFN, and may be useful for prediction of the risk of carcinogenesis.

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Distinct Geographic Distributions of Hepatitis B Virus Genotypes in Patients With Acute Infection in Japan

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Genotypes of hepatitis B virus (HBV) were determined in 145 patients with acute hepatitis B from various districts in Japan to establish their geographic distribution and evaluating the influence on the clinical illness and outcome. Genotypes were A in 27 (19%) patients, B in 8 (5%), C in 109 (75%) and mixed with B and C in the remaining one (1%). Genotype A was more frequent in metropolitan than the other areas (21/69 (30%) vs. 6/76 (8%), $P < 0.001$). On phylogenetic analysis, seven of the nine (78%) HBV/A isolates selected at random clustered with those from Europe and the United States, while the remaining two with those of subgroup A′ prevalent in Asia and Africa. Maximum ALT levels were lower (2069 ± 1075 vs. 2889 ± 1867 IU/L, $P = 0.03$) and baseline HBV DNA titers were higher (5.90 ± 1.45 vs. 5.13 ± 1.36 log genome equivalents (LGE)/ml, $P = 0.002$) in patients infected with genotype A than C. Hepatitis B surface antigen persisted longer in patients infected with genotype A than C (1.95 ± 1.09 vs. 1.28 ± 1.42 months, $P = 0.02$). HBV infection became chronic in one (4%) patient with genotype A and one (1%) with genotype C infection. Fulminant hepatic failure developed in none of the patients with genotype A, one (13%) with genotype B and five (5%) with genotype C. The point mutation in the precore region (A1896) or the double mutation (T1762/A1764) were detected in none of patients with genotype A, two (25%) with g type B and 27 (26%) with genotype C. In co sion, genotype A is frequent in patients with a hepatitis B in metropolitan areas of Japan, bably reflecting particular transmission ro and associated with longer and milder cli course than genotype C. ***J. Med. Virol. 77: 46, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: acute hepatitis; genotypes; demiology; hepatitis B v hepatitis B e antigen; sexu Japan

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INTRODUCTION

The clinical outcome in patients with acute hepatitis B varies widely. Although hepatitis is self-limited in most patients, the clinical features range from asymptomatic to fulminant hepatic failure, while some patients become carriers of hepatitis B virus (HBV) [Chan HL and Lok, 1999; Chan HLY, 1999]. Factors that determine the clinical outcome remain unknown.

Viral nucleotide (nt) mutations have been shown to influence the clinical outcome of acute hepatitis B. Mutations in the precore region (A1896) and the basic core promoter (BCP) region (T1762/A1764) are common in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996]. Viral factors other than these mutations may influence the clinical outcome of acute hepatitis B.

Eight genotypes of HBV have been identified by sequence divergence greater than 8% in the entire genome, and they are designated by capital alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Furthermore, recombinant HBV strains consisting of two different genotypes have been reported [Bollyky et al., 1996; Morozov et al., 2000]. Genotype distribution is different in different countries and even in distinct areas of the same country [Orito et al., 2001a; Kao, 2002; Kato et al., 2002; Miyakawa and Mizokami, 2003]. Therefore, surveys on genotype distribution may be helpful in identifying transmission routes and evaluating clinical relevance.

It has been shown that the clinical outcome of chronic hepatitis B is influenced by HBV genotypes. In Asian patients with chronic hepatitis B, genotype C is associated with later seroconversion of hepatitis B e antigen (HBeAg) and more severe liver damage than genotype B [Kao et al., 2000; Orito et al., 2001b; Chu et al., 2002; Ding et al., 2002; Sugauchi et al., 2002a]. Likewise, a study from India has shown that genotype D is associated with more severe liver disease than genotype A [Thakur et al., 2002]. Genotype A is peculiar in that A1896 in the precore region occurs infrequently, because it causes instability of the stem-loop structures of the pregenome encapsidation signal [Li et al., 1993; Lok et al., 1994]. These reports suggest that HBV genotypes also influence the clinical characteristics of acute hepatitis. Recent studies on small numbers of patients with acute hepatitis B suggest that the clinical course may differ among infections with distinct HBV genotypes [Mayerat et al., 1999; Kobayashi et al., 2002; Ogawa et al., 2002]. However, the association between viral genotype and severity of liver disease remains uncertain in acute HBV infection.

To evaluate the effect of HBV genotypes on the clinical characteristics of acute hepatitis B, a multi-center study

MATERIALS AND METHODS

Patients

During 1992 through 2001, serum samples v
lected from 147 patients diagnosed with acute l
B in our institutions. Only patients from whon
the onset of hepatitis were stored were incl
this study. Sixty-nine (47%) patients lived i
politan areas (Kawasaki, Tokyo and Tokorozaw
the others in Kurume, Ube, Osaka, Gifu, Nag
Sapporo. Criteria for the diagnosis of acute he
were: (1) Acute onset of liver injury without a h
liver dysfunction and detection of hepatitis B
antigen (HBsAg) in serum; and (2) IgM antibod
core (anti-HBc) in high titer. Co-infection with l
A virus or hepatitis C virus was excluded by se
tests.

Among the 147 patients, acute hepatitis B in
was complicated by hepatic encephalopathy
longed prothrombin time for the diagnosis
nant hepatic failure. Other two (1%) patients r
positive for HBsAg for longer than 6 months,
were considered to have acquired chronic infec

Sera from the 147 patients with acute hepatiti
examined virologically, and the results were co
with clinical and demographic characteristics. I
consent was obtained from each patient for the
of this study. The study protocol conforms to th
guidelines of the 1975 Declaration of Helsi
approved by the Ethics Committees of our insti

Determination of HBV DNA

Levels of HBV DNA were determined using tr
tion-mediated amplification (TMA) and hybri
protection assay (Chugai Diagnostics Science
Tokyo, Japan) after the protocol as reported [Ka
et al., 1999]. The range of detection by TMA v
3.7 log genome equivalents (LGE)/ml ($10^{3.7}$ c
corresponding to 5,000 copies/ml) to 8.7 LGE/
copies/ml). In 16 of 86 studied sera, levels of HI
were under 3.7 LGE/ml and categorized in 3.7

Genotyping HBV

HBV genotypes in most samples were det
with commercial enzyme immunoassay kits (HE
type EIA, Institute of Immunology Co. Ltd.
Japan) involving monoclonal antibodies to g
specific epitopes in the preS2-region, as repor
viously [Usuda et al., 1999, 2000; Kato et al
Genotypes in 18 (12%) samples were determ
genotype-specific probe assay (Smitest HBV Ge
Kit, Genome Science, Fukushima, Japan). In br
extracted from serum was amplified by the pol
chain reaction (PCR) with three sense primer
ACC AAC CCT CTG GGA TTC TTT CC-3′, s2
AAT CCT CTG GGA TTC TTC CC-3′ and s3:
AAT CCT CTA GGA TTC CTT CC-3′ [nt 290
and an antisense primer (as1: 5′-GAG CCT G

they were deduced from conserved sequences in the preS1 region of HBV. The biotin-labeled and amplified HBV DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one or other of the seven genotypes (A–G) immobilized on wells of a 96-well microplate. Thereafter, hybiridization was detected by staining with the streptavidine-horseradish peroxidase (HRP) conjugate [Kato et al., 2003].

Subtypes of genotype B, in terms of Ba with the recombination with genotype C and Bj without it were determined by direct sequencing of precore and core regions by the method reported previously [Sugauchi et al., 2002b].

Amplifying and Sequencing HBV DNA of Genotype A Isolates

A subgroup of genotype A is reported with the designation of A' from South Africa, Philippines, Malawi, and Belgium [Bowyer et al., 1997; Kramvis et al., 2002; Sugauchi et al., 2004]. Randomly selected HBV/A samples were classified into genotype A and subtype A' by sequencing the S region. For amplification and sequencing, the entire S region was divided into two fragments, spanning nt 3130–478 and nt 378–878, respectively, and they were amplified by two-stage PCR. The outer primers for amplification of the 1st fragment were: 5'-ACC AAT CGG CAG TCA GGA AG-3' (sense: nt 3121–3140) and 5'-CTG GAA TTA GAG GAC AAA CG-3' (antisense: nt 488–469) and the inner primers were: 5'-CAG TCA GGA AGG CAG CCT ACT-3' (sense: nt 3130–3150) and 5-AGG ACA AAC GGG CAA CAT AC-3' (antisense: nt 478–459). The outer primers for amplification of the 2nd fragment were: 5'-TGT CCT GGT TAT CGC TGG AT-3' (sense: nt 359–378) and 5'-CAA CGT ACC CCA ACT TCC AA-3' (antisense: nt 909–890) and the inner primers were: 5'-TGT GTC TGC GGC GTT TTA TC-3' (sense: nt 378–397) and 5'-ATG AAG TTT AGG GAA TAA CC-3' (antisense: nt 878–859).

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μl of the reaction mixture containing 200 μM dNTPs, 1.0 μM each of primers and 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin) and 2 U of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Connecticut). PCR products (2 μl) were subjected to the second stage of amplification under the same conditions as the first stage. Standard precautions to avoid contamination were exercised during PCR, with a negative control serum included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Wisconsin), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, California) using the PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377: PE Applied Biosystems).

The nucleotide sequences of HBV/A isolates from A strains including subtype A' retrieved from the DI EMBL/GenBank database, as well as representativ the other six major genotypes (B–G). Phylogenetic t were constructed with the mega program versior using the Kimura two-parameter matrix and neighbor-joining method [Sugita et al., 1991]. To firm the reliability of phylogenetic tree analysis, t strap resampling, and reconstruction were carried 500 times.

Detection of Point Mutations in the Precor and BCP Regions of HBV

Mutation in the precore region for A1896 was dete by enzyme-linked minisequence assay (Smitest I Pre-C ELMA, Roche Diagnostics, Tokyo, Japan) mutations in the BCP region for T1762/A1764 v detected by enzyme-linked specific probe assay (Sm HBV Core Promoter Mutation Detection Kit; Gen Science Laboratory, Tokyo, Japan) according to manufacturer's instructions, after the principles cribed previously [Orito et al., 2001b]. The results v recorded as "the wild-type" and "the mutant-t expressed dominantly by HBV isolates.

Statistical Analysis

Data were analyzed by chi-square test or Fisl exact test for categorical data and Student's *t*-tes Mann–Whitney *U*-test for continuous variables values less than 0.05 were regarded as statistic significant. Logistic regression (backward logistic gression) was used in the multivariate analysi evaluate the factors associated with differences betv genotypes A and C.

RESULTS

Distribution of HBV Genotypes

HBV genotypes were determined in 145 of the (99%) patients with acute hepatitis B; they were typeable in the remaining two patients (Table I). G type A was detected in 27 (19%) patients, B in 8 (5%), 109 (75%), and mixed genotypes with B and C in remaining one (1%). In the 69 patients with a hepatitis B from metropolitan areas (Tokyo, Kawa and Tokorozawa), genotype A was found in 21 (30% in 5 (7%), and C in 43 (63%). In the 76 patients from other areas in the mainland, by contrast, genotyp occurred in 6 (8%), B in 3 (4%), C in 66 (87%), and m genotypes with B and C in one (1%). Thus, genoty was significantly more frequent in patients with a hepatitis B from the metropolitan than the other a (30% vs. 8%, $P < 0.001$).

Demographic and Clinical Differences Among Patients Infected With HBV of Distinct Genotypes

Clinical and demographic backgrounds in pati

TABLE I. Demographic and Clinical Differences Among Patients With Acute Hepatitis Who Were Infected W of Distinct Genotypes

	Genotypes of HBV				Differences (A	
Features	A (n = 27)	B (n = 8)	C (n = 109)	B/C (n = 1)	Univariate (*P*-value)	Mu logist (/
Areas					<0.001	
Metropolitan (n = 69)	21 (30%)	5 (7%)	43 (63%)	0		
Others (n = 76)	6 (8%)	3 (4%)	66 (87%)	1 (1%)		
Age (years)	29.3 ± 8.0	35.7 ± 10.1	36.6 ± 13.6	51	0.016	
Male	25 (93%)	7 (88%)	69 (57%)	1 (100%)	0.003	
Transmission routes						
Heterosexual	15 (56%)	3 (37%)	52 (48%)	0	0.197	
Homosexual	5 (19%)	1 (13%)	2 (2%)	0	<0.001	
IV drugs	0	0	8 (7%)	0	0.280	
Unknown	7 (25%)	4 (50%)	47 (43%)	1 (100%)	0.102	
Fulminant hepatic failure	0	1 (13%)	5 (5%)	0	0.582	
ALT (IU/L)[a]	2069 ± 1075	2952 ± 1106	2889 ± 1867	646	0.030	
Bilirubin (mg/dl)[a]	10.7 ± 14.1	10.3 ± 4.9	7.8 ± 6.7	4.8	0.533	
ALP (IU/L)[a]	476 ± 161	501 ± 94	432 ± 116	No data	0.542	
HBeAg	24/26 (92%)	4/8 (50%)	57/93 (61%)	1/1 (100%)	0.357	
Precore and BCP mutations						
Precore (1896A)	0/27	1/8 (13%)	20/102 (20%)	No data	0.250	
BCP (1762T/1764A)	0/27	1/6 (17%)	14/75 (19%)	No data	0.357	
Precore or BCP	0/27	2/8 (25%)	27/102 (26%)	No data	0.096	

[a]Maximum data are shown for alanine aminotransferase (ALT), bilirubin and alkaline phosphatase (ALP).

different genotypes are compared in Table I. Patients with genotype A were younger than those with genotype C (29.3 ± 8.0 vs. 36.6 ± 13.6 years, $P = 0.016$). The proportion of male patients was higher in genotype A than C infection (93% vs. 57%, $P = 0.003$). The main route of transmission identified in the patients with acute hepatitis B was extramarital heterosexual contacts. Homosexual activity was more frequent in patients with genotype A than C (5/27 (19%) vs. 2/109 (1.8%), $P < 0.001$).

The maximum ALT levels were lower in patients with genotype A than B or C infection (2069 ± 1075, 2952 ± 1106 and 2889 ± 1867 IU/L, respectively: A vs. B, $P = 0.02$; A vs. C, $P = 0.03$). The maximum bilirubin and alkaline phosphatase levels were no different among patients infected with HBV of different genotypes. Fulminant hepatic failure developed in one (13%) patient with genotype B and five (5%) with genotype C; no patients with genotype A came down with it. Evolution into chronic infection occurred in two patients (one with genotype A and one with genotype C). The remaining 137 (96%) patients ran a non-fulminant and self-limited disease.

HBeAg was found in 24 of the 26 (92%) patients with genotype A, 4 of the 8 (50%) with genotype B and 57 of the 93 (61%) with genotype C; it was no different between genotype A than genotype C infection ($P = 0.357$). Of the six patients with fulminant hepatic failure, only one (17%) had HBeAg.

With logistic multivariate regression analysis, the variables for differences between genotypes A and C (CI), 1.378–30.213; $P = 0.0018$) and area (OR CI, 0.076–0.830; $P = 0.0024$).

Routes of transmission were compare genotypes A and C in patients with acute l from metropolitan areas. Although the mean different, frequently the proportion of ma was higher in genotype A than C infectior (95%) vs. 28/43 (65%), $P = 0.012$). Homosexu had more frequently genotype A than C inf (24%) vs. 1/44 (2%), $P = 0.012$). Additionally uals with multiple unspecific partners had i A more frequently than C infection (7/12 (26 (23%), $P = 0.035$, respectively). How logistic multivariate regression analysis these variables differed between genotyp infections.

Figure 1 compares serum HBV DNA level sion among patients infected with different HBV DNA levels were higher in patients wit A than C (5.90 ± 1.45 vs. 5.13 ± 1.36 LGE/ml,

Among the 145 patients whose HBV geno be determined, 54 (A: 15, B: 4, and C: 35) we for HBsAg in serum every 2–4 weeks unt peared. The time between the first and last HBsAg was defined as the duration of H compared between patients infected with HI types A and C (Fig. 2a). The duration of I longer in patients with genotype A than (1.95 ± 1.09 (n = 15) vs. 1.28 ± 1.42 month $P = 0.02$). When patients with fulminant hep were excluded, the mean duration of HBsAg

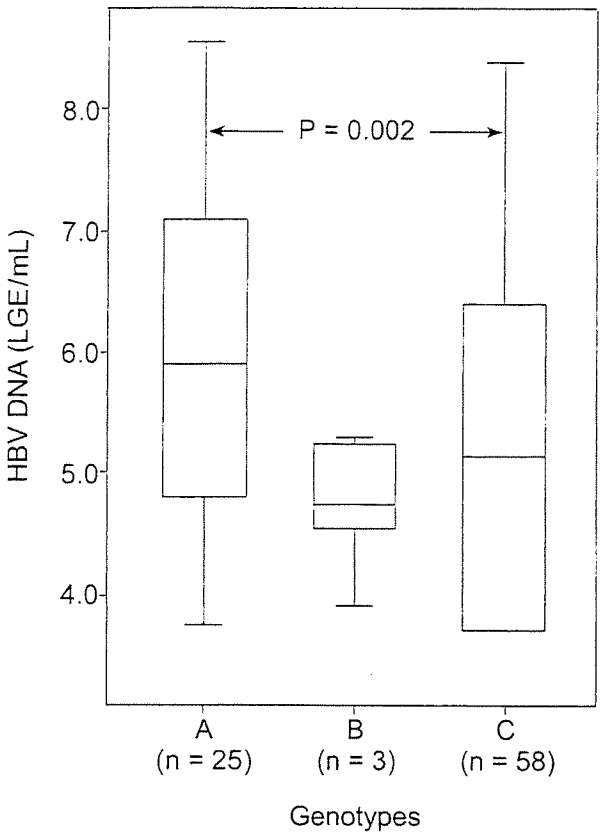

Fig. 1. HBV-DNA levels in patients with acute hepatitis B with genotypes A, B, or C at the presentation. Box plots are given with horizontal lines for the medians, upper and lower edges indicating the 25th and 75th centiles, respectively, and bars represent the extremes without including outliers. Shaded areas are outside the range of detection by the TMA method.

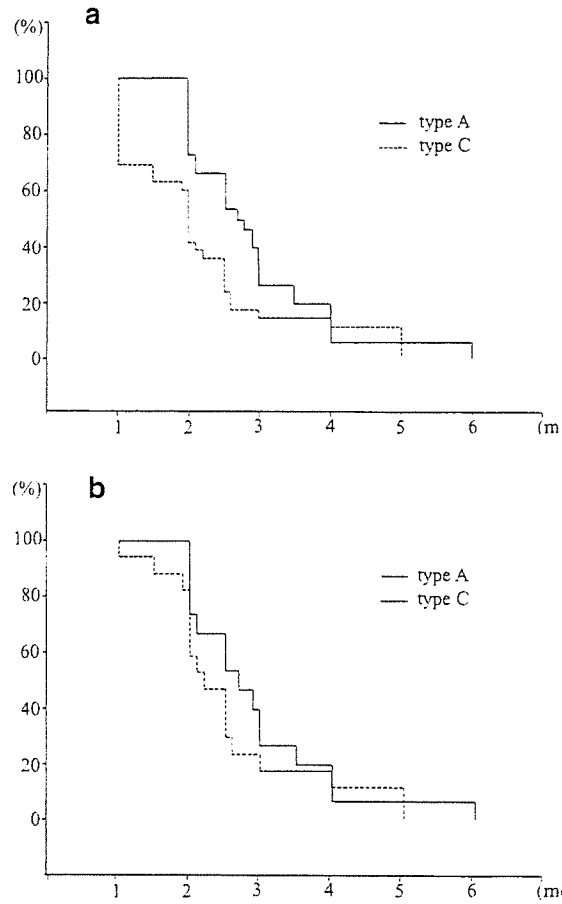

Fig. 2. The duration of HBsAg in patients with acute hepatitis I genotypes A or C. The results are shown for (a) all patients, ar patients with the wild-type sequences both in precore and BCP re of HBV.

than that in those with genotype A (1.95 ± 1.09 (n = 15) vs. 1.41 ± 1.42 (n = 31) months, $P = 0.03$).

Subtypes of Genotypes A and B

Among the 27 HBV/A isolates, 9 were selected at random and the entire S region was amplified and sequenced for them. Seven of them were classified into genotype A and the remaining 2 into subgroup A′. The sequence divergence within the seven genotype A isolates ranged from 0.12% to 2.01% in pair-wise comparison, while that between two subgroup A′ and seven genotype A isolates spanned from 5.70% to 6.53%.

A phylogenetic tree was constructed on the entire *S*-gene sequences from these nine sequences along with those from 31 HBV isolates retrieved from the database (Fig. 3). The seven (78%) HBV isolates classified into genotype A clustered with reported HBV/A isolates, while the remaining two isolates classified into subgroup A′ (cases 3 and 4) joined the branch of subgroup A′.

Six of the eight HBV/B isolates were available for analysis of subtypes. Two (both from the metropolitan cluding two from Tokyo and two from the other area Bj. One of the four patients infected with subtyp developed fulminant hepatic failure, while the rem ing three with subtype Bj as well as the two with sub Ba ran a non-fulminant course.

Point Mutations in the Precore and Basic Core Promoter Regions of HBV

All the 27 HBV isolates of genotype A in which m tions were sought had the wild-type sequences bot the precore and BCP regions. In contrast, of the genotype C isolates whose precore and BCP seque were examined, 27 (26%) had mutations in the pre or BCP regions ($P = 0.096$). Furthermore, of the genotype C isolates from patients with fulmi hepatic failure whose genetic mutations could be termined, three had mutations in the BCP re (T1762/A1764) and two had a mutation in the pre region (A1896). Only one isolate had the wild-typ

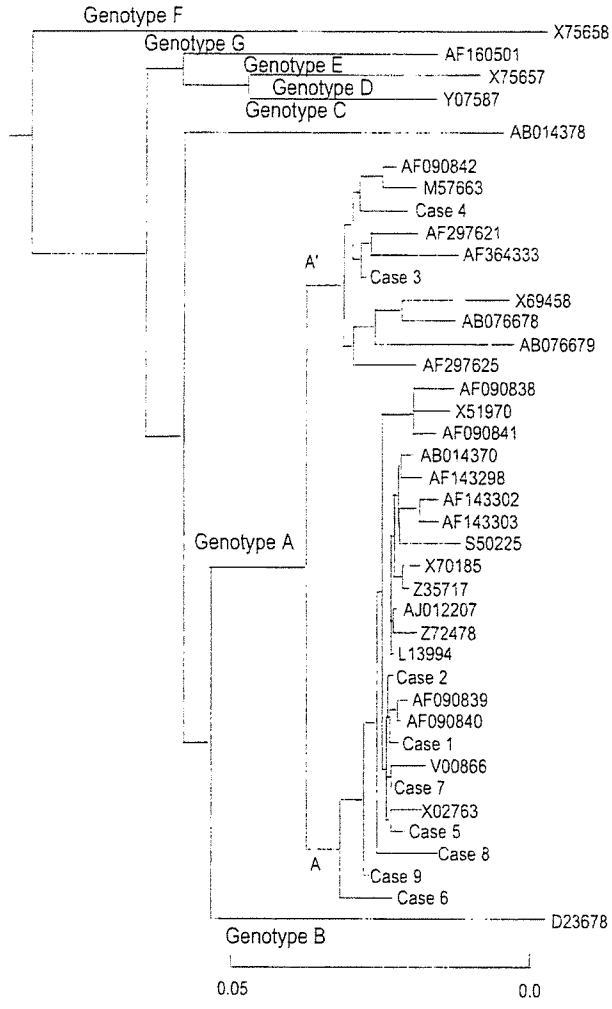

Fig. 3. A phylogenetic tree constructed on HBV DNA sequences spanning the major S-gene of all known HBV genomes, including the nine of genotype A. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates, which have been deposited in the DDBJ/EMBL/GenBank databases. HBV sequences in cases 1–9 were determined in the present study. The HBV/A sequences from cases 1, 2, and 5–9 clustered with the European-American genotype A, while those from cases 3 and 4 clustered with genotype A' that is the African subgroup of genotype A.

the eight genotype B isolates, two (25%) had mutations in the precore or BCP region (Table I).

To examine further differences between genotype A and C infections, patients infected with HBV strains with the wild-type sequences both in precore and BCP regions were compared. The maximum ALT levels were still lower in patients with genotype A than C infection (2069 ± 1075 and 2594 ± 1015 IU/L, respectively, $P = 0.02$), but the maximum bilirubin and alkaline phosphatase levels were no different amongst patients infected with HBV of distinct genotypes. There were no differences in the duration of serum HBsAg between patients with genotype A and C infections (1.95 ± 1.09

DISCUSSION

The salient finding in this study is that in HBV genotype A is frequent in patients hepatitis in Japan, lending support to previ [Kobayashi et al., 2002; Ogawa et al., 2002]. portion of patients with acute hepatitis ed with genotype A, which is detected ra patients with chronic hepatitis in Japan [2001a; Kobayashi et al., 2002]. Genotype A North-Western Europe, United States, Cer and India [Kao, 2002; Miyakawa and Mizok This genotype may be prevalent in countrie since the distribution of HBV genotypes h examined in many districts of the world. P analysis has shown that seven (78%) HBV/ the nine patients examined with acute hepa of the European-American type. Although sequences from four, (cases 1, 2, 5, and 7) clu those reported previously, those from three and 9) were separated genetically (Fig. 3), gests their distinct geographic origin.

Notably, the genotype distribution differ patients with acute hepatitis B from metrop and the others including many large cities. A is seen rarely in patients with chronic hep et al., 2001a; Kobayashi et al., 2002], it is sus genotype A in metropolitan areas has a d graphic origin. Many patients with genotype in these areas had a history of extramar contacts with plural unspecified partners. S behavior may increase the risk of infection type A. In support of this view, most homose in Tokyo who have human immunodeficienc I are coinfected with HBV genotype A [Koil 2001]. Taken together, homosexual activit crease the risk of genotype A infection in m areas. Further molecular analysis on HBV is transmitters and recipients will verify this With respect to genotype B, both Ba, and [Sugauchi et al., 2002b] were detected. Al number of studied patients was small, pa subtype Ba were found in the Tokyo metro exclusively. Whether subtype Ba intrinsic t politan area has a peculiar geographic or rently unknown and awaits further analyse

Another point made in this study is that types influence clinical features and the acute hepatitis B. It has been shown that the of patients who develop chronic HBV infecti 10% in European and American countries [1997] but rare in Japan [Kobayashi et al., 2(studies suggest that chances for evolution city may differ among patients acutely in HBV of distinct genotypes [Mayerat et al., 1 et al., 2002]. Our study has shown that pa genotype A had higher HBV DNA and lower as well as a longer duration of HBsAg in seru ment of chronic hepatitis was seen in one of

with genotype C infection. Although the number of patients studied was not large enough for statistical evaluation, the transition to chronic infection may be more frequent in infection with genotype A than the other genotypes, insofar as higher viral loads can predict chronic infection [Fong et al., 1994]. Further studies on more patients are required to evaluate whether or not viral persistence occurs more often after HBV infection with genotype A than the other genotypes.

Patients with fulminant hepatic failure in the present study were infected with either genotypes B or C; no patient with genotype A developed hepatic failure. As mutations at nt 1896 in the precore and nt 1762/1764 in the BCP regions, which are found frequently in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996], were not detected in patients with genotype A, low frequency of fulminant hepatic failure associated with genotype A infection may be attributed to the lack of these mutations. The high frequency of HBeAg in genotype A infection may also be related to low frequency of fulminant hepatic failure. However, interpretation on this data should be made carefully, because the number of patients studied was small. Further research is necessary to determine if the genotype itself affects the clinical course of acute hepatitis B.

In summary, (1) infection with HBV genotype A is common in patients with acute hepatitis in Japan; (2) patients with genotype A are more frequent in metropolitan areas and may be associated with particular sexual behavior; (3) patients with genotype A have a milder but longer course of infection, which may lead to increased risk of progression to chronic disease.

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