These observations support the clinical importance of occult HBV as a carcinogenic factor in HBsAg-negative patients with CH-C. However, it remains controversial whether occult HBV increases the risk of HCC in this population [8].

Several studies have investigated the association between HBV integration and HCC in patients with both chronic HCV infection and HCC [8–10]. However, no study has prospectively evaluated whether HBV integration in liver tissue correlates with HCC development in CH-C patients. In a prospective 12-year study, we attempted to clarify whether HBV integration promotes hepatocarcinogenesis in CH-C patients.

2. Materials and methods

2.1. Patients

A total of 67 HBsAg-negative, CH-C patients underwent ultrasonography (US)-guided fine-needle liver biopsy for histological evaluation between January and December 1994. Of these patients, 39 had chronic hepatitis with mild fibrosis (METAVIR score of F0 or F1) [11] and were included in the study. Clinical characteristics of these patients are summarized in Table 1. The patient group contained 30 men and 9 women with a mean age of 49.0 ± 7.6 years. All patients were negative for both serum HBsAg and HBV–DNA and were shown to have persistent HCV infection by nested reverse transcription-polymerase chain reaction (PCR). Sixteen of thirty-nine patients had a history of blood transfusion. No patient had a history of intravenous drug use, tattooing, or acupuncture. No patient had a history of acute hepatitis B. All patients were followed from the time of liver biopsy until October 2006. They underwent periodic US examination and analysis for HCC tumor markers, including α-fetoprotein and des-γ-carboxy prothrombin every 6 months. When a suspicious liver lesion was detected by US or a tumor marker was elevated, the patient underwent further examination by imaging such as computed tomography (CT), magnetic resonance imaging, or angiography. HCC was diagnosed on the basis of typical imaging findings, which include a mosaic pattern with a halo on B-mode US images, hypervascularity on angiographic images, or a high-density mass on arterial-phase dynamic CT images with a low-density mass on portal-phase dynamic CT images obtained with a helical or multidetector raw CT scanner. All patients who developed HCC underwent a hepatectomy; all tumors were less than 3 cm in diameter when detected under this surveillance. The final diagnosis of HCC was based on histologic examination of the tumor tissue taken from resected specimens.

The study protocol conformed to the ethics guidelines of the Declaration of Helsinki (1975). All patients provided written informed consent for analysis of the biopsy specimens, and the Hospital Ethics Committee approved the study.

Table 1
Clinical characteristics of the study patients (n = 39)

Age (years)	49.0 ± 7.6
Sex (female/male)	9(23.1)/30(76.9)[#]
History of blood transfusion	15 (38.5)
Presumed duration of HCV carriage[*]	19.0 (5–33)[##]
Alanine aminotransferase (IU/L)	60.1 ± 31.4
Aspartate aminotransferase (IU/L)	45.0 ± 23.8
Gamma glutamyl transpeptidase (mg/L)	51.2 ± 55.3
Albumin (g/dL)	4.11 ± 0.33
Total-bilirubin (mg/dL)	0.74 ± 0.33
Platelet count (×10^4/ml)	17.9 ± 6.5
HCV RNA concentration (×10^{3}IU/mL)	570 (3–4900)[##]
HCV genotype	
1b	25(64.1)[#]
2a	11 (28.2)[#]
2b	3 (7.7)[#]
HBV surface antigen	0
HBV surface antibody	6(15.4)[#]
HBV core antibody	25(64.1)[#]
Fibrosis stage[**]	
F0	14 (35.9)[#]
F1	25(64.1)[#]

HBV, hepatitis B virus; HCV, hepatitis C virus.
[*] In patients with a history of blood transfusion.
[**] According to METAVIR score.
[#] Percentages are shown in parentheses.
[##] Median; ranges are shown in parentheses.

2.2. Sample preparation

DNA was extracted from liver tissues obtained at liver biopsy on 1994 with a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were stored at −80 °C and carefully handled to avoid contamination with nucleic acids.

2.3. Detection of viral–host junctions

A PCR-based technique, Alu-PCR, one of the most effective procedures to detect junctions between integrated HBV–DNA and human DNA, was used to amplify viral–host junctions using 100 ng of genomic DNA [12–14] (Table 2). The sensitivity study for this PCR was performed using human hepatoma cell line Huh-2 cells that contain 1 copy per cell of integrated HBV (kindly provided by Dr. K Koike from Department of Gene Research, Cancer Institute, Tokyo) [15]. Amplified PCR products were analyzed by electrophoresis on 1.0% agarose gel and transferred to a Hybond-N$^+$ nylon membrane (Amersham Pharmacia, Buckinghamshire, UK). About 3.2 kb of the HBV X genome (HBV-X) was amplified according to the method of Günther et al. [16]. HBV-specific bands were then detected by hybridization with a DIG labeled HBV probe (Roche, Mannheim, Germany).

2.4. Direct sequencing

The amplified viral/host junctions were purified with an Easy Trap Kit (Takara, Otsu, Japan) and sequenced using a Prism Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Products were precipitated with ethanol and analyzed with a 377 Prism DNA Sequencer (Applied Biosystems Inc.). To identify the HBV-X integration site, we used BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to compare sequences adjacent to the integrated HBV–DNA with the human genome.

2.5. Other serological and virological tests

HBV surface antigen, surface antibody, and core antibody were measured with ARCHITECT HBsAg QT, ARCHITECT anti-HBs, and ARCHITECT anti-HBc, respectively (Abbott Japan, Tokyo, Japan). Serum HBV–DNA was measured by the Amplicor HBV test (detection limit, 400 copies/mL; Roche Diagnostics, Branchburg, NJ). HCV genotype was determined by PCR with genotype-specific primers [17,18]. HCV RNA concentration was measured by a quantitative PCR assay (detection limit, 5000 copies/mL; Amplicor GT-HCV Monitor, Version 2.0; Roche Molecular Systems, Pleasanton, CA).

2.6. Statistical analyses

Data are expressed as means ± SD or the median and range. Differences in the proportion of patients with and without HBV-X integration were analyzed by χ^2 test. Differences in quantitative values were analyzed by Mann–Whitney U test. For the incidence of HCC

Table 2
Sequences of primers for detection of viral–host junctions

Primer name	Primer sequence	HBV portion and note
UP5	5′-CAGUGCCAAGUGUUUGCUGACGCCAAAGUGCUGGGAUUA-3′	Alu-sense
T3-515	5′-AUUAACCCUCACUAAAGCCUCGAUAGAUYRYRCCAYUGCAC-3′	Alu-antisense
UP6	5′-CAAGTGTTTGCTGACGCCAAAG-3′	Alu-sense (tag)
midT3	5′-ATTAACCCTCACTAAAGCCTCG-3′	Alu-antisense (tag)
pUTP	5′-ACAUGAACCUUUACCCCGUUGC-3′	1131–1152 HB1 (HBV-X)
MD37	5′-TGCCAAGTGTTTGCTGACGC-3′	1174–1193 HB2 (HBV-X)
MD60	5′-CTGCCGATCCATACTGCGGAAC-3′	1258–1279 HB3 (HBV-X)

Numbering of nucleotides is according to Ono et al. [31]. U = dUTP; Y = (C,T); R = (A,G).

development, the date of the initial liver biopsy was defined as time zero. Data pertaining to patients who did not develop HCC were censored. The Kaplan–Meier method was used to calculate the incidence of HCC, and the log-rank test was used to analyze differences. The JMP statistical software package, version 4.0, (SAS Institute, Cary, NC) was used for all statistical analyses. All p values were derived from two-tailed tests, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Integration of hepatitis B viral genome and patient characteristics

The sensitivity of the PCR amplification was first determined with hepatoma cell line Huh-2 cells. When we made a tenfold serial dilution of Huh-2 cells with normal human PBMC without a history of liver disease, we could detect viral–host junctions at about 100 copies per reaction by the PCR (Fig. 1a).

We amplified virus–host DNA junctions from the liver of CH-C patients and detected several bands on 1.0%-agarose gels (Fig. 1b). Sequencing these PCR

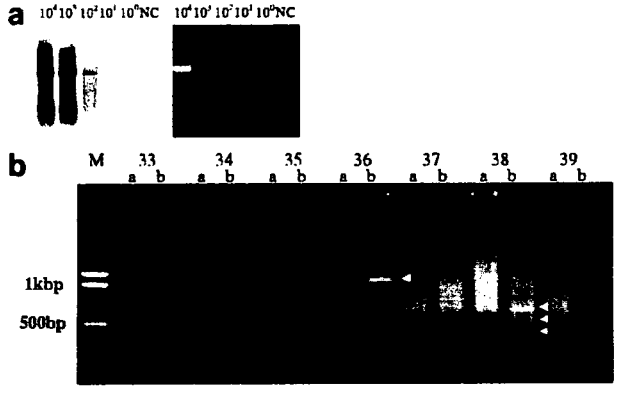

Fig. 1. The detection of HBV-X–host junction by Alu-PCR analysis. (a) The sensitivity study of Alu-PCR method. Serially diluted genomic DNA contained with HBV integrant was amplified by using HBV-X and Alu antisense primer pair. Left is Southern blot analysis from the gel electropheresis (right). (b) The numbers indicate the individual patients, and a and b indicate the primer pair used for amplification (a, HBV-X primer and Alu sense; b, HBV-X primer and Alu antisense). The PCR strategy and the primer sequences used in this study were previously described [12–14]. Arrowheads indicate PCR products with HBV-X–host junctional sequences (white) and without HBV-X–host junctions (black).

products revealed HBV-X integration in 9 of the 39 (23.1%) patients. Nineteen viral–host junctions were detected in these 9 patients. In 4 of these 9 patients, multiple integration sites (range, 2–6) were present. For example, 6 viral–host junctions were detected in patient 15, and the adjacent host sequences were from 6 different chromosomes (red circle, Fig. 2). In the other 5 cases, a single integration site was detected. The sites of HBV-X integration are shown in Fig. 2.

Clinical characteristics of patients with and without HBV-X integration are summarized in Table 3. There were no differences in the clinical characteristics. During the observation period, 4 of 9 (44.4%) patients with HBV-X integration and 13 of 30 (43.3%) patients without HBV-X integration received interferon monotherapy. These percentages did not differ significantly.

3.2. Host genome sequences at sites of HBV-X integration

The sites of host integration were divided into two groups: (1) genes already known and/or fully characterized but not previously shown to be involved in carcinogenesis (1 integration site; T cell lymphoma invasion and metastasis 1 [TIAMI] in Patient 8), and (2) unknown open reading frames (ORFs) or genes belonging to a known gene family but not functionally characterized (18 integration sites). The HBV genome ORF was integrated in both the same and opposite orientations of the host gene and both proximal to and into host genes (Table 4).

3.3. Development of HCC

Over the 12-year follow-up period, HCC developed in 6 of the 39 (15.4%) patients. HCC developed in 4 of the 30 (13.3%) patients without HBV-X integration and in 2 of the 9 (22.2%) patients with HBV-X integration (Fig. 3). The difference in the incidence of HCC between patients with and without HBV-X integration was not significant ($p = 0.8041$). Patient age, sex, and histologic data at the time of HBV-X integration analysis and at the time of HCC diagnosis are shown in Table 5. All patients who developed HCC were males. Age at the time HCC developed did not differ between patients

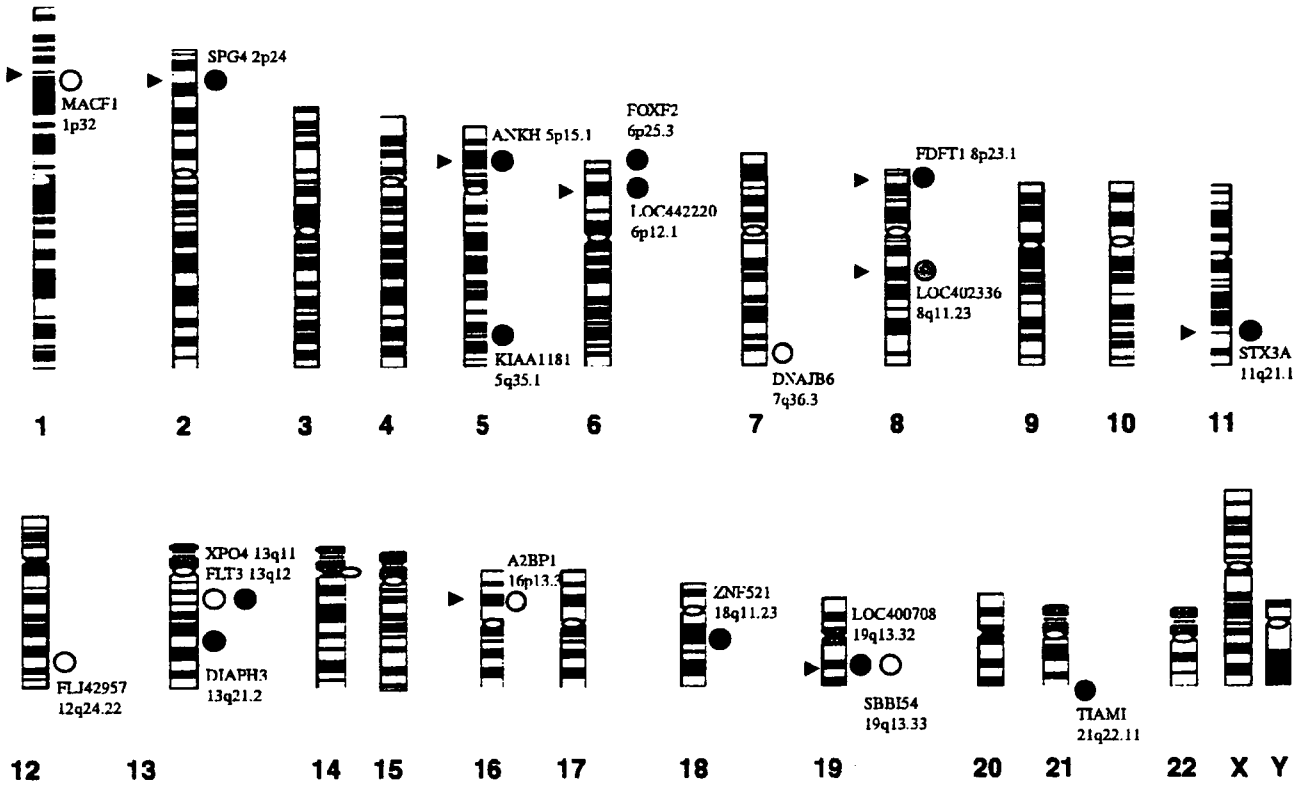

Fig. 2. Chromosomal distribution of HBV-X integration sites. Circles indicate viral integration sites, and the circle color denotes the sample. For example, the three white spots indicate three viral integration sites detected in the same specimen. Gene names and chromosomal localizations are also noted. Red arrowheads indicate DNA fragile sites [32].

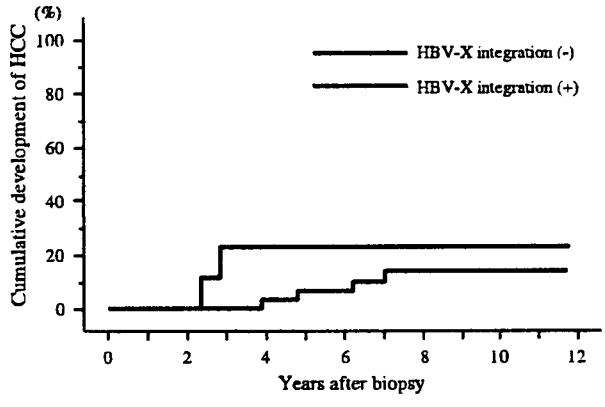

Fig. 3. Kaplan–Meier curves for the incidence of hepatocellular carcinoma (HCC). The blue and red lines represent the incidence of HCC in patients with and without HBV-X integration, respectively. No significant difference was observed between the two groups ($p = 0.8041$).

with and without HBV-X integration. Five of 6 patients who developed HCC (except for patient No. 34) had received interferon therapy, but all of them remained HCV positive. All 4 patients without HBV-X integration who developed HCC had cirrhosis at the time HCC was diagnosed. In contrast, the fibrosis stage was moderate or mild (F1 or F2) in the 2 patients with HBV-X integration who developed HCC. No patient was positive for the circulating low-level HBV–DNA analyzed with a highly sensitive HBV–DNA detection method (detection limit, 35 copies/mL; COBAS TaqMan HBV test, Roche Diagnostics) at the time of HCC diagnosis [19].

We attempted to detect HBV–host junction by the same Alu-PCR method in resected HCC materials that developed in 4 patients (patients #9, 21, 34, and 38) using paraffin-embedded samples. HBV-X integration was detected in HCC materials of none of 4 patients (data not shown).

4. Discussion

This is the first prospective study to analyze HBV integration into the host hepatocyte genome of CH-C patients with mild fibrosis and then to follow these patients over a long period for the development of HCC. Previous studies investigated HBV integration in HCC tissue of patients chronically infected with HCV [8–10] or in HCC tissue of patients without hepatitis virus infection [20]. However, in these studies, HBV integration was analyzed in cancerous and non-cancerous tissue after the development of HCC, and thus the effect of HBV integration on the development of HCC in CH-C patients was not investigated.

Table 3
Characteristics of patients with and without HBV-X–DNA integration

	HBV-X–DNA integration (−) ($n = 30$)	HBV-X–DNA integration (+) ($n = 9$)
Age (years)	48.9 ± 7.6	49.6 ± 7.7
Sex (female/male)	6 (20.0)/24 (80.0)[#]	3 (33.3)/6 (66.7)[#]
History of blood transfusion	11(36.7)[#]	4 (44.4)[#]
Presumed duration of HCV carriage[*]	19.0 (5–33)[##]	22.5 (12–33)[##]
Alanine aminotransferase (IU/L)	60.0 ± 31.8	60.4 ± 31.8
Aspartate aminotransferase (IU/L)	46.2 ± 25.9	41.0 ± 15.8
Gamma glutamyl transpeptidase (mg/L)	49.5 ± 39.0	34.6 ± 29.2
Albumin (g/dL)	4.08 ± 0.37	4.22 ± 0.14
Total-bilirubin (mg/dL)	0.70 ± 0.33	0.84 ± 0.33
Platelet count ($\times 10^4$/ml)	18.0 ± 5.3	17.6 ± 9.9
HCV RNA concentration ($\times 10^3$IU/mL)	790 (3–4900)[##]	320 (3–2100)[##]
HCV genotype		
1b	19(63.3)[#]	6 (66.7)[#]
2a	8 (26.7)[#]	3 (33.3)[#]
2b	3 (10.0)[#]	0
HBs antibody (+)	4(13.3)[#]	2 (22.2)[#]
HBc antibody (+)	20 (66.7)[#]	5 (55.6)[#]
Fibrosis stage[**]		
F0	10 (33.3)[#]	4 (44.4)[#]
F1	20 (66.7)[#]	5 (55.6)[#]

HBV, hepatitis B virus; HCV, hepatitis C virus.
[*] In patients with a history of blood transfusion.
[**] According to METAVIR score.
[#] Percentages are shown in parentheses.
[##] Median; ranges are shown in parentheses.

Table 4
Genes and sequences of HBV-X–DNA integration sites

No.	Supercontig	Position	Orientation	Chromosomal localization	Name	Location	Name/function
8.	NT034880	1375087	Same	6p25.3	FOXF2	39 kb upstream	Forkhead box F2
8.	NT086666	14245273	Opposite	5p15.1	ANKH	177 kb upstream	Ankylosis, progressive homolog
8.	NT011512	18351760	Same	21q22.11	TIAM1	21.5 kb upstream	T-cell lymphoma invasion and metastasis 1
15.	NT_022184	11183657	Same	2p24	SPG4	Intronic seq	Spastic paraplegia 4 (autosomal dominant; spastin)
15.	NT_024524	41428064	Same	13q21.2	DIAPH3	Intronic seq	Diaphanous homolog 3 (*Drosophila*)
15.	NT011109	19337933	Same	19q13.32	LOC400708	3.1 kb upstream	Similar to Serine/threonine protein phosphatase 5 (PP5)
15.	NT_077531	4155242	Opposite	8p23.1	FDFT1	Intronic seq	Farnesyl-diphosphate farnesyltransferase 1
15.	NT_010966	4345775	Opposite	18q11.2	ZNF521	23 kb upstream	Zinc finger protein 521
15.	NT_007592	46424722	Same	6p12.1	LOC442220	5.3 kb upstream	Similar to nitrogen fixation cluster-like
21.	NT_023133	17103986	Opposite	5q35.1	KIAA1181	38 kb downstream	Endoplasmic reticulum-golgi intermediate compartment 32 kDa protein
22.	NT011109	23275592	Same	19q13.33	SBBI54	Intronic seq	Hypothetical transmembrane protein SBBI54
23.	NT008183	6327145	Opposite	8q11.23	LOC402336	16.9 kb upstream	Similar to L21 ribosomal protein
24.	NT_024524	2436145	Opposite	13q11	XPO4	12.6 kb upstream	Exportin 4
27.	NT_007741	2000247	Opposite	7q36.3	DNAJB6	4 kb downstream	DnaJ (Hsp40) homolog, subfamily B, member 6 Homo sapiens
27.	NT086834	6475804	Opposite	16p13.3	A2BP1	31.9 kb upstream	Ataxin 2-binding protein 1
36.	NT_033903	4799121	Opposite	11q21.1	STX3A	29 kb downstream	Syntaxin3A
38.	NT_009775	7468765	Opposite	12q24.22	FLJ42957	71 kb downstream	FLJ42957 protein
38.	NT_024524	9545675	Opposite	13q12	FLT3	20 kb downstream	Fms-related tyrosine kinase 3
38.	NT004511	9911738	Opposite	1p32	MACF1	Intronic seq	Microtubule-actin crosslinking factor 1

In three studies of HCV-related HCC, the rates of HBV integration in tumor tissue are discrepant: 55.6% (10 out of 18 cases) [8], 29.4% (10 out of 34 cases) [10], and 0% (0 out of 21 cases) [9]. Clonal expansion of hepatocytes containing integrated HBV in association with cancer progression may increase the detection rate of HBV integration. Conversely, clonal expansion of cancerous hepatocytes without HBV integration may decrease the

Table 5
Cases of HCC development

No.	Sex	Age at biopsy	Fibrosis at biopsy	Interval between biopsy and HCC development	Age at HCC development	Fibrosis at HCC development[a]	HBV-X–DNA integration
7.	M	61	F1	4y.	65	F4	(−)
9.	M	57	F1	5y.	62	F4	(−)
21.	M	56	F1	3y.	64	F2	(+)
28.	M	56	F1	5y.	61	F4	(−)
34.	M	47	F1	7y.	54	F4	(−)
38.	M	55	F0	2y.	57	F1	(+)

[a] Non-cancerous tissue.

detection of HBV integration. Therefore, hepatocyte clonal expansion may account for discrepancies in the rates of HBV integration between studies. In contrast, clonal expansion of hepatocytes is unlikely in cases of CH-C with mild fibrosis but without HCC. The prevalence of HBV-X integration in our patient population (23.1%), therefore, represents the actual rate of HBV-X integration in CH-C patients. The number of HBV-X -host integration sites in these patients was smaller than patients with chronic hepatitis B and similar to patients with acute hepatitis B in our previous study with the same detection method for HBV integration [13].

HBV integration is detected in approximately 90% of liver tumor samples from patients with HBsAg [21]. HBV insertional mutagenesis is an important step in many cases of hepatocarcinogenesis in patients with chronic HBV infection. Chromosomal inversions, translocations, or micro deletions can occur at the integration sites, causing tumors to develop in some patients [22,23]. Several tumor-associated genes have been identified adjacent to HBV integration sites [24,25]. However, HBV does not integrate in or near a tumor-associated gene in most HBV-infected individuals. Rather, HBV–DNA integrates randomly into host DNA in HBV-related HCC [21,26,27]. This random integration also appears in patients with HCV-related HCC, although one study suggested that HBV–DNA integrates into tumor-associated genes of some HCC patients without HBsAg [8].

In the present study of CH-C patients without HCC, the HBV-X integration sites were distributed across the genome with little similarity and the host sequences adjacent to the viral genome were divergent. These data are consistent with our previous results on HBV-infected patients with the same detection method for HBV-X integration [14]. In the present study, we did not detect HBV-X integration into genes associated with carcinogenesis. Because HBV–DNA integrates randomly into host DNA and the number of HBV-integration sites was smaller in CH-C patients compared to chronic hepatitis B patients [13], the likelihood of HBV integrating into genes associated with carcinogenesis would be considerably low.

We analyzed HBV-X integration in CH-C patients with mild fibrosis and prospectively observed the patient group for 12 years. There was no statistically significant difference in the incidence of HCC between patients with and without HBV-X integration. Taken together with results from clinical observations and genetic analyses, these data suggest that testing HBV-X integration at a mild fibrosis stage may not predict the likelihood of CH-C patients developing hepatocarcinogenesis. However, the lack of statistical significance in the incidence of HCC could be partly because of the small number of study patients. Future studies with a larger patient population may detect patients with HBV integration in tumor-associated genes and a higher incidence of HCC development in CH-C patients with HBV integration.

In the present study, there was no cirrhosis in non-cancerous liver tissue surrounding the tumor at the time of HCC development, and fibrosis was not severe (stage F1 or F2) in patients with HBV-X integration. In contrast, all 4 HCC patients without HBV-X integration had cirrhosis (stage F4). In addition, the interval between the analysis of HBV-X integration and HCC development was shorter in patients with HBV-X integration than those without HBV-X integration. The stage of fibrosis, especially the presence of cirrhosis, is related closely to the incidence of HCV-related HCC [28], and most patients with HCV-related HCC have cirrhosis [10,29]. Our results showed that HCC develops in the absence of cirrhosis in some CH-C patients with HBV-X integration, and this may suggest the possibility that HBV-X integration may play a role in accelerated hepatocarcinogenesis in some cases. However, we did not detect HBV-X integration in paraffin-embedded resected HCC materials of both 2 patients with HBV-X integration at liver biopsy (patients #21 and #38). Although this can be partly due to the use of paraffin-embedded materials for analyses of integration (unfortunately frozen section was not available), we did not find the evidence that HBV-X integration directly played a role in hepatocarcinogenesis in the present study.

There are several limitations of the study. The detection of HBV integration with PCR using Alu repeats may limit the identification of HBV-X sequence integration sites that are far away from the priming site,

therefore, restricting the sensitivity of the assay as the amplicon size increases. In addition, detection of HBV integration only using the X gene-specific primers makes infeasible identification of integration sites of other virus gene sequences. Further, integrated HBV genome can limit or negate entirely the HBV X primer-binding site, because HBV sequences may be deleted upon integration. The Alu-PCR method used in the present study, therefore, may underestimate the integration of HBV in CH-C patients.

In summary, HBV-X integration was detected in 9 of 39 CH-C patients and the number of HBV-X–host integration sites in these patients was similar to patients with acute hepatitis B. They were distributed across the genome with little similarity. In the prospective observation of CH-C patients over 12 years, HBV-X integration detected at the mild fibrosis stage might not indicate a high risk for HCC during the course of CH-C. Although HBV-X integration may be associated with HCC development in the absence of cirrhosis, we did not find evidence that HBV-X integration directly plays a role for hepatocarcinogenesis in this patient population. Further studies with more sensitive and reliable method than Alu-PCR method for the detection of HBV integration are needed to elucidate the association between HBV integration and HCC development in CH-C patients without cirrhosis. Also, the analyses for HBV integration in frozen sections of resected HCC materials from CH-C patients in whom HBV integration was detected at the mild fibrosis stage may provide the evidence for direct association between HBV integration and accelerated hepatocarcinogenesis in this population. In addition, the association between genotype of integrated HBV and hepatocarcinogenesis in this population should also be investigated in the future, because the potential incidence of HCC reportedly differs according to HBV genotype in case of HBV-infected patients [30].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2007.08.016.

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HEPATOLOGY

Efficacy of antiviral therapy with lamivudine after initial treatment for hepatitis B virus-related hepatocellular carcinoma

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hepatitis B virus, hepatocellular carcinoma, lamivudine, recurrence, survival.

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Abstract

Aim: The aim of this study was to determine whether antiviral therapy with lamivudine is beneficial in patients after initial treatment for hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC).

Methods: Forty-nine consecutive patients with HBV-related HCC completely treated by hepatic resection or radiofrequency ablation were retrospectively enrolled in this study. Comparison was made between 16 patients who received lamivudine therapy at a dose of 100 mg/day after treatment for HCC (lamivudine group) and 33 patients who did not (control group) in terms of changes in remnant liver function, HCC recurrence and survival.

Results: Cumulative recurrence rates of HCC did not significantly differ between the two groups ($P = 0.622$). However, median Child–Pugh score at the time of HCC recurrence was significantly different; 5 (range 5–6) in the lamivudine group versus 7 (range 5–12) in the control group ($P = 0.005$). All patients in the lamivudine group were able to receive curative treatment for recurrent HCC. In contrast, 10 of 15 patients in the control group were unable to receive curative optimal therapy for recurrent HCC due to deterioration of remnant liver function. The cumulative survival rates of patients in the lamivudine group tended to be higher than those of patients in the control group ($P = 0.063$).

Conclusion: It is suggested that lamivudine therapy is beneficial for patients after initial treatment for HBV-related HCC because it contributes to improving remnant liver function, thus decreasing the risk of liver failure and increasing the chances of receiving available treatment modalities for recurrent HCC.

Introduction

Hepatocellular carcinoma (HCC) has a characteristically high rate of recurrence, including intrahepatic and multicentric recurrences, even if treatment for HCC results in complete curative response, due to the underlying chronic liver disease.[1–3] The frequent recurrence of HCC contributes to short survival because repeated curative treatment is often difficult due to deterioration of liver function. There are several established treatment options for HCC. Generally, hepatic resection, percutaneous ethanol injection therapy (PEIT), percutaneous microwave coagulation therapy (PMCT), radiofrequency ablation (RFA) and cadaveric or living-related liver transplantation are recognized as curative treatment options for HCC. However, patients with HCC can not always receive optimal treatment, as treatment choice is limited by remnant liver function.[4–6] Treatment options in patients with cirrhosis are often restricted, and many patients cannot receive optimal curative treatments, as such treatments may lead to severe hepatic decompensation. In order to have optimal treatment options when HCC recurs, it is very important that remnant liver function is improved or well maintained after treatment for initial HCC.

Several studies have recently reported that lamivudine, a nucleoside analog that inhibits the reverse transcriptase activity of viral DNA polymerase, has been useful for hepatitis B virus (HBV)-infected patients; not only for patients with chronic hepatitis but also those with decompensated liver cirrhosis.[7–13] These studies reported that lamivudine treatment consistently reduced HBV replication, and thus might improve remnant liver function, prevent liver failure and prolong survival.

The prevention of recurrent HCC in patients after curative treatment is important in order to improve prognosis.[14,15] Recently, there have been reports regarding the effects of lamivudine on the prevention of initial HCC.[16,17] Liaw *et al.* recently documented that lamivudine reduced not only the risk of hepatic decompensation but also the risk of initial HCC among patients with chronic HBV

and advanced hepatic fibrosis.[16] To our knowledge, however, there are very few reports that have documented whether lamivudine therapy is beneficial with regard to the incidence of recurrent HCC or survival after initial HCC treatment.[18]

In the present study, we retrospectively evaluated the efficacy of antiviral therapy with lamivudine in patients judged as having complete curative response to initial treatment for HBV-related HCC by comparing changes in remnant liver function, incidence of HCC recurrence and survival between patients who started lamivudine therapy after initial HCC treatment and those who did not.

Methods

Between December 1998 and December 2004, a total of 105 patients with chronic HBV infection were diagnosed as having initial HCC (not recurrence) and were treated at the Department of Gastroenterology, Nagoya University School of Medicine or the Department of Gastroenterology, Ogaki Municipal Hospital. All patients were positive for hepatitis B surface antigen (HBsAg) and were not positive for hepatitis C virus antibody. Serum HBV-DNA was detected in all but three patients.

The inclusion criteria for this study were as follows: (i) patients who did not receive lamivudine therapy prior to diagnosis of initial HCC; (ii) patients who underwent hepatic resection or RFA for initial HCC treatment; and (iii) patients who were judged as having complete curative response 1 month after initial HCC treatment. Dynamic computed tomography (CT) was performed at 1 month after initial treatment of HCC in all patients in order to assess the therapeutic effects; no enhancement in the treated area was considered to indicate complete curative response.

Of 105 patients, 49 patients meeting the inclusion criteria were enrolled. They comprised 41 men and 8 women (mean age, 60.6 ± 9.2 years). Thirty-one patients who underwent hepatic resection were histologically confirmed as having HCC, and 18 patients who received RFA were diagnosed as having HCC by ultrasonography (US), dynamic CT, arterial angiography and angiography-assisted CT. None of the 49 patients exhibited extrahepatic metastasis and/or vessel invasion. HCC Stage was determined according to the criteria of the Liver Cancer Study Group of Japan.[19] None of the patients received any antiviral drugs, such as interferon, for at least 1 year prior to lamivudine administration.

Of the 49 patients, 16 received lamivudine (Zeffix, Glaxo-Smith-Kline, UK) at a dose of 100 mg/day (lamivudine group) for as long as possible. The remaining 33 patients did not receive lamivudine (control group).

Biochemical tests, including alanine aminotransferase (ALT), prothrombin time (PT), albumin, total bilirubin and platelet count, were performed using standard methods. HBsAg, hepatitis B envelop antigen (HBeAg) and hepatitis B envelop antibody (HBeAb) were determined by enzyme immunoassay. HBV-DNA was quantified by PCR assay (Amplicor HBV monitor assay, Roche Diagnostics, Manheim, Germany). The lower limit of the assay was 2.6 log copies/mL. These parameters were measured every 1–3 months during lamivudine treatment.

All patients were followed primarily with abdominal US and liver function tests, as well as measurement of tumor markers, serum α-fetoprotein and des-gamma-carboxy prothrombin, at 1- to 3-month intervals after initial treatment for HCC. When suspicious findings on US or tumor markers were detected, dynamic CT was performed in order to examine recurrent HCC. Angiography-assisted CT was performed whenever possible.

Data analyses were performed using the JMP statistical software package, version 4.0 (SAS Institute, Cary, NY, USA.). Continuous data are expressed as mean values ± SD. HBV-DNA levels and Child–Pugh scores are given as median values with ranges. For qualitative variables, chi-squared test or Fisher's exact probability test was performed where appropriate. For continuous variables, Student's *t*-test or Mann–Whitney *U*-test was performed where appropriate. Cumulative recurrence and survival rates were calculated by the Kaplan–Meier method from the date of initial HCC treatment, and differences between two groups were compared by log-lank test. A *P*-value of <0.05 was considered statistically significant.

This study was approved by the ethics committees of both hospitals and was performed in compliance with the Helsinki declaration.

Results

Background characteristics

Background characteristics at the time of initial HCC treatment for the lamivudine and control groups are summarized in Table 1. There were no significant differences among the two groups with regard to age, sex, HBeAg, ALT, PT, albumin, total bilirubin, platelet count, presence of ascites, hepatic encephalopathy, Child–Pugh score, stage of initial HCC, initial HCC treatment and follow-up period. However, there was a significant difference with respect to HBV-DNA among the two groups. Median HBV-DNA levels in the lamivudine group (6.2 log copies/mL, range 2.8–8.3) were significantly higher than those in the control group (4.1 log copies/mL, range 2.6–7.1) ($P = 0.003$).

Comparison of cumulative HCC recurrence rates between lamivudine and control groups

Of the 49 patients, 22 (7 in the lamivudine group and 15 in the control group) experienced HCC recurrence during the follow-up period. The mean period until recurrence from initial treatment was 26.3 ± 21.6 months (range 4.7–56.9) in the lamivudine group and 18.7 ± 9.1 months (range 6.0–36.3) in the control group ($P = 0.250$). The sites of recurrent HCC in all 22 patients were areas of the liver distant from those initially treated for HCC.

The cumulative HCC recurrence rates in the two groups are shown in Fig. 1. Overall, cumulative HCC recurrence rates at 1, 2 and 3 years were 15.8%, 35.3% and 47.3%, respectively. The cumulative HCC recurrence rates at 1, 2 and 3 years in the lamivudine group were 13.5%, 35.1% and 35.1%, respectively, while those in the control group were 13.4%, 39.2% and 53.2%, respectively. There were no significant differences regarding the recurrence rates of HCC between the two groups ($P = 0.622$).

Biochemical and virological response, serological status and emergence of YMDD mutants in lamivudine group

In the lamivudine group, the mean lamivudine treatment period was 22.7 ± 14.2 months (range 6.3–54.8). Serial changes in bio-

Table 1 Background characteristics at the time of initial HCC treatment for the lamivudine and control groups

Characteristic	Lamivudine group ($n = 16$)	Control group ($n = 33$)	*P*-value
Age (years)	59.8 ± 7.8	61.1 ± 9.8	NS
Sex (men/women)	14/2	27/6	NS
HBeAg (+/–)	4/12	2/27	NS
HBV-DNA (log copies/mL)	6.2 (2.8–8.3)	4.1 (2.6–7.1)	0.003
ALT (IU/L)	56.6 ± 25.7	54.2 ± 44.8	NS
Total bilirubin (mg/dL)	0.8 ± 0.3	0.9 ± 0.4	NS
Albumin (g/dL)	3.7 ± 0.6	3.7 ± 0.5	NS
Prothrombin time (%)	85.3 ± 15.4	85.0 ± 15.1	NS
Platelet count ($\times 10^4$/mL)	10.8 ± 4.2	12.1 ± 5.4	NS
Ascites (+/–)	14/2	32/1	NS
Hepatic encephalopathy (+/–)	15/1	32/1	NS
Child–Pugh score	5 (5–10)	5 (5–8)	NS
Stage of initial HCC (I/II/III)	12/3/1	13/16/4	NS
Initial treatment for HCC (Ope/RFA)	13/3	18/15	NS
Follow-up period (month)	38.0 ± 21.6	32.6 ± 18.9	NS

ALT, alanine aminotransferase; HBeAg, hepatitis B envelop antigen; HBV-DNA hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation.
Values shown as mean ± SD, number of patients, or median (range).

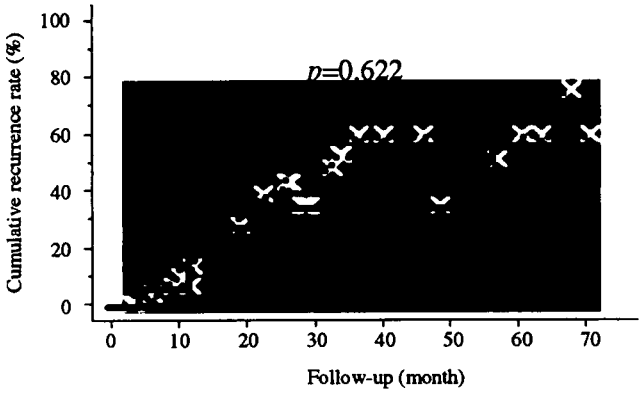

Figure 1 Comparison of cumulative hepatocellular carcinoma recurrence rates in (—) the lamivudine group, $n = 16$, and (- - -) the control group, $n = 33$.

chemical data, including ALT, total bilirubin, albumin, PT, platelet counts and serial changes in HBV-DNA levels before, and at 1, 3, 6, 9 and 12 months after lamivudine administration were investigated and are summarized in Table 2 (including 2 patients with less than 1 year of lamivudine administration). In comparison with those at the start of lamivudine administration, albumin levels gradually but significantly increased, and ALT levels gradually but significantly decreased. Total bilirubin, PT and platelet levels did not show any significant changes.

HBV-DNA levels decreased significantly when compared with those before the start of lamivudine administration. Two of four patients in the lamivudine group who were positive for HBeAg at the start of lamivudine administration exhibited seroconversion to HBeAb positivity. The emergence of YMDD mutants was observed in five of 16 patients in the lamivudine group (31.6%). Of these, three patients maintained stable liver function, while two exhibited breakthrough hepatitis, which was controlled after adefovir dipivoxil administration at a dose of 10 mg/day. There were no serious adverse effects during lamivudine therapy.

We compared the serial changes in ALT levels and HBV-DNA levels during the year of lamivudine administration between seven patients with HCC recurrence and nine patients without HCC recurrence in the lamivudine group. Serial changes are shown in Fig. 2; there were no significant differences among serial changes in ALT levels and HBV-DNA levels ($P = 0.832$ and $P = 0.290$, respectively).

Comparison of data at the time of initial HCC and at the time of recurrent HCC for the patients with recurrent HCC

We compared ALT levels, HBV-DNA levels and Child–Pugh scores at the time of initial HCC and recurrent HCC in 22 patients with recurrent HCC.

Data for the seven patients in the lamivudine group with recurrent HCC are summarized in Table 3. Mean ALT levels decreased significantly from 56.1 ± 25.3 IU/L (range 32–104) at the time of initial HCC to 36.3 ± 8.1 IU/L (range 22–48) at the time of recurrent HCC ($P = 0.028$). Median HBV-DNA levels were 6.3 log copies/mL (range 4.2–8.3) at the time of initial HCC, while those at the time of recurrent HCC were undetectable (<2.6 log copies/mL) ($P = 0.018$). Median Child–Pugh scores were not significantly different; 5 (range 5–10) at initial HCC versus 5 (range 5–6) at the time of recurrent HCC. All seven patients were able to receive curative treatment for recurrent HCC and experienced complete therapeutic response.

Data for the 15 patients in the control group with recurrent HCC are summarized in Table 4. With regard to mean ALT levels and median HBV-DNA levels, there were no significant differences between those at the time of initial HCC and those at the time of recurrent HCC. Median Child–Pugh scores increased significantly from 5 (range 5–8) at the time of initial HCC to 7 (range 5–12) at the time of recurrent HCC ($P = 0.013$). Five of 15 patients were able to receive curative treatment (RFA) for recur-

Table 2 Serial changes in biochemical data and HBV-DNA levels before, and at set time points after lamivudine administration

Variable	Pretreatment	1 month	3 months	6 months	9 months	12 months
ALT (IU/L)	56.6 ± 25.7	58.9 ± 38.3	44.1 ± 35.9*	39.9 ± 21.9*	38.7 ± 21.1*	35.9 ± 15.6*
Total bilirubin (mg/dL)	0.88 ± 0.30	0.95 ± 0.58	0.92 ± 0.35	0.86 ± 0.40	0.86 ± 0.28	0.99 ± 0.40
Albumin (g/dL)	3.66 ± 0.63	3.69 ± 0.43	3.82 ± 0.39	3.83 ± 0.35	3.89 ± 0.35	3.97 ± 0.28*
Prothrombin time (%)	85.3 ± 15.4	88.8 ± 14.3	90.6 ± 15.0	85.6 ± 12.3	90.0 ± 14.1	88.3 ± 16.9
Platelet count (x10^4/mL)	10.8 ± 4.2	11.0 ± 4.1	10.4 ± 3.8	9.8 ± 3.3	10.0 ± 4.5	10.4 ± 3.6
HBV-DNA (log copies/mL)	6.2 (2.8–8.3)	4.1 (2.6–6.2)*	2.6 (2.6–4.0)*	2.6 (2.6–3.6)*	2.6 (2.6–3.2)*	2.6 (2.6–3.7)*

*$P < 0.05$ *vs* pretreatment data. ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA.
Values shown as mean ± SD, or median (range).

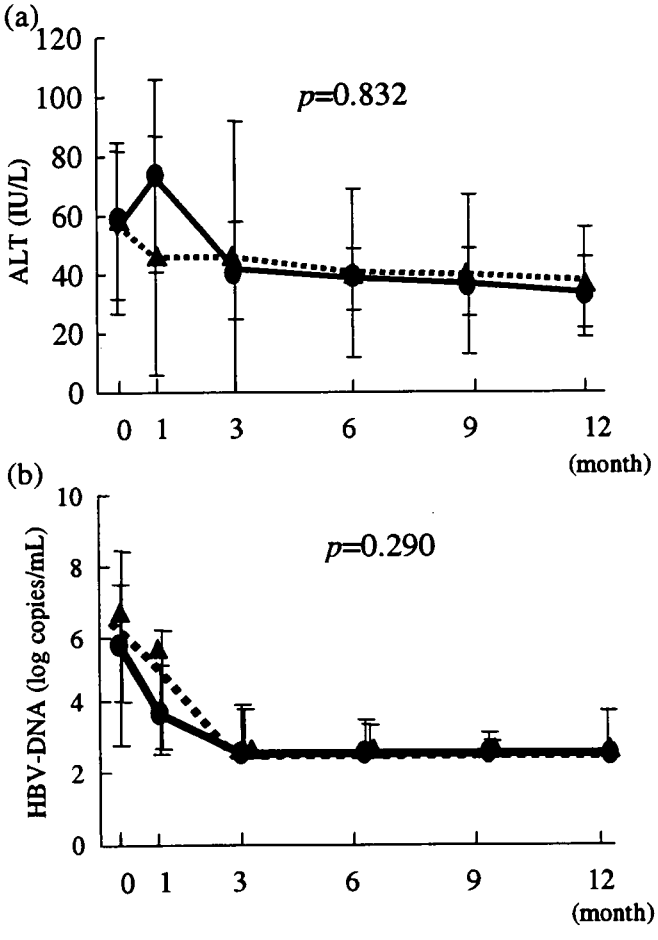

Figure 2 Serial changes in (a) alanine aminotransferase (ALT) levels and (b) hepatitis B virus (HBV)-DNA levels during the course of lamivudine administration in (●, —) seven patients with recurrent hepatocellular carcinoma (HCC) and (▲, - - -) nine patients without recurrent HCC.

rent HCC, but the remaining 10 patients were not able to receive optimal curative therapy, hepatic resection or RFA, for recurrent HCC due to deterioration of remnant liver function. Of the seven patients receiving transcatheter arterial embolization (TAE) for recurrent HCC, four received TAE that was not satisfactorily performed due to deterioration of remnant liver function. The remaining three patients were unable to receive any treatment for recurrent HCC due to poor liver function.

Data at the time of recurrent HCC comparing the lamivudine group and the control group are summarized in Table 5. When compared with data for the control group, median HBV-DNA levels were significantly reduced ($P = 0.023$) and median Child–Pugh scores were significantly lower ($P = 0.005$) in the lamivudine group. With regard to treatment for recurrent HCC, patients in the lamivudine group were significantly more able to receive curative treatments, such as hepatic resection or RFA ($P = 0.014$). There were no significant differences in mean ALT levels and stage of recurrent HCC.

Comparison of cumulative survival rates between lamivudine and control groups

The mean follow-up period was 38.0 ± 21.6 months (range 9.2–78.2) for the lamivudine group and 32.6 ± 18.9 months (range 0.5–75.7) for the control group ($P = 0.378$). The cumulative survival rates in the two groups are shown in Fig. 3. All 16 patients in the lamivudine group remained alive during the follow-up period. However, six of 33 patients in the control group died. The cumulative survival rates at 1, 2 and 3 years in the control group were 13.4%, 39.2% and 53.2%, respectively.

There were no significant differences with regard to survival rate between the two groups; however, the survival rates in the lamivudine group tended to be higher than those in the control group ($P = 0.063$). As for cause of death, in the control group, three patients died of progressive liver failure and the other three patients died of HCC progression.

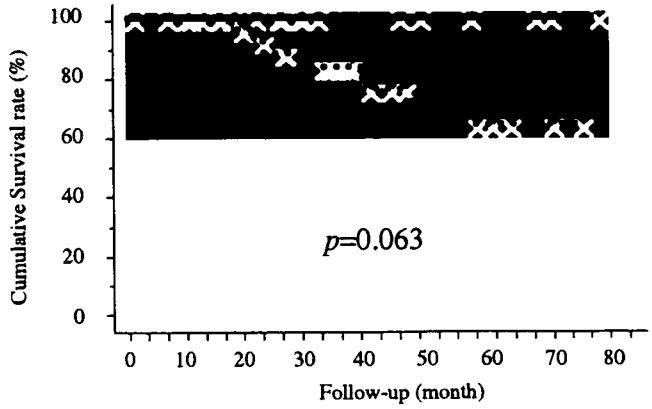

Figure 3 Comparison of cumulative survival rates in the (—) the lamivudine group, $n = 16$, and (- - -) the control group, $n = 33$.

Table 3 Comparison of data at initial and recurrent HCC for the 7 patients in the lamivudine group with recurrent HCC

Variable	Initial HCC	Recurrent HCC	*P*-value
HBV-DNA (log copies/mL)	6.3 (4.2–8.3)	<2.6	0.018
ALT (IU/L)	56.1 ± 25.3 (32–104)	36.3 ± 8.1 (22–48)	0.028
Child–Pugh score	5 (5–10)	5 (5–6)	NS
HCC stage (I/II/III)	5/1/1	4/3/0	NS
Treatment for recurrent HCC (Ope/RFA/TAE/None)	5/2/0/0	2/5/0/0	NS

ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.
Values shown as mean ± SD, number of patients, or median (range).

Table 4 Comparison of data at initial and recurrent HCC for the 15 patients in the control group with recurrent HCC

Variable	Initial HCC	Recurrent HCC	*P*-value
HBV-DNA (log copies/mL)	4.9 (3.2–6.4)	4.2 (2.6–6.1)	NS
ALT (IU/L)	66.1 ± 49.2 (17–207)	58.4 ± 41.2 (18–160)	NS
Child–Pugh score	5 (5–8)	7 (5–12)	0.013
HCC stage (I/II/III)	5/6/4	7/8/0	NS
Treatment for recurrent HCC (Ope/RFA/TAE/None)	5/10/0/0	0/5/7/3	0.001

ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.
Values shown as mean ± SD, number of patients, or median (range).

Table 5 Comparison of data at the time of recurrent HCC in the lamivudine and control groups ($n = 22$)

Variable	Lamivudine ($n = 7$)	Control ($n = 15$)	*P*-value
HBV-DNA (log copies/mL)	<2.6	4.2 (2.6–6.1)	0.023
ALT (IU/L)	36.3 ± 8.1 (22–48)	58.4 ± 41.2 (18–160)	NS
Child–Pugh score	5 (5–6)	7 (5–12)	0.005
HCC stage (I/II/III)	4/3/0	7/8/0	NS
Treatment for recurrent HCC (Ope/RFA/TAE/None)	2/5/0/0	0/5/7/3	0.014

ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.
Values shown as mean ± SD, number of patients, or median (range).

Discussion

There have been several studies investigating whether antiviral therapy with interferon is useful in reducing the incidence of HBV-related HCC.[3,20–23] Some of these studies reported that interferon was beneficial for preventing HCC only in patients achieving sustained suppression of HBV replication. Lin *et al.* conducted a randomized controlled trial in order to evaluate the effectiveness of interferon alpha with 101 HBeAg-positive Taiwanese men.[20] They found that HCC occurred in one (1.5%) of the 67 treated patients and four (12%) untreated patients ($P = 0.047$) over a period of 1–12 years. Multivariate analysis revealed that interferon therapy, preexisting cirrhosis and patient age at entry were significant independent factors for HCC development. In contrast, other reports have demonstrated that the incidence of HCC in patients undergoing interferon therapy does not significantly vary between responders and non-responders.[21,23] Thus, the effects of interferon on the prevention of HCC have not yet been adequately clarified, possibly because the therapeutic efficacy of interferon is low.

There have been some recent reports regarding the efficacy of lamivudine treatment in reducing the incidence of HCC.[16,17] Liaw *et al.* conducted a prospective randomized controlled trial in order to evaluate the efficacy of lamivudine therapy in HBV patients, with incidence of HCC as an endpoint.[16] They reported that among 651 HBsAg-positive Asian patients, HCC occurred in 17 (3.9%) lamivudine-treated patients and 16 (7.4%) placebo controls, with a hazard ratio of 0.49 ($P = 0.047$) during a median follow-up of 32 months (range 0–42). They concluded that lamivudine treatment in chronic HBV patients not only reduces the incidence of hepatic decompensation but also reduces the incidence of HCC.

With regard to the incidence of recurrent HBV-related HCC, Lin *et al.* also conducted a prospective randomized controlled study in order to evaluate the effectiveness of interferon alpha with 16 HBV patients after medical ablation therapy for primary tumors.[3] They found that HCC recurred in four of four (100%) untreated patients and in four of 12 (33.3%) interferon alpha-treated patients ($P = 0.0384$). They concluded that interferon alpha therapy may reduce HCC recurrence after medical ablation for primary HCC, although the sample size was too small to reach a firm conclusion.

There are few reports regarding the effects of lamivudine therapy on recurrent HCC.[18] In the present study, the cumulative recurrence rates of HCC after initial and complete treatment for HCC did not significantly differ between the lamivudine group and the control group. These results are consistent with the recurrence rates recently reported by Piao *et al.*[18] In the present study, among 16 patients receiving lamivudine, serial changes in ALT and HBV-DNA levels in seven patients with recurrent HCC were similar to those in nine patients without recurrent HCC. These results indicate that lamivudine treatment after initial HCC treatment reduces both ALT levels and HBV-DNA levels, but is not associated with the incidence of recurrent HCC. This is not consistent with previous reports that have documented antiviral therapy contributing to reduced incidences of HCC.[14,20,22,24] However, the present study is insufficient to support a conclusion on whether lamivudine therapy is useful in preventing recurrent HCC.

In the present study, in order to evaluate whether lamivudine contributes to preventing the incidence of recurrent HCC as accurately as possible, we selected 49 consecutive patients successfully treated for initial HCC by hepatic resection or RFA. In patients in whom HCC recurred, the locations of recurrent HCC were all distant from the site of primary HCC, but it was actually difficult to distinguish whether recurrent HCC was derived from intrahepatic metastasis or multicentric occurrence. Previous reports have documented that within a few years of HCC treatment, undetectable intrahepatic metastases are already present at distant sites of the liver.[25] In the present study, 19 of 49 patients (47.3%) had recurrence within 3 years. Our study therefore might have included patients with recurrent HCC due to intrahepatic metastasis. This may be one of the reasons for a lack of differences regarding recurrence rates between the lamivudine and control groups. Further studies with larger numbers of patients and longer follow-up periods are necessary to clarify whether lamivudine is able to prevent HCC. Such studies may clarify whether decreasing ALT and HBV-DNA levels during lamivudine administration contribute to reduced incidence of multicentric HCC recurrence.

To our knowledge, this is the first report regarding liver function and HBV-DNA levels at the time of HCC recurrence. In the lamivudine group, median Child–Pugh scores at the time of HCC recurrence were significantly lower than those in the control group. In the lamivudine group, due to good remnant liver function, all seven patients with recurrence were able to receive optimal and curative treatment, such as hepatic resection or RFA. As a result, all experienced good local control of recurrent HCC. We believe that these results account for the improvement in cumulative survival rates among patients in the lamivudine group. All seven patients with recurrent HCC in the lamivudine group had serum HBV-DNA levels of less than 2.6. In contrast, in the control group, some patients were not able to receive curative treatment at the time of HCC recurrence due to their poor remnant liver function, and three patients were unable to receive any treatment. Our most important finding was that remnant liver function in patients with lamivudine was restored or well maintained at the time of recurrent HCC, and this allowed patients to receive curative treatment, resulting in improved survival.

In the present study, there were no serious adverse effects as a result of lamivudine therapy. The most significant problem associated with lamivudine therapy is the emergence of YMDD mutants.[26,27] These induce a relapse of hepatitis (breakthrough hepatitis) and can result in fatal liver failure. Adefovir dipivoxil and entecavir have recently been introduced in an effort to treat breakthrough hepatitis.[26,27] In the present study, the emergence of YMDD mutants was observed in five of 16 patients in the lamivudine group, and two of these developed breakthrough hepatitis. However, after administration of adefovir dipivoxil, breakthrough hepatitis was successfully controlled. None of the five patients developed recurrent HCC. Because new and emerging antiviral agents are increasingly available, beginning lamivudine administration after initial HCC treatment can be recommended for patients with active viral status or ALT elevation in order to prevent deterioration of remnant liver function.

In conclusion, we speculate that lamivudine therapy is beneficial for patients after initial treatment for HBV-related HCC because it contributes to improving remnant liver function, thus decreasing the risk of liver failure and increasing the chances of receiving available treatment modalities for recurrent HCC. With regard to impact on prevention of recurrent HCC, we did not observe any differences between patients who received lamivudine therapy and those who did not. However, the present study had a small sample, a short follow-up period and was retrospective. Therefore, confirmation of our findings requires further studies with larger numbers of patients and longer follow-up periods.

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Letter to the Editor

Simultaneous multicentric occurrence of early hepatocellular carcinoma in a patient with persistent alpha-fetoprotein elevation

Dear Editor

Alpha-fetoprotein (AFP) is a tumor marker for hepatocellular carcinoma (HCC).[1,2] However, we sometimes encounter patients with persistently elevated serum AFP in whom HCC is not detected. Whether the elevation of AFP is an indicator of the presence of HCC or an indicator of a high potential for hepatocarcinogenesis is not clear. Here, we describe the clinical course of a patient with persistently elevated AFP.

A 65-year-old woman had been diagnosed with chronic hepatitis C virus infection in 1991 and has since been followed up every 2 months at the Department of Gastroenterology, Ogaki Municipal Hospital. Her serum AFP remained normal (<20 ng/dL) between 1991 and 2004, but it was elevated to >400 ng/dL on December 2004. The elevation persisted thereafter, fluctuating between 400 ng/dL and 1000 ng/dL despite the constant mild elevation of serum alanine aminotransferase (ALT) activity and the lack of elevation of *Lens culinaris* agglutinin A-reactive fraction of AFP and des-gamma-carboxy prothrombin. In January 2005, she underwent angiography and angiography-assisted computed tomography (CT) (i.e. CT during arterial portography [CTAP] and CT during hepatic arteriography [CTHA]), which are one of the most sensitive modalities for detecting hepatic tumor.[3] No liver tumor, however, was detected during this examination.

A hepatic mass lesion was detected during a routine outpatient ultrasonography in February 2006. The patient's serum AFP at that time was 970.4 ng/dL. She again underwent angiographic examination of the liver. Four small liver nodules (<2.0 cm in diameter) were found as attenuated areas during CTAP, and partially increased attenuation was detected within three of the four attenuated areas (arrows, Fig. 1a1–a3). In contrast, during CTHA, three minute enhanced areas were found that corresponded to three of the areas of increased attenuation found during CTAP (arrows, Fig. 1b1–b3). One tumor lacked an enhanced area on the CTHA. Ultrasonography-guided fine-needle biopsy of the tumors revealed four well-differentiated HCC, within one of which the component of moderate differentiation was observed. Her remnant liver function was Child–Pugh class B at diagnosis and each of the four tumors was treated by radiofrequency thermal ablation. Her serum AFP decreased to 290.8 ng/dL 1 month after the treatment.

A decrease in portal venous flow and an increase in arterial blood flow are characteristics of typical HCC.[4] However, an increase in arterial blood flow is absent in early-stage HCC, whereas a decrease in portal venous flow is present. In the nodule-in-nodule-type HCC,[5] early-stage HCC contains a part with features of typical HCC within a tumor. Three of the four HCC we found in the present case were of this nodule-in-nodule-type HCC, and the other was an early-stage HCC. These HCC are unlikely to produce intrahepatic metastases[6] and were therefore believed to develop simultaneously and independently and were considered a multicentric occurrence.[7]

Although AFP is the tumor marker that is used most widely for monitoring the development of HCC, AFP also increases in association with hepatocyte regeneration.[8] AFP therefore does not always reflect the development of HCC. Because AFP elevation indicates enhanced liver regeneration as well as advanced liver fibrosis,[9] however, it could also be a marker for a high risk of developing HCC[10] and further multicentric ocurrence of HCC. Patients with persistent AFP elevation should therefore be monitored intensively for HCC, and careful imaging studies with sensitive methods for HCC should be required in order not to miss the development of HCC only, but also the multicentric occurrence of HCC.

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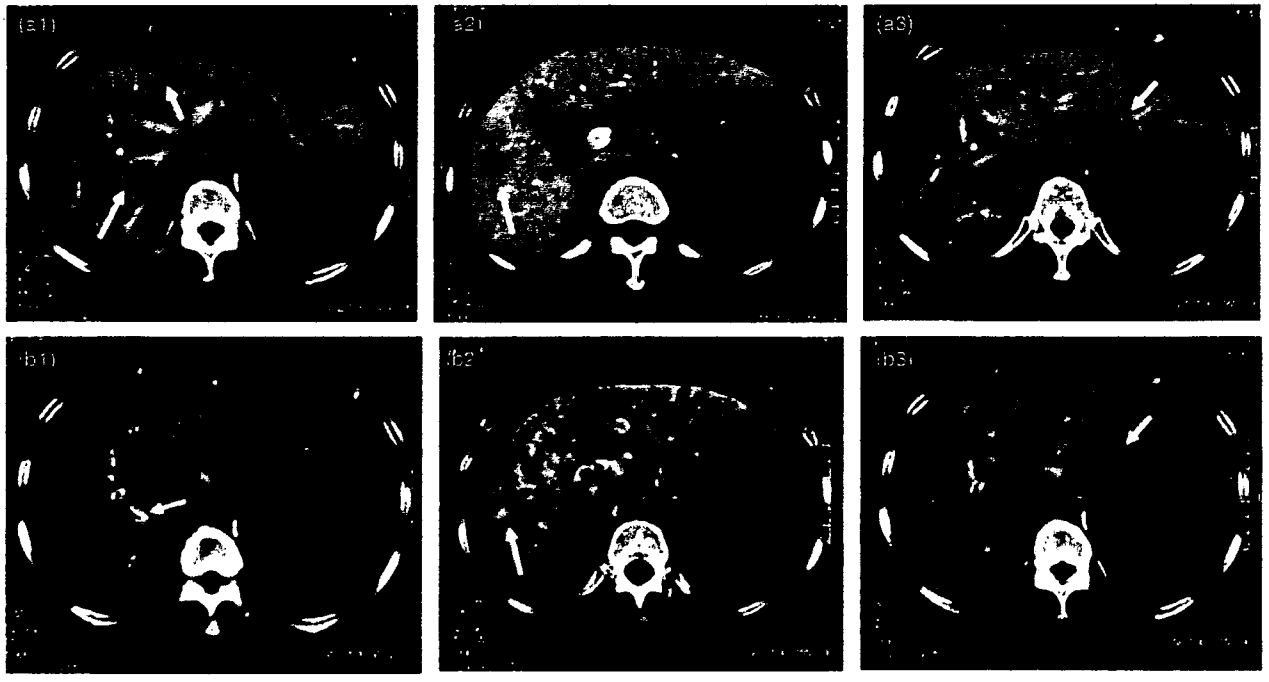

Figure 1 Findings of (a1–a3) computed tomography (CT) during arterial portography (CTAP) and (b1–b3) CT during hepatic arteriography (CTHA) in February 2006. (a1–a3) Four small liver nodules (all < 2.0 cm in diameter) were observed as attenuated nodules (low-density areas), three of which contained a small area of increased attenuation (arrows). (b1–b3) Minute high-density areas were observed that corresponded to the areas of increased attenuation on CTAP images, except in one tumor (arrows).

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Original Article

Guidelines for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet counts

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Aim: We aimed to identify the candidates for antiviral therapy, among patients who are hepatitis C virus (HCV) carriers with normal serum aminotransferase (ALT), focused on the inhibition of hepatocellular carcinoma (HCC).

Methods: Four hundred and sixty-four HCV carriers with normal serum ALT and 129 HCV carriers with persistently normal ALT (PNALT) and platelet (PLT) counts ≥150 000/μL who received liver biopsies were enrolled. HCV carriers with normal serum ALT were divided into four groups according to their ALT levels (≤30 U/L or 31–40 U/L) and PLT counts (≥150 000/μL or <150 000/μL).

Results: In 129 HCV carriers with PNALT, the rate of progression of fibrosis stage was 0.05/year and no HCC was detected during the follow up for 10 years. Approximately 20% of patients with ALT ≤40 U/L and PLT counts ≥150 000/μL were at stage F2–3; however, approximately 50% of patients with ALT ≤ 40 U/L and PLT counts <150 000/μL were at stage F2–4. An algorithm for the management of HCV carriers with normal serum ALT was advocated based on ALT and PLT counts.

Conclusion: The combination of ALT and PLT counts is useful for evaluating the fibrosis stage in HCV carriers with normal serum ALT. Most patients with PLT counts <150 000/μL are candidates for antiviral therapy, especially those with ALT levels ≥31 U/L when we focus on the inhibition of the development of HCC.

Key words: antiviral therapy, chronic hepatitis C, hepatitis C virus carriers, normal serum aminotransferase, platelet count

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) caused by hepatitis C virus (HCV) infection usually develops in patients with advanced chronic hepatitis (CH) or liver cirrhosis. The antiviral treatment for chronic hepatitis C (CH-C) is useful for inhibiting hepatic inflammation and progression of hepatic fibrosis, and consequently the development of HCC.[1–6]

Serum aminotransferase (ALT) levels are within the normal ranges in 20–40% of patients with chronic HCV infection,[7–11] defining the upper limit of normal serum ALT as ≤40 U/L. Significant hepatic fibrosis (≥F2 by the METAVIR classification) has been demonstrated in 5–30% of such patients.[9,12–16] We reported previously

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that HCV carriers with persistently normal ALT (PNALT) had histological features ranging from normal to minimal CH[17,18]; they showed slow progression of liver fibrosis and were at very low risk of developing HCC.[18]

The National Institute of Health Consensus Development Conference reported that HCV carriers with normal serum ALT are candidates for antiviral therapy.[19] A controlled study for the treatment of HCV carriers with PNALT with pegylated interferon alpha and ribavirin (PEG-IFN/Riba) for 48 weeks led to the eradication of HCV RNA in 40% of patients with genotype 1 and high viral load,[20] which is similar to the results of CH-C patients with elevated ALT levels.[21,22] However, it remains controversial whether these patients are candidates for antiviral therapy because of the limited efficacy of treatment, post-treatment flare-up, various side-effects, high cost of treatment, and their good prognoses.

In many Western countries, the upper limits of normal serum ALT are below 40 U/L;[23] however, a recent report from Italy demonstrated that the upper limit in healthy individuals was less than 30 U/L for men and 19 U/L for women.[24] We attempted to draft therapeutic guidelines for the treatment of HCV carriers with normal serum ALT. The biochemical and histological analyses were performed in HCV carriers with serum ALT levels below 40 U/L. These patients were divided into two groups based on ALT levels and then further divided into two subgroups according to their platelet (PLT) counts. We proposed an algorithm for the treatment of HCV carriers with normal serum ALT, taking into consideration the risk of progression to cirrhosis and the development of HCC. The present study demonstrated that the ranges of serum ALT and PLT counts are useful for deciding the indication of antiviral therapy for HCV carriers with normal serum ALT.

METHODS

Eligibility and definition

TWELVE HEPATOLOGISTS BELONGING to the Japanese Study Group of the Standard Antiviral Therapy for Viral Hepatitis, supported by the Ministry of Health, Labour and Welfare of Japan, which was settled on April 2004, participated in the study. Hiromitsu Kumada (Toranomon Hospital, Tokyo, Japan) serves as a chief and Takeshi Okanoue served as a researcher responsible for drafting the guidelines for the treatment of HCV carriers with normal serum ALT. In the present study, we tentatively defined the upper limit of the normal serum ALT as ≤40 U/L.

Patients with hepatitis B virus surface antigen, previous IFN treatment, history of heavy alcohol abuse, antinuclear antibody or antismooth muscle antibody, overt diabetes mellitus, or obesity (body mass index; ≥25 kg/m^2) were excluded from the study.

All of the patients underwent liver biopsy (≥2.0 cm in length) within 6 months prior to antiviral therapy, at which time their serum ALT levels were ≤40 U/L. Informed consent was obtained from every patient prior to liver biopsy and antiviral therapy.

Another study was conducted from January 1990 to August 2004 at Kyoto Prefectural University of Medicine (Kyoto, Japan). HCV carriers with PNALT were defined by serum ALT levels ≤30 U/L on at least three different occasions over a 12-month period and PLT counts ≥150 000/μL as reported previously.[18]

Study design

Among the 580 HCV carriers with normal serum ALT (≤40 U/L), 116 patients were excluded from the study because of insufficient data. Thus, 464 patients who received antiviral therapy from 1995 to 2004 were enrolled in this study (Table 1). Formalin-fixed liver specimens were stained with hematoxylin–eosin, and with Masson's trichrome. The liver specimens ($n = 262$) were also stained with Perls' Prussian blue to study hepatic iron loading. The histological findings were scored according to the classification proposed by Desmet *et al.*[25] and Ishak *et al.*[26] Steatosis was defined as fat droplets in >10% of hepatocytes. The degree of iron loading was assessed using a Perls' score of 0–4+, based on the scoring system of MacSween *et al.*[27]

The serum ALT, blood glucose level, immunoreactive insulin (IRI), serum ferritin, PLT count, serum hyaluronic acid, amount of serum HCV RNA, and the HCV genotype were examined. The homeostasis model assessment–insulin resistance was calculated as follows: plasma fasting glucose (mg/dL) × IRI (ng/mL) ÷ 405. The serum HCV RNA levels were determined using an Amplicor GT HCV monitor (Roche Diagnostic Systems, Tokyo, Japan). HCV genotype 1 (G1) and 2 (G2) were determined by a serologic genotyping assay.[28] G1 and G2 in this assay correspond to genotype 1 (1a, 1b) and 2 (2a, 2b) proposed by Simmonds *et al.*[29]

All the patients received IFN monotherapy or IFN/Riba combination therapy for 12–36 weeks. The average

Table 1 Baseline of hepatitis C virus patients with normal serum aminotransferase (ALT) received antiviral therapy

	ALT ≤ 30 U/L (group A)	ALT 31–40 U/L (group B)	*P*-value
No. patients	255	209	
Age	51.6 ± 13.0	53.5 ± 13.2	0.548*
Sex (male/female)	112/143	117/92	0.01**
BMI (kg/m^2)	21.6 ± 2.9	22.8 ± 3.0	<0.001*
HOMA-IR	2.5 ± 3.2	5.2 ± 6.5	0.093*
Genotype: 1/2/others	127/127/1	112/96/1	0.881**
Viral load: low/high	138/117	99/110	0.203**
G1 (low/high)	114/125		
G2 (low/high)	161/62		
Histology			
F stage (0/1/2/3/4)	29/166/48/11/1	22/122/57/6/2	0.169**
Grade (0/1/2/3)	25/187/41/2	7/159/43/0	0.046**
Fatty change† 0–1/2–4	232/23	161/48	0.033**
Iron load‡ 0/1–4	101/15	97/19	0.458**
Ferritin (ng/mL)	83.9 ± 103.7	118.8 ± 135.3	0.006*
PLT count (/μL)	19.2 ± 5.4	18.4 ± 6.1	0.059*
≥150 000/<150 000	204/51	141/68	0.002**
Hyaluronate (ng/mL)	60.8 ± 73.7	69.1 ± 73.0	0.249*
Duration of antiviral therapy (weeks)	25.6 ± 12.0	26.1 ± 12.1	0.297*
Effects of therapy			
SVR/non-SVR	142/113	99/110	0.075**

*P-values were calculated by Mann–Whitney-*U*-test. **Fisher-exact-test. †0: no fatty change, 1: ≤10%, 2: 11–33%, 3: 34–66%, 4: ≥67% of hepatocyte; ‡no stain by 400×, 1: few stains by 250×, 2: stains by 100×, 3: stains by 25×, 4: stains by 10×. There were significant differences in sex distribution ($P = 0.01$), BMI ($P = 0.01$), frequency of steatosis ($P = 0.033$), serum ferritin level ($P = 0.006$), grade of hepatic inflammation ($P = 0.046$), incidence of fatty change ($P = 0.033$), serum ferritin level ($P = 0.006$), and the incidence of low PLT counts ($P = 0.002$) between groups A and B. Values are expressed as mean ± SD.
ALT, alanine aminotransferase; BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance; PLT, platelet; SVR, sustained viral responders.

duration of therapy between 1995 and 2003 was 26 weeks for IFN monotherapy and 24 weeks for IFN/Riba combination therapy. In principle, 6–10 MU IFN was administered daily for 2 weeks and three times per week subsequently. The daily dosage of ribavirin was 600–1000 mg depending on body weight. Sustained viral responders (SVR) were defined as patients who were negative for serum HCV RNA 6 months after the completion of antiviral therapy.

All of the patients were divided into two groups (group A: ALT ≤ 30 U/L, group B: 31 U/L ≤ ALT ≤ 40 U/L) which were further divided into two subgroups based on PLT counts: group A-1 and B-1 (PLT counts ≥150 000/μL) and groups A-2 and B-2 (PLT counts <150 000/μL).

One hundred and twenty-nine HCV carriers with PNALT were enrolled to determine their long-term prognosis. These patients showed normal serum ALT levels (≤30 U/L) over a 12-month period on least three different occasions (PLT counts ≥150 000/μL, and body mass index [BMI] <25 kg/m^2). Thirty-nine patients received serial liver biopsies. The mean follow-up period of the 129 patients was 7.2 ± 3.2 years on 15 November 2006.

Statistical analyses

Data are expressed as mean ± SD. We compared continuous variables using the Mann–Whitney *U*-test. A frequency analysis and comparison between the groups were performed using the χ^2-test or Fisher's exact test and the Mann–Whitney *U*-test. ANOVA and Tukey's HSD procedure was used to determine the difference between multiple groups. All tests were two-tailed and *P*-values of less than 0.05 were considered significant. All statistical analyses were performed using Statistical Package of Services Solutions software, version 11.0 (SPSS, Chicago, IL, USA).

Table 2 Baseline of hepatitis C virus patients with less than 30 U/L aminotransferase who received antiviral therapy

	PLT ≥ 150 000/mL (group A-1)	PLT < 150 000/mL (group A-2)	*P*-value
No. patients	204	51	
Age	48.4 ± 12.7	58.7 ± 7.5	<0.001*
Sex (male/female)	90/114	22/29	1.000**
BMI (kg/m^2)	21.6 ± 3.0	21.3 ± 2.4	0.514*
HOMA-IR	2.8 ± 3.5	1.2 ± 0.8	0.598*
Genotype: 1/2/others	101/101/2	25/26/0	0.952**
Viral load: low/high	112/92	26/25	0.574**
Histology			
F stage (0/1/2/3/4)	29/142/27/6/0	1/25/21/3/1	<0.001**
Grade (0–1/2,3)	179/25	33/18	<0.001**
Fatty change† 0–1/2–4	188/16	44/7	0.582**
Iron load‡ 0/1–4	82/12	17/3	0.762**
Ferritin (ng/mL)	86.0 ± 112.1	73.9 ± 46.6	0.204*
PLT count (/μL)	21.0 ± 4.4	12.1 ± 2.5	<0.001*
Hyaluronate (ng/mL)	41.8 ± 56.1	112.5 ± 109.9	<0.001*
Duration of antiviral therapy (weeks)	25.7 ± 10.3	27.0 ± 9.9	0.503*
Effects of therapy			
SVR/non-SVR	115/89	27/24	0.66**

*P-values were calculated by Mann–Whitney-*U*-test. **Fisher-exact-test. †0: no fatty change, 1: ≤10%, 2: 11–33%, 3: 34–66%, 4: ≥67% of hepatocyte; ‡no stain by 400×, 1: few stains by 250×, 2: stains by 100×, 3: stains by 25×, 4: stains by 10×. There were significant differences in age ($P < 0.001$), distribution of F stage ($P < 0.001$), grade of inflammatory activity ($P < 0.001$), PLT count ($P < 0.001$), and serum-hyaluronic acid ($P < 0.001$) between groups A-1 and A-2. Frequency of F2–4 patients was 16.2% in group A-1 and 51.6% in group A-2. Values are expressed as mean ± SD.
BMI, body mass index; HOMA-IR, homeostasis model assessment–insulin resistance; PLT, platelet counts; SVR, sustained viral responders.

RESULTS

Demographic, clinical, and histological features of 464 HCV carriers with normal serum ALT

THE CHARACTERISTICS OF the 464 HCV carriers with normal serum ALT are shown in Table 1. There were significant differences in sex, frequency of steatosis, serum ferritin levels, BMI, and the incidence of low PLT counts (<150 000/μL) between groups A and B.

There were significant differences in age, fibrosis (F) stage, inflammatory activity, PLT counts, and serum hyaluronate between groups A-1 and A-2 (Table 2). The frequency of stage F2–4 patients was 16.2% in group A-1, and 49.0% in group A-2 (Table 2). In group B, there were significant differences in age, F stage, PLT counts, and serum hyaluronate between groups B-1 and B-2 (Table 3). There were no F4 patients in group A-1 and B-1, and the frequency of F3 patients was very low compared with those in groups A-2 and B-2 (2.6% *vs* 7.6%). The PLT counts decreased in proportion to the progression of liver fibrosis as follows; F0 ($n = 51$); $20.7 \pm 5.2 \times 10^4$/μL, F1 ($n = 288$); $19.8 \pm 5.6 \times 10^4$/μL, F2 ($n = 105$); $16.9 \pm 5.3 \times 10^4$/μL, F3 ($n = 17$); $15.9 \pm 4.6 \times 10^4$/μL, and F4 ($n = 3$); $11.3 \pm 3.8 \times 10^4$/μL.

Of the 464 patients, the frequency of the F0–1 stages was 80.1% and that of the F2–4 stages was 19.9% in patients with PLT counts ≥150 000/μL, and it was 50.4% and 49.6%, respectively, in patients with PLT counts <150 000/μL. In patients with PLT counts $\geq 17.0 \times 10^4$/μL, 80.8% were in stages F0–1 and 19.2% were in stages F2–4, and in patients with PLT counts $< 17.0 \times 10^4$/μL, 60.1% were in stages F0–1 and 39.9% were in stages F2–4.

The SVR rates of IFN therapy were 52.4% in F0–1 patients, 49.5% in F2–4 patients ($P = 0.896$ by Fisher's exact test), and 58.0% and 43.8% ($P = 0.592$) in IFN/Riba therapy, respectively.

In patients with genotype 1b and high viral load, the SVR rate was 12.5%. The SVR rate in genotype 2 patients was 60.4% in the IFN group and 67.7% in the IFN/Riba combination therapy group.