infected with the same genotypes of HBV. For instance, infection with HBV genotype B in Taiwan induces HCC much more frequently than that in Japan.[10,11]

Recently, two distinct subtypes of genotype B have been reported, designated Ba and Bj. Subtype Ba is ubiquitous in Asia, while Bj is restricted to Japan.[12] Notably, HBV isolates of subtype Ba possess the recombination with genotype C over the precore region plus core gene, while those of subtype Bj do not.[12] For the purpose of evaluating any clinical and virological differences, the 53 patients infected with subtypes Ba were compared with the 167 patients infected with subtype Bj at Toranomon Hospital in Tokyo, Japan.

METHODS

Patients infected with HBV

During 26 years from 1976 to 2001, 1674 patients infected with HBV visited Department of Gastroenterology, Toranomon Hospital in Tokyo Japan, and genotypes of HBV were determined in them. Genotype A was detected in 53 patients (3%), B in 224 (13%), C in 1332 (80%), D in four patients, E in one patient and F in three patients; genotypes were unidentifiable in the remaining 57 patients (3%). Subtypes of genotype B, in terms of Ba and Bj,[12] were determined in sera collected from the 224 patients infected with HBV genotype B at the presentation for evaluating any clinical and virological differences between infections with these two subtypes. Patients were considered to be in the asymptomatic carrier state when alanine aminotransferase (ALT) levels stayed normal (≤50 IU/L) throughout the observation period. Chronic hepatitis was diagnozed by liver biopsies performed under laparoscopy, and liver cirrhosis by liver biopsy as well as ultrasonographic images and laparoscopic findings. Hepatocellular carcinoma was diagnosed by imaging modalities, such as ultrasonography, computed tomography and magnetic resonance imaging, and by liver biopsy if necessary. The study design conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the hospital. An informed consent was obtained from every patient.

Serum markers of HBV infection

The HBsAg was determined with commercial kits by hemagglutination (MyCell, Institute of Immunology, Tokyo, Japan) and radioimmunoassay (AUSRIA II-125, Dinabot, Tokyo, Japan), and antibody to HBV core of IgM class was tested for by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBc-antiM RIA; Dinabot). The HBeAg was determined by ELISA (ELISA, F-HBe; Kokusai Diagnostic, Kobe, Japan). The six major genotypes of HBV (A–F) were determined by ELISA with commercial kits (HBV genotype EIA, Institute of Immunology) after the method of Usuda *et al.*[13,14] It involves the expression of seven preS2 epitopes (*b, f, g, k, m, s and u*) detected by monoclonal antibodies, the combination of which is specific for each of the six HBV genotypes: *bsu* for genotype A; *bm* for B; *bks* for C; *bksu(g)* for D, *bksu* for E and bk for F. Genotype G, which was discovered recently,[3] was determined by the combination of preS2 serotype for genotype D and subtype adw of HBsAg; it is characteristic of this genotype.[15] Serotypes of HBsAg were determined by ELISA with commercial kits (HBs Antigen Subtype EIA, Institute of Immunology).

Determination of subtypes Ba and Bj of genotype B

Nucleic acids were extracted from serum (100 μL), which had been stored at −80°C, with a Smitest EX&R kit (Genome Science, Tokyo, Japan). The core gene of HBV-DNA in extracted nucleic acids were amplified by polymerase chain reaction (PCR) with nested primers. The first-round PCR was performed with BJF3 (sense, 5′-CCG ACC TTG AGG CAT ACT TC-3′ [nt 1690–1709]) and BJF4 (antisense, 5′-GGG TCC CAC AAA TTG CTT AC-3′ [nt 2580–2606]) primers, and the second-round PCR with FJF1 (sense, 5′-GCT GTG CCT TGG GTG GCT TTG-3′ [nt 1876–1897]) and BJR2 (antisense, 5′-GCG ACG CCG TGA TTG AGA CCT-3′ [nt 2398–2411]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, CA, USA). They were then purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced in the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The core-gene sequences from patients were analyzed phylogenetically along with reference Ba and Bj sequences by 6-parameter and neighbor-joining methods.[16,17]

Nucleotide sequences of the precore region and core promoter

For determination of the wild-type or mutants in the precore region, nucleic acids extracted from serum were amplified by PCR with nested primers. The first-round PCR was performed with BCP-F7 (sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ [nt 1580–1599]) and BCP-R8 (antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ [nt 2295–2276]) primers, and the second-round PCR with BCP-F5 (sense, 5′-GCA TGG AAC CAC CGT GAA C-3′ [nt 1606–1625]) and BCP-R6 (antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ [nt 2014–1995]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis, purified and sequenced as described here. Mutations interfering with translation and transcription of HBeAg were sought in the precore region and core promoter, respectively. They included a G-to-A mutation at nucleotide 1896 (A1896) in the precore region and the double mutation in the core promoter converting the codon 1762 from A to T as well as codon 1764 from G to A (T1762/

A1764). Also examined was nt 1858 of T or C in HBV-DNA sequences.

Statistical analysis

Frequencies between groups were compared using the χ^2 test, Fisher's exact test and Mann–Whitney *U*-test. Data analysis was performed using SAS (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered significant. Differences in the progression rate of chronic hepatitis B and the frequency of HBsAg clearance were evaluated by Kaplan–Meier technique and log–rank test.

RESULTS

Clinical manifestations of the patients infected with subtype Ba or Bj of HBV genotype B

The HBV-DNA sequences were determined from nucleic acids extracted from 224 patents infected with HBV genotype B who visited Toranomon Hospital in Tokyo, Japan during 1976 through 2001, and who were subjected to phylogenetic analysis on the core gene. Subtype Ba having the recombination with genotype C was identified in 53 (24%) patients and subtype Bj without such recombination in 167 (75%); distinction between Ba and Bj was not possible in the remaining four patients (1%). Table 1 compares frequencies of acute hepatitis, asymptomatic carrier state, chronic hepatitis and liver cirrhosis with or without HCC, between 53 patients infected with HBV subtype Ba and 167 with Bj. There were no differences in the distribution of liver disease of various forms between the patients infected with subtypes Ba and Bj.

Demographic and virological characteristics of patients with chronic hepatitis who were infected with HBV subtype Ba or Bj

Demographic and virological features are compared between patients with chronic hepatitis B, 24 of whom were infected with subtype Ba and 82 with subtype Bj (Table 2). There were no differences in sex, age, duration of follow up and mothers persistently infected with HBV, between the patients infected with subtypes Ba and Bj. At presentation, however, the prevalence of HBeAg in serum was significantly higher in the patients infected with subtype Ba than Bj (63% vs 33%,

Table 1 Distribution of liver disease in patients infected with HBV subtype Ba or Bj

Disease/condition	Subtypes of genotype B		
	Ba ($n = 53$) *n* (%)	Bj ($n = 167$) *n* (%)	*P*
Acute hepatitis	0	5 (3)	NS
Asymptomatic carrier state	22 (42)	66 (40)	NS
Chronic hepatitis	24 (45)	82 (49)	NS
Liver cirrhosis or hepatocellular carcinoma	7 (13)	14 (8)	NS

NS, not significant.

Table 2 Comparison between patients with chronic hepatitis who were infected with HBV subtypes Ba or Bj

Features	Subtypes of genotype B		
	Ba ($n = 24$) *n* (%)	Bj ($n = 82$) *n* (%)	*P*
Male	19 (79)	73 (89)	NS
Age (years); median (range)	36 (23–62)	37 (21–83)	NS
Follow up (days); median (range)	3363 (50–11 642)	3475 (165–10 679)	NS
Mother with HBsAg	2 (8)	12 (15)	NS
Serotype of HBsAg			
adw	22 (92)	73 (89)	NS
adr	1 (4)	1 (1)	NS
adwr	1 (4)	0	NS
Untypeable	0	9 (11)	NS
HBeAg at presentation	15 (63)	27 (33)	$P = 0.016$
Clearance of HBeAg	10/15 (67)	21/27 (78)	NS
HBV-DNA at presentation (LEG/mL)†	7.5 ± 6.2	4.9 ± 3.6	NS

NS, not significant.

†Log equivalent genome (LEG)/mL by the transcription mediated assay.

$P = 0.016$). In contrast, the prevalence of HBeAg was no different between the asymptomatic carriers with Ba and Bj infections (3/22, 14% vs 6/66, 7%). Falling short of being significant, the mean titer of HBV-DNA was somewhat higher in the patients infected with subtype Ba than Bj.

Figure 1 depicts the development of liver cirrhosis and HCC in patients with chronic hepatitis B during follow up of up to 20 years. There were no differences in the progression of liver disease between the patients infected with subtypes Ba and Bj. No differences were noted, either, in the loss of HBsAg from serum during follow up between them, although HBsAg tended to disappear earlier in patients infected with subtype Ba than Bj up to 15 years of follow up (Fig. 2).

Mutations in the core promoter and precore region, which increase with the duration of infection and influence the severity of liver disease, were examined in the patients with chronic hepatitis at the time of presentation. Table 3 compares mutations in the core promoter and precore region between the patients infected with subtypes Ba and Bj. There were no differences in the frequency of the stop-codon mutation in the precore region, or that of the double mutation in the core promoter, between the patients infected with the two different subtypes of genotype B.

Distributions of HBsAg serotypes were no different between the patients infected with subtypes Ba and Bj. The 1858th nucleotide of T or C that influences the precore mutation (A1896) was invariably T in all 18 patients infected with subtype Ba, and in all 70 with Bj who were examined.

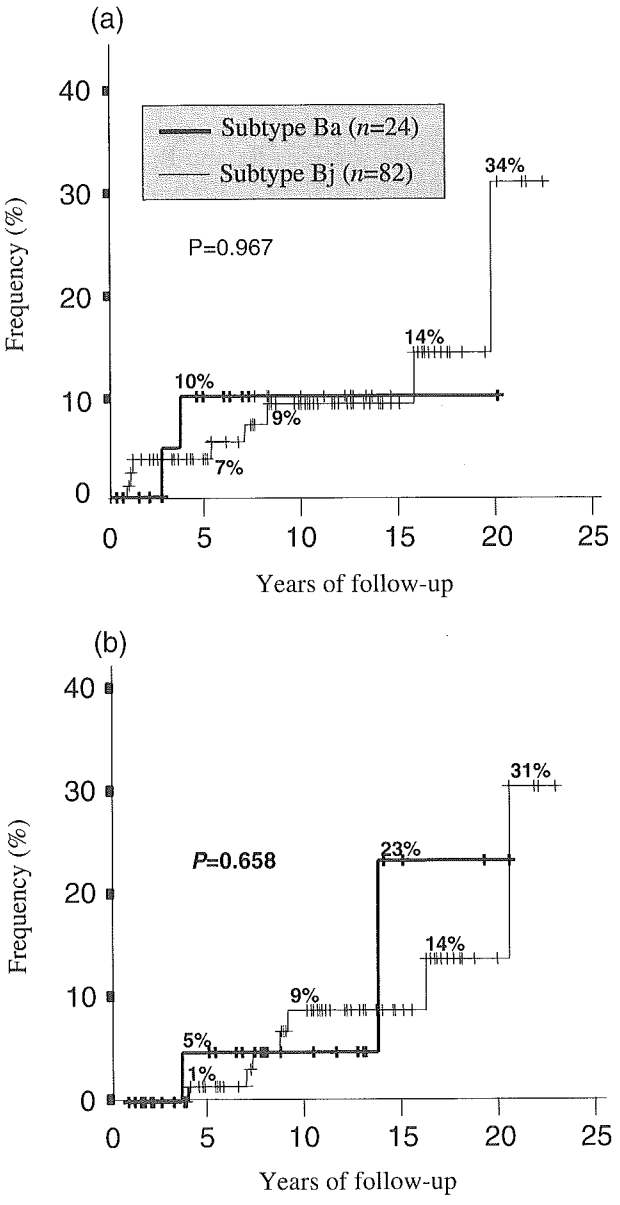

Figure 1 Evolution of chronic hepatitis in patients infected with subtype Ba or Bj of HBV genotype B. Development of liver cirrohosis (a) and hepatocellular carcinoma (b) were compared between the 24 patients infected with HBV genotype Ba and the 82 with Bj during follow up of 20 years or longer. There were no differences in the development of either liver cirrhosis of hepatocellular carcinoma by evalution of the results obtained by the Kaplan–Meier technique with the log–rank test.

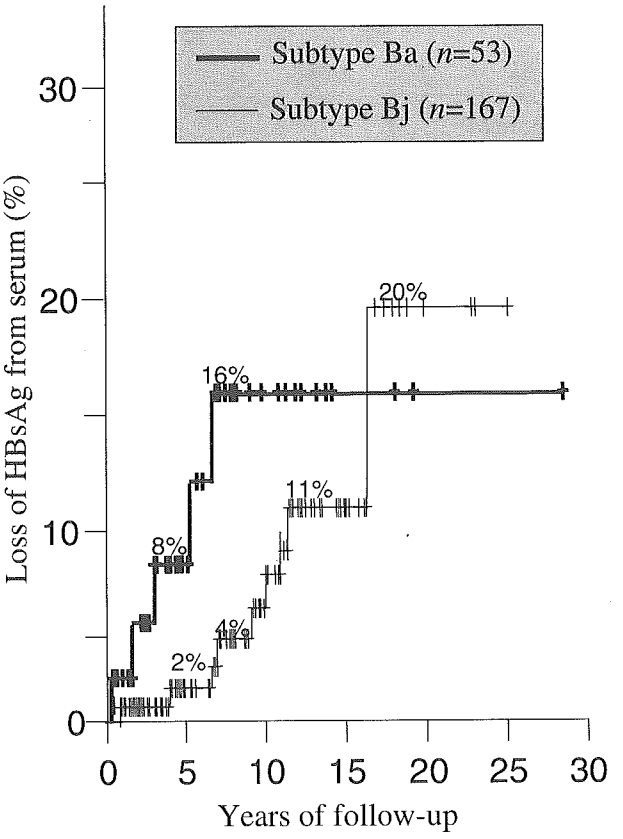

Figure 2 Loss of HBsAg from serum during long-term follow up. The 53 patients infected with subtype Ba and the 167 with subtype Bj of HBV genotype B, who presented with HBeAg in serum, were compared by the Kaplan–Meier technique, and differences were evaluated with the log–rank test.

Clinical and virological characteristics of patients with liver cirrhosis who were infected with subtype Ba or Bj

Table 4 lists demographic, histolgical and virological features of patients with liver cirrhosis, five of whom were infected with subtype Ba and 10 with subtype Bj. As for patients with chronic hepatitis B, the detection of serum HBeAg at presentation was significantly more frequent (60% vs 0%, $P = 0.022$), and the mean titer of HBV-DNA in serum tended to be higher, in the patients infected with subtype Ba than Bj.

DISCUSSION

Recombination between HBV isolates of distinct genotypes has been reported,[18,19] which may endow recombinants with a phenotype for virological characteristics or disease-inducing capacity distinct from those of parent genotypes. Because genotypes A and D are frequent in Western countries, A/D recombinants are reported there. Likewise, because genotypes B and C are common in Asia, B/C recombinants occur mostly in Asian countries. Not so many A/D or B/C recombinants have been reported, however, probably reflecting uncommon recombination events in the HBV infection.

Two subtypes of genotype B are reported, one of which has recombination with genotype C in the precore region and core gene, while the other does not.[12] It is surprising that essentially all HBV strains from Asian countries other than Japan are of the Ba subtype with the recombination (suffix 'a' representing Asia), in contrast to most of those from Japan that are of subtype Bj, without the recombination (suffix 'j' standing for Japan). Because Japan is unique in that both Ba and Bj subtypes occur in the genotype B infection, we set out to examine any demographic, virological and clinical differences between subtypes Ba and Bj. A study conducted at Toranomon Hospital in Tokyo was carried out to determine whether there would be any differences in subtypes Ba and Bj, in the same epidemiological and clinical setting in the patients of a single ethnic origin.

During 26 years from 1975 to 2001, 224 patients infected with HBV of genotype B presented to Department of Gastroenterolology, Toranomon Hospital located at the center of Tokyo, Japan. Subtypes of genotype B were determined by sequencing HBV-DNA, and Ba was found in 53 (24%) and Bj in 167 (75%); HBV isolates of genotype B from only four patients (1%) were untypeable into Ba or Bj. The 53 patients infected with subtype Ba and the 167 with subtype Bj were compared demographically, clinically and virologically.

The prevalence of subtype Ba (24%) in the patients who visited Toranomon Hospital in Tokyo was higher than that reported by Sugauchi *et al.* from Japan (7/97; 7%).[20] HBV subtype Ba is infrequent in Japan, in contrast to the other Asian countries, where subtype Ba accounts for all genotype B infections.[20] Because Toranomon Hospital is a tertiary referral hospital, selection may have occurred in favor of patients with severe disease or who were refractory to treatment. The frequency of HBsAg in mothers of patients tended to be higher in subtype Bj infection than Ba (15/167, 9% vs 2/53, 4%). Hence, the patients with subtype Ba would have had a higher chance of infection in later life than those with

Table 3 Mutations in the core promoter and precore region in the patients with chronic hepatitis who were infected with HBV subtype Ba or Bj

Mutation	Ba ($n = 18$) n (%)	Bj ($n = 69$) n (%)	P
Core promoter†			
Mutant	4 (22)	15 (22)	NS
Wild-type	14 (78)	54 (78)	
Precore region‡			
Mutant	9 (50)	36 (52)	NS
Wild-type	9 (50)	33 (48)	

NS, not significant.

Examination was possible in 18 of the 24 patients infected with subtype Ba and 69 of the 82 infected with subtype Bj of HBV genotype B.

†Double mutation for T1762/A1764; ‡A1896 mutation for a stop codon at amino acid 28.

Table 4 Comparison between patients with liver cirrhosis who were infected with HBV subtypes Ba or Bj

Features	Subtypes of HBV		
	Ba ($n = 5$) n (%)	Bj ($n = 10$) n (%)	P
Male	5 (100)	7 (70)	NS
Age (years); median (range)	44 (24–50)	37 (21–83)	NS
Follow up (days); median (range)	4505 (2001–8199)	1524 (487–5151)	NS
Mother with HBsAg	0	2 (20)	NS
HBeAg at presentation	3 (60)	0	0.022
Clearance of HBeAg	3/3 (100)	0/0	NS
HBV-DNA at presentation (LEG/mL)†	4.9 ± 4.1	4.1 ± 3.8	NS

NS, not significant.

†Log equivalent genome (LEG)/mL by the transcription mediated assay.

subtype Bj. The duration of HBV infection therefore may have been shorter in patients with subtype Ba than Bj, which needs to be taken into consideration when evaluating virological differences between them. The prevalence of HBeAg in sera is reported to be higher in patients of the same age who were infected with subtype Ba than Bj,[20] which has been confirmed in the preesnt study. These differences, however, would not readily be attributed to virological differences alone and need to be evaluated with reference to the duration of HBV infection.

Subtypes Ba and Bj did not seem to affect the severity of clinical disease. The distribution of acute hepatitis, asymptomatic carrier state, chronic hepatitis and liver cirrhosis with or without HCC was no different between the patients infected with Ba and Bj in the present series. Subtypes Ba and Bj, however, have been shown to influence resistance to lamivudine as well as virological and biochemical breakthroughs in our previous study.[21]

There was an important virological difference between Ba and Bj infection. The patients with chronic hepatitis or liver cirrhosis infected with subtype Ba possessed HBeAg in serum significantly more frequently than those infected with subtype Bj. Because HBeAg persists longer in patients infected with HBV genotype C than B,[8,9] this trait of genotype C would have borne out in HBV strains of subtype Ba that possess the recombination with genotype C over the precore region and core gene. The persistence of HBeAg over a longer period of time, before the seroconversion to anti-HBe takes place accompanied by hepatitis flares, would results in more severe disease in the patients infected with HBVgenotype C than B.[8]

Mutations in the core promoter and precore region that downregulate and abolish the synthesis of HBeAg, respectively, are under influence of HBV genotypes, and the double mutation in the core promoter (T1762/A1764) is detected more frequently in the patients infected with genotype C than B.[8,22] In so far as the core promoter region of subtype Ba is replaced by that of genotype C,[12] it would be more prone to the mutation for T1762/A1764 than that of subtype Bj. Because the T1762/A1764 mutation is implicated in hepatocarcinogenesis in patients infected with HBV,[23] a high frequency of this mutation in subtype Ba infection would be responsible, at least in part, for HCC in patients in Taiwan who develop this during youth.[10]

Very recently, Sugauchi *et al.* compared 80 patients infected with subtype Ba from Asian countries other than Japan, with 80 patients infected with subtype Bj from Japan while controlling for severity of liver disease.[20] They found a higher frequency of HBeAg in serum and the double mutation in the core promoter (T1762/A1764) in the patients infected with subtype Ba than Bj. Because the Sugauchi *et al.* study was case-controlled on patients with identical distribution of asymptomatic carrier state, chronic hepatitis, liver cirrhosis and HCC, the influence of genotype Ba and Bj on the clinical course of hepatitis B was not within the scope of the study.[20] In the present series of 53 Japanese patients infected with subtype Ba and the 157 infected with Bj, no influence of these subtypes was observed in terms of distribution of liver disease of distinct severity and the development of liver cirrhosis and HCC in patients with chronic hepatitis B during follow up of up to 26 years. These observations come as a surprise, in view of the response to lamivudine being poorer in the patients infected with subtype Ba than Bj.[21]

In conclusion, there is a significant virological difference between HBV infection of subtype Ba and Bj, which seem to be attributable to the recombination with genotype C in HBV isolates of subtype Ba. Persistence of HBeAg would influence the clinical course and response to antiviral therapies in the patients infected with subtype Ba, who would fare worse than those with subtype Bj in the long term. This would need to be confirmed in an extended series of patients who are infected with HBV of Ba or Bj in prospective studies, in view of the small number of studied patients with Ba infection in Japan, where Bj prevails.

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Usefulness of the Serum KL-6 Assay in Patients with Hepatitis C Virus

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Key Words
Chronic hepatitis C · Hepatocellular carcinoma · Idiopathic pulmonary fibrosis · KL-6

Abstract
Objective: The aim of this study is to evaluate the serum level of KL-6 in hepatitis C virus (HCV)-positive patients with chronic liver disease. ***Methods and Results:*** Subjects consisted of 502 HCV-positive patients. The serum samples of these patients stored at –80°C were measured by enzyme-linked immunosorbent assay for KL-6 at the same time. The cutoff point of the serum KL-6 level was defined as 500 U/ml. The serum KL-6 level of the 502 patients ranged between 71 and 2,295 (median, 223) U/ml. Thirty-two of the 502 (6.4%) patients showed an elevated KL-6 level of >500 U/ml. Three of the 32 (9.4%) patients with elevated KL-6 level >500 U/ml had idiopathic pulmonary fibrosis. Multivariate analysis showed that patients achieved elevated KL-6 when: (1) they had hepatocellular carcinoma (HCC; $p = 0.0007$), and (2) age was >60 years ($p = 0.0085$). The HCC rate was 37.5% (12/32) in the patients with elevated KL-6 and 8.3% (39/470) in the patients with normal KL-6 group. The median (range) age was 70 (56–77) years in the patients with elevated KL-6 group and 60 (12–92) years in the patients with normal KL-6. ***Conclusion:*** The patients with HCC aged >60 years had significantly elevated serum levels of KL-6.

Introduction

A variety of extrahepatic complications, such as essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, autoimmune thyroiditis, sialadenitis, cardiomyopathy, and idiopathic pulmonary fibrosis (IPF) have been reported in patients with chronic hepatitis C virus (HCV) infection [1–15]. Thus, it is necessary to predict these extrahepatic manifestations in the follow-up of patients with HCV. Various studies have been conducted on the diagnosis of IPF. Despite extensive research, IPF remains a disease of unknown etiology with a poor prognosis after acute exacerbation. It can progress rapidly after such exacerbation and often proves fatal, despite treatment with oral corticosteroids and intravenous high-dose corticosteroid therapy.

The serum level of KL-6 is a sensitive marker of disease activity in fibrosing lung diseases [16–19]. KL-6 is a high-molecular-weight glycoprotein and is classified as MUC1 mucin of lung tumor and differentiation antigens. The molecule consists of multiple heterogeneous submolecules. KL-6 can be detected by a murine monoclonal antibody, KL-6 antibody (IgG_1), which recognizes a sialylated sugar chain on the molecule. Although the presence of KL-6 in the serum has been reported to be a sensitive marker of disease activity in interstitial pneumonitis such as IPF, the serum KL-6 level in HCV-positive patients with hepatocellular carcinoma (HCC) sometimes increases.

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The aim of this study was to evaluate the clinical profile in HCV-positive patients with elevated serum KL-6 level.

Patients and Methods

Patients

Selected subjects consisted of 502 HCV-positive patients who fulfilled the following criteria: (1) positive HCV-RNA for more than 6 months; (2) no HBsAg, and HBc antibody in the serum (determined by radioimmunoassay); (3) outpatients of our hospital from March 20 to 27, 2001; (4) no underlying systemic disease such as systemic lupus erythematosus and rheumatic arthritis, and (5) patients provided informed consent to KL-6 level studies. The diagnosis of IPF was based on history, physical examination, chest roentgenogram, pulmonary function tests, chest high-resolution computed tomography (HRCT), according to the criteria of the American Thoracic Society [20]. Diagnosis of HCC was based on the presence of typical hypervascular characteristics on angiography, in addition to the findings on CT and ultrasonography. Microscopic examination of fine-needle biopsy material was performed in patients whose angiograms did not demonstrate a typical image of HCC. Histopathological confirmation using surgically resected specimens was made in 12 patients.

The serum samples of these 502 patients were collected and stored at –80°C until the enzyme-linked immunosorbent assay (ELISA) for KL-6 was done. The study was approved by the institutional review board of our hospital. The physicians in charge explained the purpose and method of this clinical trial to each patient.

The HCV genotype was classified by a PCR using a mixture of primers for the five subtypes known to exist in Japan, as reported by Chayama et al. [21]. Using these blood samples, HCV-RNA levels were analyzed at the same time by a branched DNA probe assay (b DNA probe assay, version 2.0, Chiron, Tokyo, Japan), and the results were expressed as mega-equivalents/ml (MEq/ml) [22].

Determination of KL-6 Concentration

The serum concentration of KL-6 antigen was measured by a sandwich-type ELISA using immobilized KL-6 antibody (Eitest® KL-6, Eisai, Tokyo, Japan) as described previously [23, 24]. Moreover, the cutoff value of KL-6 was defined as 500 U/ml.

Statistical Analysis

We used Fisher's exact test, univariate analysis and multivariate analysis (multiple logistic regression analysis) to establish which factors contributed to the elevated KL-6 level. Results for each variable were transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($p < 0.1$) in univariate analysis were subjected to multiple logistic regression to identify significant independent predictors. $p < 0.05$ was considered statistically significant. All data analyses were conducted using the Statistical Package for Social Sciences (SPSS for Windows, version 9.0). A p value <0.05 was considered to be statistically significant.

Table 1. Clinical characteristics of the 502 study patients

Characteristics	
Patients	502
Sex, male/female	306/196
Age[a], years	61 (12–92)
Posttransfusion, %	38.3
HCV genotype, % (1b/2a/2b/others)	66/23.3/9/1.7
HCV-RNA[a], MEq/ml	4.6 (<0.2–88)
AST[a], IU/l	38 (13–442)
ALT[a], IU/l	43 (7–560)
Total protein[a], g/dl	7.6 (5.8–9.5)
Platelets[a], $\times 10^4/mm^3$	14.3 (3.3–36.9)

[a] Medians and ranges.

ALT = Alanine aminotransferase; AST = aspartate aminotransferase.

Results

Clinical Background

Clinical profile characteristics of the 502 study participants are shown in table 1. Serum KL-6 levels ranged from 71 to 2,295 (median; 223) U/ml, and the distribution of serum KL-6 levels is depicted in figure 1. In 32 of the 502 (6.4%) patients, the KL-6 level exceeded 500 U/ml. Three of these 32 (9.4%) patients had a diagnosis of IPF.

Predictive Factors for Elevated Serum KL-6 Level

Next, we assessed the factors predicting KL-6 levels >500 U/ml. A logistic model was used for the analysis of nonlinear data referring to various factors potentially related to elevated KL-6 level surpassing 500 U/ml. The following factors were evaluated: sex, age, post-transfusion, HCV genotype, viral load, alanine and aspartate aminotransferases, platelet counts, HCC and IPF. Univariate analysis (table 2) disclosed two factors which were significantly associated with elevated serum KL-6 level: (1) a history of HCC ($p < 0.0001$), and (2) age >60 years ($p = 0.0052$). Because the variables were mutually correlated, multivariate analysis was performed using the four significant variables in the model. Multivariate analysis (table 3) revealed the same significant factors: (1) a history of HCC ($p = 0.0007$), and (2) age >60 years ($p = 0.0085$).

Clinical Profiles in Patients with Elevated KL-6

The differences in clinical profiles were compared between patients with normal KL-6 and patients with elevated KL-6 (table 4). Regarding IPF, all 3 patients with IPF had elevated serum KL-6 levels. The median age of

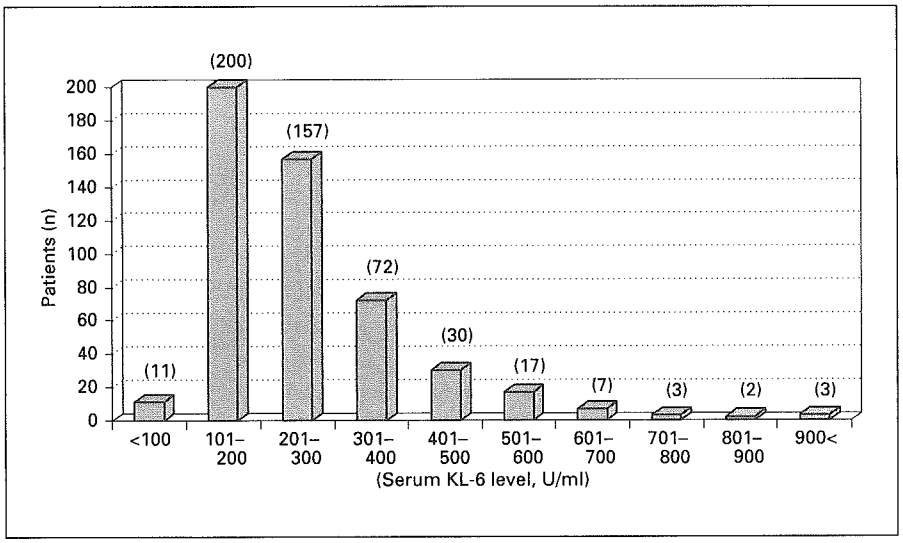

Fig. 1. Distribution of serum KL-6 levels in the patients with HCV.

Table 2. Factors associated with elevated KL-6 by univariate analysis

Factor	Category	Odds ratio	95% CI	p value
HCC	(–)/(+)	1/7.97	4.18–19.97	<0.0001
Age, years	<60/≥60	1/17.61	2.34–132.33	0.0053
Platelets, $\times 10^4/mm^3$	≥15/<15	1/2.94	0.60–14.49	0.182
AST, IU/l	≥38/<38	1/2.13	0.71–6.39	0.180
HCV genotype	2a, 2b/1b	1/2.00	0.22–18.12	0.538
ALT, IU/l	<50/≥50	1/1.58	0.49–5.00	0.450
Sex	female/male	1/1.45	0.60–5.23	0.104
HCV-RNA, MEq/ml	≥5/<5	1/1.41	0.31–6.43	0.655

CI = Confidence interval.

Table 3. Factors associated with elevated KL-6 by multivariate analysis

Factor	Category	Odds ratio	95% CI	p value
HCC	(–)/(+)	1/5.08	1.98–13.04	0.0007
Age, years	<60/≥60	1/15.37	2.01–117.66	0.0085

CI = Confidence interval.

Table 4. Difference in clinical profiles between patients with normal KL-6 and patients with elevated KL-6

Characteristics	Normal KL-6 group (<500 U/ml)	Elevated KL-6 group (≥500 U/ml)	p value
IPF (+/–)	0/470	3/29[a]	<0.0001
Median age, years (range)	60 (12–92)	70 (56–77)	0.0053
HCC (+/–)	37/431	12/20[a]	<0.0001

[a] Two patients had HCC and IPF.

the patients with elevated KL-6 exceeded that of the normal KL-6 patients by 10 years. The HCC rate was 37.5% (12/32) in the patients with elevated KL-6 and 8.3% (39/470) in the patients with normal KL-6. The median (range) age was 70 (56–77) years in the patients with elevated KL-6 and 60 (12–92) years in the patients with normal KL-6. The HCC rate of the patients with elevated KL-6 was significantly higher than that of the patients with normal KL-6. Twelve of 49 HCC patients demonstrated elevated KL-6.

Figure 2 depicts a flow chart to assess the clinical background in patients with elevated KL-6 level. Twenty-five

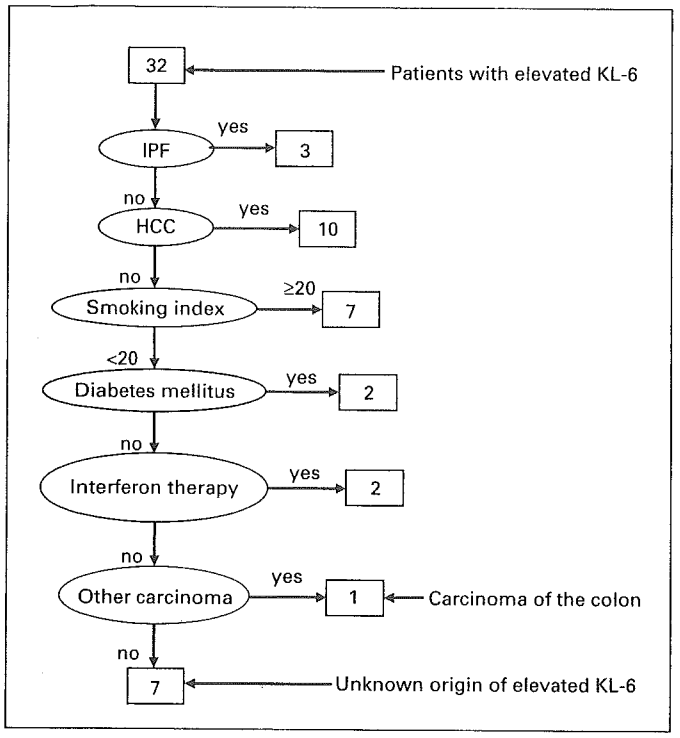

Fig. 2. Flow chart to assess the clinical background in patients with elevated KL-6 level. The smoking index was defined as the number of cigarettes smoked per day × years of smoking. Numbers in boxes indicate the numbers of patients.

of the 32 (78.1%) patients were diagnosed as having basic disease of IPF, HCC and diabetes mellitus, for example. However, the remaining 7 patients with elevated KL-6 did not have any underlying disease.

Discussion

Chest roentgenograms, CT, pulmonary function tests, gallium-67 lung scan, and bronchoalveolar lavage are used clinically for the diagnosis of IPF. However, the effect of exercise-induced changes and the repeatability of these examinations remain problems to be solved. KL-6 can be examined by blood sampling without discomfort to patients. Moreover, the serum level of KL-6 is elevated in the majority of patients with various interstitial lung diseases, including IPF [25]. In our opinion, serum KL-6 is generally a noninvasive, sensitive, diagnostic marker of IPF. The serum level of KL-6 can, therefore, provide useful information for an early diagnosis of IPF.

Ueda et al. [11] reported a higher prevalence of HCV antibody in patients with IPF compared with the general population. A total of 459 consecutive autopsy cases of chronic liver disease were examined at the Toranomon Hospital in Tokyo, Japan, from 1988 to 1998. The prevalence of IPF was 5% (13/261) in patients with HCV, 0% (0/58) in patients with hepatitis B virus (HBV) and 0.7% (1/140) in patients without HCV and/or HBV. The prevalence of IPF in HCV cases was significantly higher than those of other groups. Moreover, several studies have recently suggested that interferon therapy for chronic hepatitis C might induce interstitial pneumonitis [26, 27]. Recently, combination therapy of interferon and ribavirin has often been selected as the first choice for chronic hepatitis C in Japan. However, in about 10,000 patients treated with interferon and ribavirin, 16 patients had IFN-related IPF during therapy. Hence, preventive measure and early diagnosis of IPF are essential for the daily management of chronic hepatitis C.

Serum levels of KL-6 are generally increased in patients with fibrosing lung infection. However, serum levels of KL-6 are sometimes elevated in patients with certain malignancies such as adenocarcinoma of the lung, breast, pancreas and HCC [28]. In the present study, the serum KL-6 level in patients with HCC was higher than that of patients without HCC by univariate analysis. Therefore, we assessed the question of whether the serum KL-6 level is useful in the diagnosis of IPF in HCV-positive patients with chronic liver disease. The present study included only 3 patients with IPF of a total of 502 study patients. On the other hand, about 20% of the patients with HCC had elevated KL-6 levels. Our results indicate that serum KL-6 is a useful marker for HCC in patients with chronic liver disease and HCV. This result is in agreement with previous data reported Moriyama et al. [28].

Moreover, they have reported that serum levels of KL-6 are generally increased in patients with lung cancer compared to those without the disease. Consequently, an increase in the serum KL-6 level in patients with HCV should be associated with potential development of HCC, progression to liver disease or other mechanisms.

In conclusion, (1) patients aged ≥60 years having HCC show significantly elevated serum KL-6 levels, and (2) in patients with increasing KL-6 level and chronic hepatitis or liver cirrhosis, it is necessary to differentiate IPF, HCC, and other mechanisms.

Acknowledgments

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Hepatitis B Virus-Related Hepatocellular Carcinogenesis and Its Prevention

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Key Words
Carcinogenesis · Hepatocellular carcinoma · Liver cirrhosis · Hepatitis B virus · DNA · Interferon · Cancer prevention

Abstract
To elucidate the influence of serum hepatitis B virus (HBV) load on hepatocellular carcinogenesis in cirrhotic patients, HBV-DNA was sequentially measured. In a nested, case-control study using 96 patients without antiviral therapy, high HBV-DNA ($\geq 10^{3.7}$ copies/ml) in the last 3 years was significantly associated with carcinogenesis (a patient group without hepatocellular carcinoma (HCC) development; 0/48 vs. a patient group with eventual HCC development; 22/48, $p < 0.0001$). No patient with a continuously low HBV-DNA for the last 3 years developed HCC. Persistence of high HBV-DNA concentration suggested an increased risk of carcinogenesis. In a retrospective cohort study using 57 patients with interferon therapy, HCC developed in 2 (8.0%) of the 25 patients with HBV-DNA loss, while carcinogenesis was found in 11 (34.4%) of 32 patients without HBV-DNA loss (Fisher's exact test, $p = 0.026$). A significant decrease or loss of serum HBV-DNA stops HCC development, and its sequential analysis could be very useful both in the prediction and early detection of small HCC.

Introduction

Hepatocellular carcinoma (HCC) is a principal cause of death in many parts of sub-Saharan Africa and in Asia [1, 2]. It is also one of the most common neoplasms in Japan [3]. Abundant epidemiological and molecular biological evidence shows that hepatitis B virus (HBV) is an important factor in the development of HCC [4–6], but the precise role of HBV DNA viruses in oncogenesis is still unknown. Although increasing evidence indicates that the HBV plays an important role in the development of HCC after discovery of integrated forms of HBV [7–9], current serological and virological markers are still insufficient in establishing this relationship. Since a really curative therapy is not available for HCC at present, an accurate prediction and early detection of HBV-related HCC is essential in the current situation.

Hepatocellular carcinogenesis rates were estimated in patients with HBV-related chronic hepatitis (n = 297) and cirrhosis (n = 246), who have not received interferon (IFN), lamivudine, or steroid therapy. They were diagnosed by peritoneoscopy and/or biopsy as having chronic liver disease in the Toranomon Hospital, Tokyo, Japan, from 1974 to 1999. Cumulative carcinogenesis rates in F1 fibrosis, F2–3, and F4 were 0.5, 6.3, and 19.7% at the end of the 5th year, 2.7, 14.9, and 30.3% at the end of the 10th year, 4.1, 19.5, and 35.8% at the15th year, and 15.0, 29.6, 41.9% at the 20th year, respectively (fig. 1).

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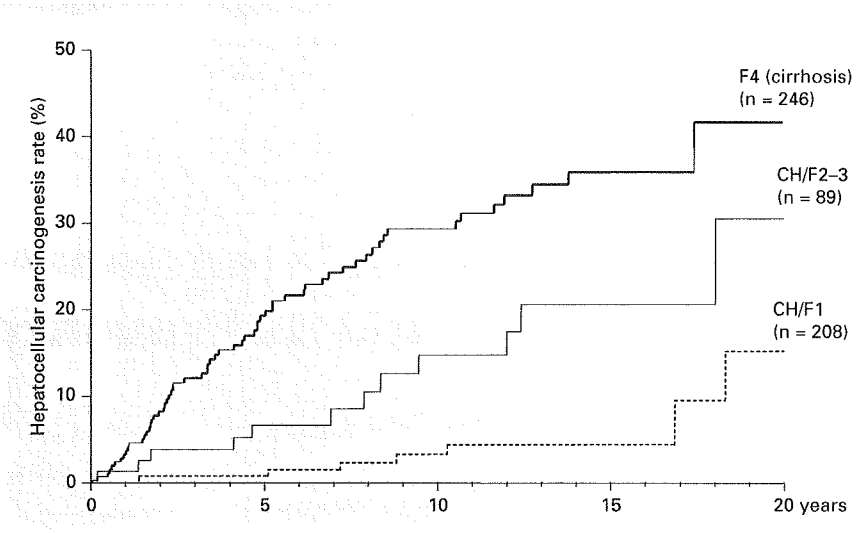

Fig. 1. Cumulative hepatocellular carcinogenesis rates in patients with chronic hepatitis or cirrhosis.

Needless to say, patients with HBV-related cirrhosis have a significantly higher risk for HCC development [10, 11], but the degree of the carcinogenesis risk in an individual patient cannot be predicted as yet. How can we recognize a super-high-risk group or a rather low-risk group in HBV-related cirrhosis? Can we predict and specify a patient who is not likely to develop HCC in the future? Hepatocellular carcinogenesis in patients with HBV infection may well be associated with persistence of aminotransferase, concentration of HBV DNA, or merely the severity of the liver disease. One of the purposes of this article is, therefore, to elucidate the relationship of hepatocellular carcinogenesis with longitudinal clinical courses of biochemical data and HBV DNA concentration in consecutive patients with cirrhosis.

IFN has been reported to be effective in patients with HBV-related chronic hepatitis, which decreases serum HBV DNA concentration and improves biochemical data on early control studies [12–14], and subsequently suppresses disease progression to cirrhosis [15, 16]. Although various effects of IFN in hepatitis B virus infection have been well investigated from virological, biochemical, and medico-economical viewpoints [17–19], the influence on a long-term outcome of liver cirrhosis or on hepatocellular carcinogenesis is still controversial [20–25]. In order to clarify the mechanism of anti-carcinogenic activity of IFN, if any, we analyzed HBV DNA concentration serially in a cohort of 60 patients with cirrhosis. The other purposes of this study are to elucidate the relationship of hepatocellular carcinogenesis with longitudinal clinical courses in consecutive cirrhotic patients with interferon therapy and to investigate an early prediction of HBV DNA elimination and the cancer preventive activity.

Factors Affecting Hepatocellular Carcinogenesis in Cirrhosis (without Anti-Viral Therapy)

Patients and Methods

Analyzable Patients without Anti-Viral Therapy

Among 217 patients who were diagnosed as having HBV-related cirrhosis by peritoneoscopy and/or liver biopsy from 1976 to 1989 in our hospital, 160 patients had not undergone interferon or other antiviral therapy. Out of the consecutive 160 patients, sequential assay of serum HBV DNA using serial sera stored at −80°C was available in 146 patients (91.3%). All 146 patients showed a positive hepatitis B surface antigen and negative anti-hepatitis C virus antibody in the assay of their initial sera. Among the 146 patients with HBV-related cirrhosis, 48 patients (32.9%) developed HCC during a median follow-up period of 7.2 years after the diagnosis of cirrhosis, and the other 98 patients (67.1%) have not developed HCC during 11.7 years.

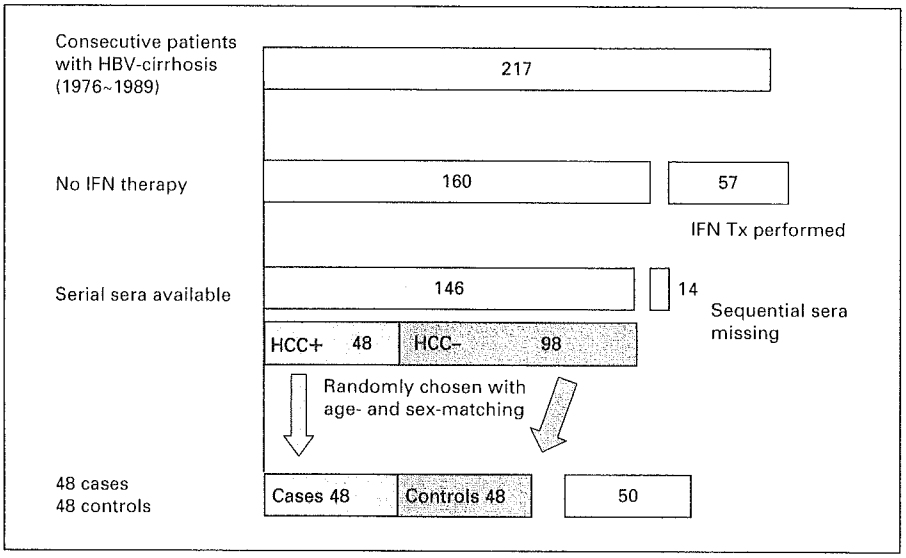

Fig. 2. Analyzed patients with HBV-related cirrhosis. Since almost all of consecutive untreated patients (146/160, 91.3%) were analyzable, a nested case-control study was established using a total of 96 patients.

Follow-Up of Patients and Diagnosis of HCC

Follow-up of the patients was made on a monthly or bi-monthly basis after diagnosis of liver cirrhosis by monitoring α-fetoprotein (AFP) and other biochemical data. Imaging diagnosis was carried out two or more times per year for each patient with computed tomography (CT), ultrasonography (US), or scintigraphy. HCC was diagnosed by typical hypervascular characteristics on angiography in addition to certain features of CT and US. A pathological confirmation of surgically resected specimens or autopsy was made in 38 (79.2%) of 48 patients with HCC development.

Nested Case-Control Study

In order to elucidate the relationship between hepatocellular carcinogenesis and longitudinal courses of clinical markers, a nested case-control study was introduced. Age- and gender-matched control patients were selected from the 98 HCC-free patients with a ratio of 1:1. The control patients were randomly selected using a computer-generated random number table, avoiding those with a short observation period of less than 3 years. Thus, a nested case-control study was made, consisting of 48 cases with cancer development (group A) and 48 demography-adjusted controls without sings of cancer (group B) (fig. 2).

Assays of HBV Markers

HBV DNA was assayed using frozen sera stored at −80°C, and quantified using transcription mediated amplification and hybridization protection assay described by Kamisango et al. [26] (TMA-HPA, Chugai Diagnostics Science, Tokyo, Japan). A lower value of HBV DNA of 3.7 LGE/ml (equivalent for $10^{3.7}$ copies/ml or 5,000 copies/ml) was considered as a low value. For annual sera from the diagnosis of cirrhosis to the end of observation period in each patient, the DNA quantification was simultaneously performed after fixation of the 48 cases and the 48 controls.

Statistical Analysis

Standard statistical measures and procedures were used. Mann-Whitney U test, χ^2 test, and Fisher's exact test were employed for examination of background characteristics of the patient groups with and without HCC development. $p < 0.05$ with the two-tailed test was considered significant. Data analysis was performed using the computer program SAS version 6.12 [27].

Results

Demography and Initial Laboratory Data of the Groups with or without HCC Development [28]

Table 1 shows the demography and initial laboratory data of the patients in groups A and B. The ratio of men was 39 of 48 (81.3%) in the both groups, and the median age was 49.5 and 49 in groups A and B, respectively. The proportion of decompensated cirrhosis, and a history of past alcohol consumption, were not significantly different

Table 1. Demography and initial laboratory data of 48 patients with HCC development and the 98 patients without HCC development during the observation period

	Group A HCC development (n = 48)	Group B no HCC (n = 48)	p
Demography			
Men:women	39:9	39:9	NS
Age, median (range)	49.5 (30–71)	49 (30–71)	NS
Decompensated cirrhosis	1 (2.1%)	7 (14.6%)	0.65
Past alcohol consumption of 500 kg or more	8 (16.6%)	9 (18.8%)	0.79
Initial laboratory data (median, range)			
Anti-HCV antibody positive	0	0	NS
HBe antigen positive	33/48 (68.8%)	17/48 (36.1%)	0.001
Bilirubin, mg/dl	1.0 (0.6–9.8)	1.0 (0.5–7.5)	0.46
Albumin, g/dl	3.95 (2.4–4.8)	4.0 (2.5–5.2)	0.23
Aspartic transaminase, IU	39.5 (15–820)	31.5 (13–376)	0.23
Alanine transaminase, IU	32 (8–740)	31 (9–313)	0.82
Platelet count, $\times 10^3/mm^3$	100 (28–225)	121 (49–255)	0.047
AFP, ng/ml	16 (3–785)	7 (3–1,520)	0.037

between the two groups. The prevalence of positive HBe antigen was, however, significantly higher in group A than that in group B. Although median platelet count was slightly lower, and alpha-fetoprotein concentration was higher in group A, there was no significant difference in bilirubin, albumin, aspartic transaminase, and ALT between the two groups.

Individual HBV DNA Concentration until the End of the Observation Period [28]

Quantitative HBV DNA assessment was sequentially performed until the diagnosis of HCC in each patient. In group A (HCC development), 9 patients showed intermittently high HBV DNA concentration and 39 patients showed a continuously high HBV DNA concentration from the diagnosis of cirrhosis to the development of HCC. All the patients experienced high HBV DNA during their clinical courses, and no patient showed low HBV DNA for a consecutive 3 years just before the detection of HCC.

Serial HBV DNA concentration of each patient was also assessed in group B (no HCC development). HBV DNA was continuously low in 9 patients, and HBV DNA concentration showed a settling down and lowering for 3 years or more until the end of observation period in 13 patients. Nine patients showed a fluctuated HBV DNA concentration, and the remaining 17 patients had a continuously high HBV DNA during the observation period. Of the 48 patients, 9 patients never experienced a high HBV DNA load, and a total of 22 patients (45.8%) showed low HBV DNA values for a successive 3 years until the end of the observation (fig. 3).

The incidences of HBV DNA patterns were significantly different between the two groups (χ^2 test, $p < 0.001$). The rates of low or a settling down trend of HBV DNA concentration was significantly lower in group A

Table 2. Demography and laboratory data of 57 patients with HBV-related cirrhosis undergoing interferon therapy

Demography	
Men:women	45:12
Age, median (range)	41 (19–60)
Decompensated cirrhosis	3 (5.3%)
Past alcohol consumption of 500 kg or more	3 (5.3%)
Laboratory data, median (range)	
Bilirubin, mg/dl	0.9 (0.4–2.6)
Albumin, g/dl	4.1 (3.0–4.9)
Aspartic transaminase, IU/l	65 (16–404)
Alanine transaminase, IU/l	74 (12–586)
Platelet count, $\times 10^3/mm^3$	125 (68–332)
Anti-HCV antibody positive	0
HBe antigen positive	41 (71.9%)
HBV-DNA, LGE/ml[1]	7.2 (3.9 to >8.7)
Observation period, years	13.6 (6.5–16.1)

[1] HBV-DNA (LGE/ml): log-genome equivalent, expressed as 10^n copies/ml.

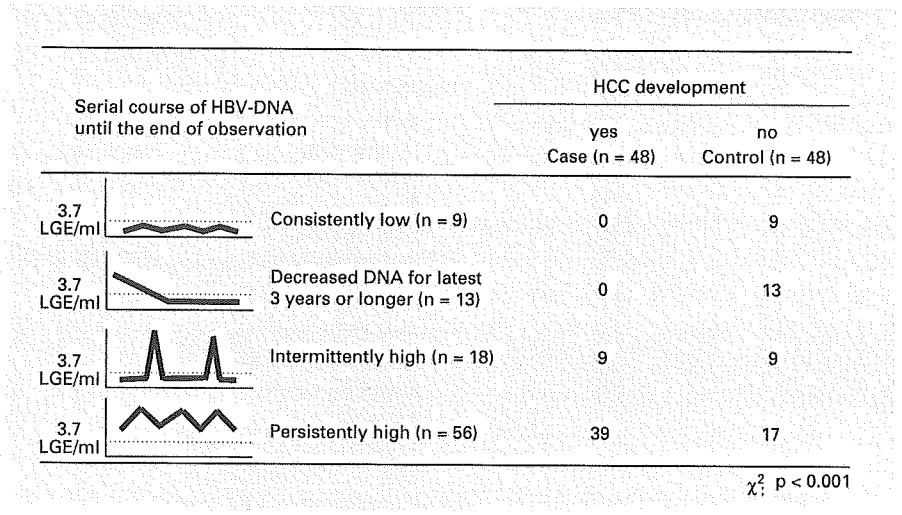

Fig. 3. Patterns of longitudinal courses of HBV-DNA in groups A and B.

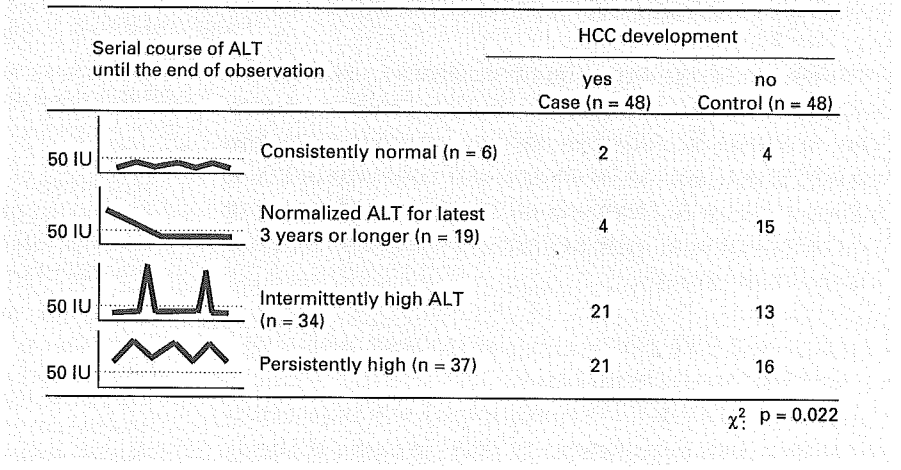

Fig. 4. Patterns of longitudinal courses of alanine transaminase in groups A and B.

than in group B (0/48 in group A vs. 22/48 in group B, Fisher's exact test, $p < 0.00001$). Any patients with a continuously low HBV DNA concentration for 3 years or longer did not develop HCC during the clinical courses.

Patterns of Longitudinal Courses of Alanine Transaminase [28]

ALT values were also assessed sequentially throughout the entire clinical courses. In group A, ALT was continuously normal in 2 patients (4.2%), ALT was high initially but normalized for the last 3 years or longer in 4 (8.3%), it showed abnormal values intermittently in 21 (43.8%), and had a continuously high value during the observation period in the remaining 21 (43.8%). In group B, 4 patients (8.3%) showed consistently normal ALT, 15 (31.3%) showed a decrease in ALT values, 13 (27.1%) intermittent elevation, and the remaining 16 (33.3%) showed continuously high ALT values during the follow-up period (fig. 4).

The incidence of HBV DNA patterns was significantly different between the two groups (χ^2 test, $p = 0.022$). While persistently or intermittently elevated ALT value slightly favored higher carcinogenesis rate (42/48 in group A vs. 29/48 in group B), statistical significance was, however, not obtained between carcinogenesis and ALT values (χ^2 test, $p = 0.077$).

Discussion

Liver cirrhosis due to hepatitis C virus usually shows a rather steady and constant clinical course, which enables us to estimate the future carcinogenesis rate from only clinical information at the time of the diagnosis of cirrhosis. Disease activity and carcinogenic potency of HBV-related liver disease, on the contrary, often change in natural clinical courses, accompanying significant fluctuation of HBe antigen system or amount of HBV DNA. When we investigate the relationship between hepatocellular carcinogenesis and its affecting and contributing factors, explanatory parameters should include not only initial demographic data but also chronological clinical data after starting the observation [29]. A longitudinal analysis is, therefore, necessary for the study of carcinogenesis in chronic liver disease caused by HBV. We, therefore, established a nested case control study using longitudinal clinical data until the end of the observation period or just before carcinogenesis, including HBV DNA quantification and ALT.

In this study, the sequential trend of serum HBV DNA concentration was significantly associated with hepatocellular carcinogenesis, and the relationship of HBV DNA to the carcinogenesis was much stronger than that of ALT. Indeed, mere initial background features and laboratory data of the patients could predict a future risk of carcinogenesis, and the chronological analysis demonstrated more discrete differentiation of a high-risk group and provided more detailed information about HBV-related carcinogenesis. Although this study illustrates that a consistence of low HBV-DNA concentration for 3 years or longer saves cirrhotic patients from carcinogenesis, the combination of 'low HBV-DNA' and '3 years' might not avoid the carcinogenesis risk sufficiently, considering the fact that hepatocellular carcinoma does develop without hepatitis, without high ALT, or without high HBV-DNA. It is, however, true that HCC scarcely develops in a patient with HBV-related cirrhosis whose HBV-DNA concentration is consistently low for the recent 3 years or longer.

Although a high load of HBV-DNA seems to promote carcinogenesis or tumor growth, the reason why a high concentration of HBV-DNA affected hepatocellular carcinogenesis remains unknown. Taking into account that hepatitis patients with positive HBe antigen and fluctuated aminotransferase values often show a high serum HBV-DNA concentration, a large amount of HBV-DNA load may be associated with a high carcinogenesis rate through an active inflammatory state and indirect cancer promotion [30]. Relationship between hosts and hepatitis virus should also be considered in future studies on carcinogenesis.

Hepatocellular Carcinogenesis in Cirrhotic Patients with Interferon Therapy

Patients and Methods

Analyzed Patients

Among 189 patients who were diagnosed as having HBV-related cirrhosis by peritoneoscopy and/or liver biopsy from 1983 to 1990 in our hospital, a total of 60 patients underwent interferon therapy from 1986 to 1990. Since 3 patients were lost to follow-up, the remaining 57 patients (95.0%) were analyzed for virological outcome, carcinogenesis, and eventual prognosis: the reason for the dropout from the observation in the 3 patients was simply house moving.

Interferon Therapy

IFN-α was administered in 35 patients (61.4%) and IFN-β in the remaining 22 patients (38.6%). The daily amount of IFN was 3 million units in 22 (38.6%) and 6 million units in 35 (61.4%), and twice a week administration was performed in 54 (94.7%) and three times a week in 3 (5.3%). All patients received an intermittent interferon therapy for a median of 18 months (range 2–132 months), but the duration of the IFN therapy was arbitrary in this pilot study. Although the amount of daily dose of IFN and the duration of the therapy varied in this study, 52 (91.2%) of the 57 patients received IFN for 6 months or longer.

Follow-Up and Diagnosis of HCC

Follow-up of the patients was made on a monthly basis after diagnosis of liver cirrhosis by monitoring virological, hematological, and biochemical data including α-fetoprotein (AFP). All these laboratory tests including HBV-markers were obtained throughout the observation period in each patient. Patients were classified into four groups according to patterns of serial concentration of HBV DNA: type A, disappearance of HBV DNA during and after IFN therapy; type B, loss of HBV DNA after cessation of IFN administration; type C, transient loss of HBV DNA only during IFN administration; type D, persistently positive HBV DNA during and after the therapy. Clinical courses of ALT fluctuation were also classified into four groups according to normalization of ALT value.

Imaging diagnosis and establishment of diagnosis of HCC were carried out as shown above.

Assay of HBV DNA

HBV-DNA was assayed using frozen sera stored at −80°C, and quantified using transcription mediated amplification and hybridization protection assay described by Kamisango et al. [26] as shown above.

Statistical Analysis

Standard statistical measures and procedures were used. Mann-Whitney's U and χ^2 tests were employed for examination of background characteristics between the groups with and without HBV DNA elimination. Fisher's exact test was also used to analyze the relationship of HBV markers with carcinogenesis. Cumulative HBV DNA disappearance rate, carcinogenesis rate, and survival rate were calculated by Kaplan-Meier technique [31], and the differences between the analyzed groups were assessed by log-rank test. $p < 0.05$ with the two-tailed test was considered to be significant. Data analysis was performed using the computer program SPSS version 11 [32].

Results

HBV DNA in Clinical Courses [33]

HBV DNA was positive in all the patients at the initiation of IFN therapy (3.9 to >8.7 LGE/ml). HBV DNA became negative (<3.7 LGE/ml) in 25 of 57 patients (43.9%) during the observation period with a median of 13.6 years. The remaining 32 patients did not show a sustained negative HBV DNA after the therapy, although 9 patients did show transient negative values for a limited period during the therapy.

Clinical courses of HBV DNA were classified into the four categories mentioned above. Nine patients (15.8%) lost HBV DNA during and after IFN therapy (type A), 16 patients (28.1%) lost HBV DNA after cessation of the therapy (type B). The other 9 patients (15.8%) showed a transient loss of HBV DNA (type C), and the remaining 23 (40.4%) retained persistently positive HBV DNA (type D).

Cumulative rate of HBV DNA disappearance was calculated using Kaplan-Meier technique. DNA became negative in 10.5% at the end of the first year after initiation of IFN, 12.3% at the third year, 21.0% at the fifth year, 43.7% at the tenth year, and 46.7% at the fifteenth year, respectively.

Hepatocellular Carcinogenesis and Serial Concentration of HBV DNA [33]

A total of 13 patients developed HCC during the observation period.

The relationship between carcinogenesis and serial concentration of HBV DNA was analyzed (fig. 5). No patients (0%) developed HCC among 9 patients in type A. Two (12.5%) of 16 patients developed HCC in type B: HCC were detected 1.2 year after disappearance of HBV DNA in one patient, and 3.6 years after disappearance of HBV DNA in the other patient. Three (33.3%) of 9 patients showed carcinogenesis in type C, and 8 (34.8%) of 23 patients developed HCC in type D during the observation. Hepatocellular carcinogenesis was significantly associated with persistent positive HBV DNA after initiation of IFN (2/25 vs. 11/32, $p = 0.019$ by χ^2 test, $p = 0.026$ by Fisher's exact test).

Cumulative carcinogenesis rates were analyzed according to the ultimate courses of serial assay of HBV DNA. Fifth-year hepatocellular carcinogenesis rate were 0% in patients with HBV DNA loss, and 9.4% in patients without HBV DNA elimination, 10-years rates were 8.0 and 22.5%, and 15-year rates were 8 and 44.0%, respectively. The carcinogenesis rate in patients with HBV DNA elimination was significantly lower than those without DNA elimination ($p = 0.011$, log-rank test).

Hepatocellular Carcinogenesis and HBe Antigen and Aminotransferase [33]

Relationship was assessed between carcinogenesis and HBeAg positivity during the clinical courses. HBeAg was positive in 41 patients (71.9%) and negative in 16 (28.1%) at the initiation of IFN therapy. Twenty-eight (68.3%) of the 41 patients showed continuous loss of HBeAg after IFN therapy. HCC developed in 4 (25.0%) of the 16 patients without HBeAg from the beginning, 4 (14.3%) of the 28 patients with HBeAg clearance, and 5 (38.5%) of 13 patients with persistent HBeAg positivity. HBeAg clearance did not significantly decrease the incidence of carcinogenesis risk ($p = 0.12$, χ^2 test with Yates' correction).

Relationship was also analyzed between carcinogenesis and a longitudinal course of ALT after IFN therapy. Four (18.2%) of 22 patients with normalization of ALT after IFN therapy developed HCC, 9 (25.8%) of 35 patients with persistent abnormal ALT developed HCC. Serial values of ALT was not significantly associated with carcinogenesis risk ($p = 0.075$, χ^2 test with Yates correction).

Cumulative HBe antigen disappearance rate, HBV-DNA disappearance rate, and ALT normalization rate

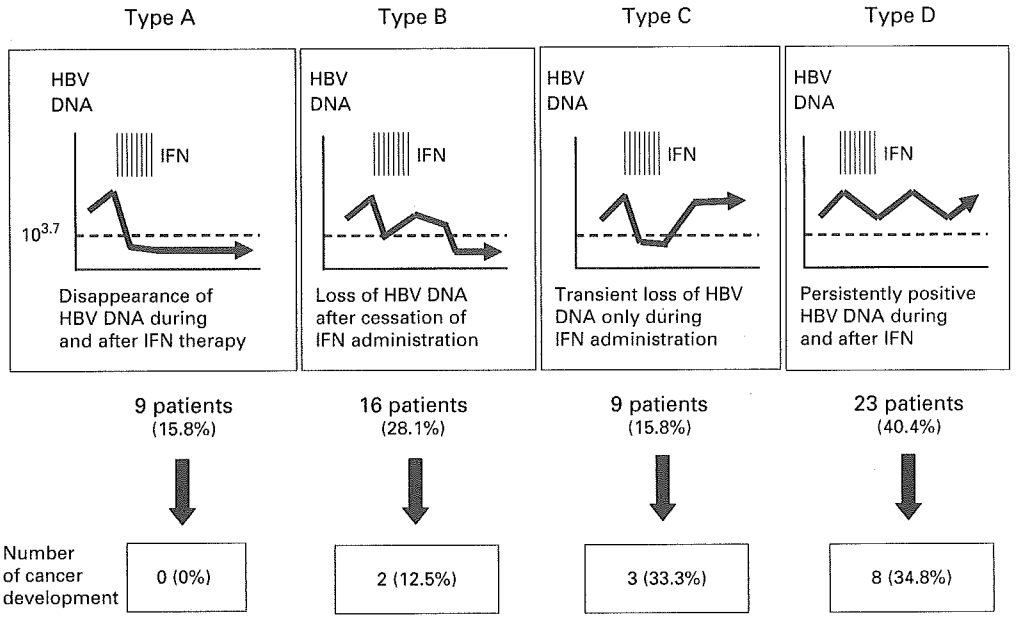

Fig. 5. Relationship between types of serial HBV-DNA concentration and carcinogenesis.

were calculated in those patients with positive HBe antigen at the beginning of IFN treatment. HBe antigen disappearance rate and DNA disappearance rate were 55.4 and 14.6% at the end of the 5th year, and 55.4 and 40.1% at the 10th year, respectively. ALT normalization rate at the 5th year was 25.4% and 10th-year rate was 41.2%. Although the incidence of virological and biochemical improvement gradually increased after the therapy, the rates evidently differed among them.

Discussion

Until recently, several authors mentioned the anti-carcinogenic activity of IFN in patients with HBV-related cirrhosis. Oon [20] and we [23] showed that IFN significantly decreased a carcinogenesis in patients with IFN therapy with a relative risk of 0.03 and 0.39, respectively. Lin et al. [25] also demonstrated an anti-tumor activity of IFN with a relative risk of 0.11 in a randomized controlled trial for patients with chronic hepatitis and cirrhosis. Mazzella et al. [21], Fattovich et al. [22], and the International Interferon-alpha Hepatocellular Carcinoma Study Group in Europe [24] demonstrated a low relative risk for carcinogenesis in patients with IFN therapy, but they could not show a statistical significance. Aside from the slightly inconsistent results after IFN therapy for cirrhosis, we tried to elucidate the relationship between virological response and HCC development, using a cohort of consecutive patients with cirrhosis who underwent IFN therapy more than 10 years ago. Considering that the disease activity and carcinogenic potency can change significantly in the course of HBV-related liver disease, a longitudinal analysis was performed for the study of clinical process and mechanism of anti-tumor activity of IFN in HBV-positive cirrhosis.

In this clinical study, sequential trends of HBV concentration were significantly associated with hepatocellular carcinogenesis, as was found in natural clinical courses of patients without IFN [28]. Although only 2 of 25 patients developed HCC who showed a disappearance of HBV-DNA during or after IFN therapy, 11 of 32 patients showed carcinogenesis who could not eliminate HBV DNA by the treatment with IFN ($p = 0.019$). Hepatocellular carcinogenesis was assessed using serial HBV DNA assay with a cut off value of 3.7 LGE/ml or $10^{3.7}$ copies/ml in this study. Although a detailed analysis of HBV-DNA concentration with more sensitive measurement may

demonstrate a better correlation with carcinogenesis rate than current one, this setting of HBV-DNA concentration as a cut-off value was significantly valuable in the prediction for HCC appearance.

Although the mere use of IFN does not guarantee the decrease of carcinogenesis in patients with HBV-related cirrhosis, a serial course of HBV DNA concentration was significantly correlated with the future HCC development during and after treatment. The value of cancer prediction was much higher in the assay of HBV DNA than that of HBe antigen. Indeed the cut-off values of HBV DNA concentration seemed to be discretionary, the advantage in clinical practice was marked and conspicuous. When more sensitive ways of HBV DNA concentration were applied to the analysis, hepatocellular carcinogenesis could be more successfully predicted.

Conclusions

Persistence of high concentration of HBV DNA was significantly associated with hepatocellular carcinogenesis in cirrhotic patients with and without IFN therapy and its sequential analysis would be useful in early detection of HCC. Further studies with a greater number of patients are required to confirm the relationship, and future studies should be aimed at defining the basic mechanism of hepatocellular carcinogenesis and the role of IFN by which the carcinogenesis rate was suppressed in the cohort.

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