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Comparison of Hepatitis B Virus Subgenotypes in Patients With Acute and Chronic Hepatitis B and Absence of Lamivudine-Resistant Strains in Acute Hepatitis B in Japan

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Hepatitis B virus (HBV) has been classified into eight genotypes and can be further divided into several subgenotypes that have different geographic distributions. Because of increased human migration, the prevalence of rare subgenotypes is increasing in Japanese patients with acute hepatitis B. Lamivudine-resistant strains of HBV have begun to emerge in association with chronic hepatitis B. The aim of this study was to investigate the distribution of HBV subgenotypes and lamivudine-resistant strains in patients in Japan with acute hepatitis B. One hundred twenty-three patients with acute hepatitis B and 123 with chronic hepatitis B were studied. HBV subgenotypes and lamivudine-resistance mutations were determined by direct sequencing of the preS and polymerase region, respectively. HBV subgenotypes Aa (n = 3), Ae (n = 23), Ba (n = 7), Bj (n = 3), Cs (n = 7), Ce (n = 76), D (n = 2), and H (n = 2) were detected in patients with acute hepatitis. In patients with chronic hepatitis, HBV subgenotypes Ae (n = 4), Ba (n = 1), Bj (n = 18), and Ce (n = 100) were found. Non-common Japanese subgenotypes, that is, non-Bj and non-Ce, were detected more frequently in patients with acute hepatitis (35.8%) than in patients with chronic hepatitis (4.1%) (Odds ratio, 0.076; 95%CI, 0.029–0.200; $P < 0.0001$). Lamivudine-resistance mutations were detected in chronic hepatitis patients with breakthrough hepatitis but not in other patients. In conclusion, the prevalence of uncommon Japanese HBV subgenotypes is expected to increase, although lamivudine-resistant strains have not yet been found in patients with acute hepatitis B. ***J. Med. Virol. 79:366–373, 2007.***

KEY WORDS: genotype; drug-resistance; phylogenetic analysis

INTRODUCTION

Approximately 350 million people worldwide are infected with hepatitis B virus (HBV) [Kao and Chen, 2002]. HBV infection causes chronic hepatitis and progresses to cirrhosis and hepatocellular carcinoma, which is the third most common cause of cancer-related death in Japan [Ganem and Prince, 2004; Kiyosawa et al., 2004]. Therefore, HBV infection is one of the most important global health problems, especially in endemic areas such as Asia. HBV has been classified into eight major genotypes on the basis of divergence of 8% in the full-length sequence [Okamoto et al., 1988; Norder et al., 2004]. Each genotype has a unique geographic distribution. Genotype A is found mainly in Europe, North America, and Africa. Genotypes B and C are predominant in East Asia. Genotype D is common in Mediterranean areas. However, several other groups recently reported that the occurrence of genotype A in

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cases of acute hepatitis B in Japan, where genotype A is rare, is increasing [Joh et al., 2003; Kobayashi et al., 2001; Yotsuyanagi et al., 2005; Takeda et al., 2006]. Therefore, the frequencies of other HBV strains that are rare in Japan may have increased among Japanese patients with chronic hepatitis B. HBV subgenotypes and their geographic distributions have also been reported [Norder et al., 2004]. HBV subgenotype Ba is found frequently in Asia, but subgenotype Bj has been found only in Japan [Sugauchi et al., 2003]. HBV subgenotype Ce is found in East Asia, including Japan and China, whereas subgenotype Cs occurs in Southeastern Asia [Huy et al., 2004, Norder et al., 2004; Chan et al., 2005; Tanaka et al., 2005]. Therefore, HBV subgenotypes can be used to study geographic distributions in greater detail than can simple genotypes. The first aim of the present study was to investigate HBV subgenotypes in patients in Japan with acute hepatitis B and to clarify the distribution of these subgenotypes.

Lamivudine has been used widely to treat chronic hepatitis B because it suppresses effectively viral replication, reduces disease activity, and improves liver histology. However, prolonged treatment with lamivudine has led to the emergence of drug-resistant strains. The underlying mutations occur in the HBV polymerase region. A lamivudine resistance mutation emerged in about 70% of patients who treated with lamivudine for 4 years [Hadziyannis et al., 2000; Liaw et al., 2000]. Lamivudine has been available since 2000, and lamivudine-resistant strains have been detected in patients in Japan with chronic hepatitis B. To date, acute hepatitis caused by a lamivudine-resistant strain has not been reported, and the clinical impact of lamivudine-resistant strains on acute hepatitis is not known. Thus, the second aim of the present study was to investigate the prevalence and clinical characteristics of lamivudine-resistant HBV strains in patients in Japan with acute hepatitis B.

MATERIALS AND METHODS

One hundred twenty-three Japanese patients with acute hepatitis B who were treated at Nagoya University Hospital or Ogaki Municipal Hospital were enrolled in this study. Patients were 88 men and 35 women, with a mean age of 38.6 ± 12.9 years (range, 16–75 years). The control group comprised 123 age- and sex-matched Japanese patients with chronic hepatitis B, including 10 patients with breakthrough hepatitis due to lamivudine-resistant strains. Acute hepatitis B was diagnosed as follows. Each patient had high titers of hepatitis B surface antigen (HBsAg) and IgM class antibody against HBV core antigen, elevated serum levels of alanine aminotransferase, and absence of antibodies against other causative viruses, such as hepatitis A virus, hepatitis C virus, Epstein-Barr virus, and cytomegalovirus. It was necessary to discriminate between development of chronic hepatitis after initial infection and acute onset of chronic infection. Thus, serum HBsAg levels noted in previous medical records pertaining to blood donation screening, labor and delivery screening, or employment health screening, for example, were obtained. Informed consent was obtained from all patients, and the study was carried out in accordance with the 1975 Helsinki Declaration.

Virologic Tests

HBsAg was measured with a commercially available kit (AxSYM HBsAg(V2); Abbott Japan, Tokyo, Japan). Antibody titers against hepatitis A virus and hepatitis C virus were measured with a commercial microparticle enzyme immunoassay (AxSYM HAVAB-M 2.0, AxSYM Anti-HCV; Abbott Japan).

HBV DNA was isolated from peripheral blood with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Nested polymerase chain reaction (PCR) analysis and direct sequencing of the preS, polymerase, and precore/core regions were performed as reported previously but with modifications [Allen et al., 1998; Lindh et al., 1999; Huy et al., 2004]. In brief, each 50 μl PCR reaction contained 100 nM each primer, 1 ng template DNA, 5 μl GeneAmp 10× PCR buffer, 2 μl dNTP, and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA). Primers for the preS region were sense (TCACCTATTCTTGGGAACAAGA) and antisense (GGCACTAGTAAACTGAGCCA); for the polymerase region were sense (CCTGCTGGTGGCTCCAGTTC) and antisense (GGTTGAGTCAGCAAACACACTTG); and for the precore–core region were sense (GTTGCATGGAGACCACCGTGAAC) and antisense (GTATGGTGAGGTGAACAATG). Amplification conditions consisted of 5 min at 94C followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The second PCR was done in the same reaction buffer with the first-round PCR product as template and the following sets of primers: for the preS region, sense (TCACCTATTCTTGGGAACAAGA) and antisense (AGAAGATGAGGCATAGCAGC); for the polymerase region, sense (GGATGTGTCTGCGGCGTTT) and antisense (ACCCCATCTTTTTGTTTTGTTAGG); and for the precore–core region, sense (CTGACTACTAATTCCCTGGATGCTGGGTCT) and antisense (ATGTCGACAACCGACCTTGA). PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under Ultra Violet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). The neighbor-joining method [Saitou and Nei, 1987] was used for phylogenetic analysis of the preS region to classify HBV into subgenotypes. Bootstrap analysis (100 replicates) was performed. Sequences of the precore and core regions were used for discrimination between subgenotypes Ba and Bj, as reported previously [Sugauchi et al., 2003].

Statistical Analyses

Data are expressed as mean ± standard deviation (SD). Contingency table analysis with Fisher's exact probability test was used for comparisons between groups. $P < 0.05$ was considered statistically significant. The statistical software used was SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

Distribution of HBV Subgenotypes

The results of phylogenetic analyses of HBV subgenotypes of the 123 patients with acute hepatitis B are shown in Figure 1. The following HBV subgenotypes were detected in patients with acute hepatitis B: Aa (n = 3), Ae (n = 23), Ba (n = 7), Bj (n = 3), Cs (n = 7), Ce (n = 76), D (n = 2), and H (n = 2). Subgenotype Aa was classified further as the Asia type in three patients. Two cases of subgenotype Ce were Okinawa variants. Results of phylogenetic analyses of HBV subgenotypes in the 123 patients with chronic hepatitis B are shown in Figure 2. In patients with chronic hepatitis, HBV subgenotypes Ae (n = 4), Ba (n = 1), Bj (n = 18), and Ce (n = 100) were found. One chronic hepatitis B patient infected with subgenotype Ae was a hemophiliac who received clotting factor concentrates from outside Japan. All cases of genotype B were confirmed by sequencing of the precore and core regions, and there were no differences between PreS and precore/core regions that allowed discrimination of Ba from Bj. Subgenotypes Bj and Ce, which were found frequently in chronic hepatitis B patients, were regarded as common Japanese subgenotypes, and subgenotypes Aa, Ae, Ba, Cs, D, and H, which were found rarely in chronic hepatitis B patients, were regarded as uncommon Japanese subgenotypes. These results are summarized in Table I. Uncommon Japanese subgenotypes were detected more frequently in patients with acute hepatitis B (35.8%) than in those with chronic hepatitis B (4.1%) (Odds ratio, 0.076; 95%CI, 0.029–0.200, $P < 0.0001$). Clinical characteristics of acute hepatitis B with uncommon Japanese subgenotypes and common Japanese subgenotypes are shown in Table II.

Distribution of Lamivudine-Resistant HBV Strains

Lamivudine resistance-associated mutations were detected within the HBV polymerase region (positions 464–562) by direct sequencing. Amino acid substitutions at position 552 (M to V or I) in the YMDD motif and at position 528 (L to M) were detected only in chronic hepatitis patients who suffered breakthrough hepatitis during lamivudine treatment, but not in other patients with acute or chronic hepatitis B.

DISCUSSION

Each HBV genotype has a unique geographic distribution; however, distributions of HBV genotypes are changing gradually due to the ease of international travel. In the United States, the prevalence of HBV precore and basal core promoter variants has increased due to the influx of immigrants from HBV-endemic countries [Chu et al., 2003]. Lamivudine has been used widely to treat chronic hepatitis B, and lamivudine-resistant strains have been reported [Hadziyannis et al., 2000; Liaw et al., 2000]. These changes in prevalence may be associated with differences in clinical course and responses to antiviral treatments. Therefore, a clear understanding of the prevalence of HBV genotypes and HBV strains is important for clinical management of HBV-related diseases. Therefore, the distribution of HBV subgenotypes and lamivudine-resistant HBV strains among patients in Japan with acute hepatitis B was investigated.

Subgenotypes Ce and Bj have accounted for the majority of HBV subgenotypes found in Japanese patients with chronic hepatitis B [Sugauchi et al., 2003; Huy et al., 2004; Norder et al., 2004; Chan et al., 2005; Tanaka et al., 2005; Takeda et al., 2006]. Genotype A has been reported in Japanese patients with chronic hepatitis B, but it is rare [Takahashi et al., 1998; Orito et al., 2001], and the origins of these strains have not been clear. In this study, HBV subgenotypes Ce and Bj together accounted for 118 of the 123 (95.9%) cases of chronic hepatitis B. Therefore, the original subgenotypes in Japan were Ce and Bj and regarded as common Japanese types, and the others identified in this study are uncommon Japanese types. Meanwhile, several recent studies [Joh et al., 2003; Kobayashi et al., 2001; Yotsuyanagi et al., 2005; Takeda et al., 2006] in Japan showed that the distributions of HBV genotypes differ between acute hepatitis B and chronic hepatitis B and that the prevalence of HBV genotype A is significantly higher among patients with acute hepatitis B than among those with chronic hepatitis B. The authors speculated that HBV genotype A, which was found in acute hepatitis B patients, originated outside Japan [Michitaka and Onji, 2004]. Genotypes B and C, which are predominant in patients with acute hepatitis B, also might be showed the same tendency as genotype A. Then genotypes B and C which origins are outside Japan would be increasing and be found in patients with acute hepatitis B. Thus, distribution of the subgenotypes for HBV genotypes B and C in patients with acute hepatitis B was examined to clarify the origin of the virus because HBV genotypes B and C include both common Japanese types (Bj, Ce) and uncommon Japanese types (Ba, Cs). Subgenotypes Ba and Cs were detected more frequently in acute hepatitis B patients (15.1%) than in chronic hepatitis B patients (0.8%) in this study (Odds ratio, 0.048; 95%CI, 0.006–0.371; $P < 0.0001$). Therefore, the trend noted for genotype A was also noted for genotypes B and C. These findings suggest that not only uncommon Japanese HBV genotype A but also uncommon Japanese HBV subgenotype Ba and Cs are becoming more common in Japanese patients with acute hepatitis B. Some attention should be paid to the existence of uncommon Japanese HBV subtypes among genotypes

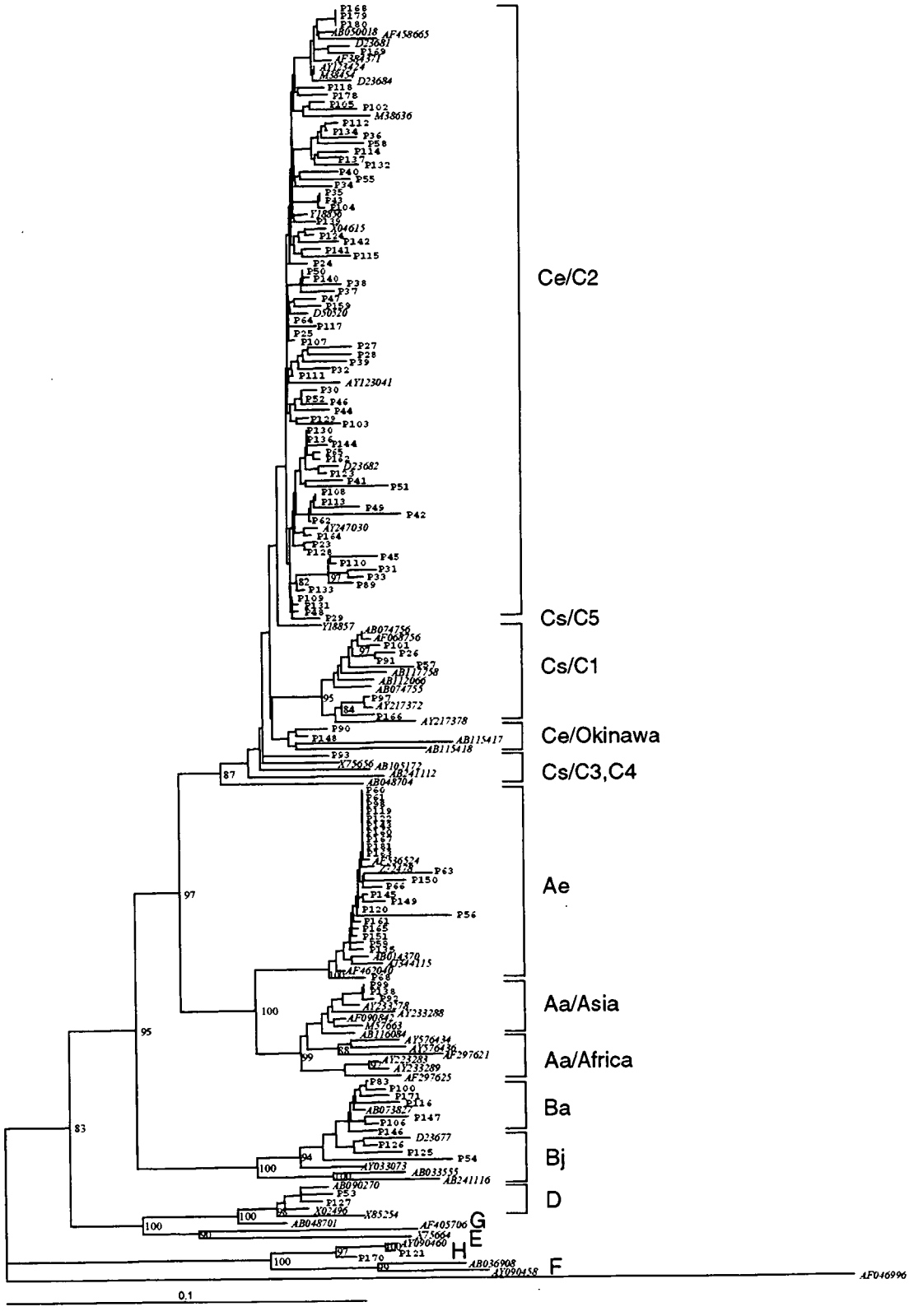

Fig. 1. Results of phylogenetic analysis of 123 sequences from the preS region of HBV of acute hepatitis patients and 59 reference strains from a database and shown by accession number. Phylogenetic analysis was performed by the neighbor-joining method with Woolly monkey HBV (AF046996) as out-group. Percentages of bootstrap values greater than 80% are shown on the nodes. The scale bar indicates genetic distance.

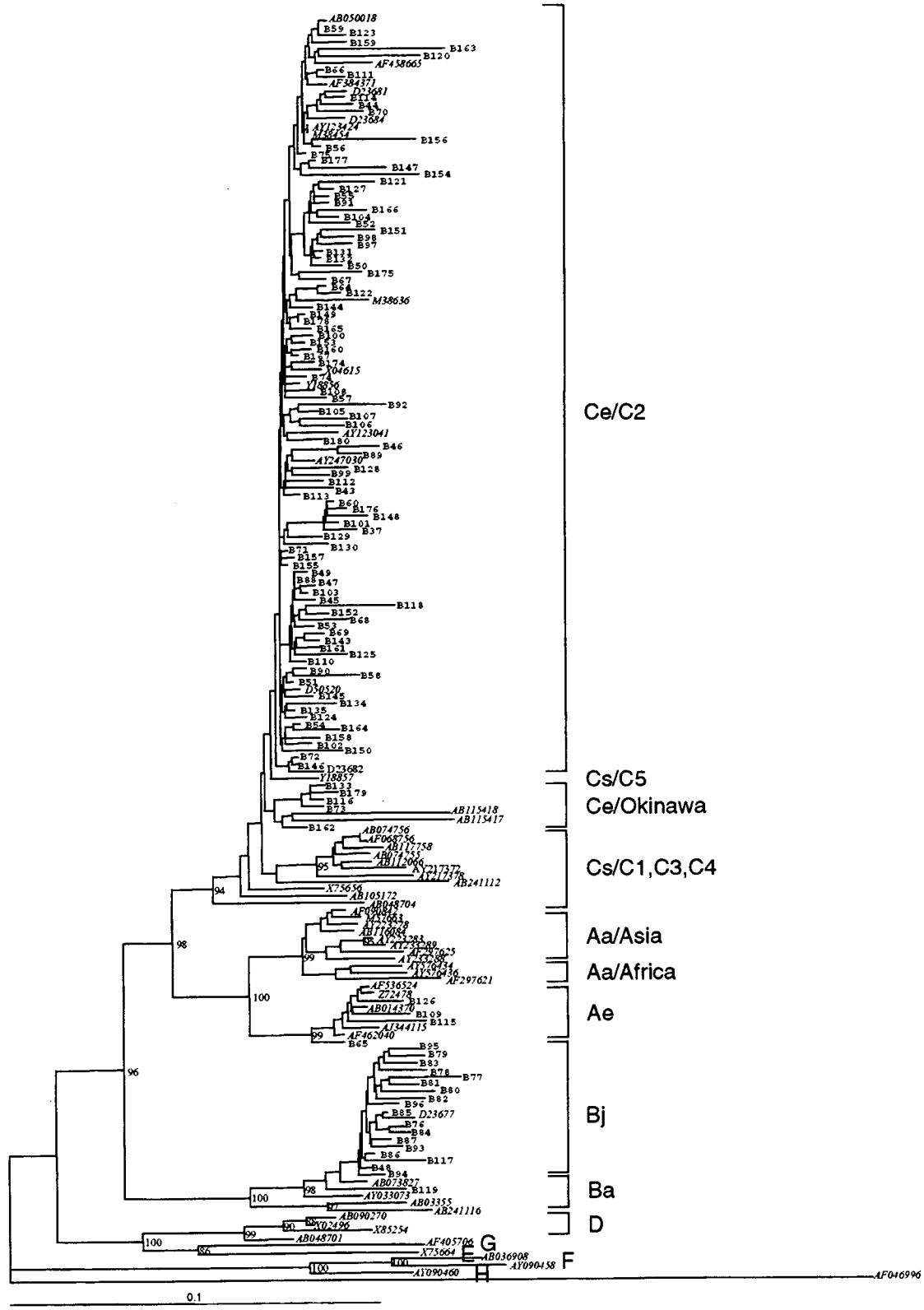

Fig. 2. Results of phylogenetic analysis of 123 sequences from the preS region of HBV of chronic hepatitis patients and 59 reference strains from a database and shown by accession number. Phylogenetic analysis was performed by the neighbor-joining method with Woolly monkey HBV (AF046996) as out-group. Percentages of bootstrap values greater than 80% are shown on the nodes. The scale bar indicates genetic distance.

TABLE I. Clinical Characteristics of 123 Acute Hepatitis B Patients and 123 Chronic Hepatitis B Patients

	Acute hepatitis (n = 123)	Chronic hepatitis (n = 123)	Odds ratio (95%CI)	*P*-value
Age	38.57+/−12.92	41.28+/−12.02		NS
Gender (male/female)	88/35	76/47	0.643 (0.377–1.098)	NS
HBV genotypes (Non-common Japanese types/ Common Japanese types)	44/79	5/118	0.076 (0.029–0.200)	0.0001

B and C, which are predominant types in Japan. A comparison between uncommon Japanese genotypes and common Japanese genotypes was undertaken. High-risk patients, such as those with multiple sexual partners and homosexuals, and patients who progressed to chronic hepatitis were found frequently to have uncommon Japanese genotypes. These results would be reflected with characteristics of genotype Ce which was majority in uncommon Japanese group and those of genotype Ae which was majority in uncommon Japanese group [Koibuchi et al., 2001; Takeda et al., 2006]. Virological and clinical differences between subgenotypes have been reported for genotypes B and C [Sugauchi et al., 2003; Chan et al., 2005]. The difference between HBV subgenotypes Ba and Bj was shown to be due to recombination with genotype C over the precore region and core gene [Sugauchi et al., 2003]. HBV subgenotype Ba leads more easily to development of hepatocellular carcinoma than does subgenotype Bj [Orito et al., 2005]. With respect to genotype C, C1858, which pairs with T1896 in the hairpin loop of the encapsidation sequence, was detected more frequently in subgenotype Cs (95%) than in subgenotype Ce (0%) [Chan et al., 2005]. The pairing of C1858 and T1896 is a stable structure and does not occur with A1896, which creates a TAG stop codon in the precore region. The presence of the A1896 mutation is associated with fulminant hepatitis [Omata et al., 1991]. These reported findings suggest clinical differences in acute hepatitis B between subgenotypes Cs and Ce. However, our sample size was too small to allow comparison of clinical differences in acute hepatitis B between HBV subgenotypes. Further studies are needed to clarify the influence of HBV subgenotypes on the clinical course of acute hepatitis B.

Lamivudine has been used widely to treat chronic hepatitis B, and lamivudine-resistant strains have been reported [Hadziyannis et al., 2000; Liaw et al., 2000]. As lamivudine use increases, the likelihood of lamivudine-resistant strains occurring in patients with acute hepatitis B increases. The prevalence of lamivudine-resistant strains in patients with acute hepatitis B and the clinical impact of lamivudine-resistant strains on acute hepatitis are unknown. Lamivudine has been used recently in patients with acute hepatitis to prevent the progression to fulminant hepatic failure or chronic hepatitis. Several studies have shown that lamivudine is safe and effective for treatment of acute hepatitis B [Kondili et al., 2004; Schmilovitz-Weiss et al., 2004]. Caution must be exercised in determining whether lamivudine should be used to treat acute hepatitis B because of the possibility of lamivudine-resistant strains. Multidrug-resistant strains of human immunodeficiency virus (HIV-1) are found in patients with primary HIV-1 infection, and this has become a serious problem [Brenner et al., 2004]. None of the patients with acute hepatitis B in the present study carried a lamivudine-resistant strain. The results of the present study indicate that lamivudine-resistant strains are not yet common among acute hepatitis B patients; therefore, lamivudine resistance is not a primary consideration in the treatment of acute hepatitis B at present. However, the possibility for acute hepatitis B caused by lamivudine-resistant strains exists. Moreover, as new antiviral drugs are developed, new drug-resistant strains will emerge. The prevalence of drug-resistant strains of HBV will increase in the future; therefore, surveillance to detect drug-resistant strains of HBV, including lamivudine-resistant strains, is important.

There are several reasons why lamivudine-resistant strains were not detected in acute hepatitis B patients in the present study. First, patients who received lamivudine also received education regarding prevention of

TABLE II. Clinical Features in Patients With Acute Hepatitis in Comparison With Common Japanese Types and Non-Common Japanese Types

	Non-common Japanese types (n = 44)	Common Japanese types (n = 79)	Odds ratio (95%CI)	*P*-value
Age	37.93+/−11.94	38.92+/−13.49		NS
Gender (male/female)	46/33	42/2	15.065 (3.404-66.667)	0.0001
Multiple sexual partners (Yes/No)	6/38	7/72	0.616 (0.193–1.962)	NS
Homosexual partners (Yes/No)	7/37	1/78	0.068 (0.008–0.571)	0.003
Progression to chronic hepatitis (Yes/No)	6/38	1/78	0.081 (0.009–0.699)	0.008
Progression to fulminant hepatitis (Yes/No)	2/42	7/72	2.042 (0.405–10.285)	NS

HBV infection so as to avoid new infection. Second, lamivudine-resistant strains did not have sufficient time to cause opportunity acute hepatitis. Chronic hepatitis B patients with breakthrough hepatitis caused by lamivudine-resistant strains were treated as soon as possible with interferon or adefovir dipivoxil [Perrillo et al., 2000, 2004; Suzuki et al., 2002] and, therefore, did not develop new infections. Third, lamivudine-resistant strains show weak infectivity and are not known to cause acute hepatitis. In vitro experiments showed that lamivudine-resistant strains had lower levels of viral replication than did wild-type strain [Melegari et al., 1998]. However, infectious experiments in an in vivo model are needed to prove this hypothesis [Wu et al., 2003]. New HBV subgenotypes and recombinants have been described recently, and therefore the prevalence of HBV strains causing acute hepatitis B will also change [Chen et al., 2004; Kurbanov et al., 2005; Wang et al., 2005]. The contributions of subgenotypes, recombinants, and lamivudine-resistant strains to the clinical features of acute hepatitis need to be clarified.

In conclusion, the prevalence of uncommon Japanese HBV subgenotypes appears to be increasing in Japan. Lamivudine-resistant mutations do not yet appear to be prevalent among patients in Japan with acute hepatitis B. Different HBV strains may have different clinical courses and responses to treatment; therefore, surveillance of HBV strains associated with acute hepatitis B will be useful for developing treatment protocols.

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Relation between incidence of hepatic carcinogenesis and integration value of alanine aminotransferase in patients with hepatitis C virus infection

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Table 1 Comparison of clinical factors between 642 anti-hepatitis C virus (HCV)-positive participants with and without clearance of serum HCV RNA

Clinical factors	Clearance of HCV RNA		p Value
	Yes	No	
Case numbers, n (%)	164 (25.5)	478 (74.5)	
Sex			0.449
Male, n (%)	56 (23.8)	179 (76.2)	
Female, n (%)	108 (26.5)	299 (73.5)	
Mean (SD) age (years)	56.4 (5.9)	56.4 (5.9)	0.987
Mean (SD) BMI (kg/m^2)	24.7 (3.3)	24.7 (3.8)	0.961
>27, n (%)	32 (22.5)	110 (77.5)	0.351
⩽27, n (%)	132 (26.4)	368 (73.6)	
Mean (SD) ALT (IU/l)	27.8 (28.3)	64.2 (60.5)	<0.001
>34, n (%)	33 (9.7)	306 (90.3)	<0.001
⩽34, n (%)	131 (43.2)	172 (56.8)	
HBsAg			<0.001
Positive, n (%)	34 (45.3)	41 (54.7)	
Negative, n (%)	130 (22.9)	437(77.1)	

ALT, alanine aminotransferease; BMI, body mass index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus.

the cut-off value (= 1 SCO), in this study, the anti-HCV tests were also rechecked if the data were 1–2 SCO. For avoiding false-positive tests, anti-HCV was also rechecked for the participants with positive anti-HCV and negative HCV RNA. When comparing clinical factors between 642 anti-HCV-positive participants, with and without clearance of serum HCV RNA, we found that patients with positive HBsAg had a significantly higher proportion of HCV RNA clearance than those negative for HBsAg (p<0.001; table 1). In a stepwise logistic regression analysis, positive HBsAg was the only independent factor significantly associated with negative HCV RNA in anti-HCV participants (OR 0.348; 95% CI 0.211 to 0.574, p<0.001). HBV carriers were observed to have a significantly higher proportion of HCV RNA clearance than non-carriers among men and women (fig 1).

Viral interferences and reciprocal viral interactions have been observed between HBV and HCV dual infection.[7 8] Our previous study also showed that there might be a reciprocal viral interaction between HBV and HCV in patients with dual viral infection treated with interferon/ribavirin combination.[9] Reports from Egypt and Japan[2 3] were conducted in countries endemic for HCV where the prevalence of HBV carriers was <8% and they did not elucidate the influence the HBV infection. In our large-scale community-based study in an area endemic for HCV infection in a country hyperendemic for HBV, the important role of HBV carriers on HCV clearance was noteworthy and especially demonstrated. Besides, we did not find the effect of gender on the HCV clearance observed in previous studies.[2 3] It seems necessary to conduct prospective, longitudinal studies in clarifying roles of gender or concurrent HBV infection on the HCV clearance rate in patients infected with HCV.

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Relation between incidence of hepatic carcinogenesis and integration value of alanine aminotransferase in patients with hepatitis C virus infection

Alanine aminotransferase (ALT) activity is the most widely used laboratory parameter in the evaluation of necroinflammatory activity in liver disease.[1–3] However, it is incorrect to evaluate the arithmetic or the annual mean values. If the ALT level is high, the measurement interval decreases, whereas if the ALT level is low, the interval increases. As a result, the arithmetic mean value increases in patients with increased ALT levels. We performed a more accurate evaluation by using the integration value of ALT. The aim of this study was to determine the utility of the integration value of ALT in predicting hepatic carcinogenesis in patients with the hepatitis C virus (HCV) infection.

A total of 1704 consecutive patients with follow-up periods of ⩾3 years, with no evidence of hepatocellular carcinoma (HCC) for ⩾3 years before the observation period, and interferon treatment completed ⩾3 year before the detection of HCC during the period from January 1995 to December 2002 were included. In all, 594 patients received interferon treatment and 1110 patients did not. All patients were followed up at Ogaki Municipal Hospital, Ogaki, Japan, at least every 6 months. During each follow-up examination, liver-function tests, including ALT, were measured. We calculated the area of a trapezoid with ALT value and the measurement interval, and added the values. We divided the integration value of ALT by the observation period to obtain the average integration value. Patients were classified into five groups according to the average integration value of ALT: group A, 0–20 IU/l (n = 217); group B, 21–40 IU/l (n = 614); group C, 41–60 IU/l (n = 446); group D, 61–80 IU/l (n = 240); and group E, ⩾81 IU/l (n = 187). The median (range) follow-up period was 7.9(3.0–16.8) years. The total number of blood examinations was 90 211, and the median (range) number of blood examinations was 33 (6–222). Factors associated with the cumulative incidence of HCC were analysed using the Cox proportional hazard model with the forward selection method.

HCC occurred in 206 of 1704 (12.1%) patients during the follow-up period. According to univariate analysis, the following were significantly associated with the development of HCC:

- Age >65 years (relative risk (RR) 2.688 (95% CI 2.020 to 3.578); p<0.001);

Table 1 Factors associated with hepatocarcinogenesis (multivariate analysis)

	RR (95% CI)	p Value
Age (years)		
≤65	1	<0.001
>65	1.964 (1.436 to 2.685)	
Sex		
F	1	0.001
M	1.675 (1.242 to 2.259)	
Average integration value of ALT (IU/l)		
0–20	1	<0.001
21–40	3.845 (1.117 to 13.298)	0.033
41–60	4.050 (1.206 to 13.597)	0.024
61–80	9.125 (2.789 to 29.857)	<0.001
≥81	18.838 (5.735 to 61.881)	<0.001
Platelets (×10⁴/mm³)		
≥12.0	1	<0.001
<12.0	3.277 (2.435 to 4.409)	
ALP (IU/l)		
≤338	1	0.003
>338	1.590 (1.167 to 2.166)	
Cholinesterase (IU/l)		
≥431	1	0.006
<431	7.856 (1.824 to 33.830)	
Albumin (g/dl)		
≥3.5	1	<0.001
<3.5	2.901 (1.973 to 4.266)	
IFN treatment		
No treatment	1	0.015
Non-SVR	0.891 (0.429 to 1.851)	0.395
SVR	0.537 (0.349 to 0.827)	0.005

ALP, alkaline phosphatase; ALT, alanine aminotransferase; F, female; IFN, interferon; M, male; SVR, sustained virologic response.

- Male sex (1.515 (1.138 to 2.018); p = 0.004);
- HCV genotype 2 (1.452 (1.003 to 2.102); p = 0.048);

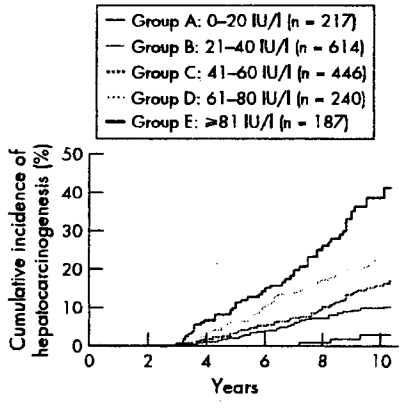

Figure 1 Incidence of hepatocarcinogenesis according to the average integration value of alanine aminotransferase (ALT). Patients were classified into five groups according to the average integration value of ALT: group A, 0–20 IU/l; group B, 21–40 IU/l; group C, 41–60 IU/l; group D, 61–80 IU/l and; group E, ≥81 IU/l. With the exception of group B vs group C, group C vs group D and group D vs group E, the cumulative incidence of hepatic carcinogenesis differed significantly between the five groups (p = 0.0020 to <0.001).

- High average integration value of ALT (group B (4.977 (1.541 to 16.075); p<0.001); group C (8.598 (2.690 to 27.486); p<0.001); group D (14.989 (4.674 to 48.062); p<0.001); group E (25.358 (7.940 to 80.985); p<0.001), fig 1);
- High aspartate aminotransferase level >40 IU/ml (3.283 (2.272 to 4.745); p<0.001), low platelet count <12.0×10⁴/mm³ (5.214 (3.953 to 6.877); p<0.001);
- Low prothrombin time level ≤70% (2.575 (1.760 to 3.768); p<0.001);
- High γ glutamyl transpeptidase level >56 IU/ml (2.615 (1.990 to 3.438); p<0.001);
- High total bilirubin level >1.2 mg/dl (1.990 (1.279 to 3.098); p = 0.002);
- High alkaline phosphatase level >338 IU/ml (3.126 (4.604 to 74.783); p<0.001);
- Low cholinesterase level <431 IU/ml (18.555 (4.604 to 79.783); p<0.001);
- High total protein level ≥6.5 g/dl (1.775 (1.065 to 2.958); p<0.001);
- Low albumin level <3.5 g/dl (4.881 (3.341 to 6.945); p<0.001);
- Low total cholesterol level <130 mg/dl (11.925 (1.671 to 85.085); p<0.001);
- Type of response to IFN treatment (sustained virologic response, 0.142 (0.074 to 0.273) p<0.001; non-sustained virologic response, 0.403 (0.260 to 0.601); p<0.001).

According to multivariate analysis, increasing age, male sex, low platelet count, high average integration value of ALT, low cholinesterase level, low albumin and type of response to interferon treatment were significantly associated with the incidence of HCC as shown in table 1.

We showed that increased liver inflammation, as assessed by increased ALT level, is associated with increased risk for development of HCC in patients with HCV infection.[1–4] The average integration value of ALT, even within the current normal range, was strongly associated with the cumulative incidence of hepatocarcinogenesis. Inhibition of ALT to a value as low as possible is necessary for the prevention of hepatic carcinogenesis.

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BOOK REVIEWS

Upper Gastrointestinal Surgery

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In the rapidly developing field of upper gastrointestinal surgery, there is often a need for access to short synopses of areas of development without the need to resort on every occasion to a literature search or standard text. This concise book brings together a selection of internationally acknowledged experts to provide such a text. The areas covered provide a rapid review of topics which would be of particular interest to specialist

Expression levels of heat shock protein 20 decrease in parallel with tumor progression in patients with hepatocellular carcinoma

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Abstract. Heat shock protein (HSP) 20, a low-molecular-weight HSP, is constitutively expressed in various tissues, such as smooth muscle, skeletal muscle, and liver. However, the characteristics and function of HSP20 have not been precisely understood. In the present study, we investigated correlations of expression levels of HSP20 in hepatocellular carcinoma (HCC) tissues and the surrounding tissues with clinical and pathologic characteristics in 53 resected HCC specimens. Although HSP20 was detected in all 53 HCC tissues, the expression levels were reduced compared with those in the adjacent non-tumor tissues. The expression levels of HSP20 were inversely correlated with tumor stage by TNM classification ($p<0.01$), presence of microvascular invasion ($p<0.05$), and tumor size ($p<0.05$). Our findings strongly suggest that HSP20 may play a role against the progression of human HCC.

Introduction

Cells produce heat shock proteins (HSPs), when exposed to various kinds of biological stress such as heat and chemicals (1). HSPs are classified into high-molecular-weight HSPs such as HSP70, HSP90 and HSP110, and low-molecular-weight HSPs with molecular masses from 10-30 kDa such as HSP20, HSP27 and αB-crystallin according to apparent molecular sizes. It is well recognized that high-molecular-weight HSPs act as molecular chaperones in protein folding, oligomerization and translocation (1). Though the functions of low-molecular-weight HSPs are not as well characterized as those of the high-molecular-weight HSPs, it is recognized that they may also have chaperone functions (1). The human genome codes for 10 low-molecular-weight HSPs (2). In their C-terminal half, these proteins share a sequence element of ~100 amino acid residues called the α-crystallin domain, and toward their N-terminal end, they share a less conserved but nevertheless similar domain (3). HSP20 was co-purified with HSP27 and αB-crystallin from skeletal muscle, and it was identified as a member of the crystallin family (4). Although HSP20 is not induced by heat or chemical stress, it is highly expressed in normal skeletal and smooth muscle, heart and liver tissues where it may be essential, but the exact role of HSP20 remains to be clarified (4).

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, and it causes more than one million deaths annually (5,6). Factors that indicate tumor progression in association with patient outcome reportedly include tumor size, number of tumors, vascular invasion that can be evaluated pathologically and imaging diagnosis (7-10). Tumor markers for HCC such as α-fetoprotein levels and *des*-γ-carboxy prothrombin levels are reported to be additional indicators of tumor progression associated with patient survival (10-13). However, these factors are not sufficient to accurately discriminate the tumor progression of HCC patients towards the accurate prediction of patient survival. It is, therefore, necessary to further investigate other indicators for the evaluation of tumor progression and for the prediction of patient outcome.

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Key words: heat shock protein 20, hepatocellular carcinoma, tumor stage

To date, it has been reported that expression of certain HSPs can be correlated with the carcinogenic process as well as with the degree of differentiation and cell proliferation, and moreover, they have been implicated in the regulation of apoptosis (14,15). In addition, evidence is accumulating about the usefulness of the prognostic implications of HSPs in certain cancer types, especially high-molecular-weight HSPs (14,15). We have recently shown that attenuated phosphorylation of HSP27 correlates with tumor progression in patients with HCC (16). Among low-molecular-weight HSPs, HSP27 has been the most extensively studied, but to the best of our knowledge there has been no report about the relationship of HSP20 and tumor progression. Therefore, in the present study, we tried to investigate the relationship between HSP20 and HCC in 53 resected HCC specimens.

Materials and methods

Patients. Fifty-three patients (46 men, 7 women, mean age: 66.9±8.4 years), having been diagnosed with HCC at the Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan underwent hepatic resection between September 2002 and August 2005. Liver cirrhosis was present in 24 patients, and chronic hepatitis was present in 29. Fourteen patients were infected with hepatitis B virus, and 34 were infected with hepatitis C virus. The remaining 5 patients had evidence of alcoholic cirrhosis. No patient had previously undergone preoperative chemotherapy.

The resected HCC specimens were obtained according to protocol approved by the Committee for the Conduct of Human Research at Ogaki Municipal Hospital. Informed consent was obtained from all patients.

Surgical specimens. Primary HCC tissues were obtained from all patients by surgical resection at the Department of Surgery, Ogaki Municipal Hospital. The excised tissue was divided into two parts, and one part was fixed with 20% neutral formalin overnight. The fixed tissue was then dehydrated with 100% methanol and xylene and embedded in paraffin wax. A three-micron-thickness of this tissue was used for immunohistochemical staining. The other part of the resected tissue was snap-frozen in liquid nitrogen and stored at -80˚C until used for Western blot analysis.

Pathological evaluations. The pathological features of HCC were evaluated by two of the authors (N.Y. and Y.K.) without knowledge of the HSP20 status of the tumor. The specimen was stained with hematoxylin and eosin, and the entire specimen was examined. Differentiation of HCC was classified as well-, moderately, or poorly differentiated HCC on the basis of the classification by the International Working Party (17). Vascular invasion and infiltration to the tumor capsule were evaluated macroscopically.

Western blot analysis. Snap-frozen samples were homogenized and sonicated in lysis buffer containing 62.5 mM Tris-HCl

Table I. Comparison of the protein levels of HSP20 with the clinical and pathological characteristics of 53 patients with HCC.

	p value	
	Tumor tissue	Non-tumor tissue
Gender male (n=46), female (n=7)	0.896	0.627
Underlying disease liver cirrhosis (n=25), chronic hepatitis (n=28)	0.957	0.010[a]
Etiology of liver disease HBV (n=13), HCV (n=35), alcoholic (n=5)	0.662	0.482
Number of tumors solitary (n=40), multiple (n=13)	0.374	0.718
Tumor size (mm) <20 (n=12), 20-50 (n=32), >50 (n=9)	0.048[a]	0.697
Vascular invasion negative (n=35), positive (n=18)	0.040[a]	0.669
Infiltration to capsule negative (n=28), positive (n=25)	0.203	0.673
Tumor stage I (n=9), II (n=25), III (n=11), IV (n=8)	0.003[a]	0.449
Histological classification (differentiation) well- (n=11), moderately (n=35), poorly (n=7)	0.858	0.636

HBV, hepatitis B virus; HCV, hepatitis C virus; [a]$p<0.05$.

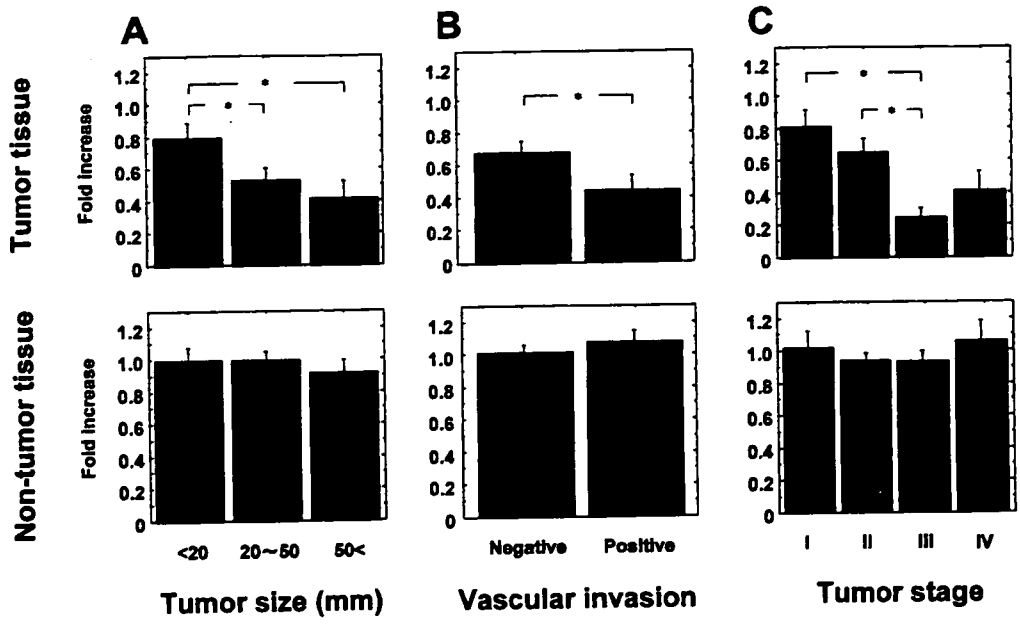

Figure 1. HSP20 levels in patients with HCC. Protein extracts from 53 HCC specimens (tumor and adjacent non-tumor tissue, respectively) were analyzed with antibodies against HSP20 and ß-actin. Signal intensities on X-ray film were quantified with NIH image software. The histograms show quantitative representations of the levels of HSP20 after normalization to levels of ß-actin. Values on the vertical axis represent the mean ± SE of independent experiments. The values were calculated as the average values with those of small (tumor size <20 mm) HCC (left column), negative vascular invasion (center column) and tumor stage I HCC (right column) equal to 1.0. *$p<0.05$.

(pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. Western blot analysis was performed as described previously (18) with polyclonal antibodies against HSP20 and HSP27 (Stressgen Biotechnologies, Victoria, British Columbia, Canada). Peroxidase-conjugated antibodies against rabbit IgG were used as secondary antibodies against the above-mentioned primary antibodies. Primary antibodies against ß-actin (Sigma-Aldrich Co, St. Louis, MO) were detected with peroxidase-conjugated antibodies against mouse IgG as secondary antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film with the ECL Western blotting detection system (GE Healthcare UK Ltd, Buckinghamshire, UK). Protein band intensities were determined by integrating the optical density over the band area (band volume) with NIH image software. HSP20 levels were normalized to those of ß-actin.

Immunohistochemical analysis. Immunohistochemical staining of some specimens was performed with the streptavidin-biotin complex method to investigate expression and localization of HSP20. Primary antibodies were anti-HSP20 rabbit polyclonal antibodies (Stressgen Biotechnologies, Golden, CO). Briefly, deparaffinized sections were treated with 3% H_2O_2 in methanol for 10 min to inhibit endogenous peroxidase activity. Sections were immersed in 0.05 M citrate buffer (pH 6.0), heated in a microwave oven for 15 min, and then incubated with primary antibodies for 2 h at room temperature. Each section was treated sequentially with biotinylated secondary antibodies (anti-rabbit-IgG) and streptavidin-peroxidase complex (Dako Chem Mate, Kyoto, Japan). Finally, immune complexes were visualized with 3,3'-diamino-benzidine tetrahydrochloride as a chromogen. Mayer's hematoxylin was used as a counterstain.

Statistical analysis. Patient clinical data were expressed as mean ± SD. The data were analyzed with the SPSS software program (Release 11.5.1J standard version; SPSS Japan, Tokyo, Japan). One-way analysis of variance (ANOVA) was used to determine the significance of differences between protein expression and grade of tumor differentiation or tumor stage. Nonparametric data were analyzed with the Mann-Whitney U test, Kruskal-Wallis test, or Spearman's correlation coefficient (r). All p values were derived from two-tailed tests and $p<0.05$ was accepted as statistically significant. A Spearman's correlation coefficient of $r\geq0.400$ was accepted as a positive correlation.

Results

Correlations of HSP20 levels according to characteristics of HCC. The levels of HSP20 were compared with the clinical and pathological characteristics of 53 patients with HCC, including gender, underlying liver disease, etiology, number of tumors, tumor size, vascular invasion, infiltration to the tumor capsule, and tumor stage (evaluated according to the TNM classification of the International Union Against Cancer) (19), and histological classification (Table I). Comparisons of the levels of HSP20 revealed significant differences with respect to tumor size ($p=0.048$), vascular invasion ($p=0.040$) and tumor stage ($p=0.003$) in tumor tissues, while there were no significant differences in HSP20 levels in adjacent non-tumor tissues, except in those tissues with underlying liver disease (Table I). In the non-tumor tissues, the levels of HSP20 in liver cirrhotic tissue were significantly higher than those in chronic hepatitis patient tissue (Table I).

HSP20 levels according to tumor size, vascular invasion and tumor stage are shown in Fig. 1. A trend toward decreased expression levels of HSP20 in tumor tissues was

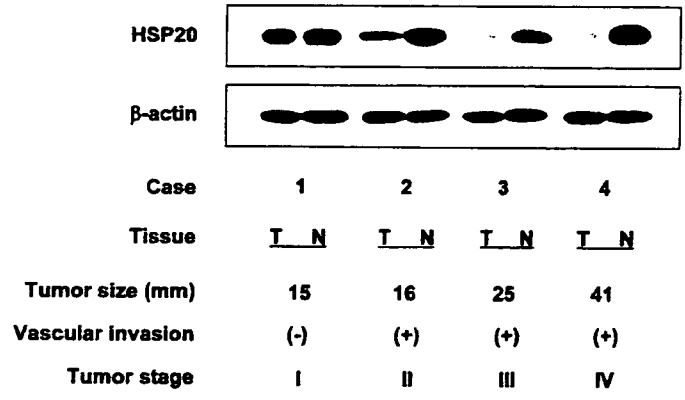

Figure 2. HSP20 levels in four representative patients with HCC according to tumor size, vascular invasion and tumor stage. Comparison with those in non-tumor tissue. Protein extracts were analyzed with antibodies against HSP20 and ß-actin. T, tumor tissue; N, adjacent non-tumor tissue.

observed as tumor size, vascular invasion and tumor stage increased, suggesting that the levels of HSP20 in the adjacent non-tumor tissues were higher than those in the tumor tissues (Fig. 1A, B and C; upper panel). On the other hand, HSP20 levels in the adjacent non-tumor tissues were not correlated with these factors, suggesting that the levels of HSP20 in the tumor tissues were attenuated in parallel with HCC progression (Fig. 1A, B and C; lower panel). Western blot images of HSP20 expression in 4 representative patients with HCC according to tumor size, vascular invasion and tumor stage are shown in Fig. 2.

Immunohistochemical analysis of HSP20 in HCC specimens. To confirm our results from Western blot analysis, we performed immunohistochemical analysis of HSP20 in HCC tumor and non-tumor tissues. Immunohistochemical staining of HSP20 in stage-IV-HCC specimens containing tumor and non-tumor tissue is shown in Fig. 3. Immunoreactivity for HSP20 in tumor tissue was markedly lower than that in non-tumor tissue.

Comparisons between the levels of HSP20 and the levels of phosphorylated HSP27 in HCC tumor tissues. HSP27, a low-molecular-weight HSP, is phosphorylated at three serine residues (Ser-15, Ser-78 and Ser-82) (1). We previously reported that attenuation of phosphorylated HSP27 (Ser-15, Ser-78 and Ser-82) in tumor tissue correlates with HCC progression (16). Therefore, we investigated the correlation between the levels of HSP20 and the levels of phosphorylated HSP27 that had been determined in the previous study. The levels of phosphorylated HSP27 (Ser-15) were significantly correlated with the levels of HSP20 ($r=0.505$, $p<0.001$; Fig. 4A). On the contrary, the levels of phosphorylated HSP27 (Ser-78), phosphorylated HSP27 (Ser-82) or total HSP27 were not correlated with those of HSP20 (Fig. 4B, C and D, respectively).

Discussion

In the present study, we showed that attenuation of HSP20 levels correlated with tumor progression in tumor tissues of patients with HCC. In addition, the HSP20 levels correlated inversely with tumor size and vascular invasion of HCC, both of which are indications of an advanced tumor. To the best of our knowledge, this is the first report of a significant relation between HSP20 levels and progression of HCC.

Recently, we reported that attenuation of phosphorylated HSP27 is correlated with HCC progression (16). It is recognized that HSP27, HSP20 and αB-crystallin form one type of complex (3,20). It has been shown that phosphorylation of HSP27 is associated with the disassembly of HSP27 complexes (21,22). In the present study, we found significant correlation between the levels of HSP20 and that of phosphorylated HSP27 (Ser-15), but not Ser-78 and Ser-82. Although the differential role of the three phosphorylation sites are not known, our findings suggest that HSP20 and phosphorylated HSP27 (Ser-15) may have suppressive effects on HCC progression. In addition, these results suggest that phosphorylated HSP27 (Ser-78) and phosphorylated HSP27 (Ser-82) may have different roles in HCC progression. Further investigations are required not only to clarify the exact role of HSP20, but also to determine whether these HSPs can be prognostic factors in HCC. Moreover, HSPs not only have prognostic implications but also have therapeutic implications for cancer (14). Among HSPs, the use of the HSP90 inhibitor,

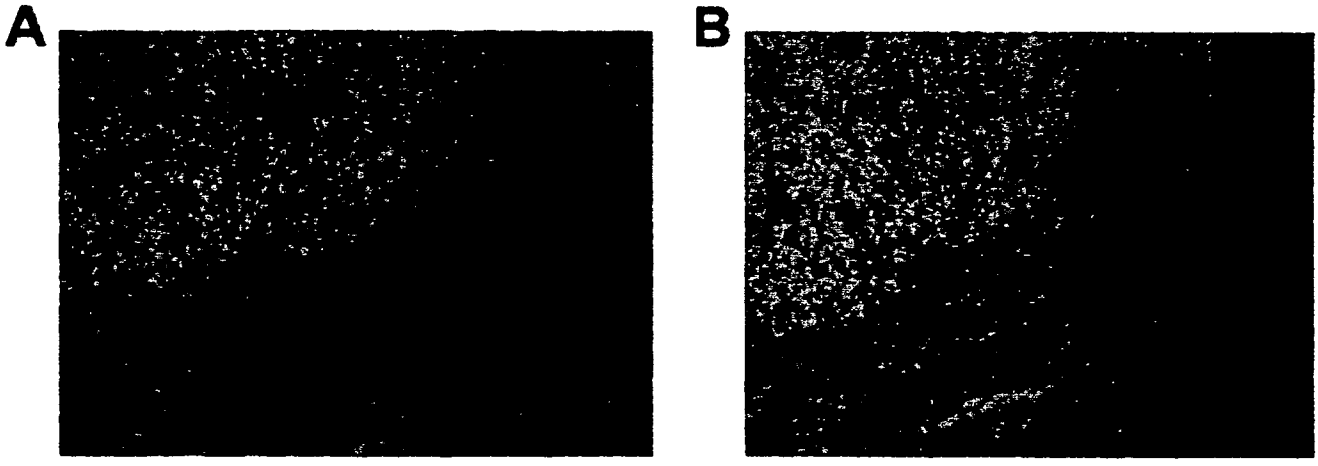

Figure 3. Immunohistochemical analysis of HSP20 in a patient with HCC (tumor stage IV) and adjacent non-tumor tissue (chronic hepatitis) (A). The same patient specimen stained with hematoxylin and eosin. (B).

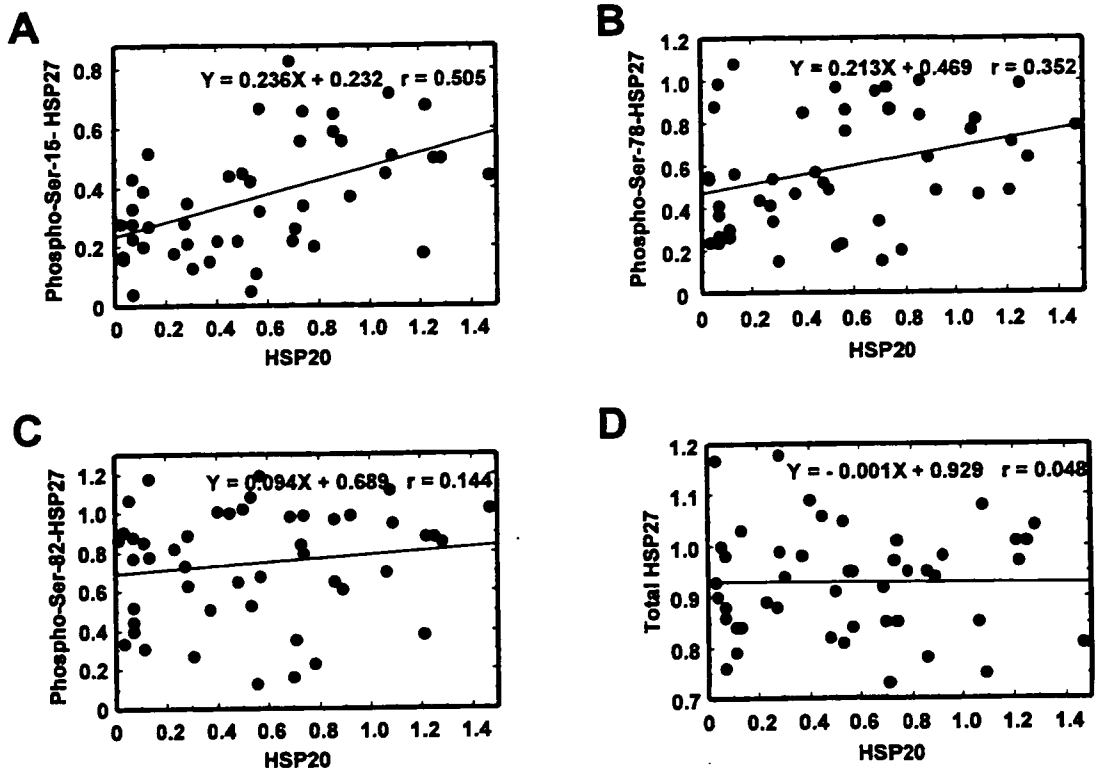

Figure 4. Correlations between the levels of HSP20 and the levels of (A) Ser-15-phosphorylated HSP27, (B) Ser-78-phosphorylated HSP27, (C) Ser-82-phosphorylated HSP27, and (D) total HSP27. The expression levels of HSP20, phosphorylated HSP27, and total HSP27 were determined by the band intensities obtained from Western blot analysis, and then normalized to those of ß-actin.

which is under phase I trial has been extensively studied (14,15). Although the role of HSP20 in HCC is not precisely known, further investigations would help us to use HSP20 as a target for cancer therapy.

In conclusion, our present results strongly suggest that expression levels of HSP20 decrease with progression in tumor stages in patients with HCC and that HSP20 may have a suppressive effect on the advancement of human HCC.

Acknowledgements

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HEPATOLOGY

Efficacy of ribavirin plus interferon-α in patients aged ≥60 years with chronic hepatitis C

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Key words
age group, hepatitis C virus, interferon, ribavirin, sustained virologic response.

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Abstract

Background: In Japan, patients with hepatitis C virus (HCV)-associated liver disease are getting older, and thus the number of deaths due to such disease is increasing. The efficacy of combination therapy with ribavirin and interferon for chronic HCV infection in elderly patients has not been fully clarified. The aim of the present study was to evaluate the efficacy and tolerability of combination therapy in such patients.

Methods: Two hundred and twenty consecutive patients with chronic hepatitis C were treated with combination therapy. These patients were divided into two groups according to age: patients ≥ 60 years ($n = 66$) and patients < 60 years ($n = 154$). Clinical characteristics, the sustained virologic response (SVR) rate obtained by intention-to-treat analysis, and the rate of reduction or discontinuation of ribavirin were compared between the two groups.

Results: The ribavirin discontinuation rate was significantly higher in the patients aged ≥60 years than in the patients aged <60 years. However, the SVR rates did not differ significantly between patients aged ≥60 years and those aged <60 years (31.8% *vs* 38.3% by intention-to-treat analysis). According to multivariate analysis, genotype and HCV viral load were significantly associated with SVR while patient age did not affect SVR.

Conclusions: Treatment of chronic hepatitis C with combination therapy was comparably effective between patients aged ≥60 years and those aged <60 years, although the ribavirin discontinuation rate was higher among the older patients than the younger patients.

Introduction

Hepatitis C virus (HCV) infection is a widespread viral infection that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The need for treatment of chronic HCV infection in the elderly is increasing in Japan and is expected to increase in the USA and other Western countries.[1]

Sustained virologic responders who are negative for serum HCV-RNA 6 months after treatment with interferon (IFN) are reported to be likely to remain in virologic and biochemical remission with histologic improvement.[2,3] Moreover, IFN therapy reduces the risk of hepatocellular carcinoma among virologic or biochemical responders.[4–6] Ribavirin is now generally used in combination with IFN for the treatment of chronic hepatitis C, and this therapy has been reported to be more effective than IFN monotherapy, with a higher rate of HCV eradication.[7–10]

Efficacy of IFN monotherapy in elderly patients with chronic hepatitis C has been reported,[11,12] but efficacy of combination ribavirin and IFN therapy in elderly patients has not been established. We retrospectively evaluated the efficacy and tolerability of ribavirin plus interferon in patients aged ≥60 years with chronic hepatitis C.

Methods

Patients

Two hundred and twenty consecutive patients with chronic hepatitis C with a high viral load (we defined high viral load as serum HCV-RNA level >100 KIU) were treated with IFN and ribavirin in combination between January 2002 and April 2003 at 14 institutions: Nagoya University Hospital and affiliated hospitals. One hundred and twenty-two of 220 patients were naïve patients. All met the following inclusion criteria: <75 years old; positivity for anti-HCV antibody; and serum HCV-RNA level > 100 KIU/mL on

Table 1 Patients treated by combination therapy

	Total patients ($n = 220$)	Age <60 years ($n = 154$)	Age ≥60 years ($n = 66$)	*P*
Sex ratio (male/female)	147/73	109/45	38/28	0.0567
Baseline serum ALT (IU/L)	94.0 ± 68.6	92.4 ± 71.4	97.6 ± 62.0	0.6081
Hemoglobin (g/dL)	14.3 ± 1.3	14.5 ± 1.3	13.9 ± 1.4	0.0056
Creatinine clearance (mL/min)	101.6 ± 24.5	106.5 ± 24.6	85.3 ± 15.2	<0.0001
Genotype (1/2/other)	169/50/1	115/38/1	54/12/0	0.4510
HCV-RNA (KIU/mL)	648.7 ± 339.4	638.8 ± 342.3	671.9 ± 333.9	0.5090
Activity (A0/A1/A2/A3)	6/77/63/18	5/55/44/14	1/22/19/4	0.8405
Fibrosis (F0/F1/F2/F3/F4)	8/74/45/26/10	6/54/34/17/7	2/20/11/9/3	0.9199

ALT, alanine aminotransferase; HCV-RNA, hepatitis C virus RNA.

quantitative polymerase chain reaction (PCR) assay (Amplicor Monitor Assay; Roche Molecular Systems, Pleasanton, CA, USA) within 12 weeks preceding the therapeutic period. Exclusion criteria included pretreatment hemoglobin level < 10 g/dL, positivity for serum hepatitis B surface antigen, drug addiction, alcohol abuse, autoimmune hepatitis, primary biliary cirrhosis, coexisting serious psychiatric or medical illness, and pregnancy. To exclude any patient bias, only complete cohorts from each hospital were enrolled. HCV genotypes were determined by PCR with genotype-specific primers.[13,14]

All patients were treated with 6–10 MU IFN-α-2b (Intron A, Schering Plough, Osaka, Japan) daily for 2 weeks, followed by the same dose of IFN three times a week for 22–46 weeks. We conducted 24 weeks of treatment at first. In the last 44 patients treatment duration was elongated to 48 weeks because this produced higher efficacy than 24 weeks of treatment. Oral ribavirin (Rebetol, Schering-Plough, Kenilworth, NJ, USA) was administered for 24 weeks at 600 mg/day for patients who weighed ≤60 kg and at 800 mg/day for those who weighed >60 kg during the treatment period. The dose of ribavirin was reduced by 200 mg/day when the patient's hemoglobin concentration fell below 10 g/dL because of hemolytic anemia induced by the drug. Ribavirin was discontinued when IFN therapy was discontinued. In Japan, combination with interferon and ribavirin therapy was approved for medical insurance coverage in 2001 with a limit in ribavirin administration of up to 24 weeks. Combination therapy with peg-interferon and ribavirin was not approved for medical insurance coverage in Japan until November 2004.

Liver histology

Pretreatment liver biopsy specimens were classified in terms of fibrosis on a scale of F0–F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; F4, cirrhosis) and in terms of necroinflammatory activity on a scale of A0–A3 (A0, no histologic activity; A1, mild activity; A2, moderate activity; A3, severe activity).[15]

Assessment of efficacy

Virologic response was assessed by qualitative HCV-RNA assay with a lower sensitivity limit of 100 copies/mL (Amplicor HCV version 2.0; Roche Molecular Systems). According to the qualitative HCV-RNA results, responses were defined as follows: sustained virologic response (SVR), no HCV-RNA detected at the end of the 24-week follow-up period after completion of treatment; relapse, no HCV-RNA at end of treatment and reappearance of serum HCV-RNA during the 24 week follow-up period; or non-response (NR), persistent positive serum HCV-RNA throughout treatment.

Comparison of characteristics and efficacy of treatment according to age

Patients were divided by age into two groups: those aged ≥60 years ($n = 66$) and those aged <60 years ($n = 154$). Sex ratio, baseline serum alanine aminotransferase level, pretreatment hemoglobin level, creatinine clearance, HCV genotype and viral load, histologic activity and fibrosis were compared between the two groups (Table 1). End-of-treatment virologic response (ETR) rate and SVR rate obtained by intention-to-treat analysis and per-protocol analysis, and the rate of reduction or discontinuation of ribavirin were compared between the two groups (Table 2).

Comparison of treatment efficacy between combination therapy and monotherapy in older patients

We examined efficacy of combination therapy in comparison to that of monotherapy in patients aged ≥60 years. For this purpose, we included as historical controls 257 patients with chronic hepatitis C with a high viral load treated with IFN-α alone. These were 168 men and 89 women aged 18–69 years (mean ± SD, 50.1 ± 9.9 years) treated at Nagoya University Hospital or Ogaki Municipal Hospital from 1989 to 2001. Forty-seven patients out of 257 were >60 years. All patients were treated with 6–10 MU IFN-α daily for 2 weeks, followed by the same dose of IFN-α three times a week for 22–46 weeks.

The study protocol was approved by the ethics committee of each hospital, and written informed consent was obtained from each patient before therapy.

Statistical analysis

Values are expressed as mean ± SD. Between-group differences in mean quantitative values were analyzed using Student's *t*-test, and differences in non-parametric data were analyzed by Mann–Whitney *U*-test. Differences in proportions were tested using χ^2 test. Multiple logistic regression analysis was used to identify factors related to SVR. All statistical analyses were performed