Table 1. Background of the patients studied

Patient No.	Clinical treatment response	Gender	HCV genotype	Age years	Pretreatment		
					RNA level KIU/ml	ALT IU/l	Hb mg/dl
M1	SVR	M	1b	43	>850	361	14.0
M2	Rel	M	1b	53	560	54	14.6
M3	NR	M	1b	42	700	212	14.5
M4	NR	F	1b	44	620	209	13.8
M5	NR	M	1b	51	>850	108	13.6
C1	SVR	M	1b	54	830	112	15.1
C2	SVR	F	1b	60	>850	51	14.2
C3	SVR	M	1b	35	>850	134	16.6
C4	SVR	F	1b	54	>850	26	12.6
C5	Rel	M	1b	32	410	51	14.2
C6	Rel	M	1b	67	190	154	13.9
C7	Rel	M	1b	68	460	164	13.0
C8	Rel	M	1b	68	>850	98	12.7
C9	Rel	M	1b	47	560	50	15.6
C10	Rel	F	1b	64	>850	16	13.7
C11	Rel	M	1b	44	>850	56	15.5
C12	Rel	F	1b	70	>850	142	12.2
C13	NR	M	1b	61	>850	61	13.5
C14	NR	M	1b	67	260	139	13.1
C15	NR	M	1b	52	>850	113	11.7
C16	NR	M	1b	70	400	101	16.7
C17	NR	M	1b	29	>850	55	14.1
C18	NR	F	1b	60	>850	90	14.3
Mean ± SD				54 ± 13	697 ± 217	111 ± 77	14.1 ± 1.3

M1–5 = Patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy; ALT = serum alanine transaminase; Hb = hemoglobin.

Reverse Transcriptase (Invitrogen Corp., Carlsbad, Calif., USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) as described previously [13].

The complete HCV NS5B sequence was determined in all samples. Briefly, cDNA was amplified by a long polymerase chain reaction (PCR) with LA Taq (Takara Shuzo Co. Ltd, Tokyo, Japan). The long PCR fragments (around 2.1 kbp) were generated with primers 7355F-NK (CCTGACAGAGTCCACCGTGTCTTCTGCCTT) and 9440R-NK (GTTGGGGAGCAGGTAGATGCCTACCCCTAC). The first-round PCR product was further amplified using hemi-nested primers including 7378F-NK (AGCTCGCTACTAAGACCTTTGGCAGCTCCG) and 9440R-NK. The amplicons were then cloned into pCR2.1-TOPO vector (Invitrogen Corp), and nucleotide sequences were determined using Prism Big Dye (Applied Biosystems, Foster City, Calif., USA) with an ABI 3100 DNA automated sequencer.

The sequences generated were used to confirm HCV genotypes and to identify specific nucleotide mutations and amino acid substitutions that may be associated with interferon-α/ribavirin treatment. Additionally, to determine whether ribavirin serves as a RNA mutagen, two models were used to estimate evolutionary distances: Tamura-Nei model for all codon substitutions, and Pamilo-Bianchi-Li model for synonymous and non-synonymous substitutions.

Amino acid sequences of the HCV NS5B region in various HCV genotypes were obtained from GenBank/DDBJ database. They were aligned to show amino acid 415 of the HCV NS5B region.

Statistical Analysis

The χ^2 test, Fisher's exact test and Student's t test were used where appropriate.

Results

Nucleotide Substitutions in the HCV NS5B Region before and after Therapy

The mean pair-wise genetic distances of all codon substitutions among 14 patients (4/18 had a SVR and were negative during follow-up) receiving combination therapy were 0.00668 ± 0.00702, which was not different from

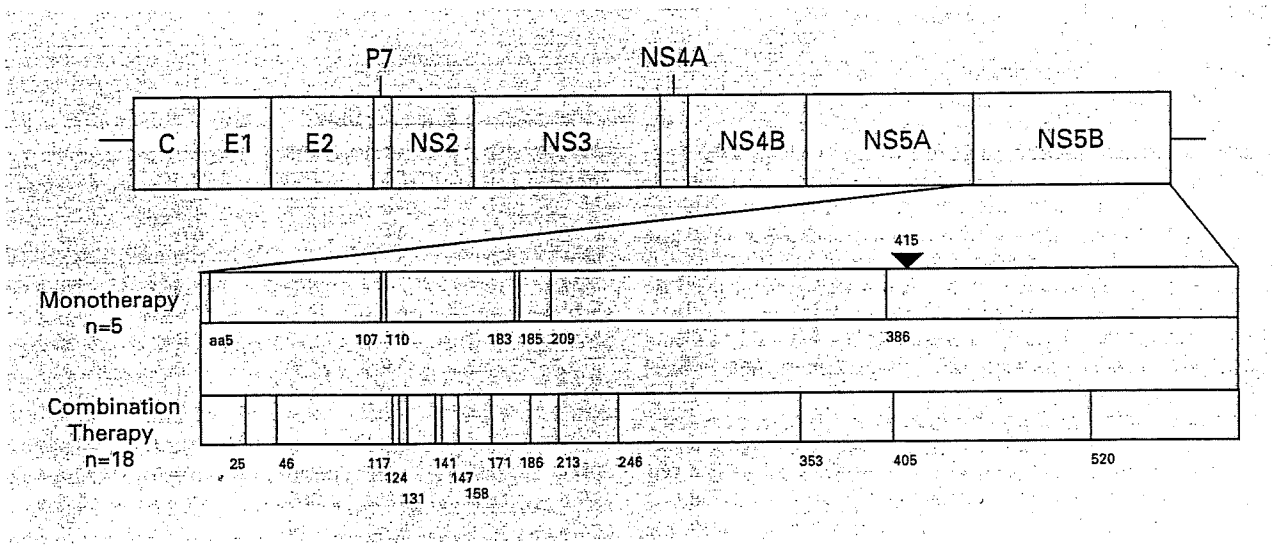

Fig. 1. The site map of amino acid mutations in the NS5B region in patients receiving interferon-α monotherapy versus interferon-α/ribavirin combination therapy. The vertical bars indicate the sites of amino acid substitutions after therapy.

the 4 patients (1/5 had a SVR) receiving interferon-α monotherapy 0.00627 ± 0.00444 (p = NS). The nucleotide substitution rate was also not different between the 2 groups after taking account of the bias towards synonymous substitutions (0.01778 ± 0.0237 vs. 0.01603 ± 0.01208, p = NS).

Amino Acid Substitutions in the NS5B Region

In the 5 patients receiving interferon-α monotherapy (1 was SVR and negative for HCV RNA during follow-up), 7 amino acid substitutions in the NS5B region were detected. In the 18 patients treated with interferon-α/ribavirin combination therapy (4 were SVR and negative for HCV RNA during follow-up), 17 amino acid substitutions were detected (fig. 1; table 2–4). There were no amino acid substitutions specific to the clinical treatment response identified. Note that with reference to the amino acid residue at the NS5B 415th site, all 18 in 23 (since 5/23 were SVRs) patients with HCV genotype 1b infection had Y before and after treatment.

There was also no difference in the number of sites of amino acid substitutions in the HCV NS5B region between 17 in the patients treated with combination therapy and 7 in the patients receiving interferon-α monotherapy (0.2055% (17/591 × 14) vs. 0.2961% (7/591 × 4); the NS5B region consists of 591 amino acids).

Amino Acid Alignment in the NS5B Region of Different HCV Genotypes

A total of 150 complete amino acid sequences derived from the nucleotide sequences of the HCV NS5B region from different HCV genotypes (from genotype 1a to 6) were obtained from the GenBank/DDBJ database. All 17 HCV-1a sequences had F at position 415 of the NS5 region. In contrast, 104/106 HCV genotype 1b sequences had Y at position 415 of NS5B. For other HCV genotypes, only HCV genotype 1c and 1 of 6 genotype 3 sequences had F at position 415 of the NS5B region (fig. 2; table 5).

Discussion

This study showed three important points. First, most HCV genotypes 1b had Y, instead of F in position 415 of the HCV NS5B region. Second, interferon-α/ribavirin treatment did not induce any specific amino acid substitutions in the HCV NS5B region in Japanese patients with HCV genotype 1b infection. Third, interferon-α/ribavirin treatment did not induce more nucleotide mutations or amino acid substitutions compared to interferon-α monotherapy in Japanese patients with HCV genotype 1b infection.

Table 2. Amino acid substitutions in HCV NS5B in the patients studied

NS5B	aa	5th		25th		46th		107th		110th		117th		124th		131th	
		before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after
M1	SVR	T	ND	A	ND	S	ND	D	ND	N	ND	N	ND	K	ND	E	ND
M2	Rel	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
M3	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
M4	NR	S	T	P	P	G	G	D	N	S	N	N	N	K	K	E	E
M5	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C1	SVR	T	ND	P	ND	G	ND	D	ND	N	ND	N	ND	K	ND	D	ND
C2	SVR	T	ND	A	ND	S	ND	D	ND	N	ND	N	ND	K	ND	D	ND
C3	SVR	T	ND	A	ND	G	ND	D	ND	S	ND	N	ND	K	ND	E	ND
C4	SVR	T	ND	A	ND	G	ND	D	ND	S	ND	N	ND	K	ND	E	ND
C5	Rel	T	T	A	A	G	G	D	D	N	N	N	N	K	K	D	D
C6	Rel	T	T	P	P	S	S	D	D	S	S	N	N	K	K	E	E
C7	Rel	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C8	Rel	T	T	P	P	S	S	D	D	N	N	N	N	E	E	E	E
C9	Rel	T	T	P	P	S	S	D	D	N	N	N	N	K	K	E	E
C10	Rel	T	T	A	S	C	S	D	D	N	N	N	N	K	K	E	E
C11	Rel	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C12	Rel	T	T	A	A	G	G	D	D	S	S	N	N	K	K	E	E
C13	NR	T	T	P	A	S	S	D	D	N	N	D	N	K	E	E	E
C14	NR	T	T	A	P	S	S	D	D	N	N	N	N	E	E	E	E
C15	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	V	V
C16	NR	T	T	A	A	S	S	D	D	S	S	N	N	K	K	E	E
C17	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	E	E
C18	NR	T	T	A	A	S	S	D	D	N	N	N	N	K	K	D	E

ND = Not detected; M1–5 = patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy.

Table 3. Amino acid substitutions in HCV NS5B in the patients studied

NS5B	aa	141th		147th		158th		171th		183th		185th		186th		209th	
		before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after
M1	SVR	K	ND	V	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
M2	Rel	K	K	V	V	R	R	E	E	P	L	A	A	V	V	K	K
M3	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
M4	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
M5	NR	K	K	V	V	R	R	E	E	P	P	A	V	V	V	R	K
C1	SVR	K	ND	V	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C2	SVR	K	ND	V	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C3	SVR	K	ND	I	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C4	SVR	K	ND	I	ND	R	ND	E	ND	P	ND	A	ND	V	ND	K	ND
C5	Rel	K	V	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C6	Rel	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C7	Rel	E	K	V	I	R	R	E	E	P	P	A	A	V	V	K	K
C8	Rel	K	K	I	I	R	R	E	E	P	P	A	A	V	V	K	K
C9	Rel	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C10	Rel	K	K	I	I	R	R	E	E	P	P	A	A	V	V	K	K
C11	Rel	K	K	V	V	R	R	E	E	P	P	A	A	G	G	K	K
C12	Rel	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C13	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C14	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C15	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C16	NR	K	K	V	V	R	G	E	K	P	P	A	A	V	G	K	K
C17	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K
C18	NR	K	K	V	V	R	R	E	E	P	P	A	A	V	V	K	K

ND = Not detected; M1–5 = patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy.

Table 4. Amino acid substitutions in HCV NS5B in the patients studied

NS5B	aa	213th		246th		353th		386th		405th		415th		520th	
		before	after	before	after	before	after	before	after	before	after	before	after	before	after
M1	SVR	C	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
M2	Rel	C	C	A	A	P	P	R	C	V	V	Y	Y	T	T
M3	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
M4	NR	C	C	A	A	L	L	R	R	V	V	Y	Y	T	T
M5	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C1	SVR	C	ND	A	ND	L	ND	R	ND	V	ND	Y	ND	T	ND
C2	SVR	T	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
C3	SVR	N	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
C4	SVR	C	ND	A	ND	P	ND	R	ND	V	ND	Y	ND	T	ND
C5	Rel	N	N	A	V	P	P	R	R	V	V	Y	Y	T	T
C6	Rel	C	C	A	A	L	L	R	R	V	V	Y	Y	T	I
C7	Rel	N	N	A	A	P	L	R	R	V	V	Y	Y	T	T
C8	Rel	C	C	A	A	L	L	R	R	V	V	Y	Y	T	T
C9	Rel	N	N	A	A	P	P	R	R	V	V	Y	Y	T	T
C10	Rel	N	N	A	A	L	L	R	R	V	V	Y	Y	T	T
C11	Rel	S	S	A	A	P	P	R	R	V	V	Y	Y	T	T
C12	Rel	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C13	NR	S	N	A	A	P	P	R	R	V	V	Y	Y	T	T
C14	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C15	NR	T	T	A	A	P	P	R	R	V	V	Y	Y	T	T
C16	NR	R	C	A	A	P	P	R	R	I	V	Y	Y	T	T
C17	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T
C18	NR	C	C	A	A	P	P	R	R	V	V	Y	Y	T	T

ND = Not detected; M1–5 = patients treated with interferon-α monotherapy; C1–18 = patients treated with interferon-α/ribavirin combination therapy.

Table 5. Amino acid residues at position 415 of NS5B in various HCV genotypes

HCV genotype	No.	Position 415 of NS5B			
		F	%	Y	%
1a	17	17	100	0	0
1b	106	2	2	104	98
1c	2	2	100	0	0
2a	18	0	0	18	100
2b	3	0	0	3	100
3	6	1	17	5	83
4	1	0	0	1	100
5	2	0	0	2	100
6	5	0	0	5	100

Understanding the molecular mechanism(s) of action of interferon-α/ribavirin therapy and determinants/resistance to therapy may lead to the design of better treatment strategies. The currently proposed mechanisms of action of ribavirin in combination therapy with interferon-α for HCV include: (1) inducing a Th2 to Th1 bias in favor of a host antiviral response; (2) blocking the host enzyme inosine monophosphate dehydrogenase to reduce the availability of the guanosine pool; (3) inhibition of viral RNA-dependent RNA polymerase, and (4) serving as a RNA mutagen to introduce mutations into the HCV genome [14]. Recently, it was observed that a HCV variant with amino acid substitution from F to Y at the NS5B 415th position was consistently detected in patients with HCV genotype 1a infection during ribavirin monotherapy. It was suggested that this amino acid substitution was selected by ribavirin therapy and was responsible for viral resistance during therapy [12].

This study showed that amino acid substitution at the NS5B 415 position is irrelevant in Japanese patients with HCV genotype 1b infection. First, all the Japanese patients with HCV genotype 1b that we studied had Y at the 415 position of the NS5B region. Second, this is confirmed by the HCV gene and deduced amino acid sequences available in the Genbank/DDBJ database. Such a pattern with F at the 415 position of HCV NS5B region was commonly observed in HCV genotypes 1a and 1c, and only occasionally in genotypes 1b and 3. To argue that F at position 415 is an important phenotype as a re-

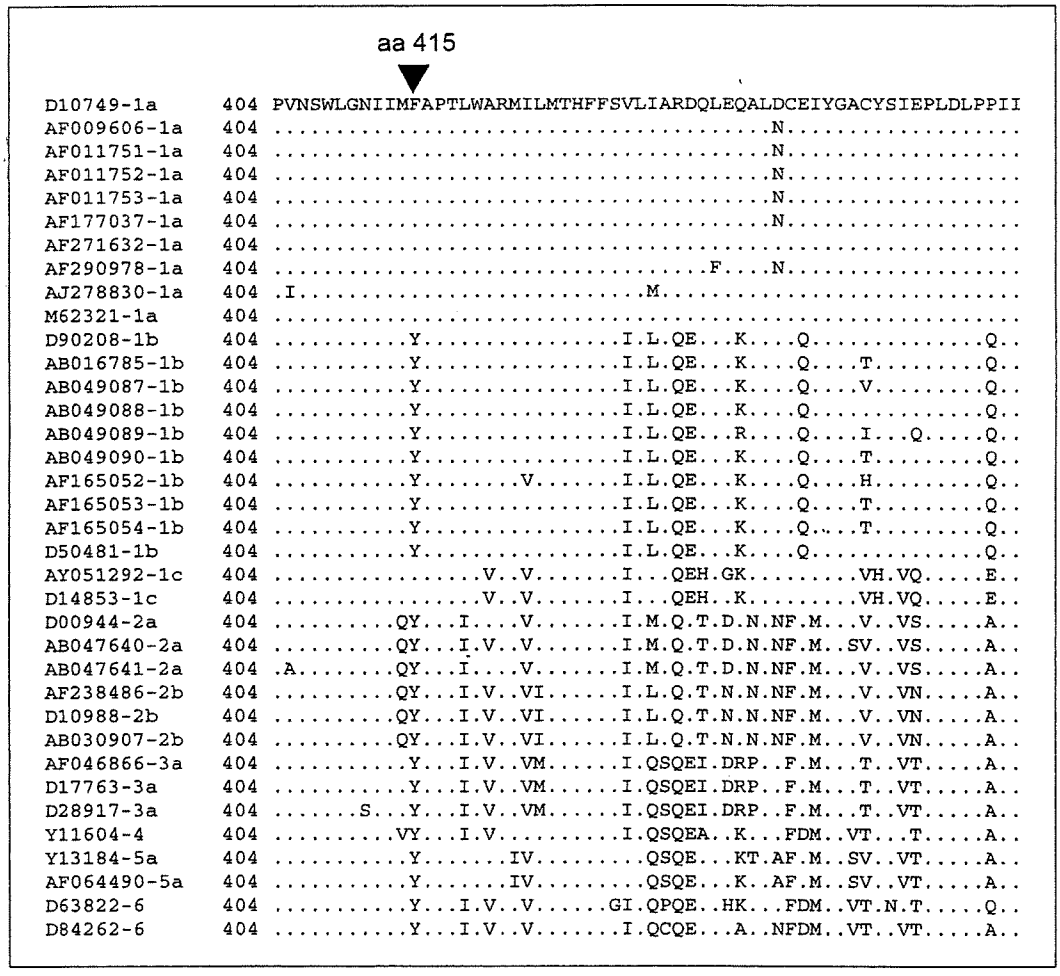

Fig. 2. The alignment of the deduced amino acid sequences of various HCV genotypes in the NS5B region based on 150 sequences available in the GenBank/DDBJ database.

sponse to therapy determinant is difficult since most isolates of HCV genotypes 2 and 3, which are known to respond very well to combination therapy, have Y at position 415 of NS5B. One has to postulate that there are other significant viral or host factors related to HCV genotypes 2 and 3 that make them more susceptible to response to combination therapy to offset the Y factors at position 415 of HCV NS5B. Also, the clinical observation that HCV genotype 1a (with F in position 415) and genotype 1b (with Y in position 415) had a similar clinical treatment response rate to interferon-α/ribavirin therapy also suggests that the amino acid in position 415 is not a key determinant to clinical response to interferon-α/ribavirin combination therapy.

The entire NS5B region was studied before and after therapy and no specific amino acid substitutions were found to be associated with relapse or no response. Therefore, this study ruled out the possibility of a viral amino acid substitution in the NS5B region as a major viral determinant for response to therapy or a viral resistance factor.

Finally, ribavirin has been suggested to be a viral mutagen. In this study, the number of nucleotide mutations and amino acid substitutions in the NS5B region did not increase with interferon-α/ribavirin combination therapy compared with interferon-α monotherapy. Certainly, the best approach is to study ribavirin monotherapy in Japanese patients with HCV genotype 1b infection. However,

the risk to benefit ratio precludes this type of therapy for testing in Japan. Nevertheless, the present study showed that the mutagen effect of ribavirin was not observed in Japanese patients with genotype 1b infection after a 6-month course of interferon-α/ribavirin combination therapy.

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MAJOR ARTICLE

T1653 Mutation in the Box α Increases the Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Genotype C Infection

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Background. Most patients with chronic hepatitis B virus infection become carriers of inactive virus after hepatitis B e antigen seroconversion; however, a subgroup of patients have persistent abnormal transaminase levels and develop hepatocellular carcinoma after seroconversion.

Methods. In an age-matched case-control study, 40 carriers of inactive virus (mean age ± standard deviation [SD], 50.9 ± 11.1 years), 40 patients with chronic hepatitis (mean age ± SD, 50.2 ± 8.9 years), and 40 patients with hepatocellular carcinoma (mean age ± SD, 50.7 ± 9.4 years) who were infected with hepatitis B virus genotype C and had test results positive for antibody to hepatitis B e antigen were analyzed.

Results. The prevalence of T1653 in the box α was significantly higher among patients with hepatocellular carcinoma than among carriers of inactive virus who did not have hepatocellular carcinoma (70% vs. 25%; $P < .0001$) or chronic hepatitis (70% vs. 35%; $P = .003$). Mutations in the basic core promoter region (T1762/A1764) were frequently found in all groups, regardless of clinical status (in 77.5% of carriers of inactive virus, 77.5% of patients with chronic hepatitis, and 90% of patients with hepatocellular carcinoma). In the multivariate analysis, the presence of T1653, an alanine aminotransferase level of ≥37 U/L, and a platelet count of $<18 \times 10^4$ platelets/mm^3 were independent predictive values for hepatocellular carcinoma (odds ratio [95% confidence interval], 5.05 [1.56–16.35], 12.56 [3.05–51.77], and 11.5 [3.47–38.21], respectively). High α-fetoprotein level was the only independent predictive value for T1653 in patients with hepatocellular carcinoma (odds ratio, 12.67; 95% confidence interval, 1.19–134.17]). Among patients with test results positive for antibody to hepatitis B e antigen who had hepatocellular carcinoma and were infected with different genotypes of hepatitis B virus, the prevalence of T1653 was 40%, 15%, 25%, 25%, 67%, and 23% in patients infected with hepatitis B virus genotypes Aa, Ae, Ba, Bj, C, and D, respectively ($P < .05$ for genotype C vs. genotypes Ae, Ba, Bj, or D).

Conclusions. Our data indicate that the addition of T1653 mutation in the box α to the basic core promoter mutation increases the risk of hepatocellular carcinoma in patients with hepatitis B virus genotype C.

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated prevalence of >500,000 cases worldwide per year [1]. It is now accepted that hepatitis B virus (HBV) has a carcinogenic potential in humans. Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there has been increasing evidence of an association between these molecular alterations and the development of HCC in patients with HBV infection.

During persistent HBV infection, carriers frequently undergo seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe). Most patients who acquire chronic HBV infection with HBV genotype C (which is a common genotype in East

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Asian countries) by perinatal transmission become carriers of inactive virus after seroconversion. A subgroup of patients have persistent abnormal serum transaminase levels and develop HCC in the anti-HBe–positive phase. Many of these patients have active viral replication and are infected with several mutant viruses. The association between different clinical events after seroconversion and specific HBV genomic mutations has not been clearly defined.

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore (preC) region at nt 1896 (A1896) are associated with seroconversion and persistent viral replication. It is noteworthy that both BCP and preC mutations are often found in patients with advanced liver disease, (e.g., HCC) [2–8]. The T1762/A1764 mutation alters HBeAg production at the transcription level, and the A1896 in the preC region terminates translation of the precursor protein, abrogates HBeAg production, and results in seroconversion. A1896 was also reported previously to be associated with severe forms of chronic liver disease [7,8].

HBV has been classified into 8 major genotypes with use of the complete nucleotide sequence of the viral genome [10]. HBV genotypes not only have distinct geographical distributions [7, 11, 12] but also have different clinical manifestations and responses to therapy (e.g., IFN therapy). Furthermore, HBeAg positivity and levels of HBV DNA, which are controlled by specific mutations, differ between HBV genotypes (e.g., the BCP double mutation is more prevalent among strains of HBV genotype C, followed by HBV genotype A, and the A1896 mutation is frequently found in HBV genotypes B and D) [13–16].

There have been many studies involving viral mutations associated with clinical features, but most previous studies have ignored age, sex, HBeAg status, and HBV genotypes. In Japan, most patients with HCC experience seroconversion (i.e., they are anti-HBe positive) and have HBV genotype C; therefore, we performed an age-matched case-control study among anti-HBe–positive patients infected with HBV genotype C (including carriers of inactive virus, patients with chronic hepatitis, and patients with HCC) to determine the specific HBV genome mutations associated with disease progression.

PATIENTS AND METHODS

Serum samples. Serum samples were obtained from 211 patients from different regional areas worldwide. A total of 120 patients from Japan who were infected with HBV genotype C (40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC) were matched with control subjects according to age and HBe status. Control serum samples were obtained from patients with HCC who were positive for anti-HBe and who were infected with HBV genotype Aa (10 subjects), Ae (13), Ba (20), Bj (20), C (15), and D (13). Control subjects were from Hong Kong (19 subjects), Japan (36), and the United States (36). The majority of patients infected with HBV genotypes Aa, Ba, Bj, and C were Asian, and the majority of patients infected with HBV genotypes Ae and D were white and black. None of the subjects had serological test results positive for markers of infection with hepatitis C virus or HIV-1.

The study protocol was approved by ethics committees of the participating institutions in accordance with the 1975 Helsinki declaration. Informed consent was obtained from each patient.

Serological assays for HBV markers. HBeAg and anti-HBe were detected by chemiluminescent EIA (Lumipulse *f*, Fujirebio). HBV genotypes were determined by the restriction fragment–length polymorphism method on the S gene sequence amplified by PCR [29] and ELISA with monoclonal antibodies directed to distinct epitopes on the preS2 region products [18], with use of commercial kits (HBV genotype EIA; Institute of Immunology). The genotypes were also confirmed with use of a phylogenetic tree analysis. α-Fetoprotein and serum protein induced by the absence of vitamin K (antagonist II) were examined with use of chemiluminescent EIA.

Amplification and sequencing of the core promoter and the precore region plus core gene. HBV DNA sequences bearing the core promoter and preC or core regions were amplified by PCR with heminested primers by the method described elsewhere [19]. Thereafter, PCR products were sequenced directly with Prism Big Dye (Applied Biosystems) in the ABI 3100 DNA automated sequencer (Applied Biosystems). Accession numbers for all strains are AB236515–AB236634.

Case-control study. A carrier of inactive virus was defined as an HBsAg-positive individual with normal alanine aminotransferase (ALT) levels for a 2-year period (with at least 4 evaluations at 3-month intervals) and without the presence of portal hypertension. Chronic hepatitis was defined as persistent elevation of ALT levels (> 1.5 × upper limit of normal [35 U/L]) during a 6-month period (with at least 3 evaluations at 2-month intervals) without a decrease in platelet count or albumin level, and hypersplenism (splenomegaly on ultrasonographic examination). Twenty-one patients were confirmed to have chronic hepatitis by means of a fine-needle biopsy of the liver. Staging and grading (expressed as mean value ± SD [95% CI]) were 1.24 ± 0.64 (0.99–1.58) and 1.36 ± 0.58 (1.07–1.59), respectively, as previously described [30]. None had received antiviral treatment during the follow-up period. Of 40 patients with HCC, 23 patients received a diagnosis of HCC on the basis of a pathologic examination, and 17 patients received a diagnosis of HCC on the basis of results of abdominal ultrasonography, angiography, CT, or MRI, as well as an elevated serum α-fetoprotein level (≥400 ng/mL).

Statistical evaluation. Data were expressed as mean ±

SD. Statistical analyses were performed using χ^2 test and Fisher's exact test for categorical variables. Mann-Whitney U test or 1-way analysis of variance were used for continuous variables, as appropriate. Mantel-Haenszel χ^2 test was used to analyze the trend of frequencies of viral mutations. Multivariate analyses with logistic regression were used to determine the independent factors associated with HCC and T1653. Differences were considered to be significant for P values <.05. The statistical analysis software used was Stata software, version 8.0 (StataCorp).

RESULTS

Table 1 compares ALT level, platelet count, and HBV DNA level, as well as mutations in the box α (enhancer II), core promoter, and preC region, among 40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC who were infected with HBV genotype C in an age-matched case-control study. ALT and HBV DNA levels were significantly lower among carriers of inactive virus than among patients with chronic hepatitis or patients with HCC ($P<.0001$ and $P = .001$, respectively). Platelet count was lower among patients with HCC than among carriers of inactive virus or patients with chronic hepatitis ($P<.0001$).

The frequency of the T1653 mutation in the box α was significantly higher among patients with HCC (70%) than among carriers of inactive virus (25%) or patients with chronic hepatitis (35%; $P<.0001$) (table 1). Of interest, the T1653 mutation had an opposite correlation with the M1753 mutation. The prevalence of T1762/A1764 was high in all clinical status groups, with no statistically significant difference between groups (table 1). The trend of the frequency of T1653, increasing from carriers of inactive virus to patients with chronic hepatitis to patients with HCC, was analyzed by Mantel-Haenszel χ^2 test (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$) (figure 1). The trend of the frequency of T1762/A1764 was not statistically significant ($P = .1502$) (figure 1).

The attributable risk of multiple factors, including sex, HBV DNA level, ALT level, platelet count, and the presence of the T1653, M1753, T1762/A1764, and A1896 mutations for HCC in the HBV carriers was determined by multiple logistic regression analysis (table 2). There was a statistically significant association between development of HCC and ALT level >37 U/L (OR, 12.56; 95% CI, 0.55–6.21; $P<.0001$) and platelet count $<18 \times 10^4$ platelets/mm^3 (OR, 11.5; 95% CI, 3.47–38.21; $P<.0001$). The T1653 mutation was still significantly associated with the development of HCC (OR, 5.05; 95% CI, 1.56–16.35; $P = .007$).

The attributable risk of multiple factors, including HBV DNA level, ALT level, platelet count, α-fetoprotein level, protein in-

Table 1. Demographic, clinical, and virologic characteristics of patients infected with hepatitis B virus (HBV) genotype C who were matched for age and hepatitis B e antigen (HBeAg) status.

Variable	Clinical status: Carriage of inactive virus (n = 40)	Chronic hepatitis (n = 40)	Hepatocellular carcinoma (n = 40)	P
Male sex	31 (77.5)	37 (92.5)	36 (90)	.171
Age, years	50.9 ± 11.1	50.2 ± 8.9	50.7 ± 9.4	Matched
HBeAg positive	0 (0)	0 (0)	0 (0)	Matched
Anti-HBeAg positive	40 (100)	40 (100)	40 (100)	Matched
HBV genotype C	40 (100)	40 (100)	40 (100)	Matched
Alanine transaminase level, U/L[a]	20.8 ± 7.6	102 ± 108.7	83.2 ± 84.8	.0001
Platelet count, $\times 10^4$ platelets/mm^3[b]	20.7 ± 3.1	17.4 ± 4.1	12.8 ± 5.7	.0001
HBV DNA level, LGE/mL[c]	4.3 ± 0.8	5.9 ± 1.5	5.4 ± 1.5	<.0001
Mutation in the box α: T1653[d]	10 (25)	14 (35)	28 (70)	<.0001
Mutation in the core promoter				
M1753	10 (25)	6 (15)	9 (22.5)	.609
T1762/A1764	31 (77.5)	31 (77.5)	36 (90)	.289
Mutation in the precore region: A1896	25 (62.5)	26 (65)	25 (62.5)	1.0

NOTE. Data are no. (%) of patients or mean value ± SD. Anti-HBeAg, antibody to HBeAg; LGE, log genome equivalents.

[a] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[b] $P<.0001$ for patients with hepatocellular carcinoma vs. carriers of inactive virus or patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with chronic hepatitis.

[c] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[d] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

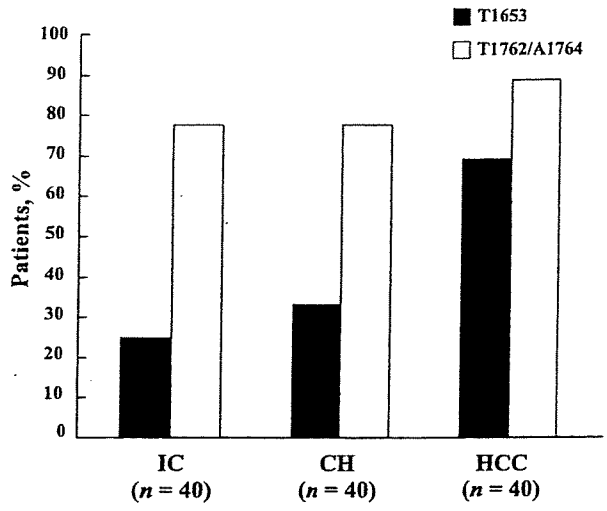

Figure 1. Prevalence of T1653 box α and T1762/A1764 basic core promoter mutations among patients with chronic hepatitis B virus infection, stratified by clinical status. The trend of the frequency of the T1653 mutation was analyzed by Mantel-Haenszel χ^2 test. The OR estimate is an approximation of the OR for carriers of inactive virus (IC), patients with chronic hepatitis (CH), and patients with hepatocellular carcinoma (HCC) having a strain with the mutation (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$). The trend of the frequency of the T1762/A1764 mutation was not statistically significant according to the Mantel-Haenszel χ^2 test ($P = .1502$).

duced by the absence of vitamin K (antagonist II) level, for T1653 in patients with HCC with HBV genotype C infection was determined by multiple logistic regression analysis (table 3). An α-fetoprotein level >300 ng/mL was the only independent predictive value for the presence of the T1653 mutation in patients with HCC with HBV genotype C infection (OR, 12.67; 95% CI, 1.19–134.17; $P = .035$).

Table 4 compares sex, age, and mutations in the box α, core promoter, and preC region among patients infected with HBV genotypes Aa (10 patients), Ae (13), Ba (20), Bj (20), C (15), and D (13) with the same variables among patients with HCC. Mean age was significantly higher among patients with HBV genotype Bj infection, compared with patients with HBV genotype Ba, genotype C, and genotype D infection ($P < .05$). The prevalence of T1653 among patients with HBV genotype C infection (66.7%) was significantly higher than it was among patients infected with other genotypes (15%–25%; $P < .05$), excluding patients infected with HBV genotype Aa. The prevalence of T1762/A1764 among patients with HBV genotype Ba infection (85%) and HBV genotype C infection (86.7%) was also significantly higher than it was among patients infected with other genotypes (20%–50%; $P < .05$). The prevalence of A1896 among patients with HBV genotype Aa infection and HBV genotype Ae infection was significantly lower than it was among patients infected with other genotypes ($P < .05$).

DISCUSSION

Many previous studies have reported that the clinical course of chronic HBV infection may be modified by several specific viral mutations [5, 20, 21], although the significance of such specific mutations in patients with chronic hepatitis B remains controversial. Because most studies have not controlled for different variables, such as age, HBV genotype, and HBe status, it is unknown whether the mutations were associated with disease progression, greater age of the patient, the specific HBV genotype or subtype, or HBe status. In this study, to exclude any biases, we performed an age-matched case-control study involving only anti-HBe–positive patients infected with HBV genotype C.

In the present case-control study, the prevalence of T1653 was found to be significantly higher among patients with HCC, compared with carriers of inactive virus and patients with chronic hepatitis with HBV genotype C infection; however, the prevalence of T1762/A1764 was high in all clinical status groups. During the anti-HBe–positive phase of infection, T1653 was more reliable than T1762/A1764 as a predicting factor for

Table 2. Multivariate analysis of variables with independent predictive value for development of hepatocellular carcinoma among a group of 120 patients with hepatitis B virus infection.

Variable	OR (95% CI)	P
Sex		
Female	1	
Male	5.06 (0.85–30.15)	.075
HBV DNA level		
<4.8 LGE/mL	1	
≥4.8 LGE/mL	0.34 (0.09–1.21)	.096
Alanine transaminase level		
<37 U/L	1	
≥37 U/L	12.56 (3.05–51.77)	.0001[a]
Platelet count		
≥18 × 10^4 platelets/mm^3	1	
<18 × 10^4 platelets/mm^3	11.51 (3.47–38.21)	.0001[a]
T1653 mutation		
No	1	
Yes	5.05 (1.56–16.35)	.007[a]
M1753 mutation		
No	1	
Yes	1.23 (0.31–5.04)	.770
T1762/A1764 mutation		
No	1	
Yes	2.67 (0.57–12.54)	.214
A1896 mutation		
No	1	
Yes	0.96 (0.29–3.11)	.943

NOTE. Each OR was adjusted for age and other variables in the analysis. LGE, log genome equivalents.

[a] Statistically significant.

Table 3. Multivariate analysis of variables with independent predictive value for the presence of the T1653 mutation among 40 patients with hepatocellular carcinoma.

Variable	OR (95% CI)	P
HBV DNA level		
<4.9 LGE/mL	1	
≥4.9 LGE/mL	0.89 (0.16–4.79)	.899
ALT level		
<53 U/L	1	
≥53 U/L	1.72 (0.29–9.96)	.541
Platelet count		
≥12 × 10^4 platelets/mm^3	1	
<12 × 10^4 platelets/mm^3	1.39 (0.28–7.02)	.683
α-Fetoprotein level		
<300 ng/mL	1	
≥300 ng/mL	12.67 (1.19–134.17)	.035[a]
PIVKA-2 level		
<50 mAU/mL	1	
≥50 mAU/mL	0.25 (0.05–1.43)	.120

NOTE. Each OR was adjusted for age and other variables in the table. PIVKA-2, protein induced by the absence of vitamin K (antagonist II).

[a] Statistically significant.

the development of HCC. In fact, in the multivariate analysis, the presence of T1762/A1764 was not an independent predictor of HCC, but ALT level >37 U/L, platelet count <18 × 10^4 platelets/mm^3, and the presence of T1653 were independent predictors of HCC. The T1653 mutation had also been reported by Takahashi et al. [17]; they reported that this specific mutation was prevalent among Japanese patients with HCC, although their study was not a case-control study. These results do not deny that T1762/A1764 is associated with hepatocarcinogenesis, because poor prognosis associated with HBV genotype C infection, compared to that associated with HBV genotype B (Ba and Bj) infection, correlated with a high prevalence of T1762/A1764 [2, 9, 16], indicating that the BCP double mutation is associated with a high potential for hepatocarcinogenesis. The appearance of the T1653 mutation after the occurrence of the T1762/A1764 mutation (the T1762/A1764 mutation usually occurs earlier than the T1653 mutation) could indicate that the virulence of HBV is increasing, which could result in the development of HCC. In the multivariate analysis, however, HBV DNA level was no longer a predicting factor for HCC. One of the reasons for this is that the HBV DNA data used in this study were obtained at the time of diagnosis of HCC. A recent prospective study from Taiwan has indicated that high HBV DNA levels at baseline and infection with HBC genotype C were independent predictors for HCC, but the mean viral load at the time of diagnosis of HCC was significantly lower than at baseline [27]. Although our data could not indicate an association between HBV DNA level and hepatocarcinogenesis, if we could measure the HBV DNA level before diagnosis of HCC, it might found to be be a predicting factor for HCC. Furthermore, an examination of the characteristics of patients with HCC who had the T1653 mutation showed that an elevated α-fetoprotein level (≥300 ng/mL) was the only predictor for the development of HCC in patients with the T1653 mutation. It has been reported that α-fetoprotein level is useful not only for diagnosis but also as a prognostic indicator for patients with HCC [22, 23], suggesting that the T1653 mutation might be associated with poor prognosis for patients with HCC.

The prevalence of several mutations among patients with HCC differed from that among patients with different HBV genotypes (Aa, Ae, Ba, Bj, C, and D) (table 4). The prevalence

Table 4. Demographic and virological characteristics of patients with hepatocellular carcinoma who were positive for antibody to hepatitis B e antigen (anti-HBe), by hepatitis B virus (HBV) genotype.

	HBV genotype						
Variable	Aa (n = 10)	Ae (n = 13)	Ba (n = 20)	Bj (n = 20)	C (n = 15)	D (n = 13)	P
Male	10 (100)	12 (92.3)	18 (90)	15 (75)	15 (100)	13 (100)	.10
Age, years[a]	54.4 ± 7.7	55.3 ± 4.4	54.4 ± 14.8	64.9 ± 9.6	47.9 ± 7.6	53.5 ± 8.3	.0002
HBeAg positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	Matched
Anti-HBe positive	10 (100)	13 (100)	20 (100)	20 (100)	15 (100)	13 (100)	Matched
Mutation in the box α: T1653[b]	4 (40)	2 (15.4)	5 (25)	5 (25)	10 (66.7)	3 (23.1)	.039
Mutations in the core promoter region							
M1753	3 (30)	3 (23.1)	5 (25)	4 (20)	2 (13.3)	1 (7.7)	.759
T1762/A1764[c]	5 (50)	6 (46.2)	17 (85)	4 (20)	13 (86.7)	5 (38.5)	<.0001
Mutation in the precore region: A1896[d]	0 (0)	0/13 (0)	9/20 (45)	15/20 (75)	9/15 (60)	8/13 (61.5)	<.0001

NOTE. Data are no. (%) of patients or mean value ± SD. HBeAg, hepatitis B e antigen.

[a] $P < .05$ for Bj vs. Ba or D; $P < .0001$ for Bj vs. C.

[b] $P < .05$ for C vs. Ba or Bj or D; $P < .01$ for Ae vs. C.

[c] $P < .05$ for Ae vs. Ba or C; $P < .01$ for D vs. Ba or C; $P < .0001$ for Bj vs Ba or C.

[d] $P < .05$ for Ba vs. Aa or Ae; $P < .005$ for Aa vs. C or D and for Ae vs. Ba or C or D; $P < .0001$ for Bj vs Aa or Ae.

of T1653 was the highest among patients with HBV genotype C infection, followed by those with HBV genotype Aa infection, although the number of patient with HBV genotype Aa infection was too small for any conclusions to be drawn. The prevalence of T1762/A1764 was higher among patients with HBV genotype Ba and HBV genotype C infection than among patients infected with other genotypes. HBV genotype Ba has a sequence that closely resembles that of HBV genotype C in the core promoter region, because it is recombinant HBV between HBV genotype Bj and HBV genotype C from nucleotides 1740 to 2485. Although A1896 was not found in HBV genotype Aa and HBV genotype Ae, as has been reported elsewhere [15], HBV genotype Aa had some specific mutations upstream of the preC initiation codon and encapsidation signal site. Therefore, several HBV genotype–specific mutations would be associated with different mechanisms on seroconversion or HBV replication for each genotype or subtype.

Buckwold et al. [24] reported that T1762/A1764 can no longer bind liver-enriched transcription factors and that the transcription of precore RNA and the expression of HBeAg were reduced. Thereafter, Li et al. [25] reported that this mutation not only removed the nuclear receptor–binding site but also created a hepatic nuclear factor 1 transcription factor–binding site. As for a factor correlated with BCP, the core upstream regulatory sequence, which has a strong stimulation effect on the BCP, was reported. In an earlier article by Yu et al. [28], the box α elements (nucleotides 1646–1668) individually stimulated promoter activity >100-fold. The T1653 mutation converts the box α binding site for CCAAT/enhancer-binding protein and related factors into the perfect palindromic sequence 1648-TCTTATATAAGA, which might enhance binding affinity and core promoter/enhancer II activity. Therefore, it is possible that the mutation in the box α influenced the HBe production and viral replication through the BCP activity. In addition, the T1653 mutation corresponds to an amino acid change from histidine to tyrosine at aa 94 of the X protein, so this alteration of X protein might be hepatocarcionogenesis. Gunther et al. [26] analyzed T1653, T1762, and A1764 mutations in the context of an in vitro study involving wild-type HBV (genotype D, AF043594), and they reported that the preC mRNA and HBeAg secretion was reduced, but the amount of progeny virus DNA in the cells and in the culture medium increased only marginally (if at all), as determined by Southern blot analysis. However, because the genotype was different from that in our study (genotype D vs. genotype C) and the mutant type included not only T1653, T1762, and A1764 mutations but also other mutations in the core promoter, it is possible that some other mutation influenced the results in the earlier study.

In conclusion, the addition of the T1653 mutation in the box α to the BCP mutation increases the risk of HCC in patients with HBV genotype C infection, suggesting that HBV with both the T1653 mutation and the BCP double mutation in patients with chronic hepatitis B should be eradicated by antiviral therapy. Functional analyses of HBV strains with the T1653 mutation are needed in vitro and in vivo.

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High Frequency of Hepatocellular Carcinoma in Mongolia; Association With Mono-, or Co-Infection With Hepatitis C, B, and Delta Viruses

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To investigate the association between viral infection pattern and hepatocellular carcinoma (HCC), 292 chronic hepatitis patients, including 108 with developed HCC were screened using serological and molecular genetics methods. Viral etiology was established in 267 (91.4%), anti-HCV detected in 198 (67.8%), and HBsAg in 124 (42.5%) including 93 (74.4%) cases with HDV co-infection. HCV mono-infection predominated in both, "non-HCC" and "HCC" groups (54% and 39%, respectively) with higher frequency in the first group ($P=0.011$), whereas HBV in co-infection with HDV was more frequent in HCC group (14% vs 25%, $P=0.017$). Patients with HCV mono-infection were older than those with co-infection ($P<0.02$), had higher frequency of HCV-viraemia (82% vs 7%, $P<0.0001$), and yet had significantly lower prevalence of HCC (29.6% vs. 49.1%, $P=0.003$). Alpha-fetoprotein (AFP) and protein induced by vitamin K antagonist-II (PIVKA-II) were specifically elevated in 71% of HCC patients. In conclusion, although HCV monoinfection pattern predominates in Mongolia, co-infection with HBV and HDV had stronger association with HCC development at younger age. Liver tumor markers; AFP and PIVKA-II are useful tools for complex HCC-screening and clinical follow-up for chronic hepatitis patients in Mongolia. ***J. Med. Virol. 78:1688–1695, 2006.***

KEY WORDS: Mongolia; HCC; co-infection; HBV; HCV; HDV

INTRODUCTION

Primary liver cancer account for about 5.6% of all human cancers (7.5% for men and 3.5% for women) in the world, with more than 0.5 million new cases registered in 2000. The vast majority of primary liver cancer is hepatocellular carcinoma (HCC), which is the sixth most common malignancy in world, but because of a very poor prognosis, it is also the third most common cause of death from cancer in the world [Bosch et al., 2004; Parkin et al., 2005]. The age adjusted incidence rate estimated per 100,000 men is divergent geographically, ranging from 2.1 in Central America up to 35.5 in Eastern Asia with the highest level in Mongolia; 61.8–98.93, according to different estimates [Bosch et al., 2004; Dulamsuren, 2004]. Overall, 75% to 80% of cases of HCC are attributable to persistent viral infections with either hepatitis B virus (HBV) or hepatitis C virus (HCV). A recent study from Mongolia indicated a very high prevalence of HCV, HBV and hepatitis Delta virus (HDV) co-infections among chronic liver disease patients [Tsatsralt-Od et al., 2005a]. HCV, HBV and HDV share common transmission routes through exposure to infected blood or blood products, thus co-infection is common in areas of high endemicity [Panigrahi et al., 1994; Sheen et al., 1994; Tsatsralt-Od et al., 2005b]. Studies on the influence of the HCV and HBV co-infection indicated various phenomena of viral interference; where the strongest suppressing

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effect was induced by either, HCV [Pontisso et al., 1996; Crespo et al., 1997; Jardi et al., 2001], HBV [Wang et al., 1999] or mutual interference of both [Koike et al., 1995; Ohkawa et al., 1995]. Contrasting results accumulated regarding triple-infection, indicated the predominance of HCV [Yeh et al., 1994; Liaw, 2001], or HDV [Mathurin et al., 2000; Jardi et al., 2001] or not systematical fluctuations among them throughout the time [Raimondo et al., 2006]. The role of co-infection in the development of HCC is also controversial; significant increase in cancerogenesis was observed in co-infection of HCV and HBV in comparison to single infection with any of these viruses [Donato et al., 1998]. On the other hand, no association with progression to HCC has been found in triple-infection with the conclusion that only one predominant virus is involved in pathogenesis [Huang et al., 1998]. The patterns of interference of hepatitis viruses and their association with cancerogenesis remain unclear, and any new data is a step toward understanding the underlying mechanisms of HCC development.

Currently, the vast majority of the hepatitis virus carriers are concentrated in the developing countries where proper epidemiological surveillance for HCC is complicated due to the lack of modern diagnostic equipment (computer tomography, magnetic resonance imaging and biopsy). In his context screening for serological tumor markers is a very useful tool for early HCC diagnosis in high risk groups, differential diagnosis, clinical follow-up of the patients, and epidemiological surveillance [Fujiyama et al., 2002].

The aims of the present study, beyond the confirmation of previous observations, were to find a possible association of the high HCC rate in Mongolia with a specific pattern of HCV, HBV and HDV co-infection, and reveal prognostic significance of the combination of the viruses and their virological features wih regard to cancerogenesis, and additionally, to evaluate the reliability of the combination of two serological tumor markers as additional inexpensive and easy to perform clinical laboratory methods for use in Mongolia and other developing countries with potentially high incidence of HCC.

MATERIALS AND METHODS

Patients

The collection of the material for this study was carried out by the Laboratory of Molecular Biology, Institute of Biology of Mongolian Academy of Sciences. A total of 292 consecutive patients were selected from those attending the Liver Clinics and the National Cancer Center, Ulaanbaatar, Mongolia between April and August, 2005. Written informed consent was obtained from all the patients enrolled. After an interview with the clinician, physical and laboratory examination, a diagnosis was assigned to each patient. Blood serum specimens obtained from the subjects at the first day were collected and stored in −70°C until examination.

Clinical-Laboratory Methods

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) tests were done immediately using kits from Human GmbH by the Raitmana Frenkle kinetic method on Humalyser 3000 (Germany). Values up to ALT 37 IU/l, AST 32 IU/l for female and ALT 42 IU/l, AST 32 IU/l for male were considered to be normal. Confirmation of HCC was based on ultrasound, computer tomography, and/or histological liver investigation. Alpha-fetoprotein (AFP) (Fujirebio, Tokyo, Japan) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) (Sankojunyaku, Tokyo, Japan) were measured by chemiluminescent enzyme immunoassay. The values obtained were assumed normal if less or equal 10 ng/ml for AFP, and less or equal 40 mAU/ml for PIVKA-II.

Serological Methods

HBV surface antigen (HBsAg), hepatitis B e antigen (HBeAg) (Fujirebio CO LTD, Tokyo, Japan) and hepatitis C virus antibody (Anti-HCV) (Ortho Clinical Diagnostics, Tokyo, Japan) were detected using the chemiluminescent enzyme immunoassay. HBsAg was quantified in IU/ml. HBsAg-positive serum samples were subjected to HBV genotyping using the EIA commercial kits (HBV GENOTYPE EIA, Institute of Immunology, Tokyo, Japan). The method allows discrimination among the seven major HBV genotypes (A–G) by monoclonal antibodies targeted to the preS2 epitopes [Usuda et al., 1998]. Presence of the hepatitis Delta antibody (Anti-HDV) in the serum was assessed using the recombinant HDVAg peptide [Semiletov Iu et al., 2002] using a diagnostic kit ELISA-AntiHDV (RPC Diagnostics Systems, Nizhnyi Novgorod, Russia).

Detection of HBV DNA, HCV RNA, HDV RNA, and Genotyping

The total DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Inc., Hilden, Germany) from 100 μl of all HBsAg-positive serum samples, where the volume was available. HBV DNA was detected by polymerase chain reaction (PCR) in the S gene allowing detection from 100 HBV DNA copies/ml [Abe et al., 1999; Tanaka et al., 2004].

Total RNA was extracted from serum, reverse transcribed into cDNA using Random hexamer primer as described previously [Ohno et al., 1997]. HCV genotyping performed using genotype specific primers for the core region [Ohno et al., 1997]. A part of HDVAg coding region of the HDV was amplified using previously reported specific primers [Nakano et al., 2001] and directly sequenced in both forward and reverse direction.

Statistical Analyses

Statistical differences were evaluated by Fisher's exact probability test and Chi square test with Yates'

correction, where appropriate, using the STATA software version 8.0 (StataCorp. LP, College Station, TX). Differences were considered significant for P-values less than 0.05.

RESULTS

Prevalence of Hepatitis Viruses and Co-Infection Patterns Among Chronic Hepatitis and HCC Patients

After clinical and laboratory examination, a total of 292 chronic hepatitis patients were divided into two groups; 108 (37%) with established diagnosis of hepatocellular carcinoma (HCC group) and remaining 184 (63%) were assigned into the "non-HCC" group. The overall male/female ratio was 0.8, and mean age (standard deviation) was 45.8 (±14.9). These estimations in each of the clinical groups are summarized in the Table I. The chronic hepatitis patients with HCC were significantly older than those without HCC (54.3 vs. 41.1, respectively, $P < 0.0001$). Male/female ratio was 0.66 in non-HCC group, whereas in HCC group it was 1.04, indicating expected general inclination for HCC to progress with age especially for men. ALT, AST, AFP and PIVKA-II were significantly higher in the HCC group ($P < 0.0001$), as expected. The seroprevalence of HBsAg was significantly higher in the HCC group (51.9% vs. 37.0, $P = 0.018$). HBeAg and HBV DNA positivity were remarkably low in both, HCC (17.9% and 46.4%) and non-HCC (19.1% and 41.2%) groups. All HBsAg positive cases were examined for anti-HDV and/or HDV-RNA by nested PCR; overall the seroprevalence was 74.4% (69.1% in non-HCC vs. 82.1% in HCC) and 97.7% had HDV viremia (100% in non-HCC vs. 95.8% in HCC). The seroprevalence of anti-HCV was also equally high in both HCC and non-HCC groups (63% and 70.7%), and among the carriers 56.7% were viraemic in HCC group and 49.3% in non-HCC. When the result of serological and molecular examination were combined; according to the pattern of the hepatitis viruses carried, studied patients were categorized into five groups; HCV only (48.6%), HBV+HDV (17.8%), HBV+HDV+HCV (14.4%), HBV only (5.8%) and HBV+HCV (4.8%) (Table I). There were 25 cases (8.6%) in which no evidence of the viral infection was found. Thereby, HCV alone (53.2%, 142/267) and HBV in co-infection with HDV and/or HCV (40.4% 108/267) are predominating among liver disease patients with determined viral etiology.

Although HCV monoinfection was the most predominant pattern among the studied cohort, the prevalence of this pattern was significantly higher in the non-HCC group when compared to HCC (54.3% vs. 38.9%, respectively, $P = 0.011$), whereas HBV+HDV pattern was significantly prevalent in HCC versus non-HCC (25% vs. 13.6%, respectively, $P = 0.016$) (Table I and Fig. 1A). As graphically shown in Figure 1B, when non-HCC/HCC ratio was compared among groups with different pattern, patients infected with HBV+HDV had HCC with significantly higher frequency than those with HCV or HBV-monoinfection ($P < 0.03$) as did patients with triple-infection in comparison to HCV only-infected ($P = 0.046$). Interestingly, there was no difference in non-HCC/HCC ratio between HBV+HDV and HBV+HDV+HCV groups. Although, no statistically significant difference was observed in the HBV monoinfection and HBV+HCV coinfection patterns, this was probably due to a small number of the cases as these patterns were found in the minority of our patients. Thereby, the majority of the patients with established HCC had HCV-monoinfection (39%), followed by HBV+HDV (25%), HBV+HDV+HCV (19%), HBV+

TABLE I. Studied Patients in Mongolia

	TOTAL (n = 292)	Chronic hepatitis (n = 184)	Hepatocellular carcinoma (n = 108)	P
M/F	128/164	73/111	55/53	NS
Age: MEAN+SD	45.9+14.7	41.1+13.7	54.3+12.5	<0.0001
ALT Median (range)	79 (17-320)	63 (17-282)	127 (23-320)	<0.0001
AST Median (range)	70 (15-318)	52 (15-219)	105 (19-318)	<0.0001
AFP Median (range)	4.9 (0.9-625900)	3,8 (0,9-335)	23 (1.3-625900)	<0.0001
PIVKA-II Median (range)	24 (9-921600)	22 (9-176)	215 (12-921600)	<0.0001
AFP (>10 ng/ml)	96 (32.9)	28 (15.2)	68 (63.0)	<0.0001
PIVKA-II (>40 mAU/ml)	70 (24.0)	7 (3.8)	63 (58.3)	<0.0001
Viral hepatitis markers				
HBsAg	124 (42.5)	68 (37.0)	56 (51.9)	0.0107
HBeAg	23/124 (18.4)	13/68 (19.1)	10/56 (17.9)	NS
HBV DNA	54/124 (43.5)	28/68 (41.2)	26/56 (46.4)	NS
Anti-HDV	93/124 (74.4)	47/68 (69.1)	46/56 (82.1)	NS
HDV RNA	46/47 (97.9)	23/23 (100)	23/24 (95.8)	NS
Anti-HCV	198 (67.8)	130 (70.7)	68 (63.0)	NS
HCV RNA	72/134 (54.0)	33/67 (49.3)	38/67 (56.7)	NS
Patterns of mono/co-infection				
HCV only	142 (48.6)	100/184 (54.3)	42/108 (38.9)	0.0112
HBV+HDV	52 (17.8)	25/184 (13.6)	27/108 (25.0)	0.0157
HBV+HDV+HCV	42 (14.4)	22/184 (11.9)	20/108 (18.5)	NS
HBV only	17 (5.8)	14/184 (7.6)	3/108 (2.8)	NS
HBV+HCV	14 (4.8)	8/184 (4.3)	6/108 (5.6)	NS

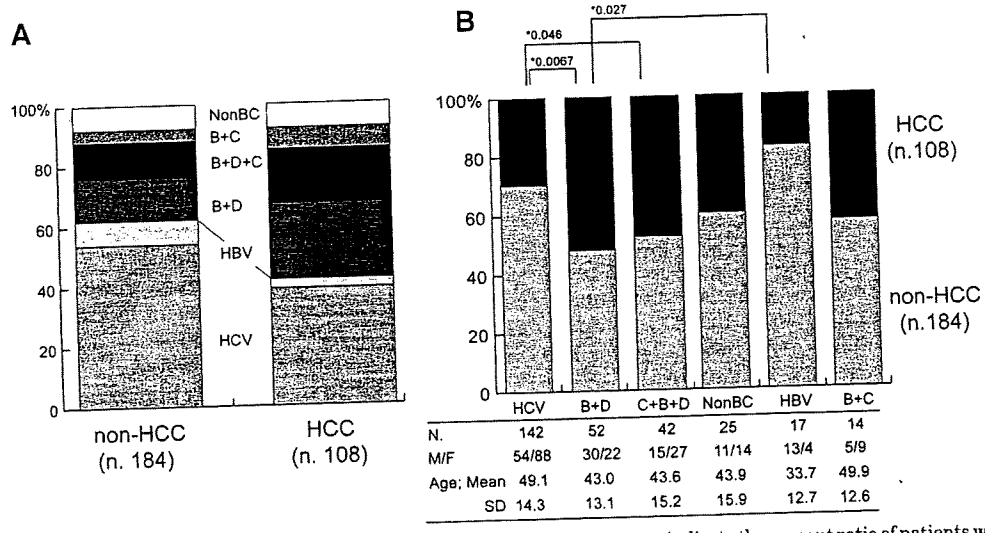

Fig. 1. Coinfection patterns among chronic hepatitis patients with and without HCC. **A**: Stacked columns indicate changes in percent ratio of coinfection pattern from non-HCC to HCC groups. "HCV" and "HBV" indicate groups of patients with monoinfection with either virus, and "B+D, B+D+C and B+C" indicate groups where patients had dual, or triple coinfection of HBV with HDV and/or HCV, respectively. **B**: Stacked columns indicate the percent ratio of patients with non-HCC or HCC in each group with infection pattern. The groups aligned from left to right according to decreased epidemiological prevalence in studied population. The characteristics of the analyzed patients attached in the table below the graph.

HCC (6%) and HBV (3%) (Fig. 1A). However HBV+HDV pattern had stronger association with HCC since the majority ($P < 0.05$) of patients in this group had HCC (27/52, 52%), followed by HBV+HDV+HCV (20/42, 46%), in contrast to patients with monoinfection; HCV (42/142, 30%), and HBV (3/17, 18%) (Fig. 1B). When groups of patients with dual- and triple-infection with similar age distribution were combined, a significantly high prevalence of HCC in younger age was observed in coinfected-patients group versus HCV monoinfected (49.1%, 50.9 years vs. 29.6%, 59.3 years, $P = 0.003$, data not shown). Only three of the patients with HCC had HBV monoinfection that limited statistical evaluation. Interestingly, the male/female ratio among HCC patients was almost 1.0 among all four categories.

The mean age of the subjects in each category is shown in the Figure 2, indicating that investigated patients with HCV-monoinfection were elder than those with dual-, and triple infection within the non-HCC and HCC groups ($P < 0.03$), and no significant difference observed among patients with coinfection in each of the clinical groups. Patients with HBV monoinfection were younger overall and in the non-HCC ($P < 0.02$), but not in the HCC group.

HBV, HDV, and HCV Genotypes Among Non-HCC and HCC Patients

In total, 124/292 (42.5%) patients were found to be HBsAg positive. To investigate the prevalence of the HBV genotypes among different categories and clinical groups of the patients, the serum specimens were subjected to EIA genotyping; HBV/D was determined in 88.7% (110/124), HBV/A in 3.2% (4/124), cases and 10/124 (8.1%) samples with the border-line levels of the HBsAg were negative by EIA (data not shown). This indicated that HBV/D was predominant among all clinical groups and HBV/A was found in exceptional minority.

All HBsAg-positive specimens were tested for HDV; 74.4% (93/124) were positive serologically, 47/93 were selected at random for RNA extraction, transcription to cDNA and were examined by PCR; 97.9% (46/47) were positive. Sequencing of the amplified cDNA in part of HDV Ag coding region and subsequent phylogenetic analysis revealed phylogenetic relatedness to genotype I of all cases (tree not shown and available from authors

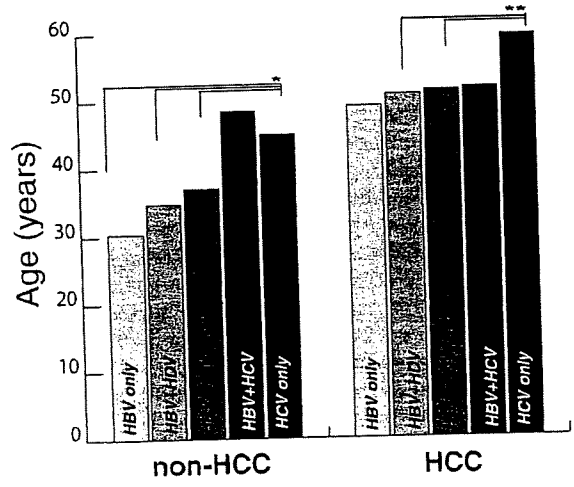

Fig. 2. Mean age among chronic hepatitis patient with and without HCC harboring different patterns of infection. B—HBV monoinfection, B+D—HBV and HDV co-infection, C—HCV monoinfection, B+D+C—triple infection with HBV, HDV and HCV, B+C—co-infection with HBV and HCV. $*P < 0.05$, $**P < 0.002$.

TABLE II. Characteristics of HCC Patients

			Coinfection			
	HCV only	HBV only	HBV+HDV	HBV+HDV+HCV	HBV+HCV	Non-B,C
Number (%)	42 (38.9)	3 (2.8)	27(25.0)	20 (18.5)	6 (5.6)	10 (9.3)
Gender (M/F)	21/21	3/0	17/10	8/12	2/4	4/6
Age (Mean+SD)	59.3 ± 12.5[a]	49.0 ± 14.0	50.6 ± 11.5	51.3 ± 12.7	51.3 ± 9.4	52.3 ± 12.1
ALT	129.5 ± 70.3	117 ± 56.9	153.8 ± 62.1[b]	110.7 ± 46.3	96.3 ± 60.1	128.0 ± 58.7
AST	114.3 ± 65.2	110.3 ± 54.9	139.1 ± 62.3	106.4 ± 58.2	96.8 ± 51.8	115.4 ± 54.6
HCV RNA	34/41 (82.9)	nt	nt	1 (5.0)[c]	3 (50)	nt
HBV DNA	nt	1 (50)	9 (33.3)	14 (70.0)[d]	2 (33.3)	0
HDV RNA	nt	nt	4/4 (100)	19 (95.0)	nt	nt
HBeAg	nt	0	5 (18.5)	4 (20.0)	1 (16.7)	nt
AFP (>10 ng/ml)	26 (61.9)	1 (33.3)	20 (74.1)	15 (75.0)	5 (83.3)	1 (10.0)[e]
PIVKA-II (>40 mAU/ml)	27 (64.3)	1 (33.3)	18 (66.7)	13 (65.0)	3 (50.0)	1 (10.0)[f]
AFP and/or PIVKA	32 (76.2)	1 (33.3)	20 (74.1)	17 (85)	5 (83.3)	1 (10.0)[f]

[a]Age; HCV vs B+D, B+D+C, $P < 0.02$.
[b]ALT; B+D vs B+D+C, B+C, $P < 0.05$.
[c]HCV RNA; B+D+C vs HCV, $P < 0.0001$.
[d]HBV DNA; B+D vs B+D+C, $P = 0.02$.
[e]AFP; NonB,C vs B+C, $P = 0.02$.
[f]PIVKA; NonB,C vs HCV, B+D, B+D+C, B+C, $P < 0.02$.

by request). Thereby, HDV genotype I found in 100% cases examined.

Anti-HCV detected in total 198/292 (67.8%), and 134/198 were selected at random for RNA extraction, transcription to cDNA and detection of the highly conserved part in the HCV 5'UTR; 53% (71/134) were found positive, of them HCV/1b was determined in 97.2% (69/71). Thereby, out of 68% of subjects found in this study to be infected with HCV about 53% had detectable viraemia with HCV/1b in 97% (data not shown).

HBV, HDV, and HCV Interference Among Patients With HCC

Detailed summary of the findings among HCC patients with different patterns of coinfection are shown in the Table II; mean level of ALT was the highest in HCC patients with HBV+HDV coinfection when compared to HCV-monoinfection, HBV+HCV and HBV+HCV+HDV groups ($P < 0.05$). Interestingly, HCV viraemia was found frequently among HCV monoinfected HCC patients (82.9%) and those with HCV+HBV co-infection (66.7%), whereas the vast majority of the patients with triple infection HBV+HDV+HCV (95%) had undetectable level of HCV RNA ($P = 0.005$). Similarly, among chronic hepatitis patients with triple-infection, HCV RNA was also rare finding (9%, 2/22). In order to confirm the result, 20 serum samples (where sera were available) were examined for HCV core Ag; 19/20 (95%) had undetectable levels and one was weakly positive.

In HCC group, the HBV DNA positivity of patients with HBV+HDV coinfection (9/27, 33.3%) was similar to that of HBV-monoinfected patients (2/3, 33.3%), probably due to small number of cases. However, when patients in non-HCC and HCC groups were analyzed altogether the difference in HBV DNA positivity between HBV-monoinfected and HBV+HDV-coinfected became evident (12/17, 70.6% vs. 19/52, 36.5%, respectively; $P = 0.02$). Interestingly, in the HCC (differently from non-HCC) group HBV viraemia was significantly rare among HBV+HDV as compared to HBV+HDV+HCV (9/27, 33.3% vs. 14/20, 70%, $P = 0.004$). In the patients studied HDV viraemia was found frequently in all patients with no respect to the clinical stage and coinfection pattern.

AFP and PIVKA II in Clinical Diagnostics

In order to assess the significance of the combined evaluation of the Alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) among patient with and without HCC, the result of the examination plotted in two dimensional graphs (Fig. 3). Patients were subdivided into non-HCC and HCC groups, respective value of PIVKA-II (x-axis) and AFP (y-axis) were plotted in logarithmic units. No correlation was observed between two markers. Abnormal levels of PIVKA-II were found in 57% of HCC and 2% of non-HCC, for AFP these were in 66% and 16%, respectively. Both markers were negative in 84% of non-HCC but 29% of HCC, and were positive in 51% of HCC but 1.6% of non-HCC. The PIVKA-II alone, was elevated specifically in 5.6% of HCC patients who had AFP within normal range. And 15% of HCC with abnormal AFP did not have elevation of PIVKA-II, together with14% of CH. This indicates that using both of the markers would help to diagnose HCC at least in 70% of this cohort according to evaluation of both markers, and using of PIVKA-II in addition to AFP would be reasonable to increase the specificity and sensitivity. No significant difference in positivity for AFP and/or PIVKA-II was found among HCC patients with different pattern of mono and co-infection (Table II).

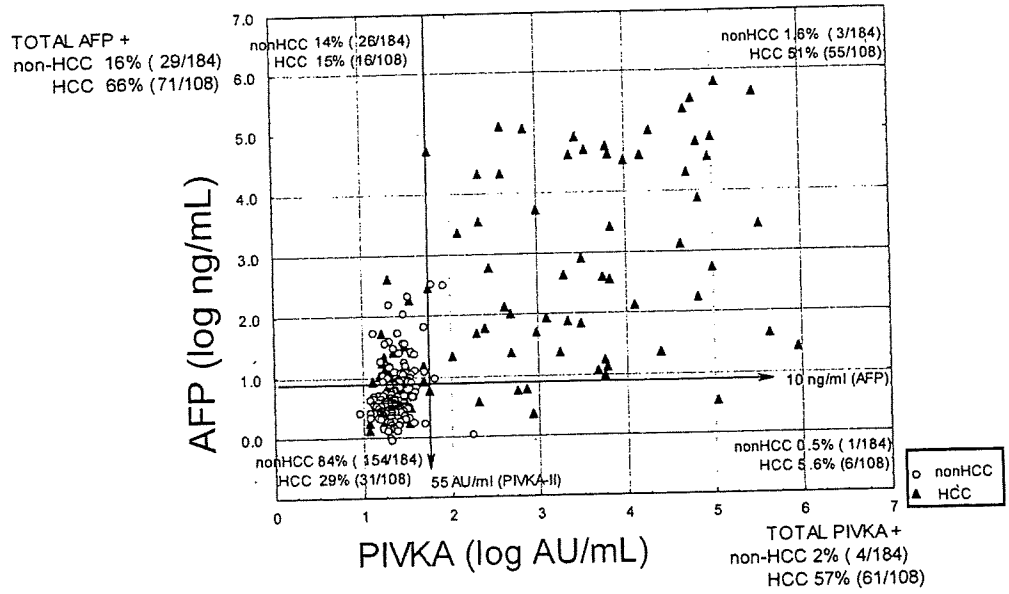

Fig. 3. Correlation between AFP and PIVKA-II. Y axis—AFP (log ng/ml), X axis PIVKA-II (log mAU/ml), Cutoff values indicated by dotted lines; 10 log ng/ml for AFP and 55 log AU/mL for PIVKA.

DISCUSSION

Recent review of worldwide surveys indicate that age adjusted HCC incidence in Mongolia was 98.93 per 100,000 men, although local public health estimates were lower; 61.8 [Dulamsuren, 2004] the rate is still one of the highest in world [Bosch et al., 2004]. In previous reports a very high prevalence of hepatitis viruses in this country was identified [Kondo et al., 1997; Kato et al., 1999], but no data were available concerning the role played in HCC development by a particular virus or coinfection pattern. Another study, reporting the very high prevalence of dual and triple infection among chronic hepatitis patients especially those with HCC has been published during the preparation of the manuscript [Tsatsralt-Od et al., 2005a]. In present study we aimed to extend the analysis of association between the patterns of hepatitis viruses' mono-, dual-, and triple-infections and the HCC development. Two groups were formed, including chronic hepatitis patients with established hepatocellular carcinoma (HCC group) and those with no signs of the pathology (non-HCC group). Because these cohorts consist of consecutively diagnosed patients with no selection bias, we believe that the results obtained in this group are valid to be extrapolated into a total population of the chronic hepatitis patients in the country. Hepatitis virus infection was determined in 91% of the chronic liver disease patients examined; 42.5% carried HBsAg and 67.8% carried anti-HCV. Furthermore, HCV monoinfection (53.2%, 142/267) and HBV in co-infection with HDV and/or HCV (40.4% 108/267) were predominant among the liver disease patients with determined viral etiology. An interesting observation made in this study is that the majority of the patients with HCC had HCV-monoinfection (42/108, 39%), followed by HBV+HDV (27/108, 25%), HBV+HDV+HCV (19/108, 18%), HBV+HCV (7/108, 7%) and HBV-monoinfection (3/108, 3%). At the same time when the association of these patterns were compared with the younger non-HCC group, HBV+HDV revealed strongest association with cancerogenesis since the greater part of patients with this pattern had HCC (27/52, 52%, $P < 0.02$), followed by HBV+HCV (7/15, 47%), HBV+HDV+HCV (19/41, 46%), in contrast to HCV-monoinfection which prevalence was significantly higher in non-HCC group (100/142, 70.4%, $P < 0.02$). This might indicate that the patients with HBV+HDV pattern are more likely to develop HCC but equal contribution of HCV is associated with its higher prevalence. However previous studies indicated that seroprevalence of HCV and HBV with HDV are equally high in Mongolian population [Takahashi et al., 2004; Tsatsralt-Od et al., 2005b], thus one of the possible explanation would be a different spread-time of these viruses in the population, that may be associated with HCC incidence as recently shown on example of different HCV genotypes in the world populations [Tanaka et al., 2006]. This is supported by observation that patients with HBV monoinfection were tending to be younger those with HCV-monoinfection in the present study (Fig. 1) as well as in a previous report [Fujioka et al., 1998]. Further investigation using epidemiological-mathematical modeling based on the effective population size to trace the history of the hepatitis viruses in this population will provide more information.

The second interesting point of the present results is the association of the viraemia with the co-infection pattern. Significant reduction of the HCV viraemia was observed among patients with triple-infection in comparison with HCV-monoinfection in both non-HCC and HCC groups (non-HCC; from 81% to 9% and HCC; from 83% to 5%, $P < 0.005$). Similar tendency was observed among those with HBV+HCV coinfection pattern. Furthermore, HBV viraemia among those with triple infection was higher than those with HBV+HDV double-infection (14/20, 70% vs. 9/27, 33%, $P < 0.02$) in HCC group but not in non-HCC group (10/22, 45.5% vs. 11/25, 44%). That might indicate that HDV suppresses HBV viraemia in dual-infection, however triple-infection (+HCV) may affect negatively this suppression and may be associated with faster progression to HCC. Due to small number of HCC cases with HBV monoinfection, statistical evaluation of the HBV DNA was unavailable in this study. Of note, stronger suppressive effect of HDV on HCV rather than on HBV was indicated previously in patients with triple-infection in contrast to those with HBV+HDV co-infection [Jardi et al., 2001]. The effect of the inhibition of inhibition was also suggested for interference of HDV and HIV, where HDV was deprived of its suppression capability on HBV (HBV+HDV double infection) by HIV coinfection (HBV+HDV+HIV) [Morante et al., 1989; Govindarajan, 1990]. The result of the present study discrepant with previous report from Mongolia in respect to rates of viraemia among HCC patients with triple-infection, that was previously reported as high [Tsatsralt-Od et al., 2005a]. This was possibly associated with reoccurring superinfections with HDV or HCV among patients studied in previous report, as was also suggested by the authors [Tsatsralt-Od et al., 2005a]. The different prevalence of the coinfection patterns among HCC patients is probably associated with different age of studied population (54.3 ± 12.5 in present study and 58.8 ± 9.2 in previous) and different recruitment conditions. Thereby, results of the present study suggested that HCV monoinfection and HBV in coinfection with HDV are associated with the high HCC incidence in Mongolia. Additionally, HDV plays a significant role in the interference with HBV and HCV during course of triple- and dual-infection. These results indicate that efficient screening and follow-up for HCC in Mongolia is essential and serological tumor markers, such as AFP and PIVKA-II might be a very important tool for early diagnosis. In this study we demonstrated no correlation between these markers (Fig. 2), thus they can complement each other. The mutual complementation works more efficiently for detecting HCC at stage of small size, most of which raise serum levels of either PIVKA-II or AFP, but not both [Fujiyama et al., 2002]. It is noted that 29% of HCC patients did not have elevation of any of these markers, this rate is higher, than in Japan (14%) [Fujiyama et al., 2002], and it indicates that besides tumor makers, using other methods (as Ultrasonography, CT, MRI and liver biopsy) are very important.

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