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Nucleotide Mutations Associated With Hepatitis B e Antigen Negativity

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One hundrded and forty four patients with chronic hepatitis B were tested to identify new mutations associated with hepatitis B e antigen (HBeAg) negativity, using a full genome sequence analysis. All the patients were Chinese and had hepatitis B virus infection of genotype C. Patients with none of the pre-core or core promoter mutations were significantly ($P < 0.001$) less common in the group with anti-HBe (13%) than in the group with HBeAg (56%). The complete nucleotide sequence was determined in four anti-HBe-positive patients who had neither pre-core nor core promoter mutations and in five HBeAg-positive patients who also had neither of these mutations (the groups were matched for age and sex). Six mutations were found to be significantly more common in the former group than in the latter: G529A (3/4 vs. 0/5), C934A (4/4 vs. 1/5), A1053G (4/4 vs. 1/5), G1915T/A (4/4 vs. 0/5), T2005C/A (4/4 vs. 0/5), and C3026T (3/4 vs. 0/5). Three of the six mutations were significantly more common in the four anti-HBe-positive patients who had neither pre-core nor core promoter mutations, compared to 11 HBeAg-positive patients who had pre-core and core promoter mutations, and also compared to 15 anti-HBe-positive patients who had pre-core and core promoter mutations, suggesting further the specificity of these mutations. Of the six mutations, two resulted in amino acid substitution in the polymerase protein, and one is located near the enhancer I region. The results suggest that the six newly discovered mutations are associated with HBeAg negativity. ***J. Med. Virol. 76:170–175, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: hepatitis B e antigen (HBeAg); genotype; nucleotide mutation

INTRODUCTION

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide [Maynard, 1990; Maddrey, 2000]. Chronic HBV infection is the cause of up to 50% of cirrhosis and 70–90% of hepatocellular carcinomas (HCC) in China, South-East Asia, and Africa [Lok, 1992; Fattovich, 1998], and in Asian countries, almost all patients with chronic HBV infection have been infected perinatally from hepatitis B e antigen (HBeAg)-positive mothers [Okada et al., 1976]. HBeAg is considered to be a marker for viral replication, but some HBeAg-negative patients remain viremic and continue to have active liver disease [Hadziyannis et al., 1983; Lok et al., 1984; Bonino et al., 1986]. Many of these patients are found to have a G to A change at nucleotide 1896, which creates a stop codon (TAG) in the precore (Pre-C) open reading frame, which in turn prevents translation of the Pre-C protein and aborts HBeAg production [Carman et al., 1989]. Other patients have mutations in the core promoter (CP) region, including an A to T mutation at nucleotide 1762 and a G to A mutation at nucleotide 1764 [Okamoto et al., 1994]. In vitro studies of this double mutation show decreased transcription of Pre-C messenger RNA and hence a resultant decrease in HBeAg production by 70% [Buckwold et al., 1996; Chan et al., 1999]. A recent follow-up study on Pre-C and CP mutations has also

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shown that the presence of these mutations is useful for predicting seroconversion [Yamaura et al., 2003].

Besides the G1896A mutation and the A1762T/G1764A mutation, a number of point mutations, as well as deletions and insertions of nucleotides, have been detected in the Pre-C region and CP region that could correlate with seroconversion [Okamoto et al., 1990; De Castro et al., 2001]. In the present study, the complete HBV genome was examined for other nucleotide mutations associated with HBeAg negativity, in addition to mutations in the Pre-C and CP regions.

MATERIALS AND METHODS

Patients

A cohort of 193 Chinese patients with chronic HBV infection who visited the Liver Disease Clinic of the Second Hospital of HeBei Medical University in Shijiazhuang city, North China, between June and August 2001 were enrolled in the study. These patients comprised 124 men and 69 women and had a median age of 29.1 years old (range: 5–73 years old). Patients who were co-infected with hepatitis C or D virus or with the human immunodeficiency virus and patients with other concomitant causes of chronic liver disease were excluded. According to the consensus diagnostic criteria for HBV infection, 182 patients were diagnosed with chronic hepatitis B. The remaining 11 patients had persistently normal alanine aminotransferase (ALT: normal range 10–21 IU/L) levels, suggesting an inactive HBV carrier stage. None of the 193 patients were treated with antiviral agents such as interferon or lamivudine. Of the 193 patients, 169 (87.6%) were of genotype C, 21 (10.9%) of genotype B, and 3 (1.5%) of genotype A. For the mutation analysis, 144 patients who were positive for either HBeAg or anti-HBe were selected from the 169 genotype C patients. Informed consent was obtained from each patient.

Conventional HBV Markers and Genotyping of HBV

Hepatitis B surface antigen (HBsAg), HBeAg and anti-HBe were measured using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan). Serum concentration of HBV DNA was measured using the AMPLICOR HBV Monitor test (Roche Diagnostics K.K., Tokyo, Japan), which has a quantitative range of 2.6–7.6 log copies/ml. When the concentration to be tested was beyond this range, the actual concentration was determined using a serum sample diluted 100-fold with normal human serum. The HBV genotype was determined using the restriction fragment length polymorphism (RFLP) method on an S-gene sequence amplified by polymerase chain reaction (PCR) with nested primers [Mizokami et al., 1999].

Determination of Pre-C and CP Mutations

The 1,896th nucleotide in the Pre-C region of G or A was detected with an enzyme-linked mini-sequence assay kit (Roche Diagnostics), and the results were expressed as the percentage mutation rate, as defined by Aritomi et al. [1998]. If the mutation rate was 0%, the strain was considered to be Pre-C mutation-negative, while a Pre-C mutation-positive strain was recorded when the mutation rate exceeded 0%. The double mutation in the CP region (A1762/T1764) was detected using an HBV CP mutation detection kit (Smitest: Genome Science Laboratories, Tokyo, Japan), and the results were classified into three categories: wild, mixed, and mutant types. A wild type strain was considered to be CP mutation-negative, while mixed and mutant types were recorded as CP mutation-positive strains. The detection limits of the pre-C and the CP mutation detection kits are both 1,000 copies/ml.

Determination of Nucleotide Sequence

The complete genome sequence was determined according to the method described by Rokuhara et al. [2000]. Briefly, nucleic acids were extracted from a serum sample of 100 μl with a DNA/RNA extraction kit (Smitest EX-R&D: Genome Science Laboratories Co., Ltd.). Two microliters of each DNA solution were used for amplification by PCR. The reaction was carried out in 25 μl of PCR-mixture containing 250 μmol/L of each dNTP, 1× PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 0.001% gelatin], 0.25 U EX-Taq DNA polymerase (TaKaRa, Tokyo), and 0.25 μM of a primer pair. The PCR was initiated using the hot-start technique.

To determine the full-length nucleotide sequence of HBV, two fragments (fragments A and B) were amplified by PCR, using the primers shown in Table I. Fragment A (1,498 bases in length; nt 457–nt 1954) was amplified with nested pairs of outer (SB1 and CB2) and inner primers (SB3 and CB4), while fragment B (2162 bases in length; nt 1611–nt 557), was amplified with nested pairs of outer primers (es2 and PS4) and inner primers (is2 and PS3). The first round of PCR was performed with an outer primer set for 40 cycles (94°C for 1.5 min, 55°C for 1 min, and 72°C for 2 min), and was followed by an extension reaction at 72°C for 7 min. The second round was undertaken with an inner primer set for 30 cycles, and was also followed by an extension reaction. PCR products were subjected to electrophoresis on a 1.0% agarose gel with ethidium bromide staining and visualization with an UV transilluminator. The band containing the target sequence was removed and DNA was isolated using GFX™ PCR DNA and a Gel Band Purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The nucleotide sequence was directly determined by the dideoxy method, using the sequencing primers shown in Table I. The accuracy of the sequence was ensured by comparison of the sequence data for the complete genome obtained with sense-sequencing primers and that obtained with anti-sense-sequencing primers.

Statistical Analysis

Mann–Whitney's *U* test was utilized for quantitative data, and Fisher's exact test and a Chi-square test were

TABLE I. Primers Used for PCR and Sequencing of HBV DNA

Primer		Sequence	nt position
Primers for PCR of fragment A			
SB1	Sense	5-TGCTGCTATGCCTCATCTTC	(414–433)
CB2	Anti-sense	5-GGAAAGAAGTCAGAAGGCAA	(1974–1955)
SB3	Sense	5-AGGTATGTTGCCCGTTTCTC	(457–476)
CB4	Anti-sense	5-AAAAGAGAGTAACTCCACAG	(1954–1935)
Primers for PCR of fragment B			
es2	Sense	5-ACGTCGCATGGAGACCACCG	(1601–1620)
PS4	Anti-sense	5-CAGTTTCCGTCCGAAGGTTTTG	(594–573)
is2	Sense	5-GAGACCACCGTGAACGCCCA	(1611–1630)
PS3	Anti-sense	5-GAAACATAGAGGTGCCTTGAGCAG	(557–534)
Primers for sequencing			
SB3	Sense	5-AGGTATGTTGCCCGTTTCTC	(457–476)
as1	Anti-sense	5-TGCGAAAGCCCAGGATGATG	(631–612)
s2	Sense	5-TGCGAAAGCCCAGGATGATG	(760–783)
as2	Anti-sense	5-AGTTGGCGAGAAAGTGAAAGCCTG	(1107–1084)
s3	Sense	5-CTCTGCCGATCCATACTGCGGAA	(1256–1278)
as3	Anti-sense	5-CGGGACGTAGACAAAGGACGT	(1434–1414)
is2	Sense	5-GAGACCACCGTGAACGCCCA	(1611–1630)
ea1	Anti-sense	5-TGAAAAAGTTGCATGGTGCTGGTG	(1827–1804)
s4	Sense	5-TATCGGGAGGCCTTAGAGTCTCCG	(2012–2035)
as4	Anti-sense	5-ATAGGGGCATTGGTCT	(2314–2298)
s5	Sense	5-CGCAGAAGATCTCAATCTCGG	(2417–2437)
as5	Anti-sense	5-GGATAGAACCTAGCAGGCAT	(2654–2635)
s6	Sense	5-GGGTCACCATATTCTTGGGAA	(2814–2834)
as6	Anti-sense	5-GGGTTGAAGTCCCAATCTGGATT	(2987–2965)
is1	Sense	5-AAGCTCTGCTAGATCCCAGAGT	(18–39)
ea2	Anti-sense	5-TAGAAAATTGAGAGAAGTCCACCA	(280–257)
s1	Sense	5-CATCCTGCTGCTATGCCTCATC	(409–430)
as1	Anti-sense	5-TGCGAAAGCCCAGGATGATG	(631–612)

Nucleotides are numbered from the unique *Eco*RI site of HBV.

used for qualitative data. P values less than 0.05 were considered significant. Analyses were carried out using SPSS version 10.0J (SPSS Inc., Chicago, IL).

RESULTS

Of the 144 patients selected for mutation analysis, 90 (62.5%) were HBeAg-positive and the remaining 54 (37.5%) were anti-HBe-positive. The clinical and virological backgrounds of the two groups of patients are compared in Table II. The 90 HBeAg-positive patients tended to be younger and have a higher concentration of HBV DNA than the 54 anti-HBe patients. Patients with none of the Pre-C and CP mutations were significantly ($P < 0.001$) more common in the HBeAg-positive patients (56%) than in the anti-HBe-positive patients (13%).

A comparison of the clinical background of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and 47 anti-HBe-positive patients who had at least one of the mutations is shown in Table III. Distributions of age, gender, ALT level, and HBV DNA concentration did not differ between the two groups.

Nucleotide sequences of the complete genome were determined in four out of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and in 5 out of 50 HBeAg-positive patients who also had neither mutation. All nine of the genome sequences

TABLE II. Comparison of Clinical and Virological Backgrounds of Patients With HBeAg and Those With Anti-HBe

	HBeAg-positive n = 90	Anti-HBe-positive n = 54	P
Age[a]	25 (5–53)	36 (11–73)	<0.001[b]
Gender (M:F)	58:32	30:24	>0.2[c]
ALT[a]	89 (11–2100)	62 (13–458)	>0.2[b]
HBV DNA (log copies/mL)[a]	8.3 (4.4–7.9)	5.0 (3.2–8.8)	<0.001[b]
Pre-C/CP mutations			
Both negative	50 (56%)	7 (13%)	<0.001[c]
Pre-C mutation only	13 (14%)	20 (37%)	
CP mutation only	12 (13%)	5 (9%)	
Both positive	15 (17%)	22 (41%)	

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

TABLE III. Comparison of Clinical and Virological Backgrounds of Anti-HBe-Positive Patients With Neither Pre-C nor CP Mutations and Anti-HBe Patients With at Least one of These Mutations

	Pre-C and CP mutation-negative n = 7	Pre-C and/or CP mutation-positive n = 47	*P*
Age[a]	37 (18–60)	36 (11–73)	>0.2[b]
Gender (M:F)	4:3	26:21	>0.2[c]
ALT[a]	44 (18–86)	65 (13-458)	0.17[b]
HBV DNA (log copies/ml)*	4.7 (3.3–5.5)	5.0 (3.2–8.8)	>0.2[b]

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

determined had nucleotide lengths of 3,215 bases, and thus there were no insertions or deletions. When the full genome sequences were compared, the six mutations shown in Table IV were significantly more common in the four anti-HBe-positive patients than in the five HBeAg-positive patients. The positions of the six mutations in the HBV genome are shown in Figure 1. Of the four mutations located in the polymerase gene, the G529A and C934A mutations cause amino acid substitutions in the polymerase protein. The C3026T mutation does not cause an amino acid substitution in the polymerase, but rather in the pre-S1 protein, while the A1053G mutation does not lead to an amino acid substitution, but the mutation is located near the enhancer I region. The G1915T/A and T2005C/A mutations are located in the core gene, but do not result in an amino acid substitution. Patients with at least one of the three mutations (G529A, C934A, and A1053G) which might affect HBV replication had a significantly ($P = 0.029$) lower level of HBV DNA (n = 22, median 5.3 copies/ml, range 3.8–8.9) than those patients who had no mutations (n = 13, median 8.5 copies/ml, range 3.8–8.9).

To examine further the specificity of the six mutations, these mutations were also determined in 11 HBeAg-positive patients who were positive for Pre-C and CP mutations and in 15 anti-HBe-positive patients who were also positive for Pre-C and CP mutations. The frequencies of the six mutations were compared between groups of patients classified according to their HBeAg/anti-HBe and Pre-C/CP mutation status. Three (G1915T/A, T2005C/A, and C3026T) of the six mutations were found to be significantly more common in anti-HBe-positive patients who had neither a Pre-C nor a CP mutation than in the two groups of patients with Pre-C and CP mutations, as shown in Table V.

The nucleotide sequence data reported in this paper have been registered in the DDBJ/EMBL/GenBank nucleotide sequence databases, with the accession numbers AB198076-84.

DISCUSSION

Studies to date have shown that the stop codon mutation in the Pre-C region (G1896A) and the double mutation in the CP region (A1762T/G1764A) are independently associated with the seroconversion of HBeAg, and that the Pre-C mutation is more directly associated with seroconversion than the core promoter mutation [Okamoto et al., 1994; Yamaura et al., 2003]. Only a small number of anti-HBe-positive patients (13%) were both negative for the Pre-C and CP mutations, and in the present study this rate was significantly lower than that (56%) in HBeAg-positive patients. These results are consistent with previous reports, suggesting that the two mutations are the main causes of seroconversion. However, there are also patients in whom HBeAg secretion discontinues without

TABLE IV. Comparison of Full Nucleotide Sequences of HBV With Neither Pre-C nor CP Mutations for HBeAg-Positive and Anti-HBe-Positive Patients

Nucleotide mutation	Amino acid substitution (viral protein)	HBeAg Pre-C and CP mutation-negative n = 5	Anti-HBe Pre-C and CP mutation-negative n = 4	*P*
G529A	D480N (P) None (S)	0	3	0.048
C934A	L615I (P)	1	4	0.040
A1053G	None (P)	1	4	0.040
G1915T/A	None (C)	0	4	0.008
T2005C/A	None (C)	0	4	0.008
C3026T	A60V (Pre-S1) None (P)	0	3	0.048

Six mutation sites with significant differences are shown. Data are expressed as the number of positives. Statistical analysis was performed with a chi-square test. P, polymerase protein; S, surface protein; C, core protein; Pre-S1, pre-surface 1 protein.

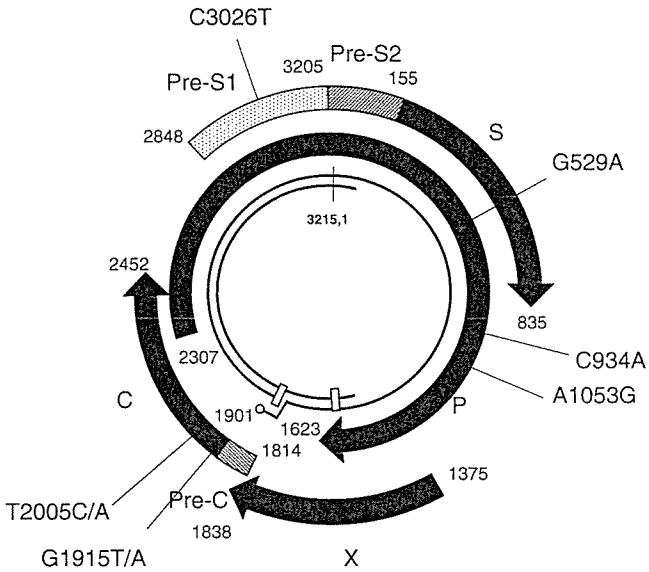

Fig. 1. Organization of the hepatitis B virus genome (genotype C) and the approximate positions of the six nucleotide mutations in the HBV genome. The inner circles represent the minus and plus DNA strands of the viral genome. The different open reading frames encoded by the genome, designated as S, C, P, and X, are indicated by the arrows. Abbreviations: S, surface antigen; C, core; P, polymerase; Pre-C, precore.

appearance of Pre-C and/or CP mutations. Thus, we speculated that some other mutations might be associated with HBeAg seroconversion. A variety of other mutations in the CP and Pre-C regions have been detected in previous studies [Carman et al., 1989; Tillmann et al., 1995; Baumert et al., 1996; Laras et al., 1998; Chan et al., 2000; De Castro et al., 2001; Yoo et al., 2003], but other regions of the HBV genome have not been analyzed sufficiently for mutations associated with HBeAg seroconversion.

When the full nucleotide sequences of HBV genomes of HBeAg-positive and anti-HBe-positive patients with neither Pre-C nor CP mutations were compared, six mutations (G529A, C934A, A1053G, G1915T/A, T2005C/A, C3026T) were found to be significantly more common in the anti-HBe-positive patients. The six mutations were also more common in anti-HBe-positive patients who had neither Pre-C nor CP mutations than in HBeAg-positive patients or in anti-HBe-positive patients who had Pre-C and CP mutations, with the results being statistically significant for three (G1915T/A, T2005C/A, C3026T) of the six mutations. These results suggest that the six mutations are associated with HBeAg negativity.

The mechanisms through which the six mutations facilitate HBeAg negativity were not investigated in the present study. However, some possible mechanisms can be considered, based on the locations of these mutations in the HBV genome. The G529A and C934A mutations cause amino acid substitutions in the polymerase protein. Thus, these two mutations may attenuate HBV replication through changes in the enzymatic activity of the polymerase. The A1053G mutation is located near the enhancer I region, which may affect the replication of HBV [Bock et al., 2000]. Patients who had at least one of the three mutations associated with HBV replication tended to have a lower level of HBV DNA than those who had none of these mutations, providing further support for a replication-associated mechanism. It has been reported that amino acid substitutions in immunogenic epitopes in the core protein are found most frequently during or after seroconversion from HBeAg to anti-HBe [Akarca and Lok, 1995; Carman et al., 1995]. We found two mutations in the core gene, but these mutations did not cause amino acid substitutions. Thus, the mechanisms through which the G1915T/A and T2005C/A mutations exert their effects remains unclear.

In anti-HBe-positive patients, the clinical background, including the mean age, gender distribution, ALT level and HBV DNA level, were similar in patients with and without Pre-C and/or CP mutations. Although these comparisons were cross-sectional, the results suggest that mutations other than those in the Pre-C and CP regions have a similar impact in patients in whom seroconversion occurs, compared to Pre-C and CP mutations.

The six mutations identified in the present study have not been described previously. These mutations

TABLE V. Comparison of Six Mutations Among Three Groups Classified According to Their HBeAg/anti-HBe and Pre-C/CP Mutation Status

Mutation site	Anti-HBe Pre-C and CP mutation-negative n = 4	HBeAg Pre-C and/or CP mutation-positive n = 11	Anti-HBe Pre-C and/or CP mutation-positive n = 15
G529A	3	3	3
C934A	4	6	10
A1053G	4	4	9
G1915T/A	4[a]	3	1
T2005C/A	4[b]	3	4
C3026T	3[c]	0	1

Data are expressed as the number of positives. Statistical analysis was performed with Fisher's exact test. Other comparisons were not statistically significant.
[a]$P = 0.026$ versus 11 patients with HBeAg, and $P = 0.001$ versus 15 patients with anti-HBe.
[b]$P = 0.026$ versus 11 patients with HBeAg, and $P = 0.018$ versus 15 patients with anti-HBe.
[c]$P = 0.009$ versus 11 patients with HBeAg, and $P = 0.016$ versus 15 patients with anti-HBe.

are thought to be associated with HBeAg negativity because they were found specifically in anti-HBe-positive patients with neither a Pre-C nor a CP mutation. However, several issues remain to be resolved to clarify the real significance of the six mutations, including the mechanisms through which they facilitate HBeAg negativity, their universality in genotypes other than genotype C, and their clinical relevance. Furthermore, it is possible that immune-based selection pressures that cause loss of HBeAg are responsible for the selection of the mutations identified in the present study [Locarnini, 2004]. Therefore, it is not possible to conclude that the new mutations are definitely associated with seroconversion, but they do provide new clues regarding the nature of seroconversion.

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REFERENCES

Akarca US, Lok AS. 1995. Naturally occurring core-gene-defective hepatitis B viruses. J Gen Virol 76:1821–1826.

Aritomi T, Yatsuhashi H, Fujino T, Yamasaki K, Inoue O, Koga M, Kato Y, Yano M, 1998. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. J Gastroenterol Hepatol 13:1125–1132.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ. 1996. Two core promoter mutations in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest 98:2268–2276.

Bock CT, Malek NP, Tillmann HL, Manns MP, Trautwein C. 2000. The enhancer I core region contributes to the replication level of hepatitis B virus in vivo and in vitro. J Virol 74:2193–2202.

Bonino F, Rosina F, Rizzetto M, Rizzi R, Chiaberge E, Tardanico R, Callea F, Verme G. 1986. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. Gastroenterology 90:1268–1273.

Buckwold VE, Xu Z, Chen M, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis virus basal core promoter on precore gene expression and viral replication. J Viral 70:5845–5851.

Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet ii:588–590.

Carman WF, Thursz M, Hadziyannis S, McIntyre G, Colman K, Gioustoz A, Fattovich G, Alberti A. 1995. Hepatitis B e antigen negative chronic active hepatitis: Hepatitis B virus core mutations occur predominantly in known antigenic determinants. J Viral Hepat 2:77–84.

Chan HLY, Hussain M, Lok ASF. 1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 29:976–984.

Chan HLY, Leung NW, Hussain M, Wong ML, Lok AS. 2000. Hepatitis B e antigen-negative chronic hepatitis B in Hong Kong. Hepatology 31:763–768.

De Castro L, Niel C, Gomes SA. 2001. Low frequency of mutations in the core promoter and precore regions of hepatitis B virus in anti-HBe positive Brazilian carriers. BMC Microbiol 1:10.

Fattovich G. 1998. Progression of hepatitis B and C to hepatocellular carcinoma in Western countries. Hepatogastroenterology 45:1206–1213.

Hadziyannis SJ, Lieberman HM, Karvountzis GG, Shafritz DA. 1983. Analysis of liver disease, nuclear HBcAg, DNA viral replication, and hepatitis B virus DNA in liver and serum of HBeAg vs. anti-HBe positive carriers of hepatitis B virus. Hepatology 3:656–662.

Laras A, Koskinas J, Avgidis K, Hadziyannis SJ. 1998. Incidence and clinical significance of hepatitis B virus precore gene translation initiation mutations in e antigen-negative patients. J Viral Hepatitis 5:241–248.

Locarnini S. 2004. Molecular virology of hepatitis B virus. Semin Liver Dis 24:3–10.

Lok ASF. 1992. Natural history and control of perinatally acquired hepatitis B virus infection. Dif Sis 10:46–52.

Lok ASF, Hadziyannis S, Weller IVD, Karvountzis MV, Monjardino J, Karayiannis P, Montano L, Thomas HC. 1984. Contribution of low level HBV replication to continuing inflammatory activity in patients with anti-HBe positive chronic hepatitis B virus infection. Gut 25:1283–1287.

Maddrey WC. 2000. Hepatitis B: An important public health issue. J Med Virol 61:362–366.

Maynard JE. 1990. Hepatitis B: Global importance and need for control. Vaccine 8:S18–20.

Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. 1999. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS lett 450:66–71.

Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y. 1976. E antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N Engl J Med 294:746–749.

Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. 1990. Hepatitis B viruses hosts along with seroconversion to the antibody against e antigen. J Virol 64:1298–1303.

Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 68:8102–8110.

Rokuhara A, Tanaka E, Yagi S, Mizokami M, Hashikura Y, Kawasaki S, Kiyosawa K. 2000. De novo infection of hepatitis B virus in patients with orthotopic liver transplantation: Analysis by determining complete sequence of the genome. J Med Virol 62:471–478.

Tillmann H, Trautwein C, Walker D, Michitaka K, Kubicka S, Boker K, Manns M. 1995. Clinical relevance of mutations in the precore genome of the hepatitis B virus. Gut 37:568–573.

Yamaura T, Tanaka E, Matsumoto A, Rokuhara A, Orii K, Yoshizawa K, Miyakawa Y, Kiyosawa K. 2003. A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. J Med Virol 70:545–552.

Yoo BC, Park JW, Kim HJ, Lee DH, Cha YJ, Park SM. 2003. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. J Hepatol 38:98–103.

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Impact of daily high-dose IFNα-2b plus ribavirin combination therapy on reduction of ALT levels in patients with chronic hepatitis C with genotype 1 and high HCV RNA levels

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Abstract

The possibility of delaying progression to hepatocellular carcinoma in chronic hepatitis C patients with genotype 1 and high viral titers with baseline ALT levels of ≥50 IU/L was examined by administration of IFN plus ribavirin combination therapy using ALT normalization as index and IFN monotherapy as control. The rate of sustained ALT normalization (ALT normal at 24 weeks after the end of treatment) was 28.1% with combination therapy and 10.5% with IFN monotherapy ($P = 0.001$). Furthermore, the number of patients with sustained viral response (SVR) and with sustained ALT normalization in non-SVR patients was also significantly higher in the combination therapy versus monotherapy group. Mean ALT values during treatment and for 6 months after the end of treatment were significantly lower with combination therapy versus monotherapy even in virological nonresponders, as well as significantly lower during the post-treatment observation period in patients who relapsed after the end of treatment. Since increase in the rate of sustained ALT normalization and SVR were successfully achieved, inhibition of progression to hepatocellular carcinoma should be studied with long-term IFN and ribavirin combination therapy.

Keywords: Chronic hepatitis C; IFNα-2b; Ribavirin; ALT; Hepatocellular carcinoma prevention

1. Introduction

With the aging of the chronic hepatitis C patient population in Japan, a rapid increase in the incidence of hepatocellular carcinoma (HCC) is being observed [1]. Deaths due to HCC number over 30,000 per year [1], and prevention of progression to HCC is now an urgent issue. Many reports on the efficacy of interferon (IFN) in preventing progression to HCC in patients with chronic hepatitis C have been published by Japanese researchers [2–8]. At first, normalization

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of serum alanine aminotransferase (ALT) levels while on IFN therapy was thought to inhibit progression to HCC [3]. However, the results of long-term follow-up studies clearly indicate that sustained viral response (SVR) and/or sustained normalization of ALT after the end of treatment are necessary for the long-term inhibition of progression to HCC [4,6–8]. Results indicating that such inhibition is possible if ALT levels are maintained long-term within about twice the upper limit of normal (80 IU/L) have also been reported [9,10].

The standard of treatment of chronic hepatitis C worldwide is pegylated-IFN (PEG-IFN) in combination with ribavirin. The addition of ribavirin has been shown radically to increase the rate of eradication of HCV [11–13], and this is thought to result in increased inhibition of progression to HCC in patients with chronic hepatitis C. The efficacy of PEG-IFN plus ribavirin in the prevention of histologic progression using fibrosis as index has already been reported although these studies did not directly examine the effect on inhibition of progression to HCC [14,15]. Based on an average follow-up period of 20 months, one-stage improvement in METAVIR score was observed in 73% of patients on 1-year administration of PEG-Intron 1.5 μg plus ribavirin. One-stage exacerbation was observed only in 8%. Ribavirin alone has almost no effect on reducing HCV levels, but is reported to normalize ALT levels during treatment [16,17], and similar effects may be expected with combination therapy. The focus worldwide is on antiviral efficacy, and there are very few reports of detailed examination of the effect of combination therapy on liver function [18].

Recently, the efficacy of combination therapy with ribavirin in the context of inhibition of progression to HCC has started to be investigated although the number of patients involved is small compared with the numbers enrolled in clinical studies of IFN in Japan. Yang et al. [19] reported that the 7-year cumulative HCC rate is 1.4% in patients receiving IFN plus ribavirin, which is much lower than the 10.2% reported in patients receiving IFN alone, although the difference was not statistically significant due to the small sample size. It has been reported elsewhere that factors contributing to inhibition of progression to HCC include absence of liver cirrhosis before the start of treatment and sustained viral response although the data include both combination therapy and monotherapy cases [20]. The above Japanese data indicate that prevention of progression to HCC can be expected with sustained normalization of ALT, although the presence of this factor does not necessarily indicate that liver histology is normal [18]. Hence we tested the hypothesis that combination therapy consisting of IFNα-2b plus ribavirin for 24 weeks in difficult-to-treat HCV genotype 1 patients leads not only to the eradication of HCV but ultimately to the prevention of histological progression with increased normalization of liver function.

2. Materials and methods

2.1. Patient selection

Two randomized comparative clinical studies of IFNα-2b plus ribavirin combination therapy versus IFNα-2b alone were initiated in Japan in 1998 in chronic hepatitis C patients; one in difficult-to-treat genotype 1 and high viral titer (> 100 kcopies/mL) patients [21] and the other in nonresponders and relapsers to previous IFN therapy [22]. From these two studies, data on patients with ALT levels ≥50 IU/L (i.e. about 1.5 times the mean upper limit of normal) at the start of treatment were extracted and the effects of treatment on ALT improvement were retrospectively analyzed. In both studies, IFN α-2b (Intron A; Schering Plough, Kenilworth, NJ) was administered at doses of 6 or 10 MIU six times per week for 2 weeks followed by 6 MIU three times per week for 22 weeks. Patients in the combination treatment groups additionally received ribavirin (Rebetol, Schering Plough, Kenilworth, NJ) at a dose of 600 mg/day (three capsules) and 800 mg/day (four capsules) in those weighing <60 kg and ≥60 kg, respectively, for 24 weeks. Patients in the control groups took ribavirin placebo capsules. In both studies, patients were randomized to either treatment.

The studies were approved by the institutional review boards of each study site and all patients provided written informed consent to participate. Inclusion criteria were as follow: (1) HCV RNA-positive and ALT abnormal in tests conducted within 12 weeks prior to the start of treatment; (2) HCV genotype 1 or if genotype 2 nonresponder or relapser to previous IFN treatment; (3) age between 20 and 64 years; (4) hemoglobin ≥ 12 g/dL and platelets ≥ 100,000 mm^{-3} in the most recent test conducted within 12 weeks prior to the start of treatment; (5) available for hospitalization for 4 weeks after the start of treatment; and (6) contraception possible both during and for 6 months after the end of treatment. Exclusion criteria were as previously reported [23]. The database for this retrospective study included information on sex, age, body weight, histological stage and activity index, IFN treatment history, HCV RNA levels, aspartate aminotransferase, ALT, hemoglobin, white blood cells (WBC), red blood cells, platelets, and creatinine.

2.2. Study design

Pretreatment ALT levels were classified into three grades: 50 to <100 IU/L, 100 to <150 IU/L, and ≥150 IU/L. The effect of timing of initial ALT normalization on sustained ALT normalization was examined as well as the association between virological efficacy and improvement in liver function. ALT was measured before and 1–4, 6, 8, 12, 16, 20, and 24 weeks after the start and 2, 4, 8, 12, 16, 20, and 24 weeks after the end of treatment. The judgment of ALT normalization and less than two times of upper normal ALT levels were made based on the normal values at each study site (median 37.5 IU/L, range 21–50 IU/L), and the timing of

initial ALT normalization was recorded as the day when the judgment of normal ALT was made for the first time during treatment. HCV RNA was measured before and 4, 12, and 24 weeks after the start and 24 weeks after the end of treatment. HCV RNA was measured by qualitative Amplicor assay (Mitsubishi Kagaku BCL, Tokyo, Japan), and genotype determined before the start of treatment by reverse transcriptase polymerase chain reaction (Mitsubishi). Evaluation of liver histology was conducted by a single evaluator based on liver tissue samples taken within 48 weeks prior to the start of treatment.

2.3. Definition of response

ALT normalization at 24 weeks after the end of treatment was considered "effective" and was the primary endpoint of this examination. Separately, the association with virological efficacy was also examined. HCV RNA negativity by qualitative assay at 24 weeks after the end of treatment was defined as sustained viral response. Virological relapsers were patients who were HCV RNA-negative by qualitative assay at the end of treatment but who became HCV RNA-positive after the end of treatment. Nonresponders were patients who were never HCV RNA-negative during or after treatment.

2.4. Statistical analysis

After confirming the absence of interaction in efficacy by the Breslow–Day test, comparison of sustained ALT normalization rate by pretreatment ALT levels was conducted using the Mantel–Haenszel test. The log-rank test was used to analyze the timing of initial ALT normalization, and *t*-test or Wilcoxon test was used for mean ALT values during the treatment and posttreatment observation periods. Significance level was two-sided 5%. All calculations were performed by SAS program version 6.12 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics

The study included 167 patients given combination therapy and 105 assigned monotherapy. Main patient characteristics are shown in Table 1. Mean age was 48–49 years and the majority of patients had extremely high HCV RNA levels exceeding the upper limit of quantitation of 850 kcopies/mL. No imbalance in patient background was observed between the two treatment groups.

3.2. ALT normalization rate

Sustained ALT normalization rate was 28.1% (47/167) in the combination therapy group and 10.5% (11/105) in the monotherapy group; combination therapy was significantly superior to monotherapy ($P=0.001$; Fisher's direct probability test). Sustained ALT normalization rate taking into account baseline ALT levels is shown in Fig. 1. In this respect, combination therapy was again significantly superior to monotherapy ($P=0.001$; Mantel–Haenszel test). Sustained ALT normalization rate was also significantly superior in the combination therapy group in patients whose pretreatment ALT was 100 to <150 IU/L ($P=0.001$; Fisher's direct probability test). In the combination group, the frequency of patients with ALT levels sustained within twice the upper limit of normal (i.e. an index of inhibition of progression to HCC [9,10]) was 34.1% (29/85), 44.7% (17/38), and 25.0% (9/36) in those whose pretreatment ALT was 50 to <100 IU/L, 100 to <150 IU/L, and ≥150 IU/L, respectively; in the monotherapy group the frequency was 22.9% (11/48), 0% (0/23), and 14.3% (4/28), respectively (Fig. 2). Combination therapy was hence significantly superior to monotherapy ($P=0.001$; Mantel–Haenszel test).

Table 1
Patient demographics at baseline

	IFN + ribavirin	IFN alone	*P*-value
n	167	105	–
Sex (M/F)	135/32	81/24	0.538 (F)
Age (years), mean (S.D.)	47.9 (10.1)	49.1 (9.3)	0.391 (T)
HCV levels (kcopies/mL)			
<500	37	25	0.677 (MH)
500 to <850	46	21	
≥850	84	59	
ALT levels (IU/mL)			
50 to <100	87	48	0.228 (MH)
100 to <150	41	26	
≥150	39	31	
IFN treatment history			
Naïve	39	17	0.060 (MH)
Relapser	82	53	
Nonresponder	40	35	

F, Fisher's exact test; T, *t*-test; MH, Mantel–Haenszel test.

3.3. Association between virological efficacy and sustained ALT normalization

The patients judged to have sustained ALT normalization were divided into SVR and non-SVR patients based on virological efficacy and the effect of the addition of ribavirin to IFN assessed (see Table 2). The sustained ALT normalization

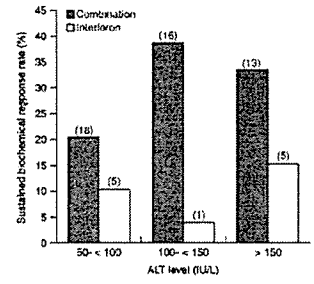

Fig. 1. Rate of sustained biochemical response to treatment by baseline ALT levels. The rate of sustained ALT normalization was significantly better with combination therapy than with interferon monotherapy (Mantel–Haenszel test: $P<0.01$). Numbers of patients in the parenthesis.

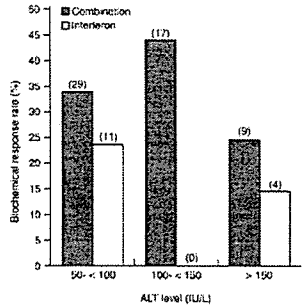

Fig. 2. Rate of patients with ALT levels sustained at less than twice the upper limit of normal (Mantel–Haenszel test: $P < 0.01$). ALT levels were measured ≥4 times during the post-treatment follow-up period. Numbers of patients in the parenthesis.

Table 2
Rate of sustained ALT normalization by virological response at end of follow-up and baseline ALT levels

ALT level (IU/L)	IFN + ribavirin		IFN alone	
	SVR	Non-SVR	SVR	Non-SVR
50 to <100	80% (8/10)	13% (10/77)	–	10% (5/48)
100 to <150	75% (6/8)	30% (10/33)	–	4% (1/26)
≥150	90% (9/10)	14% (4/29)	100% (2/2)	10% (3/29)
Total	82% (23/28)	17% (24/139)	100% (2/2)	9% (9/103)

rate in non-SVR patients was 17.3% (24/139) in the combination therapy group and 8.7% (9/103) in the monotherapy group. The results of the Mantel–Haenszel test taking ALT levels into account showed that combination therapy was significantly superior to IFN alone ($P = 0.034$). Logistic regression analysis determined the factors for sustained ALT normalization in both treatment groups, and are shown in Table 3. The risk for not achieving sustained ALT normalization in nonresponders to previous IFN treatment was four times higher than in IFN-treatment-naïve patients and relapsers. Among non-SVR patients with sustained ALT normalization, low pretreatment WBC count was the only significant influencing factor (data not shown).

3.4. *Effect of timing of initial ALT normalization*

The timing of initial ALT normalization with respect to pretreatment ALT levels is shown in Fig. 3. Log-rank test

Table 3
Multiple logistic regression analysis of factors associated with sustained ALT normalization

Variables	Odds ratio (adjusted)	95% CI	*P*-value
IFN nonresponder vs. relapser/naïve	0.250	0.093–0.669	0.0148
ALT	1.005	1.001–1.009	0.0116
WBC	1.000	0.999–1.000	0.0416
Serum creatinine	7.959	0.915–69.213	0.0598
IFN relapser vs. nonresponder/naïve	0.558	0.261–1.193	0.1277
Platelet	1.056	0.972–1.148	0.1931

indicated that the timing of normalization was significantly earlier in the combination group of patients with ALT levels ≥100 IU/L. ALT normalization rate at the end of treatment was directly correlated to pretreatment ALT levels: 83.9% (73/87), 80.5% (33/41), and 61.5% (24/39) of those whose baseline ALT was 50 to <100 IU/L, 100 to <150 IU/L, ≥150 IU/L, respectively, in the combination group and 79.2% (38/48), 53.8% (14/26), and 45.2% (14/31), respectively, in the monotherapy group showed normalization. ALT normalization rate at the end of treatment was significantly lower in the monotherapy group versus the combination group ($P = 0.015$; Mantel–Haenszel test).

3.5. *Change in ALT levels by virological efficacy*

Figs. 4 and 5 show mean ALT values during and after treatment in relapsers and nonresponders, respectively. No significant difference was observed between the combination and monotherapy groups in pretreatment ALT levels in either relapsers or nonresponders. When change in ALT levels during treatment in the monotherapy and combination therapy groups was compared, no difference in effect of HCV RNA-negativity during treatment was observed among relapsers. However, when the two treatment groups were compared at all time points after the end of treatment, ALT values were significantly lower in the combination therapy group ($P < 0.001$; Wilcoxon test). Furthermore, the mean value for all measurement time points was <80 IU/L in this treatment group. Moreover, in virological nonresponders all-time point ALT values were significantly lower in the combination group compared

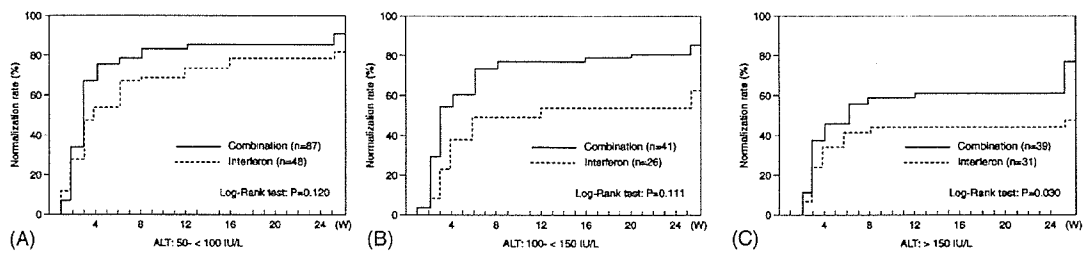

Fig. 3. Initial timing of ALT normalization by pretreatment ALT levels. No significant difference was observed between the combination and monotherapy groups in the timing of initial ALT normalization in patients with low pretreatment ALT levels (50–100 IU/L). In patients with pretreatment ALT levels of ≥100 IU/L, timing of initial ALT normalization was significantly earlier in patients receiving combination therapy than in patients receiving monotherapy.

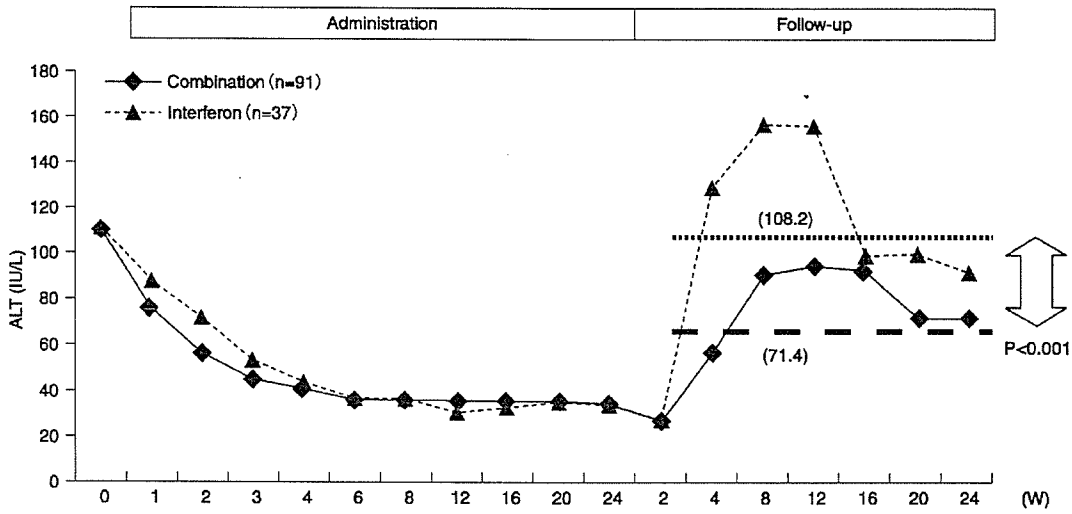

Fig. 4. Changes in ALT levels by viral response: relapsers. No difference was observed between patients receiving combination therapy and monotherapy during the treatment period. After the end of treatment, whereas ALT levels averaged within twice the upper limit of normal in patients receiving combination therapy, a period of marked increase in ALT levels was observed in patients receiving monotherapy. Mean ALT level over the entire period was significantly lower in the combination vs. monotherapy group.

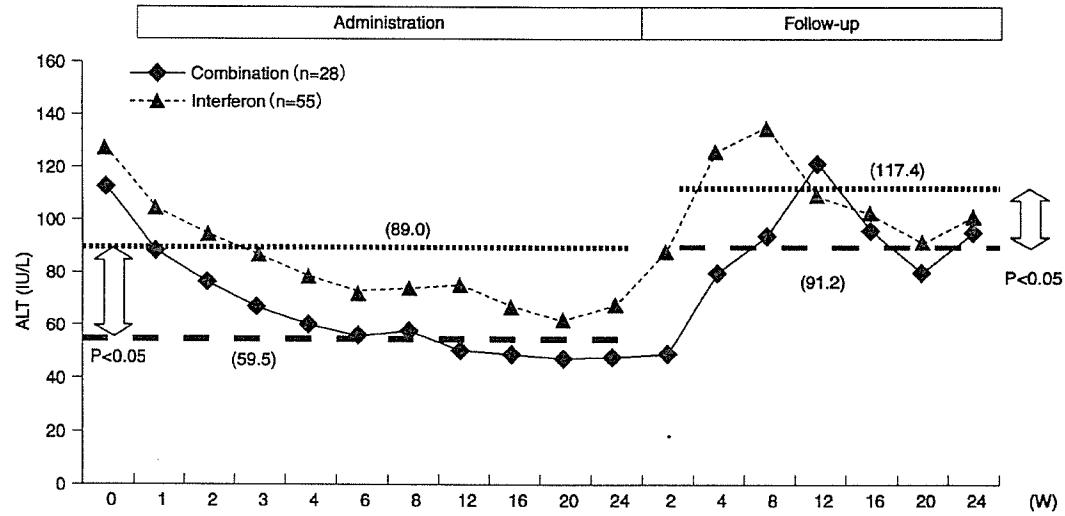

Fig. 5. Changes in ALT levels by viral response: nonresponders. Mean ALT level over the entire period was significantly lower in nonresponder patients receiving monotherapy than in patients receiving combination therapy.

with in the monotherapy group ($P = 0.036$; Wilcoxon test). The mean value for all measurement time points during treatment was <80 IU/L.

4. Discussion

Pretreatment liver function affects progression to liver cirrhosis [24] and gender, alcohol consumption, ALT levels, and histological activity index are factors influencing progression to this condition in HCV-infected patients. This is perhaps related to the observation by Takimoto et al. [8] that achieving SVR in patients with high ALT levels is relatively easy. In the current study, SVR rate was observed to increase in correlation with higher pretreatment ALT levels in both the combination and monotherapy groups (data not shown). On the other hand, Yabuuchi et al. [7] have shown that ALT concentrations in patients with sustained ALT normalization, even in those who become HCV RNA-positive, are significantly lower than in SVR patients and virological nonresponders. In the present study, ALT normalization during treatment occurred at a higher rate in correlation with lower baseline ALT levels, but earlier timing of ALT normalization was not necessarily associated with sustained ALT normalization. However, sustained ALT normalization was achieved more easily in patients with high pretreatment ALT levels regardless of

treatment group. In contrast to non-Japanese reports [25,26], we found that achieving sustained ALT normalization was difficult in nonresponders and relapsers. Older age, longer disease duration, and difference in IFN dose may have been causative factors regarding this result; the reason could not be clarified in this study.

When IFN was first introduced for the treatment of chronic hepatitis C in Japan, the ALT normalization rates reported in genotype 1 patients ranged at about 18–32% [2,3,6]. Many of the patients included in the present study were nonresponders and relapsers to previous treatment, but nevertheless the observed sustained ALT normalization rate (15%) was not very different from those reported previously. In large-scale Japanese clinical studies, the incidence of sustained ALT normalization in non-SVR patients with genotype 1 was about 7–16% [2,4,6,7]. The incidence is estimated about 10% at maximum with IFN monotherapy. The present study not only indicates that addition of ribavirin improves SVR rate but also increases the incidence of sustained ALT normalization including in non-SVR patients compared with IFN monotherapy. Hence sustained liver function normalization was improved by about 30%. Furthermore, ribavirin add-on therapy significantly boosted the number of patients whose ALT was maintained within twice the upper limit of normal, which may reduce the risk of progression to HCC.

An issue that is gaining increasing importance for the future is the method of prevention of progression to HCC in virological nonresponders [27]. In Japan, the long-term rate of hepatocarcinogenesis is considered not greatly different between virological nonresponders to IFN therapy and untreated patients [5,8]. The 5-year incidence of cancer in virological nonresponders has been reported variously at 5–14% [6,7]. Yearly incidence rates of about 1–2% [4,8,28] including a high 5.1% [5] have also been reported. In studies conducted in the USA and Europe, the 5-year incidence rate of liver cirrhosis and HCC in virological nonresponders was 27.8% and 27%, respectively [29,30]. Furthermore, the incidence of progression to HCC in patients with advanced histology followed for 5–7 years was 9.6% [30]. We observed significantly lower on-treatment ALT levels in combination therapy patients versus those on monotherapy, which remained significantly lower after the end of treatment. The timing of ALT flare-up was also delayed in the combination group. This result cannot be explained by differences in change of HCV levels in the two groups since these were not significant (data not shown). However, whether the sustained low ALT levels associated with IFN plus ribavirin combination therapy will lead to less HCC will only be revealed with longer follow-up. Since improved liver function by continued ribavirin monotherapy in virological nonresponders to IFN plus ribavirin has been reported [31], long-term residual effects of ribavirin even after the end of treatment are a possibility. A large-scale clinical study of the effect of long-term treatment with PEG-IFN on prevention of progression to HCC in virological nonresponders to PEG-IFN plus ribavirin combination therapy is ongoing [32]. Long-term IFN monotherapy was reported to enhance ALT normalization in patients without HCV-RNA negative after previous IFN therapy [33].

On the other hand, in patients who relapsed after the end of treatment, mean ALT after the end of treatment was significantly lower in combination versus monotherapy patients. Relapse was observed delayed at 4 weeks after the end of treatment in the combination group (data not shown), suggesting a contribution of HCV to difference in the pattern of change in ALT levels. Viral relapse rate is known to be lowered by long-term combination therapy [12,13,34], and there is much expectation for increased efficacy by this regimen. The results of studies designed to test the hypothesis that time to onset of HCC is prolonged by combination therapy are awaited.

The present study was limited in that while it conclusively demonstrates that combination therapy with ribavirin increases the rate of sustained ALT normalization compared with IFN monotherapy, it was not powered to show prevention of progression to HCC in the long term. Large-scale clinical trials are necessary to examine this postulate. In particular, it is important to determine whether the period of prevention to progression to HCC is extended in virological nonresponders to combination therapy.

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References

[1] Kiyosawa K. Characteristics of Japanese hepatocellular carcinoma—its position in worldwide status of hepatocellular carcinoma. White paper for hepatocellular carcinoma. Tokyo: Japanese Society for Hepatology; 1999. p. 5–9 [in Japanese].
[2] Iino S. Incidence of progression to hepatocellular carcinoma following interferon treatment of chronic hepatitis C using a survey by questionnaire. Annual report from Non-A, Non-B Hepatitis Research Group sponsored by the Ministry of Health and Welfare. Tokyo; 1997. p. 49–52 [in Japanese].
[3] Kasahara A, Hayashi N, Mochizuki K, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Hepatology 1998;27:1394–402.
[4] Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. Ann Intern Med 1999;131:174–81.
[5] Okanoue T, Itoh Y, Minami M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. J Hepatol 1999;30:653–9.
[6] Ikeda K, Saitoh S, Arase Y, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. Hepatology 1999;29:1124–30.
[7] Yabuuchi I, Imai Y, Kawata S, et al. Long-term responders without eradication of hepatitis C after interferon therapy: characterization

of clinical profiles and incidence of hepatocellular carcinoma. Liver 2000;20:290–5.
[8] Takimoto M, Ohkoshi S, Ichida T, et al. Interferon inhibits progression of liver fibrosis and reduces the risk of hepatocarcinogenesis in patients with chronic hepatitis C. Dig Dis Sci 2002;47:170–6.
[9] Tarao K, Rino Y, Ohkawa S, et al. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. Cancer 1999;86:589–95.
[10] Nishiguchi S, Shiomi S, Nakatani S, et al. Prevention of hepatocellular carcinoma in patients with chronic active hepatitis C and cirrhosis. Lancet 2001;357:196–7.
[11] Davis GL, Esteban-Mur R, Rustig V, et al. Interferon alfa-2b or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. New Engl J Med 1998;339:1493–9.
[12] Poynard T, Marcellin P, Lee SS, et al. Randomised trial of interferon α2b plus ribavirin for 48 weeks or for 24 weeks versus interferon α2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 1998;352:1426–32.
[13] McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alfa-2b or in combination with ribavirin as initial treatment for chronic hepatitis C. New Engl J Med 1998;339:1485–92.
[14] Poynard T, McHutchison J, Davis G, et al. Impact of interferon alfa-2b and ribavirin on progression of liver fibrosis in patients with chronic hepatitis C. Hepatology 2000;32:1131–7.
[15] Poynard T, McHutchison J, Manns M, et al. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. Gastroenterology 2002;122:1303–13.
[16] Di Bisceglie AM, Conjeevarum HS, Fried MW, et al. Ribavirin as therapy for chronic hepatitis C. A randomized, double-blind trial. Ann Intern Med 1995;123:897–903.
[17] Duseiko G, Main J, Thomas H, et al. Ribavirin treatment for patients with chronic hepatitis C: results of a randomized-controlled study. J Hepatol 1996;25:591–8.
[18] Hung CH, Lee CM, Lu SN, et al. Is delayed normalization of alanine aminotransferase a poor prognostic predictor in chronic hepatitis C patients treated with a combined interferon and ribavirin therapy? J Gastroenterol Hepatol 2002;17:1307–11.
[19] Yang HC, Lai MY, Chen PJ, et al. The effect of interferon plus ribavirin or interferon alone on the development of hepatocellular carcinoma in non-cirrhotic patients with chronic hepatitis C. J Hepatol 2002;36(Suppl. 1):250 [Abstract 899].
[20] Yu ML, Chuang WL, Dai CY, et al. Preventive effects of antiviral therapy on progression of chronic hepatitis C virus infection to liver cirrhosis and hepatocellular carcinoma in Taiwan. J Hepatol 2003;38(Suppl. 2):183 [Abstract 632].
[21] Iino S, Matsushima T, Kumada H, et al. Comparison of ribavirin (SCH18908) and interferon α-2b combination therapy and interferon α-2b monotherapy in chronic hepatitis C patients of genotype 1b and high viral load—a double-blind parallel study to determine dosage and administration. Rinsho-Iyaku 2002;18:565–91 [in Japanese].
[22] Toyota J, Sainokami S, Yasuda K, et al. Comparison of interferon α-2b and ribavirin (SCH18908) combination therapy and interferon α-2b monotherapy in chronic hepatitis C patients who have not responded or relapsed to previous interferon therapy—double-blind comparative study to examine concomitant efficacy. Rinsho-Iyaku 2002;18:539–63 [in Japanese].
[23] Iino S, Tomita E, Kumada H, et al. Prediction of treatment outcome with daily and high dose interferon α-2b plus ribavirin combination therapy in the treatment of chronic hepatitis C with genotype 1b and high HCV RNA levels: relationship of baseline viral levels and viral dynamics during and after therapy. Hepatol Res 2004;30:63–70.
[24] Freeman AJ, Law MG, Kaldor JM, et al. Predicting progression to cirrhosis in chronic hepatitis C virus infection. J Viral Hepat 2003;10:285–93.
[25] Di Bisceglie AM, Thompson J, Smith-Wilkaitis N, et al. Combination of interferon in chronic hepatitis C: re-treatment of nonresponders to interferon. Hepatology 2001;33:704–7.
[26] Bonkovsky HL, Stefancyk D, McNeal K, et al. Comparative effects of different doses of ribavirin plus interferon-α2b for therapy of chronic hepatitis C. Results of a controlled, randomized trial. Dig Dis Sci 2001;46:2051–9.
[27] Ueda E, Enomoto N, Sakamoto N, et al. Changes of HCV quasispecies during combination therapy with interferon and ribavirin. Hepatol Res 2004;29:89–96.
[28] Suzuki K, Ohkoshi S, Yano M, et al. Sustained biochemical remission after interferon treatment may closely be related to the end of treatment biochemical response and associated with a lower incidence of hepatocarcinogenesis. Liver 2003;23:143–7.
[29] Galeras JA, Crera I, Coll S, et al. Long-term follow-up of chronic hepatitis C patients non-responders to antiviral treatment. Hepatology 2003;38:442A [Abstract 583].
[30] Pradat P, Tillman HL, Braconier JH, et al. Long-term follow-up of chronic hepatitis C patients—response to therapy and incidence of liver-related complications. Hepatology 2003;38:431A [Abstract 562].
[31] Hoofnagle JH, Ghany MG, Kleiner DE, et al. Maintenance therapy with ribavirin in patients with chronic hepatitis C who fail to respond to combination therapy with interferon alfa and ribavirin. Hepatology 2003;38:66–74.
[32] Shiffman ML, Di Bisceglie AM, Lindsay KL, et al. Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment. Gastroenterology 2004;126:1015–23.
[33] Nomura H, Tanimoto H, Sou S, et al. Pilot study of prolonged interferon-α retreatment in chronic hepatitis C patients with genotype 1b. Hepatol Res 2003;27:266–71.
[34] Hiramatsu N, Kasahara A, Nakanishi F, et al. The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan. Hepatol Res 2004;29:142–7.

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Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

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Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance: Negative ($n = 54$)	Positive ($n = 27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

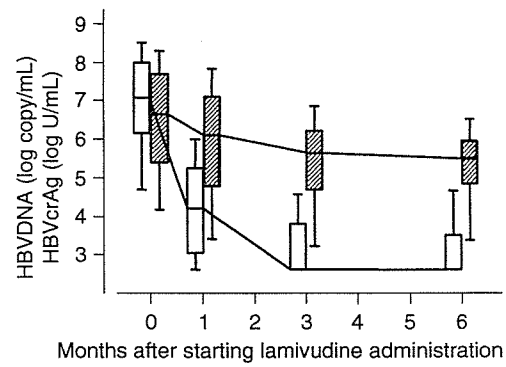

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

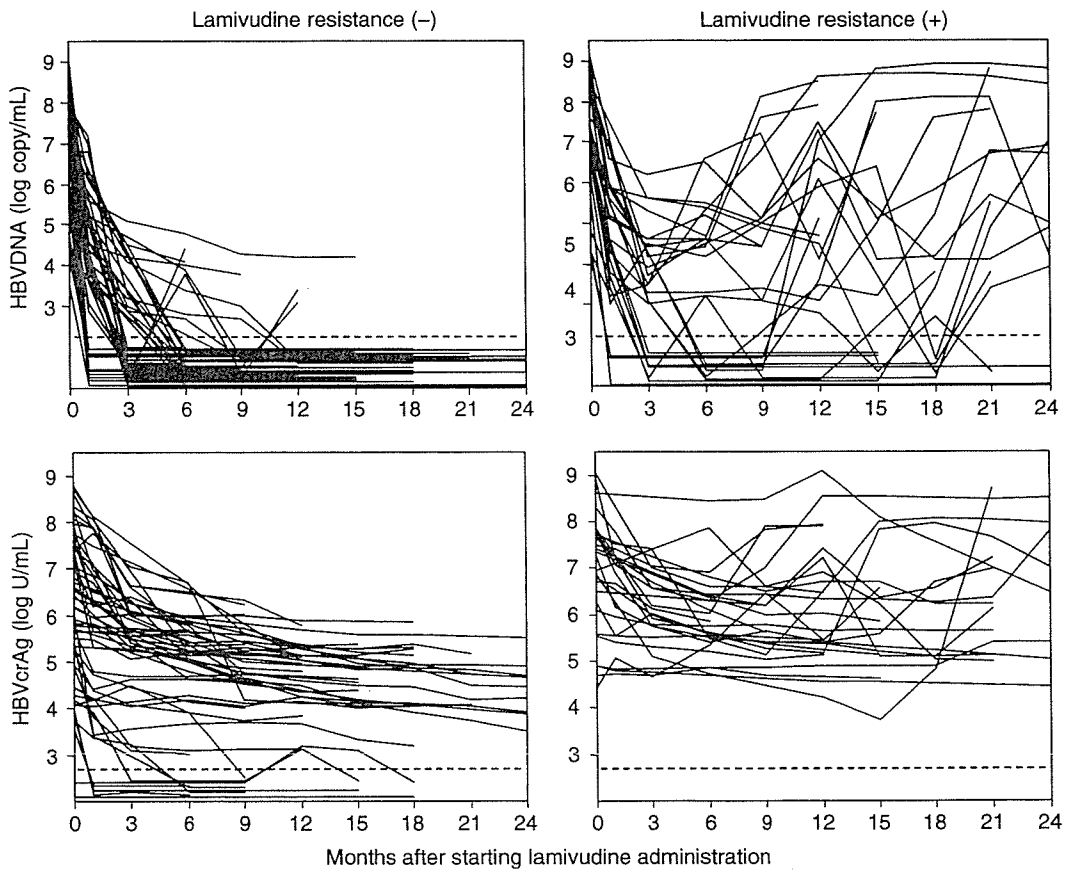

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

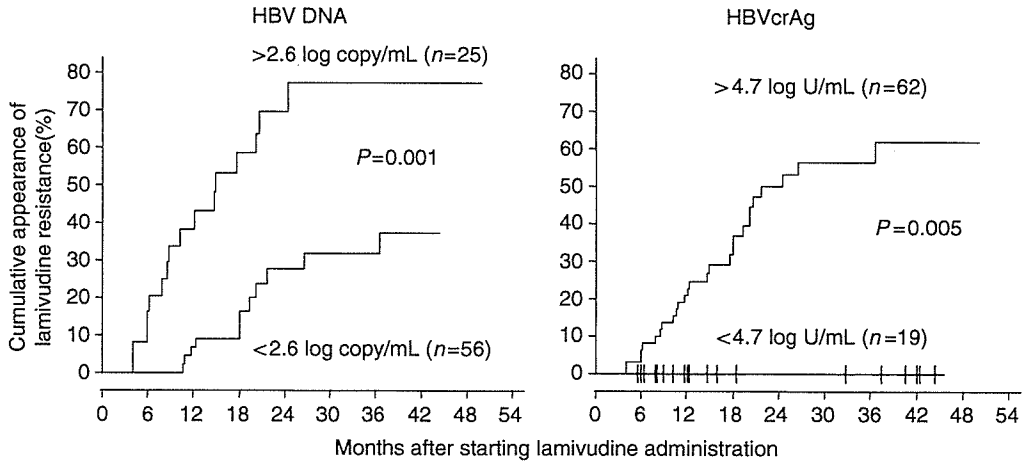

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

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References

1. Doong S L, Tsai C H, Schinazi R F, Liotta D C, Cheng Y C. Inhibition of the replication of hepatitis B virus in vitro by 2′, 3′-dideoxy-3′-thiacytidine and related analogues. Proc Natl Acad Sci USA 1991; 88: 8495–9.
2. Benhamou Y, Dohin E, Lunel-Fabiani F, Poynard T, Huraox J M, Katlama C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995; 345: 396–7.
3. Dienstag J L, Goldin R D, Heathcote E J, Hann H W, Woessner M, Stephenson S L, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003; 124: 105–17.
4. Dienstag J L, Perrillo R P, Schiff E R, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995; 333: 1657–61.
5. Lai C L, Chien R N, Leung N W, Chang T T, Guan R, Tai D I, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339: 61–8.
6. Liaw Y F, Sung J J, Chow W C, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004; 351: 1521–31.
7. Matsumoto A, Tanaka E, Rokuhara A, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2,795 patients. Hepatol Res 2005; 32: 173–84.
8. Ling R, Mutimer D, Ahmed M, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996; 24: 711–3.
9. Lok A S, Lai C L, Leung N, Yao G B, Cui Z Y, Schiff E R, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 2003; 125: 1714–22.
10. Tipples G A, Ma M M, Fischer K P, Bain V G, Kneteman N M, Tyrrell D L. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996; 24: 714–7.
11. Liaw Y F, Chien R N, Yeh C T, Tsai S L, Chu C M. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 1999; 30: 567–72.
12. Suzuki F, Tsubota A, Arase Y, Suzuki Y S, Akuta N, Hosaka T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 2003; 46: 182–9.
13. Zollner B, Schafer P, Feucht H H, Schroter M, Petersen J, Laufs R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. J Med Virol 2001; 65: 659–63.
14. Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 2002; 40: 439–45.
15. Rokuhara A, Tanaka E, Matsumoto A, Kimura T, Yamaura T, Orii K, et al. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J Viral Hepatol 2003; 10: 324–30.
16. Bruss V, Gerlich W H. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. Virology 1988; 163: 268–75.
17. Garcia P D, Ou J H, Rutter W J, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J Cell Biol 1988; 106: 1093–104.
18. Lee W M. Hepatitis B virus infection. N Engl J Med 1997; 337: 1733–45.
19. Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999; 450: 66–71.
20. Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). Hepatol Res 2000; 17: 31–42.
21. Mason W S, Halpern M S, England J M, Seal G, Egan J, Coates L, et al. Experimental transmission of duck hepatitis B virus. Virology 1983; 131: 375–84.
22. Summers J, Smith P M, Horwich A L. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J Virol 1990; 64: 2819–24.
23. Tuttleman J S, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 1986; 47: 451–60.

24. Moraleda G, Saputelli J, Aldrich C E, Averett D, Condreay L, Mason W S. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. J Virol 1997; 71: 9392–9.
25. Werle-Lapostolle B, Bowden S, Locarnini S, Worsthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 2004; 126: 1750–8.
26. Zhu Y, Yamamoto T, Cullen J, et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. J Virol 2001; 75: 311–22.
27. Lau D T, Khokhar M F, Doo E, Ghany M G, Herion D, Park Y, et al. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000; 32: 828–34.
28. Yuen M F, Sablon E, Hui C K, Yuan H J, Decraemer H, Lai C L. Factors associated with hepatitis B virus DNA breakthrough in patients receiving pro longed lamivudine therapy. Hepatology 2001; 34: 785–91.
29. Mihm U, Sarrazin C, Herrmann E, Teuber G, von Wagner M, Kronea berger B, et al.Response predictors and results of a long-term treatment with lamivudine in patients with chronic hepatitis B. Z Gastroenterol 2003; 41: 249–54.