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Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism

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Abstract

Recently hepatitis B virus genotype C (HBV/C) has been classified into geographically typical two subtypes (subgenotypes); HBV/C1 in Southeast Asia (Cs) and HBV/C2 in East Asia (Ce). Our aim is to develop a rapid subtyping assay and to examine the virological features of these two subtypes. Based on 171 HBV/C strains retrieved from the database, 17 single nucleotides polymorphisms (SNPs) were found between two subtypes. Taking advantage of five SNPs in non-overlapping polymerase region, a restriction fragment length polymporphism method with three endonucleases was newly developed for distinguishing between HBV/Cs and HBV/Ce. The method was applied to 49 HBV/C carriers from Japan and Hong Kong. The 24 in Hong Kong were classified into HBV/Cs, and the 25 in Japan were HBV/Ce, confirmed by sequencing. Some specific mutations were detected in the encapsidation signal; precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858, was found in HBV/Ce strains, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found in HBV/Cs. Especially, two closely linked mutations (A1896 and A1899) in HBV/Ce could stabilize the epsilon loop structure more efficiently and influece viral replication. Hence, these virological differences between the two subtypes might influence clinical features.

Keywords: Hepatitis B virus; Single nucleotides polymorphisms; Subgenotypes

1. Introduction

HBV genotypes have a distinct geographical distribution and correlate with severity of liver disease [1,2]. Genotypes B and C are prevalent in Asia, and genotype C causes more serious liver disease than genotype B [3,4]. HBV strains even of the same genotype may differ both virologically and clinically. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j" for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Additionally, there have been some lines of evidence for virological and clinical differences between HBV/Aa in Africa and HBV/Ae in Europe and the US [8,9]. Infection with HBV/Aa is associated with low serum levels of HBV DNA as well as low prevalence of hepatitis B e antigen (HBeAg) in serum, and is implicated in the high incidence of HBV-induced hepatocellular carcinoma (HCC) in Africa [10,11].

Recently, phylogenetic analysis of the pre-S1/pre-S2 genes revealed two major groups within genotype C: one for strains from southeast Asia including Vietnam, Myanmar and Thailand (named HBV/C1) and the other for strains from

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(far) East Asia including Japan, Korea and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [12], and by a recent independent study in Hong Kong [13]. The latter paper designated the two subtypes (subgenotypes) as HBV/Cs in Southeast Asia and HBV/Ce in the (far) East Asia that have different epidemiological distributions [13]. However, further studies are required to evaluate clinical and virological significance between HBV/C1 (Cs) and HBV/C2 (Ce), and development of a simple and efficient method for classification is essential.

In this study, we investigated single nucleotides polymorphisms (SNPs) between HBV/Cs and HBV/Ce at complete genome levels, and developed a novel polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method in the non-overlapping polymerase region involving five SNPs to distinguish between HBV/Cs and HBV/Ce precisely.

2. Materials and methods

2.1. Subjects

A total 49 sera containing HBV/C determined by the ELISA on preS2-region products [14,15], with the results confirmed by PCR-RFLP of the S gene [16], were obtained from chronic carriers of HBV who visited Nagoya City University hospital in Japan or Queen Mary Hospital in Hong Kong. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and an informed consent was obtained from each HBV carrier. To determine SNPs between HBV/Cs and HBV/Ce, 34 HBV/Cs and 137 HBV/Ce complete sequences were additionally recruited from DDBJ/EMBL/GenBank database.

2.2. PCR-RFLP for distinguishing between subtypes (subgenotypes) Cs and Ce of HBV genotype C

Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for specific determination of HBV/C consisted of two PCR cycles with hemi-nested primers followed by RFLP with the restriction site specific for HBV/Cs or Ce. The first-round PCR was performed with a sense primer (HBV964F: 5′-ATT AGA CCT ATT GAT TGG AAA GT-3′ [nt 964-986]) and an antisense primer (HBV1272R: 5′-AGT ATG GAT CGG CAG AGG AG-3′ [nt 1272-1253]) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2: 5′-CCT ATT GAT TGG AAA GTA TGT CA-3′ [nt 970-992]) and an antisense primer (HBV1272R). To determine HBV/Cs, a portion (5 μl) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *Ase*I at 37 °C and *Bst*EII at 60 °C for 1 h each. For HBV/Ce digestion, *Nci*I was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (w/v) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

2.3. Amplification and sequencing of the core promoter as well as the precore region plus core gene

To confirm the results by PCR-RFLP, HBV DNA sequences bearing the core promoter and precore/core regions were amplified by PCR with hemi-nested primers by the method described previously [17], with slight modifications. In brief, the first round of PCR was performed with sense primer (HB7F-2: 5′-CAT GGA GAC CAC CGT GAA CGC-3′ [nt 1607–1627]) and antisense primer (HB8R-2: 5′-ATA GGG GCA TTG GTC T-3′ [nt 2314–2299]) for 40 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min [6 min in the last cycle]) in a 96-well cycler (GeneAmp 9700, Perkin-Elmer Cetus, Norwalk, CA). The second round of PCR was performed with sense primer (HB7F-2) and antisense primer (HB7R-2: 5′-CCT GAG TGC TGT ATG GTG AGG-3′ [nt 2072–2052]) for 35 cycles, under the same conditions as in the first-round PCR. The standard precautions for avoiding contamination during PCR were exercised carefully, and a negative control serum was included in each run of tests to ensure the specificity. Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

2.4. Molecular evolutionary analyses of HBV

Reference sequences were retrieved from the DDBJ/EMBL/GenBank database and their accession numbers for identification. Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the six-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/). Based on these values, a phylogenetic tree was constructed by the neighbor-joining method with the mid-point rooting option.

3. Results

3.1. SNPs for distinguishing between HBV/Cs and HBV/Ce in complete genomes

When the 171 HBV/C (34 HBV/Cs and 137 HBV/Ce) strains, retrieved from the DDBJ/EMBL/GenBank database were compared over the complete genomes, 17 SNPs were found between two subtypes (subgenotypes) (Table 1). Of them, five SNPs in non-overlapping polymerase region include restricted enzyme sites: *Bst*EII site (nt 1041 of T [T1041] and C1044), *Ase*I site (A1050 and A1053) and *Nci*I site (C1155). Interestingly, the 34 HBV/Cs strains possessed *Bst*EII site (G/GTNACC [nt 1039–1045]) and/or *Ase*I

Table 1
Subtype-specific mutations in the complete genomes of HBV/Cs and HBV/Ce

SNPs no.	Nucleotide position	Cs ($n=34$)	Unmatched	Amino acids/region	Ce ($n=137$)	Unmatched	Amino acids/region	Enzymes
1	166	C	0	Thr/S, **His/P**	A	1	Thr/S, **Asn/P**	
2	312	T	2	**Leu/S**, Phe/P	C	1	**Ser/S**, Phe/P	
3	400	C	3	Ile/S, **Leu/P**	A	0	Ile/S, **Ile/P**	
4	**1041**	**T**	**6**	**Gly/P**	**C**	**10**	**Gly/P**	*BstE*II
5	**1044**	**C**	**0**	**Thy/P**	**T**	**1**	**Thy/P**	*BstE*II
6	1047	A	0	Pro/P	T	3	Pro/P	
7	**1050**	**A**	**2**	**Ala/P**	**C**	**20**	**Ala/P**	*Ase*I
8	**1053**	**A**	**1**	**Leu/P**	**A/G**	**1**	**Leu/P**	*Ase*I
9	**1155**	**T**	**0**	**Ala/P**	**C**	**9**	**Ala/P**	*Nci*I
10	1721	A	1	Val/X	G	0	**Leu/X**	
11	2065	A	0	Leu/C	C	7	Leu/C	
12	2158	A	2	Val/C	C	5	Val/C	
13	2559	A	0	Lys/P	C	3	**Gln/P**	
14	2561	A	1	Lys/P	G	5	**Gln/P**	
15	2633	G	0	Leu/P	A	0	Leu/P	
16	2958	T	2	**Phe/P**, Asn/PreS1	C	4	**Leu/P**, Asn/PreS1	
17	3008	C	1	**Ser/P, Ala/PreS1**	A	5	**Arg/P, Asp/PreS1**	

(AT/TAAT [nt 1050–1055]), while the 137 HBV/Ce strains had neither *BstE*II nor *Ase*I sites. On the other hand, 128 of 137 (93%) HBV/C2 strains possessed *Nci*I site (CC/SGG [nt 1154–1158]) and none of the HBV/C1 strains had *Nci*I site due to T1151. Additionally, according to the SNPs, eight amino acids differences were found between two subtypes (subgenotypes) (Table 1).

3.2. PCR-RFLP for distinguishing between HBV/Cs and HBV/Ce

Geographically, typical genetic representatives for HBV/Cs and HBV/Ce (eight strains each) were selected. The partial genome sequence alignment including restriction sites is shown in Fig. 1. HBV/Cs strains were obtained

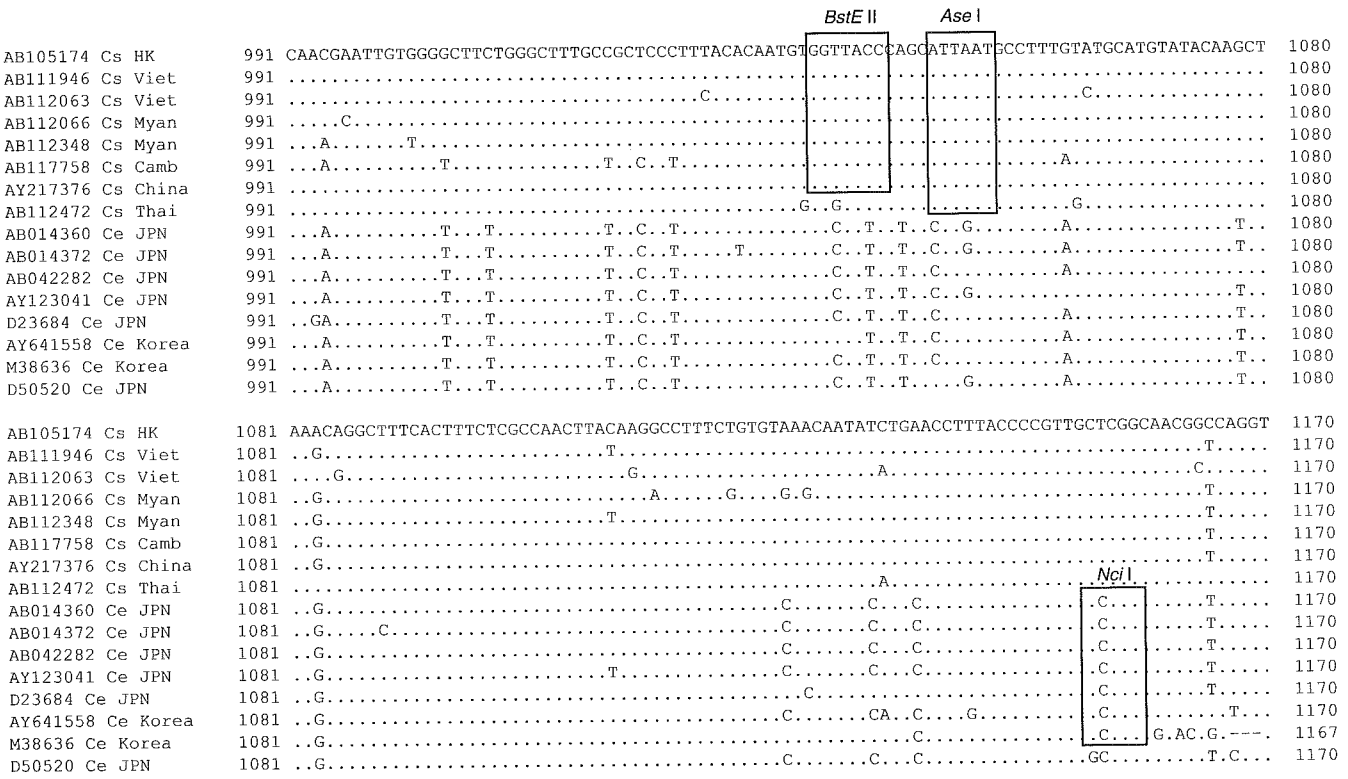

Fig. 1. Alignment of 8 HBV/Cs (C1) and 8 HBV/Ce (C2) sequences in non-overlapping polymerase region. The specific *BstE*II and *Ase*I sites are specific for HBV/Cs strains, while *Nci*I site is found in HBV/Ce strains. All sequences from the database are identified with accession numbers, followed by subtype and the country of origin in abbreviation for Cambodia (Camb), Hong Kong (HK), Japan (JPN), Myanmar (Myan) and Vietnam (Viet).

from Vietnam, Thailand, Myanmar, China, Hong Kong and HBV/Ce from Japan, Korea. The subtypes (subgenotypes) of the 16 strains were confirmed by a phylogenetic analysis of the complete genome (Fig. 2a). Taking advantage of the five SNPs of T1041, C1044, A1050, A1053 and C1155, a RFLP method with three endonucleases was developed for distinguishing between HBV/Cs and HBV/Ce. PCR products of 309 bp in size (nt 964–1272), amplified on HBV/Cs strains, were split by *Ase*I digestion into two fragments of 88 and 221 bp and/or *BstE*II digestion into two fragments of 76 and 233 bp (Fig. 3), while those on HBV/Ce strains were not. In contrast, the products of 309 bp, amplified on HBV/Ce strains, were broken down by *Nci*I digestion into two fragments of 192 and 117 bp, while those on HBV/Cs strains were not.

Total 49 HBV/C samples, consisting of 24 in Hong Kong and 25 in Japan, were examined for the specificity of the novel PCR-RFLP method. Based on the PCR-RFLP, the 24 strains from Hong Kong were classified into HBV/Cs, and the 25 from Japan were HBV/Ce. To confirm the reliability of the PCR-RFLP method, the precore region plus core gene was sequenced directly on all 49 samples. All the 24 HBV/Cs and 25 HBV/Ce samples determined by PCR-RFLP were

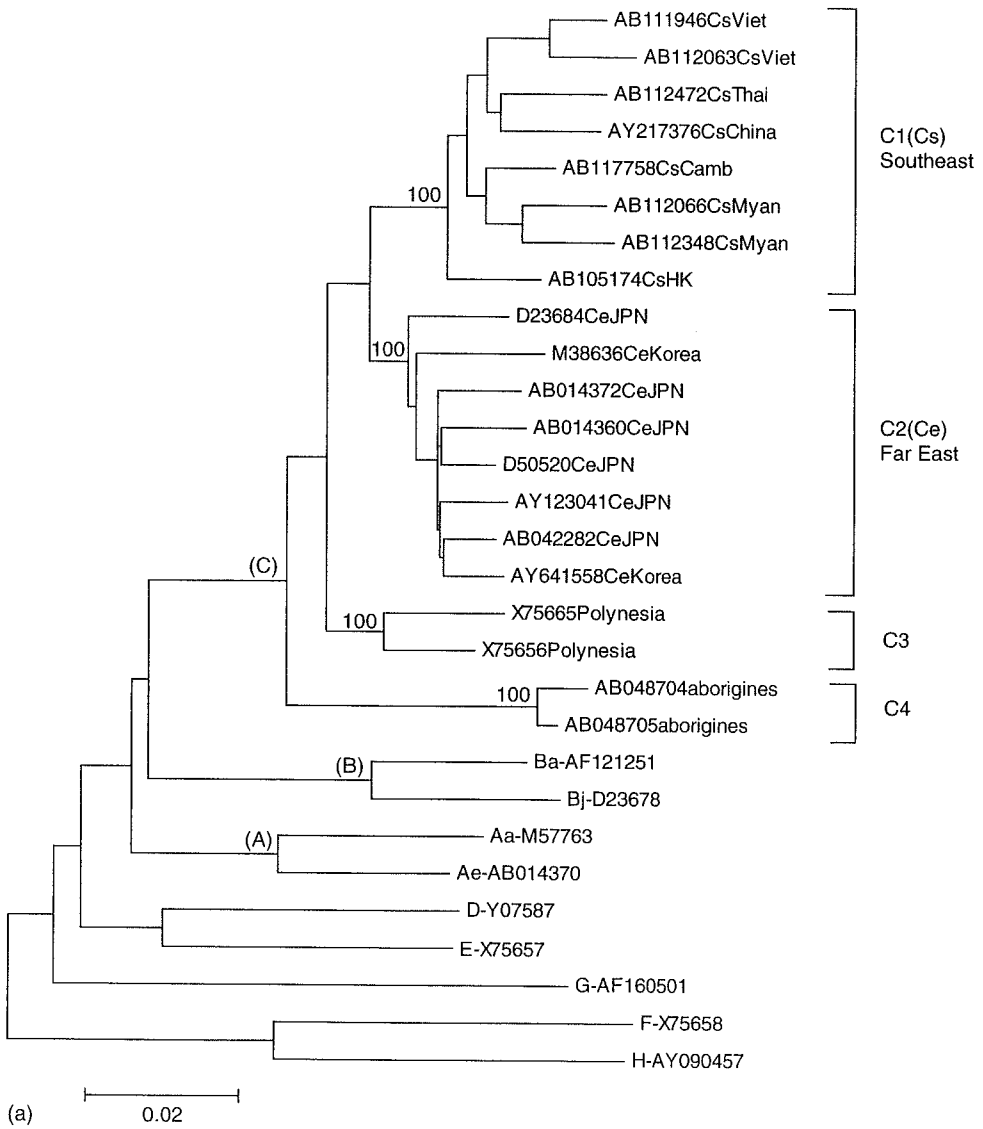

Fig. 2. (a) A phylogenetic tree constructed on the complete genome sequences of 29 HBV strains. Eight HBV/C1 (Cs) and eight HBV/C2 (Ce) strains (shown in Fig. 1) are compared along with four other HBV/C (C3 and C4) and nine HBV strains representative of the other seven genotypes (Aa, Ae, Ba, Bj, D–H). (b) A phylogenetic tree constructed on the X gene, precore and core gene sequences spanning 398 bp. Together with the above 29 representative sequences retrieved from database, 24 HBV/C1 (Cs) strains determined by PCR-RFLP belong to HBV/C1 (Cs) and 25 HBV/C2 (Ce) strains by PCR-RFLP had a cluster with the representative HBV/C2 (Ce) strains from database. All strains in this study are shown in bold. Each representative strain from the database are identified with accession numbers, followed by subtype and the country of origin in abbreviation for Cambodia (Camb), Hong Kong (HK), Japan (JPN), Myanmar (Myan) and Vietnam (Viet). The length of the horizontal bar indicates the number of nucleotide substitution per site.

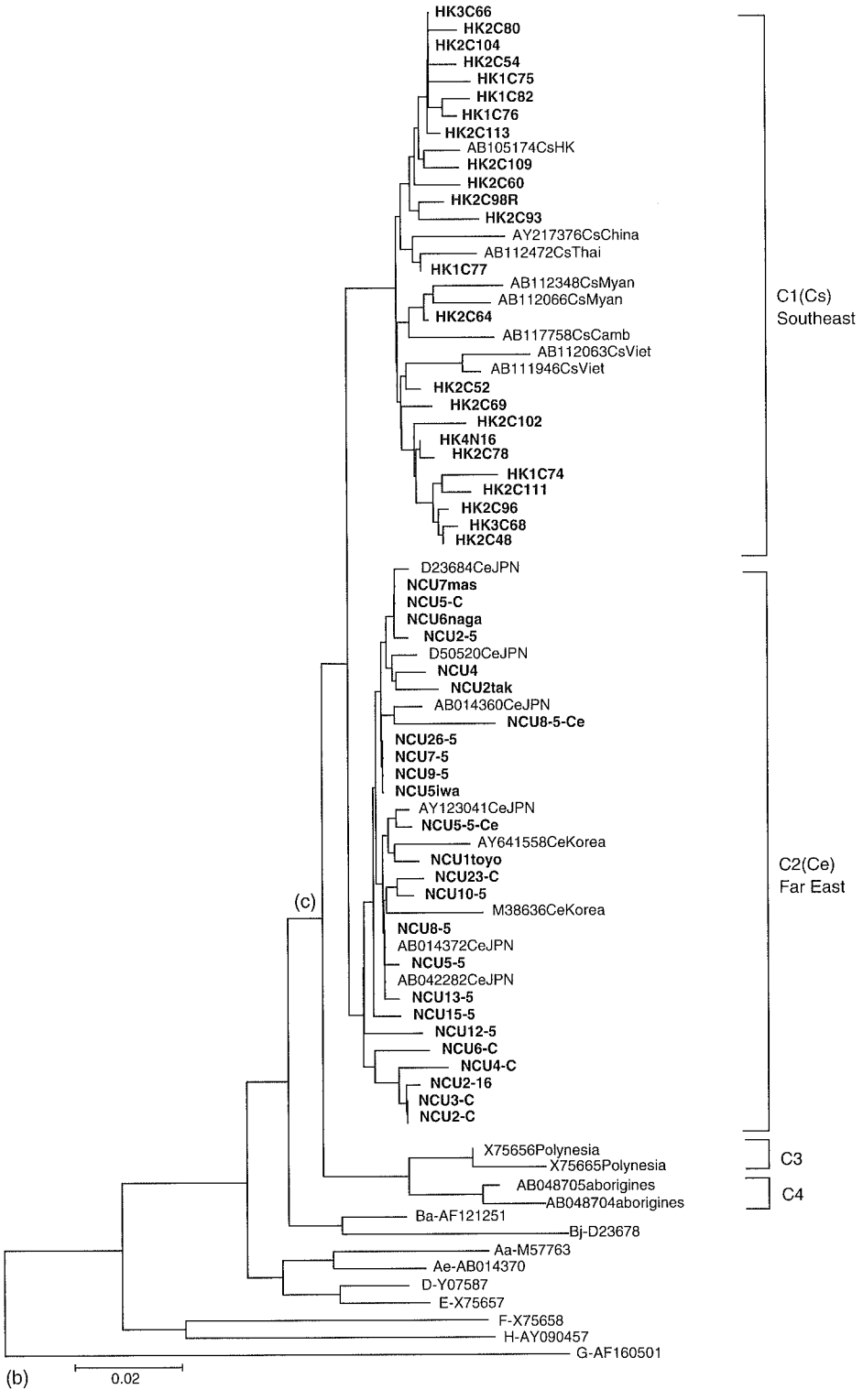

Fig. 2. (*Continued*).

completely classified into each subtype (subgenotype) by sequencing. To evaluate the sensitivity of the method, serial dilution of each HBV/Cs and HBV/Ce clones was used for the hemi-nested PCR, and its detection limit was five copies per assay.

3.3. Mutations in the enhancer, BCP and precore region in patients infected with HBV/C1 and C2

An alignment of sequences covering the BCP and the encapsidation signal (ε) in HBV/Cs and HBV/Ce allowed

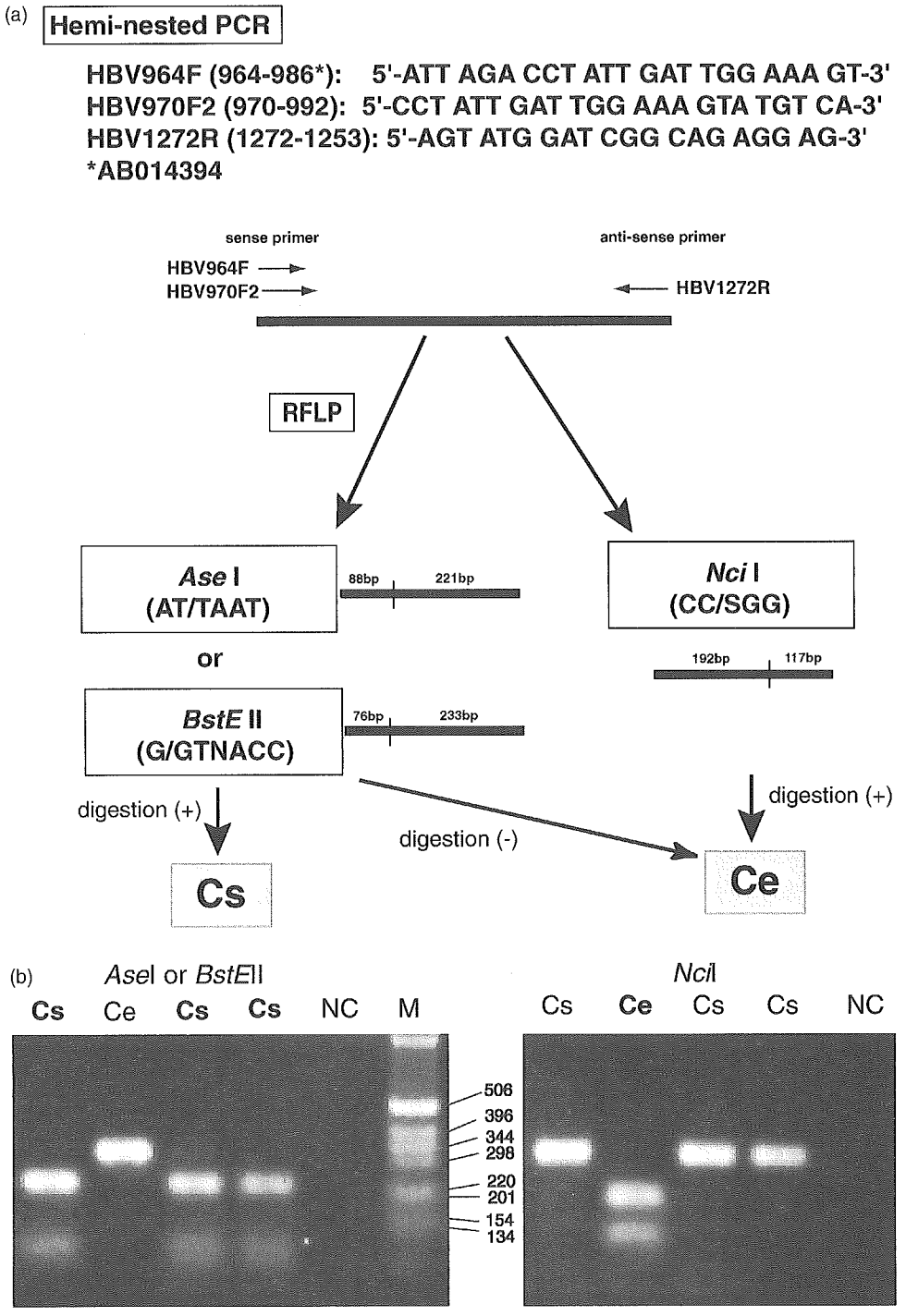

Fig. 3. (a) The strategy of a novel subtyping assay of HBV/C based on PCR-RFLP with *Bst*EII, *Ase*I, *Nci*I restriction enzymes. HBV/Cs is digested by *Bst*EII and/or *Ase*I, while HBV/Ce is digested by only *Nci*I. (b) Identification of restriction patterns obtained by restriction endonuclease digestion. Using hemi-nested PCR followed by cleavage with three kinds of restrict enzyme, it was possible to distinguish between HBV/Cs and HBV/Ce.

the identification of specific substitutions for HBV/C1 and HBV/C2 strains at nt 1721, 1757, 1775, 1856 and 1858 (Table 2). The prevalence of T1653, A1896 and A1899 substitutions was significantly higher in HBV/Ce than that in HBV/Cs, while the prevalence of A1727 and A1898 substitutions was higher in HBV/Cs. Double mutation in BCP (T1762/A1764) was highly prevalent in both subtypes (subgenotypes). Interestingly, the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found only in HBV/Ce strains (45/162, 28%), whereas no mutation was found in HBV/Cs strains due to C1858. Another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found in HBV/Cs strains (7/58, 12%) (Table 2).

Table 2
Subtype-specific mutations in basic core promoter and encapsidational signal of HBV/Cs and Ce strains

Nucleotide position	Cs	This study ($n=24$)	Database ($n=34$)	Ce	This study ($n=25$)	Database ($n=137$)	P-value
1653	T	2 (8%)	0	T	7 (28%)	37 (27%)	<.0001
1721	A	22 (92%)	33 (97%)	G	24 (96%)	137 (100%)	<.0001
1727	A	19 (79%)	30 (88%)	A	13 (52%)	59 (43%)	<.0001
1757	A	13 (54%)	10 (29%)	G	25 (100%)	137 (100%)	<.0001
1762/1764	T/A	21 (88%)	13 (38%)	T/A	20 (80%)	65 (47%)	NS
1775	G	15 (63%)	28 (82%)	A	25 (100%)	132 (96%)	<.0001
1856	T	9 (38%)	6 (18%)	C	25 (100%)	137 (100%)	<.0001
1858	C	23 (96%)	23 (68%)	T	25 (100%)	137 (100%)	<.0001
1896	A	0	0	A	6 (24%)	39 (28%)	<.0001
1898	A	7 (29%)	0	A	0	0	<.0001
1899	A	1 (4%)	1 (3%)	A	4 (16%)	19 (14%)	0.029

4. Discussion

Chronic patients infected with HBV/C have a more aggressive clinical course than those infected with HBV/B [3,18]. In this study, we focused on HBV/C because it is prevalent mainly in Asia and seems to contribute to progressive liver disease and poor clinical outcomes in infected patients. Phylogenetic analyses of the complete genome show at least 4 subtypes (subgenotypes) of HBV/C (C1–4) with different geographic distribution (Fig. 2a) [19,20]. HBV/C1 was found only in Southeast Asia including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong and southern China, while HBV/C2 was found in far East Asia including Japan, Korea and northern China. Additionally, two another subtypes (subgenotypes) of HBV/C were named as C3 and C4 [19,20]. C3 was found in a large area of the Pacific from New Zealand to Polynesia, while C4 was isolated from Aborigines in Northeast Australia [17]. However, as C3 and C4 strains were rarely found in most Asian countries, we focused the classification between Cs (C1) and Ce (C2) in the present study.

A total of 118 complete genome sequences of the HBV/C strains isolated in the different geographic regions were analyzed phylogenetically in the recent study [13]; the phylogenetic subclusters within HBV/C were subsequently designated respectively to the geographic regions, i.e. "Cs" for Southeast Asian (Vietnam, Thailand, Myanmar and Southern China), and "Ce" for far East Asia (Korea, Japan, and Northern China). According to this classification, 80% of the patients in Hong Kong were belonged to the Cs and 20% to the Ce [13]. When taken in account both facts, i.e. evident geographic origins of these subtypes (subgenotypes) and the phylogenetic confirmation, the designation using the small letters (indicating possible origins) appears to be logical, similarly to the previously reported Asian "Ba" and Japanese "Bj" [5,7], Africa/Asian Aa and European Ae [8]. Hence, "Cs" and "Ce" designation was applied to the present study.

Based on five SNPs between HBV/Cs and HBV/Ce, we developed a novel PCR-RFLP method for distinguishing between HBV/Cs and HBV/Ce with high reliability. All 49 samples examined were completely classified by the PCR-RFLP. This method allows the classification between these subtypes (subgenotypes) without using expensive, labor- and time-consuming methods such as sequencing and molecular evolutionary analyses. Examining additional 171 complete sequences from database, only 9 sequences of HBV/Ce have exceptional mutations at the restriction site of *Nci*I, indicating that less than 5% of the strains known up to date are unclassified by this method, and require sequencing as previously described [17].

Some specific mutations were detected in the encapsidation signal site; the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found only in HBV/Ce strains, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found only in HBV/Cs strains (Fig. 4). These mutations could stabilize the ε loop structure and the former HBeAg-negative mutants bearing a TAG stop codon mutation at codon 28 (A1896) uniformly replicate at least 20-fold better than mutants bearing a TGA stop codon at the same amino acid position enhance viral replication [21]. This C1858 variant was frequntly found in HBV/A and HBV/F [22]. Additionally, A1899 mutation was more prevalent in the HBV/Ce. As previously reported, the effects caused by these two closely linked mutations (A1896 and A1899) on viral replication are not independent each other [21]. The stringent selection for a highly efficient RNA encapsidation element may play a crucial role in the natural occurrence of these two closely linked precore mutations. Our replication model also shows that the combined mutations can induce higher replication in vitro (unpublished data). Hence, these several virological differences between the two subtypes (subgenotypes) might influence clinical outcomes such as fulminant hepatitis or hepatocarcinogemesis.

The biologic function of HBeAg remains controversial. Although HBeAg is not required for viral replication, it appears to be necessary for the establishment of chronic infection in animal models [23]. The most common mutation in the precore sequence that abrogates the synthesis of HBeAg is a stop-codon mutation (G1896A). As all HBV/Ce strains possessed T1858 and most HBV/Cs had C1858, the HBV/Cs with C1858 might be responsible for a delayed seroconversion for the loss of HBeAg in the carriers of HBV/Cs. The clinical significance of C1858 and T1858 among HBV/C

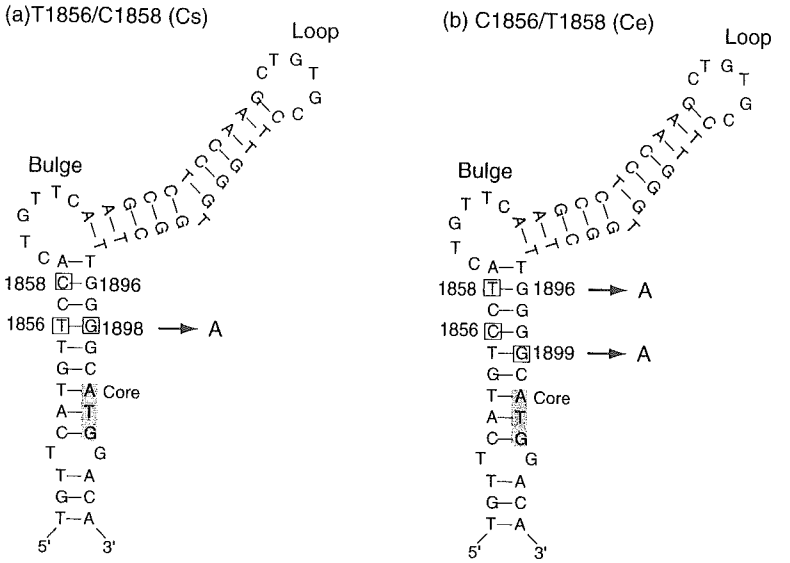

Fig. 4. Conformation of the pregenome encapsidation (ε) signal for (a) HBV/Cs and (b) HBV/Ce. The precore mutation, G1898A accompanied by T1856 forming a base pair with it, was found only in HBV/Cs strains. In contrast, the precore stop mutation, G1896A accompanied by T1858, was found only in HBV/Ce strains. A1899 mutation is significantly predominant in HBV/Ce strains.

is not well known. A previous study among multi-ethnic carriers in Hawaii indicated no significant difference in clinical characteristics between C1858 and T1858 variants [24]. However, as the number of patients was not enough to clarify the significance of this variation, further clinical studies would be required on a case–control study with large-scale cohorts.

A previous study [12] indicated that the amino acid changes specific to HBV/Cs and HBV/Ce were concentrated in the pre-S1, S and P regions, but not in the X and core regions. The pre-S1 region contains the HBV receptor for entering hepatocytes [25] and also has sites for transcriptional factors [26]. Another study [13] showed three amino acids differences in polymerase region. Therefore, the relationship between HBV/Cs and HBV/Ce and their virulence in chronic liver diseases including hepatocellular carcinoma are of great interest, since the prevalence of HBV-related hepatocellular carcinoma is extremely high in Asia compared with other regions.

In conclusion, a new PCR-RFLP method involving 5 SNPs was developed for specifically distinguishing between HBV/Cs and HBV/Ce. The two subtypes (subgenotypes) have distinct geographic distribution and virological characteristics. The novel PCR-RFLP would be useful in evaluating clinical, epidemiological and virological differences between HBV/Cs and HBV/Ce infections in countries where HBV genotype C endemic.

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Effect of Long-Term Postoperative Interferon Therapy on Intrahepatic Recurrence and Survival Rate after Resection of Hepatitis C Virus-Related Hepatocellular Carcinoma

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Key Words
Hepatocellular carcinoma · Liver resection · Interferon · Recurrence · Hepatitis C virus · Survival rate

Abstract
Objective: This study was aimed at evaluating the effects of interferon (IFN)-α on survival rate after resection of hepatocellular carcinoma. ***Methods:*** In a randomized, controlled trial by the University Hospital, Medical Center and affiliated hospital in Osaka, Japan, 30 men were after surgery randomly allocated to an IFN-α group (15 patients) and to a control group. Patients in the IFN group received 6 MIU of IFN-α intramuscularly daily for 2 weeks, then three times a week for 14 weeks, and finally twice a week for 88 weeks. The incidence of recurrence and survival rate were then studied. ***Results:*** The response to IFN was sustained viral response (SVR) in 2 patients, biochemical response (BR) in 6, partial response (PR) in 5, and no response (NR) in 2. In the control, 8 of the 15 patients demonstrated continuous abnormally high levels of ALT. At the end point of the study, intrahepatic recurrence was detected in 9 of the IFN group and in 13 of the control (p = 0.065, log-rank test). The cumulative survival rate was higher in the IFN group than in the controls (p = 0.041). ***Conclusion:*** Postoperative IFN therapy improves the outcome after resection of hepatitis C virus-related hepatocellular carcinoma.

Introduction

The outcome after surgical treatment of hepatocellular carcinoma (HCC) related to hepatitis C virus (HCV) is still unsatisfactory because of high rates of the recurrence of HCC and the progress of the underlying liver disease to chronic hepatitis or cirrhosis [1]. We have reported that interferon (IFN)-α helps to prevent the development of HCC in patients with HCV-related cirrhosis in a prospective randomized controlled study [2] and that postoperative IFN therapy demonstrates a decrease in the incidence of recurrence after resection of HCV-related HCC [3]. Recently, IFN has been found to prevent exacerbation of compensated cirrhosis and to inhibit the development of HCC, resulting in higher survival rates [4]. Here we report on the effects of postoperative IFN therapy on the outcome after resection of HCV-related HCC.

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Table 1. Clinical characteristics in each group

Characteristics	IFN group	Control group	p
Mean age ± SD, years	61.9±5.8	60.0±4.8	0.33
Men:women	15:0	15:0	>0.99
Albumin, g/dl	3.6 (3.3, 4.3)	3.6 (3.3, 4.1)	0.77
ALT activity, IU/l	105 (45, 131)	86 (58, 151)	0.49
Platelets $\times 10^4$ cells/l	14.7 (7.5, 23.1)	11.2 (6.1, 20.9)	0.14
AFP > 100 ng/l	4	4	>0.99
Child-Pugh score A:B	11:4	12:3	>0.99
Tumor size, cm	2.5 (1.9, 3.5)	2.6 (2.4, 3.5)	0.68
Differentiation of tumor			
Well/moderate/poor	1/11/3	2/11/2	0.77
Histological findings			
Grade, 1/2/3	4/6/5	1/7/7	0.33
Stage(s), 1–3/4	8/7	7/8	>0.99

ALT = Alanine aminotransferase; AFP = alpha-fetoprotein; IFN = interferon. Results of laboratory tests are given as medians, with 10th and 90th percentiles in parentheses.

Methods

Patients and Trial Profile

The protocol of this study was as described [3] and comprised the same 30 patients. Thirty male patients who underwent HCC resection were randomly allocated to an IFN-α group (15 patients) and a control group. The criteria for eligibility were (1) single tumor less than 5 cm in maximum diameter disclosed by preoperative imaging; (2) detectable HCV RNA without hepatitis B surface (HBs) antigen or HIV antibodies; (3) chronic hepatitis or a Child-Pugh score of A or B [5] for compensated cirrhosis, and (4) no severe thrombocytopenia. Clinical characteristics and laboratory test results were similar in the two groups, as were the surgical procedures (table 1). Most of the variables in the enrolled patients were similar to those of eligible patients who declined participation in the study. In the IFN group, patients received 6 MIU of IFN-α (human lymphoblastoid interferon; Sumiferon, Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly every day for 2 weeks, then 3 times weekly for 14 weeks, and finally twice weekly for 88 weeks (total dose, 1,572 MIU). None of the patients in the control group received treatment for carcinoma or liver disease after the resection until the detection of recurrence; none of the patients in the IFN group received chemotherapy or any treatment other than IFN administration before the detection of recurrence. Methods for the treatment of the recurrence were determined on the basis of the number and location of the recurrent tumor(s) and on the results of liver function tests. One or two recurrent tumors were treated with ablation therapy such as resection or microwave coagulation therapy. For multiple recurrent tumors or in cases where ablation therapy was considered difficult because of the location of the recurrent tumor(s) or because of liver function, transarterial therapy (transcatheter arterial embolization and hepatic arterial infusion chemotherapy) was applied.

This study was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Committee of our institutions. Written informed consent was obtained from each patient.

Statistical Analysis

For all cases of recurrence, laboratory data before surgery were compared with those upon detection of recurrence; the latter were also compared between the two groups. The Mann-Whitney test was used to evaluate differences among the laboratory test results. The length of time until death was considered the interval between resection and death. The cumulative survival rates (which included data on patients who did not undergo or complete IFN therapy in the IFN group) were calculated by the Kaplan-Meier method, and significant differences between the groups were assessed by the log-rank test. All data were analyzed with SAS statistical software (version 6.12, SAS Institute, Cary, N.C., USA).

Results

The median follow-up period (from the resection to death or to the end point of the study) was 1,817 days (25th and 75th percentiles, 1,579 and 2,008 days) for patients receiving IFN-α and 1,487 days (1,194 and 2,055 days) for the 15 controls. As previously reported [3], IFN therapy was not administered to 1 patient because of premature ventricular contractions, was not completed by 3 patients because of adverse events, and was stopped prematurely in 4 patients with recurrence so that the tumor(s) could be treated. The response to IFN was sustained viral response (SVR) in 2 patients, biochemical response (BR) in 6 patients, partial response (PR) in 5 patients, and no response (NR) in 2 patients (fig. 1). In the controls, 8 of the 15 patients showed continuous abnormally high ALT activity. Intrahepatic recurrence was detected in 9 patients in the IFN group and in 13 controls at the end point of the study (fig. 2, p = 0.065). One or two

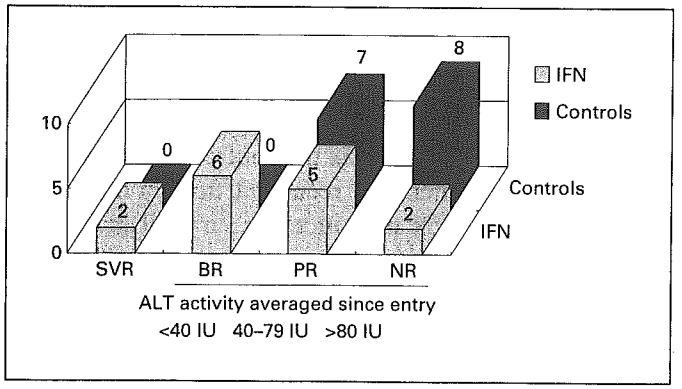

Fig. 1. Effects of IFN on viral clearance and on alanine aminotransferase (ALT) activity averaged since the end of treatment. The response to IFN was a SVR in 2 patients, a BR in 6 patients, a PR in 5 patients, and no response (NR) in 2 patients. In the controls, 8 of the 15 patients continued to have abnormally high ALT activities.

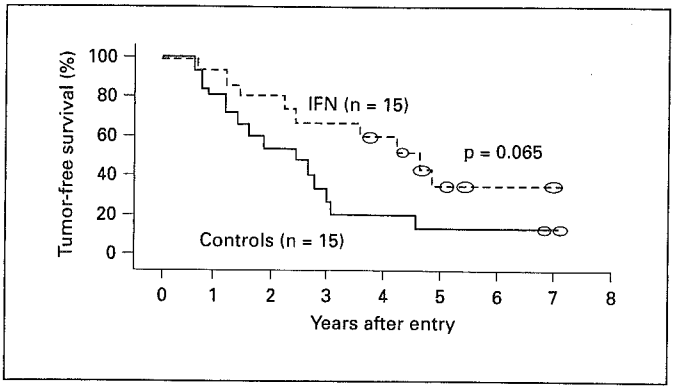

Fig. 2. Nonrecurrence rates after resection in patients given IFN or not. The median follow-up has been 5.0 years for patients receiving IFN-α and 4.1 years for the controls. Recurrences had been detected in 9 patients in the IFN group and in 13 control patients.

recurrent tumors were detected in 8 patients of the IFN group and in 7 of the control; three or more recurrent tumors were found in one of the former group and in six of the latter. Ablation therapy was carried out on 6 patients of each group. Transarterial therapy was used on 3 patients in the IFN group and on 6 control patients. One patient in the control group could not undergo any treatment for recurrent tumors because of decompensation. IFN therapy was completed after treatment of the recurrent tumors at the request of 3 of the 4 patients in whom recurrence was detected during IFN therapy.

In the IFN group, the serum concentration of total bilirubin and ALT activity had decreased by the time recurrence was detected (p = 0.062 and 0.066, respectively). In the control group, the serum concentration of total bilirubin had increased (p = 0.042).

At recurrence, the median serum concentration of albumin was 40 g/l in the IFN group and 37 g/l in the control (table 2, p = 0.052). The total bilirubin concentration was significantly lower in the IFN group than in the control (p = 0.044).

All of the patients who died (3 in the IFN group and 9 in the control) had recurrences of HCC. The cumulative survival rate was significantly higher in the IFN group than in the control (fig. 3, p = 0.041, log-rank test).

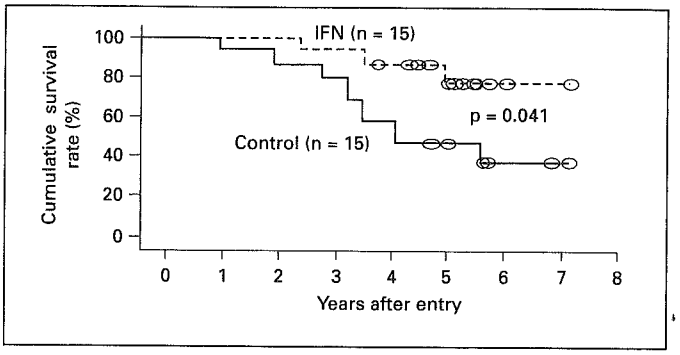

Fig. 3. Cumulative survival rates. Three patients in IFN group and 9 patients in the control group have died. All patients who died had recurrence of HCC.

Table 2. Results of laboratory tests at the detection of recurrent tumor(s)

	IFN group (n = 9)	Control group (n =13)	p
Albumin, g/l	40 (36, 42)	37 (28, 39)	0.052
Total bilirubin, mol/l	12 (10, 17)	18 (10, 35)	0.044
ALT, IU/l	57 (26, 110)	70 (59, 160)	0.12
Platelet count, $\times 10^9$/l	115 (79, 202)	123 (68, 215)	0.85

ALT = Alanine aminotransferase; IFN = interferon. Results of laboratory tests are given as medians, with 10th and 90th percentiles in parentheses.

Discussion

In this study, the cumulative survival rate after resection of HCC was significantly higher in the IFN group than in the control: 1 of the 9 patients in the former group

and 6 of the 13 in the latter had multiple recurrent tumors. Some recurrent tumors were intrahepatic metastases from primary tumors that were not detected at the resection, and other tumors were new carcinomas that developed after the resection. The prevalence of multicentric carcinogenesis increases during progression to active hepatitis and hepatic fibrosis [6, 7]. IFN suppresses the development of HCC by inducing remission of active hepatitis and by improving hepatic fibrosis in patients infected with HCV [2, 4, 8, 9]. IFN may by such effects suppress multiple recurrences arising from multicentric carcinogenesis after surgery. IFN-α has shown antiproliferative effects on a human hepatoma cell line [10]. IFN can enhance natural-killer cell activity and demonstrates antiangiogenic properties [11, 12]. Ikeda et al. [13] have reported that IFN-β prevents recurrence of HCC after treatment, suggesting that it acts as an antitumor agent. A combination of anticancer agents and IFN-α has shown improvement in the survival rate of patients with advanced HCC [14]. Thus, the anticancer effects of IFN may be another reason for the small number of recurrent tumors in our IFN group, which may have contributed to the length of survival time, especially that the occurrence of multiple recurrent tumors is a risk factor for the short survival time reported in other studies [15, 16].

Previous studies on survival after treatment for recurrence have shown that a low serum concentration of albumin and a high one of total bilirubin are risk factors for short survival [15, 16]. Risk factors for short survival in patients with HCV-related cirrhosis are low albumin concentration, high concentration of total bilirubin, and absence of IFN therapy [17]. IFN-α prevents exacerbation of compensated cirrhosis [4]. In this study, laboratory test results showed that liver function improved in the IFN group, but did not change, or even worsened in the control. At the detection of recurrence, the serum concentration of total bilirubin was significantly lower in the IFN group. Moreover, 1 patient in the control group could not undergo any treatment for recurrent tumors because of decompensation. IFN may increase survival in patients given IFN by improving both liver function and indications for radical treatment of recurrences.

In this study, a low platelet count ($<100 \times 10^9$/l) and a high histologic activity index of grade 3 or 4 were not significant factors by the log-rank test ($p = 0.66$ and 0.53, respectively). In a study of 3-year survival, a low platelet count has been shown as a possible risk factor after the detection of recurrence [15]. Although the platelet count at recurrence was not different between our groups, it may have been a contributing factor to the short survival in the control group; the number of patients was too small for a clear conclusion.

This study suggests that postoperative IFN therapy improves the outcome after resection of HCV-related HCC not only because of suppression of recurrence but also because of improvement in liver function. Patients with early-stage HCC and with liver function good enough to undergo liver resection and IFN therapy would be candidates for postoperative IFN therapy.

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Journal of Medical Virology 77:77–82 (2005)

Chemiluminescence Enzyme Immunoassay for Monitoring Hepatitis C Virus Core Protein During Interferon-α2b and Ribavirin Therapy in Patients With Genotype 1 and High Viral Loads

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This study evaluated an updated chemiluminescence enzyme immunoassay (CLEIA) for hepatitis C virus (HCV) core protein for monitoring viral kinetics during treatment with interferon (IFN)-α and ribavirin. Using the CLEIA, serum levels of HCV core protein were measured in 17 patients with genotype 1 and high baseline viral loads during the first 4 weeks of combination therapy. HCV RNA was measured by the Amplicor Monitor test for comparison. At the start of therapy, the median HCV level (interquartile range) was 700 (540–940) kIU/ml of viral RNA and 11,310 (5,528–14,238) fmol/L of core protein. HCV RNA was above the upper limit of the linear range of the Amplicor Monitor test in 13 of the 17 patients, while the core protein level was within the linear range of the CLEIA in all patients. During therapy, the proportion of patients with HCV levels below the cutoff values at each time point was less with the Amplicor Monitor test than with CLEIA. Serum HCV core protein level decreased rapidly during the first 24 hr of therapy and more slowly thereafter, with median exponential decays of 1.08 and 0.046 log10/day, respectively. In the second phase, between day 1 and 28, the median decrease in HCV core protein level was higher in four patients with sustained virologic response (0.13 log10/day) than in 13 patients with no response (0.028 log10/day, $P = 0.042$). The wide linear range of the HCV core protein assay is appropriate for measuring viral loads during therapy with IFN-α and ribavirin. ***J. Med. Virol. 77:77–82, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: core protein; enzyme immunoassay; hepatitis C; interferon; ribavirin; viral kinetics

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease worldwide [Lauer and Walker, 2001]. Persistent infection with HCV often progresses to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma over the course of several decades. Since the report by Hoofnagle et al. [1986], describing the effects of interferon (IFN) therapy on chronic hepatitis C, this drug has been approved for the eradication of HCV, and may reduce the incidence of hepatocellular carcinoma [Nishiguchi et al., 1995; Yoshida et al., 1999; Kubo et al., 2001]. Ribavirin is a synthetic guanosine nucleoside analog that inhibits replication of various RNA and DNA viruses. In patients with chronic hepatitis C, the combination of IFN-α and ribavirin yields a higher rate of sustained virologic response than IFN-α alone [Davis et al., 1998; McHutchison et al., 1998; Poynard et al., 1998; Reichard et al., 1998]. However, the rate of sustained eradication of HCV achieved by combination therapy remains unsatisfactory for patients with HCV genotype 1 and high baseline viral loads.

Analysis of the dynamics of HCV during the early phase of IFN-based therapy is important for monitoring the response to therapy, and sometimes for modifying treatment regimens. Beginning 7–10 hr after initiation of IFN administration, the serum level of HCV declines

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rapidly, with an estimated half-life of 5.0–7.2 hr during the first 1 or 2 days of therapy, and then declines more slowly [Neumann et al., 1998; Yasui et al., 1998]. This second-phase decrease in HCV level is especially important when analyzing viral kinetics for prediction of long-term response to therapy [Enomoto et al., 2002a,b, 2004].

A simple and inexpensive method with a wide linear range of quantitation is needed for frequent measurement of serum HCV levels to assess responses to antiviral therapy. Several assays for measurement of serum HCV levels are available commercially, including reverse-transcription polymerase chain reaction (PCR) for HCV RNA [Zeuzem et al., 1994; Lee et al., 2000] and enzyme immunoassay (EIA) for HCV core protein [Tanaka et al., 1995, 1996; Nishiguchi et al., 2002]. Although the sensitivity of the conventional EIA for HCV core protein was previously inferior to that of PCR, EIA has been improved in its analytical sensitivity [Aoyagi et al., 1999; Tanaka et al., 2000; Zanetti et al., 2003]. In particular, a chemiluminescence enzyme immunoassay (CLEIA) has been developed recently utilizing a partially automated system with specialized equipment that is both simple to perform and can yield results rapidly. However, the usefulness of this assay for monitoring serum HCV levels during IFN-α and ribavirin therapy has not been assessed clinically.

The aim of this study was to evaluate whether the CLEIA for HCV core protein can be used to examine viral kinetics in the early phase of treatment with IFN-α and ribavirin. CLEIA was used to monitor serum levels of HCV core protein in patients with genotype 1 and high viral loads during the first 4 weeks of combination therapy. HCV RNA was measured by the PCR-based Amplicor Monitor test for comparison.

MATERIALS AND METHODS

Patients

There were 17 patients with chronic hepatitis C (10 men and 7 women; mean age, 54 ± 10 years) who began combination therapy with IFN-α2b and ribavirin at our hospital between March 1999 and December 2002. The inclusion criteria were as follows: persistent elevation of serum alanine aminotransferase for at least 6 months before therapy; presence of genotype 1 of HCV in serum; presence of serum HCV RNA at levels above 200 kIU/ml as determined by the Amplicor Monitor test; absence of serum hepatitis B surface antigen and of signs of other likely causes of chronic liver disease; histological features of chronic hepatitis in liver biopsy specimens obtained within 6 months before the start of therapy; absence of anemia (hemoglobin concentration less than 12 g/dl in women and less than 13 g/dl in men); and no evidence of hepatocellular carcinoma on ultrasonographic or computed tomographic examinations. Serum samples were obtained from the patients before administration of the drug(s) on the first day of therapy (day 0) and on day 1, 7, 14, and 28. The samples were stored at −80°C before being tested. The procedures of the study were in accord with the Declaration of Helsinki of 1975 (1983 revision) and were approved by the ethics committee of our hospital.

Treatment

Patients received recombinant IFN-α2b (Intron A, Schering-Plough, Kenilworth, NJ) by intramuscular injection at a dosage of 6 MU every day for 2 weeks, followed by 6 MU three times a week for 46 weeks. Ribavirin (Rebetol, Schering-Plough) was given orally twice a day for the first 24 weeks at a total daily dose of 600 mg in the nine patients who weighed 60 kg or less and 800 mg in the remaining eight patients, who weighed more than 60 kg. This protocol was commonly used in this country at the time of this study. The response to therapy was assessed virologically by repeated PCR assays for serum HCV RNA. A sustained virologic response was defined as one in which serum HCV RNA was not found more than 6 months after the end of therapy. Patients who did not meet these criteria were considered to have no response to therapy.

Assays

Routine hematological and biochemical tests were performed using standard procedures. Serum HCV RNA was measured by the Amplicor Monitor test (Roche Diagnostics, Branchburg, NJ) [Zeuzem et al., 1994; Lee et al., 2000], which exhibits good linearity between 0.5 and 500 kIU/ml. When HCV RNA was not detected by this method, the serum was tested again using the more sensitive, qualitative Amplicor test [Lee et al., 2000]. Genotypes of HCV were identified by direct sequencing of the amplification products generated during the Amplicor Monitor test with an ABI 3700 DNA sequencer (Perkin Elmer Corp./Applied Biosystems, Foster City, CA) [Kuboki et al., 2000].

Serum HCV core protein was measured by CLEIA (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. In brief, 200 μl of serum sample was mixed with 100 μl of a pretreatment solution containing 0.3% Triton X-100 and 15% sodium dodecyl sulfate. After incubation at 56°C for 30 min, 100 μl of the pretreatment solution was added to a well, coated with monoclonal antibodies to the HCV core antigen, and filled with 160 μl of reaction buffer. The mixture was incubated with agitation for 10 min at 37°C and then washed with buffer. Alkaline phosphatase-conjugated monoclonal antibodies to HCV core antigen were then added to the well, which was then incubated for 10 min at 37°C. After washing, 200 μl of a substrate buffer was added, and the mixture was incubated for 5 min at 37°C. The reactive chemiluminescence unit was measured, and the concentration of HCV core antigen was determined according to a standard curve generated using the recombinant HCV core antigen. All steps of this assay after the first incubation (at 56°C for 30 min) were performed on a fully automated chemiluminescence analyzer system (Lumipulse-f, Fuji Rebio, Tokyo,

Japan). The total assay time was 30 min. The linear range of the assay was 15–50,000 fmol/L.

Histological Evaluation

Liver biopsy was performed for each patient within 6 months before the start of therapy. Histopathological findings were assessed by grading inflammatory activity and staging fibrosis according to the classification of Desmet et al. [1994]. All evaluations were done by an experienced pathologist blinded to the clinical data.

Statistical Analysis

Statistical analysis was performed with the Statview SE + Graphics program, version 5.0 (SAS Institute, Cary, NC). The significance of correlations was evaluated by Spearman's rank analysis. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. A two-tailed *P*-value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Baseline Characteristics of Patients

Of the 17 patients, 9 had a history of IFN monotherapy. All patients were infected with genotype 1b of HCV, which is the most common kind in Japan. At the start of treatment with IFN-α2b and ribavirin, the median alanine aminotransferase activity (interquartile range) was 80 (46–114) IU/L. The median HCV level (interquartile range) was 700 (540–940) kIU/ml of viral RNA and 11,310 (5,528–14,238) fmol/L of core protein. The baseline HCV RNA level was above the upper limit of linear range of the Amplicor Monitor test in 13 of the 17 patients, but the core protein level of all patients was within the linear range of the CLEIA. The grade of inflammatory activity was mild in nine patients and moderate in seven. The stage of fibrosis was mild in eight patients, moderate in six, severe in one, and cirrhotic in one. In one patient, the biopsy sample was too small to evaluate.

Relationship Between HCV RNA Level and Core Protein Concentration

The relationship between the results of measuring HCV RNA by the Amplicor Monitor test and those of measuring HCV core protein by CLEIA is shown in Figure 1. In three samples taken from one patient during therapy, HCV core protein was above the cutoff value for the CLEIA (534, 221, and 55.4 fmol/L, respectively), while HCV RNA was below the cutoff value for the Amplicor Monitor test. In eight samples taken from five patients, HCV RNA was above the cutoff value for the Amplicor Monitor test (median, 2.7 kIU/ml; range, 0.6–20 kIU/ml), while HCV core protein was below the cutoff value for CLEIA. Samples below the cutoff value of each assay were assigned the viral load of the cutoff value for calculation. There was a significant correlation between the results obtained with the two techniques at the start of therapy ($r = 0.589$, $P = 0.011$).

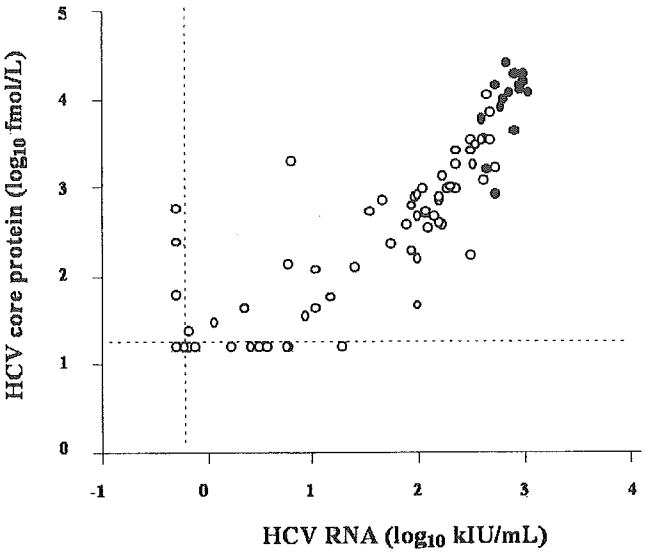

Fig. 1. Correlation between serum HCV RNA level measured by the Amplicor Monitor test and HCV core protein level measured by the CLEIA. Broken lines show the cutoff value for each assay. ●, samples taken at the start of therapy with IFN-α2b and ribavirin. ○, samples taken during therapy.

The correlation between results was also significant when all serum samples taken during therapy were included in analysis ($r = 0.859$, $P < 0.0001$).

Changes in HCV Levels During the First 4 Weeks of Treatment

On day 1, 7, 14, and 28 of treatment with IFN-α2b and ribavirin, HCV RNA was below the cutoff value by the Amplicor Monitor test in zero (0%), one (6%), four (24%), and four (24%) patients, respectively, while HCV core protein was below the cutoff value by CLEIA in 0 (0%), 1 (6%), 5 (29%), and 8 (47%) of the 17 patients. The proportion of patients with HCV levels below the respective cutoff values during therapy was lower with the Amplicor Monitor test than with CLEIA.

Changes in serum HCV core protein in all patients monitored by the CLEIA are shown in Figure 2. Changes in HCV RNA as measured by the Amplicor Monitor test paralleled those in core protein (data not shown). As reported previously, HCV core protein decreased rapidly during the first 24 hr of therapy and more slowly thereafter. We defined the period between 0 and 24 hr of therapy (day 0) as "the first phase," and the period from day 1 to 28 (day 14 if core protein was below the cutoff value on day 28, or day 7 if core protein was below the cutoff value on day 14) as "the second phase." The median rate of exponential decay of serum HCV core protein (interquartile range) in the first and in second phases were 1.08 (0.69–1.34) and 0.046 (0.016–0.11) log10/day, respectively.

Of the 17 patients treated with combination therapy, 4 had a sustained virologic response and 13 had no response. The median rate of decay in the first phase (interquartile range) was 1.06 (0.93–1.24) log10/day in

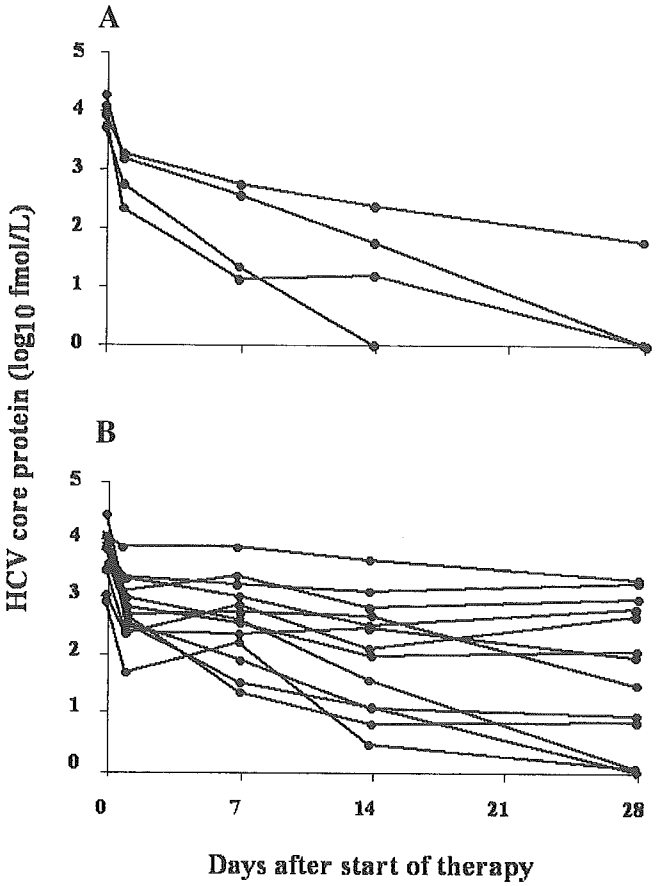

Fig. 2. Time course of serum HCV core protein level monitored by CLEIA during the first 4 weeks of IFN-α2b and ribavirin treatment (**A**) in the four patients with sustained virologic response, and (**B**) in the 13 patients with no response. In the second phase between day 1 and 28, the median decrease in HCV core protein was larger in patients with sustained virologic response (0.13 log10/day) than in patients with no response (0.028 log10/day, $P = 0.042$).

patients with sustained virologic response and 1.11 (0.69–1.34) log10/day in patients with no response; the differences in first-phase viral decline were not significant ($P = 0.73$). The median rate of decay in the second phase (interquartile range) was 0.13 (0.045–0.21) log10/day in patients with sustained virologic response and 0.028 (0.0048–0.11) log10/day in patients with no response; the differences in second-phase viral decline between the sustained-response and no-response groups were significant ($P = 0.042$).

DISCUSSION

Among commercial assays for the measurement of serum HCV levels, the Amplicor Monitor test is used widely, as a sensitive PCR-based method [Zeuzem et al., 1994; Lee et al., 2000]. However, it yields results with limited reproducibility and requires expensive equipment. The cumbersome procedures make testing of many samples difficult and increase the risk of contamination. High levels of HCV RNA may be underestimated with it, and, in addition, its analytical sensitivities may be affected by the genotype, of the resulting in underestimation of viral loads of genotype 2 or 3.

The serum concentration of HCV core protein is correlated with HCV RNA titer [Tanaka et al., 1995, 1996]. Viral protein is more resistant to multiple freezing and thawing than viral RNA. In the new version of the EIA, HCV core antigens are released from the virion and antibodies to HCV core are inactivated by pretreatment with sodium dodecyl sulfate. This pretreatment step increases sensitivity by approximately 100-fold. Aoyagi et al. [1999] demonstrated high intra-assay precision and inter-assay reproducibility of the EIA over a wide range of values. This method, which uses specific monoclonal antibodies specific to the conserved region of core protein, can evaluate the viral loads of different genotypes with equal sensitivity. In particular, the partially automated CLEIA assay requires less than 1 hr to yield results. Clinically, rapid availability of results allows viral loads to be accurately monitored and treatment regimens to be modified as required. The low cost of the assay enables serum samples to be frequently tested to evaluate viral kinetics.

The cutoff value of the CLEIA was set at 15 fmol/L of HCV core protein, based on the results of measurement of serially diluted standard samples. This value is equivalent to 1–2 kIU/ml of viral RNA (unpublished observations). Although the proportions of patients with HCV levels below the cutoff values during therapy were smaller with the Amplicor Monitor test than with the CLEIA, the analytical sensitivity of the CLEIA is nearly the same as that of the Amplicor Monitor test. In addition, the baseline HCV core protein level of all patients with genotype 1 and high viral loads was within the linear range of the CLEIA, whereas in a majority of the patients the viral RNA was above the upper limit of the linear range of the Amplicor Monitor test. The wide linear range permitted by the HCV core protein assay is appropriate for accurate measurement of the high viral loads present before and during the first few days after the start of therapy.

Davis [2002] showed that a decrease in serum HCV level by 2 log10 units within the first 12 weeks of therapy with IFN-α and ribavirin can be used as the optimal definition of an early virologic response which, if not achieved, is associated with a low likelihood of sustained virologic response. Discontinuation of therapy should be recommended for patients with genotype 1 who do not achieve an early virologic response. This type of prospective assessment also requires methods with wide linear ranges to precisely evaluate viral loads before and during antiviral therapy.

Previous studies of the dynamics of HCV in the first few weeks of IFN treatment have revealed biphasic viral decline [Neumann et al., 1998; Yasui et al., 1998], as we also observed. Neumann et al. [1998] suggested that the rapid viral decrease in the first phase reflects the dose-dependent effects of IFN on HCV production, and that the slower decrease in the second phase arises from the

death of hepatocytes infected with HCV. It was found previously that the second-phase decrease in HCV RNA monitored by quantitative PCR was correlated with the long-term effects of IFN therapy [Enomoto et al., 2002a,b]. In the present study, CLEIA also exhibited significant correlation between the second-phase decrease in HCV core protein and the sustained virologic response to treatment with IFN-α and ribavirin. The changes in serum HCV core protein monitored by the CLEIA early during IFN-α and ribavirin treatment can be used to predict long-term therapeutic response.

Randomized controlled trials have shown that weekly treatment with pegylated IFN-α plus ribavirin yields higher rates of sustained virologic response than treatment with unmodified IFN-α plus ribavirin [Manns et al., 2001; Fried et al., 2002]. Owing to advances in treatment, IFN is now indicated even for difficult-to-treat patients with HCV genotype 1 and high baseline viral loads. Evaluation of HCV levels by assays with wide linear ranges will most likely become more important in the future.

In summary, the CLEIA is a simple, sensitive, specific, reproducible, and inexpensive method for the measurement of HCV core protein. The wide linear range of the HCV core protein assay is appropriate for monitoring viral loads in patients with genotype 1 and high viral loads during therapy with IFN-α and ribavirin.

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