PATIENTS AND METHODS

Patients

A cross-sectional study was performed in 115 consecutive Vietnamese HBV infected patients within 1 year, between January 2000 and December 2000. There were 87 men and 28 women, aged from 16 to 83, with the mean age 45.62 ± 15.8 years. These patients were recruited from in-patients Gastroenterology wards at Cho Ray Hospital (Ho Chi Minh city, Vietnam) and Bach Mai Hospital (Hanoi, Vietnam). The diagnoses of HBV-related liver diseases were established based on clinical data, laboratory tests, and imaging studies (ultrasonography, computerised tomography (CT-scan), and/or Magnetic Resonance Imaging (MRI)). Among 115 patients, 39 were diagnosed as acute hepatitis B based on either the novo appearance of HBsAg or the presence of Immunoglobulin M antibody to hepatitis B core antigen (IgM anti-HBc). Patients with a prolonged prothrombin time over than 50% of control and/or hepatic encephalopathy during their acute hepatitis (AH) were diagnosed as FH. Seventy-six patients were diagnosed as chronic HBV infection; and the persistence of HBsAg of these patients were followed in more than 1 year. The chronic group included asymptomatic carriers (ASCs) with normal or mild elevated alanine transferase (ALT) (<2 times of upper normal limit); chronic hepatitis (CH) with mild symptoms and abnormal ALT; liver cirrhosis (LC), and HCC. Cirrhosis and HCC were defined on liver function test, alpha-fetoprotein level, imaging studies, and histology. None of the patients had co-infection with hepatitis C virus (HCV) and/or hepatitis D virus (HDV) and their serum samples were stored at −70°C until used. Informed consent was obtained from all patients, and the study was approved by the local ethical committee.

Serologic Markers

All sera were screened for HBsAg, HBeAg, anti-HCV antibody, and anti-HDV antibody by enzyme linked immunosorbent assay (ELISA), using commercially available kits from Abbott (Abbott Laboratories, North Chicago, IL). Diagnosis of acute hepatitis was reconfirmed by IgM anti-HBc assay in all cases.

Extraction of DNA

Viral DNA was extracted from 100 μl of serum using the DNA/RNA extraction Kit (SepaGene RV-R, Sanko Junyaku Co., Ltd., Tokyo, Japan). The resulting pellet was eluted in 50 μl of RNase-free water and kept in −20°C until use.

HBV Genotyping by PCR

Genotyping of HBV was identified by PCR using type-specific primers designed from pre-S1 through S genes of HBV [Naito et al., 2001]. Six genotypes (A to F) of HBV could be identified by specific bands of second PCR. To avoid false-positive results, instructions to prevent cross contaminations were strictly followed, and the results were considered valid only when they were consistently obtained in duplicate.

Amplification of the CP/PC Regions

Partial gene covering 282 nucleotides (nt) (from nt 1689 to 1970) of CP/PC region were amplified by nested PCR. Primer pair eP11: 5′-GCATGGAGACCACCGTGAAC-3′ (sense) and BG1R: 5′-ATAGGGGCATTTGGTGGTCT-3′ (antisense) was used for the first round PCR; and primer pairs PC1: 5′-CATAAGAGGACTCTTGGACT-3′ (sense), PC2: 5′-AAAGAATTCAGAAGGCAAAAAAGA-3′ (antisense) for the second round PCR. The PCR reaction was performed in 40 cycles (94°C 20 sec, 55°C 20 sec, and 72°C for 30 sec) followed by extension at 72°C for 7 min. PCR products were separated by 2% agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, CA).

Nucleotide Sequencing and Phylogenetic Analysis

Purified PCR products were subjected to direct sequencing using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The inner primer pair was used as sequencing primers. Sequences of amplified DNA were determined using automated DNA sequencer ABI 377 (Perkin Elmer, Norwalk, CT). Nucleotide sequences were multiple-aligned, analysed using Genetyx for Windows ver. 6.0 software (GENETYX, Tokyo, Japan), and corrected manually by visual inspection. Nucleotide consensus sequences of CP/PC regions of HBV genotypes B and C were taken from GenBank for multi-aligment and mutant analysis. Tree construction was analysed by neighbour-joining method with bootstrap resampling (1,000 times), using MEGA version 2.1 [Kumar et al., 2001].

Quantitation Assay of Viral Load

Quantitation of HBV DNA was performed by real-time PCR method [Chen et al., 2001]. The detection limit of this assay was 3.73×10^2 genome equivalents per ml. Sequences of primers and probes were HBc1: 5′-AGTGTGGATTCGCACTCCT-3′ (sense, nt 2269–2287), HBc1R: 5′-GAGTTCTTCTTCTAGGGGACCTG-3′ (antisense, nt 2387–2365) and HBcP1: 5′-CCAAATGCCCCTATCTTATCAACACTTCC-3′ (TaqMan probe, nt 2303–2331).

Statistical Analysis

Proportions of each factor were compared between the groups using Fisher exact 2-tail test, and the group means were compared using the Student's *t*-test. Differences were considered to be significant for $P < 0.05$. Mean of HBV DNA levels were compared after logarithmic transformation of the HBV DNA values from the real-time PCR assay.

RESULTS

Patients With Acute and Chronic HBV-Infected Diseases

Among 39 acute HBV-infected patients including acute hepatitis and fulminant hepatitis, there were 29 (74.3%) with genotype B and 10 (25.7%) with genotype C. Their mean age was 35.2 ± 12.8 years and 36 of them were men. In 76 chronic HBV-infected patients, there was an equal distribution of genotype B (39; 51%) and genotype C (37; 48.7%). The mean age of this group was 50.9 ± 14.4 years. The characteristics of the patients in each group and each diagnosis category were described in Table I. The acute group had a younger age ($P < 0.001$), a higher ALT ($P < 0.001$), and a higher HbeAg +ve rate ($P < 0.05$) than that of the chronic group. HBV DNA level among acute HBV infected patients was higher than that of chronic infection, however, there was no statistically significant difference ($P = 0.26$). Of note, the frequency of genotype B was found to be higher than that of genotype C in the acute group, in comparison with the chronic group (74.3% vs. 51.3%, $P < 0.05$).

CP/PC Mutant in Acute HBV-Infected Patients

In the CP region, the occurrence rate of the CP mutant was 15/39 (38.4%), in which the T1762/A1764 and deletion mutants accounted for 30.7 and 7.6%, respectively (Table II). Two out of three cases of fulminant hepatitis had a deletion in this region, spanning from 20 to 21 nucleotides (Fig. 1). The total rate of CP mutants in genotype B (9; 31%) was lower than that of genotype C (6; 60%) but the difference was not statistically significant ($P = 0.14$). In the PC region, 70% of genotype C isolates carried the C at nucleotide 1858 (C-1858) ($P < 0.001$), and there was no A1896 mutant among these isolates (Fig. 1B and Table II). However, genotype B isolates carried only T at nucleotide 1858 (T-1858), and the A1896 mutant was determined in 34.4% cases ($P < 0.05$) (Fig. 1A and Table II).

CP/PC Mutant in Chronic HBV-Infected Patients

In the CP region, the occurrence rate of the CP mutant was 51/76 (67.1%). Three kinds of mutants were detected in genotype B, i.e., T1762/A1764 (33.3%), T1762A alone (10.2%) and deletion mutant (7.6%) (Table III). The frequency of the T1762/A1764 double mutant was found to be higher in genotype C (81%) than in genotype B isolates (33.3%) ($P < 0.001$). In the PC region, the A1896 mutant was seen in 25/76 (32.8%). As reported in the acute group, C-1858 also possessed a strong link to genotype C (70.2%) ($P < 0.001$). The A1896 mutant, therefore, was less detectable in genotype C (5.4%) than in genotype B (58.9%) ($P < 0.001$). Furthermore, when only the T-1858 isolates were taken into account, the A1896 mutant rate was also lower in genotype C than in genotype B (2/11 (22.2%) and 23/39 (58.9%), respectively). In addition, there were correlations between cirrhosis and HCC with a high occurrence rate of T1762/A1764 in genotype C ($P < 0.01$); and A1896 in genotype B ($P < 0.05$) (Table III).

CP/PC Mutant, Virological Manifestations, and Liver Injury

In the acute group, the mean age, HBV DNA, and ALT level were not significantly different between the wild type and the CP/PC mutant type (Table IV). Conversely, in the chronic group, the CP mutant was detected more frequently in older age cases and associated with a lower HBV DNA level than that of the wild type. However, there was no statistically significant difference ($P = 0.6$ and 0.6, respectively). The same insignificant different finding was observed with the A1896 mutant, although it was detected in cases with a higher mean age, higher HBV DNA, and ALT level ($P = 0.7$; $P = 0.4$; and $P = 0.6$,

TABLE I. Characteristic of Patients of Acute and Chronic HBV Infection

Diagnosis	n	Sex (M/F)	Age (year)[a]	ALT (UI/L)	HBeAg (+ve/−ve)	HBV DNA (log_{10} copies/ml)	Genotype	
							B	C
Acute infection	39	36/3	35.2 (12.8)*	1,089 (892)**	9/30***	5.48 (1.33)****	29 (74.3)†	10 (25.7)
AH	36	26/10	35.2 (12.9)	1,137 (908)	8/28	5.50 (1.36)	28 (77.7)	8 (22.3)
FH	3	2/1	34.6 (15.1)	516 (373)	1/2	5.31 (1.38)	1 (33.3)	2 (66.7)
Chronic infection	76	59/17	50.9 (14.4)*	85 (120)**	5/71***	4.98 (1.21)****	39 (51.3)†	37 (48.7)
ASC	10	8/2	36.5 (18.1)	40 (5)	2/8	6.30 (0.74)	6 (60)	4 (40)
CH	4	3/1	47.2 (8.3)	69 (16)	0/4	4.45 (0.54)	0	4 (100)
LC	39	29/10	52.9 (12.8)	108 (163)	1/38	4.98 (1.36)	21 (53.8)	18 (46.2)
HCC	23	19/4	54.3 (12.7)	61 (28)	2/21	4.79 (0.93)	12 (52.1)	11 (47.9)

[a]Age, ALT, HBV DNA were denoted in mean with the standard deviation in parenthesis; sex, HBeAg were denoted in number of cases; and genotype was denoted in number of cases with percentage in parenthesis.
*$P < 0.001$.
**$P < 0.001$.
***$P < 0.05$.
****$P = 0.26$.
†$P < 0.05$.

TABLE II. Core Promoter and Precore Mutant in Acute HBV Infected Patients

	Core promoter (CP) region[a]			Precore region	
	T1762/A1764	T1762 alone	Deletion in CP	C-1858	A1896
Genotype B (n = 29)	8 (27.5)*	0	1 (3.4)	0***	10 (34.4)**
AH (n = 28)	7 (25.0)	0	1 (3.5)	0	9 (32.1)
FH (n = 1)	1 (100)	0	0	0	1 (100)
Genotype C (n = 10)	4 (40.0)*	0	2 (20.0)	7 (70.0)***	0**
AH (n = 8)	4 (50.0)	0	0	6 (75.0)	0
FH (n = 2)	0	0	2 (100)	1 (100)	0

[a]Number of cases with percentage in parenthesis.
*$P = 0.69$.
**$P < 0.05$.
***$P < 0.0001$.

respectively). HBeAg was still detected in patients with CP/PC mutants, however, the rate of HBeAg loss was found more frequent in CP mutant infected patients than those with A1896 mutant.

Phylogenetic Analyses

As shown in Figure 2, all of 115 analysed CP/PC sequences (282 nucleotides) were clustered in major branches of genotype B (68 isolates) and C (47 isolates). All genotype C isolates in this study was belonged to sub-branches that differed from Japanese isolates of genotype C (Accession D50520 and D50517). These genotype C isolates were closely related to branches including isolates from Vietnam and Thailand strains from database (AF223957 and AF068756, respectively). Genotype B isolates, however, were shown branching off from genotype C branch rather than from a more proximal node, with low bootstrap value (28%). The C-1858 strains which were only detected in genotype C isolates, assembled closely but did not form a unique phylogenetic entity.

DISCUSSION

It is known that the CP/PC plays a central role in HBV replication. CP directs the transcription of both pre-genomic RNA and precore mRNA [Kramvis and Kew, 1999]. PC and core genes are essential for the pre-genome encapsidation signal and for the core protein assembly [Tong et al., 1992]. The CP mutants have been found to correlate with the HBV genotypes, viral replication, and liver damage in East Asian HBV carriers [Lindh et al., 1999]. The PC stop codon mutant, A1896, has been considered an important factor for fulminant hepatitis and progressive liver disease [Lok et al., 1994; Hunt et al., 2000]. On the other hand, Vietnam has a high rate of endemic HBV infection, with an HBsAg carrier rate between 9–14% in urban areas [Tran et al., 1993; Nakata et al., 1994] and 12–20% in rural areas [Hipgrave et al., 2003]. More than 3.5 million Vietnamese are currently at risk of a premature death due to HBV infection [Ngoan Le et al., 2002; Hipgrave et al., 2003]. Therefore, virologic characterisation of this virus and the CP/PC mutant may be helpful for the understanding of HBV pathogenesis in this country.

In this study, genotype B was found to be the predominant genotype in the acute group (74.3%). As reported previously, genotypes B and C of HBV were equally distributed in Vietnam [Tran et al., 2003] and a similar result was also confirmed in chronic infected patients in the present study (51.3 and 48.7%, respectively). Interestingly, it was known that genotype B in Japan was linked to the acute form, specifically to fulminant but not acute hepatitis [Imamura et al., 2003]. Moreover, genotype B in Hong Kong patients was strongly associated with chronic hepatitis B exacerbations [Yuen et al., 2003a]. This finding suggested a correlation between genotype B with the acute forms of HBV infection. Recent studies have shown that genotype B might be more immunogenic, and patients infected with this genotype have earlier HBeAg seroconversion, in comparison to patients with genotype C [Chu et al., 2002; Yuen et al., 2003b]. Hence, an in-depth genomic sequence analysis of HBV in acute cases could be required to address this matter.

Among the investigated sequences of 39 acute and 76 chronic HBV-infected patients, there were different effects of genotypes on the CP/PC mutants. In the acute group, genotype B was found to correlate with the A1896 mutant. In the chronic group, genotype B was associated with the A1896 mutant, whereas genotype C was correlated with CP mutants. Interestingly, the T1762/A1764 double mutant in genotype C was found to be associated with cirrhosis and HCC. However, due to the small number of asymptomatic carriage and chronic hepatitis in this study, this result needs further confirmation. Nevertheless, similar findings were also reported by other Asian studies, suggesting that the high prevalence of the CP mutant in genotype C isolates could be one of the important factors causing a detrimental effect on the evolution of HBV infection [Takahashi et al., 1995; Lindh et al., 1999; Fang et al., 2002; Yotsuyanagi et al., 2002].

It has also been known that genotype C in Southeast Asian countries has a high prevalence of the C-1858 variant, which is base-paired to nucleotide 1896 and prevents the occurrence of the A1896 mutant [Lok et al.,

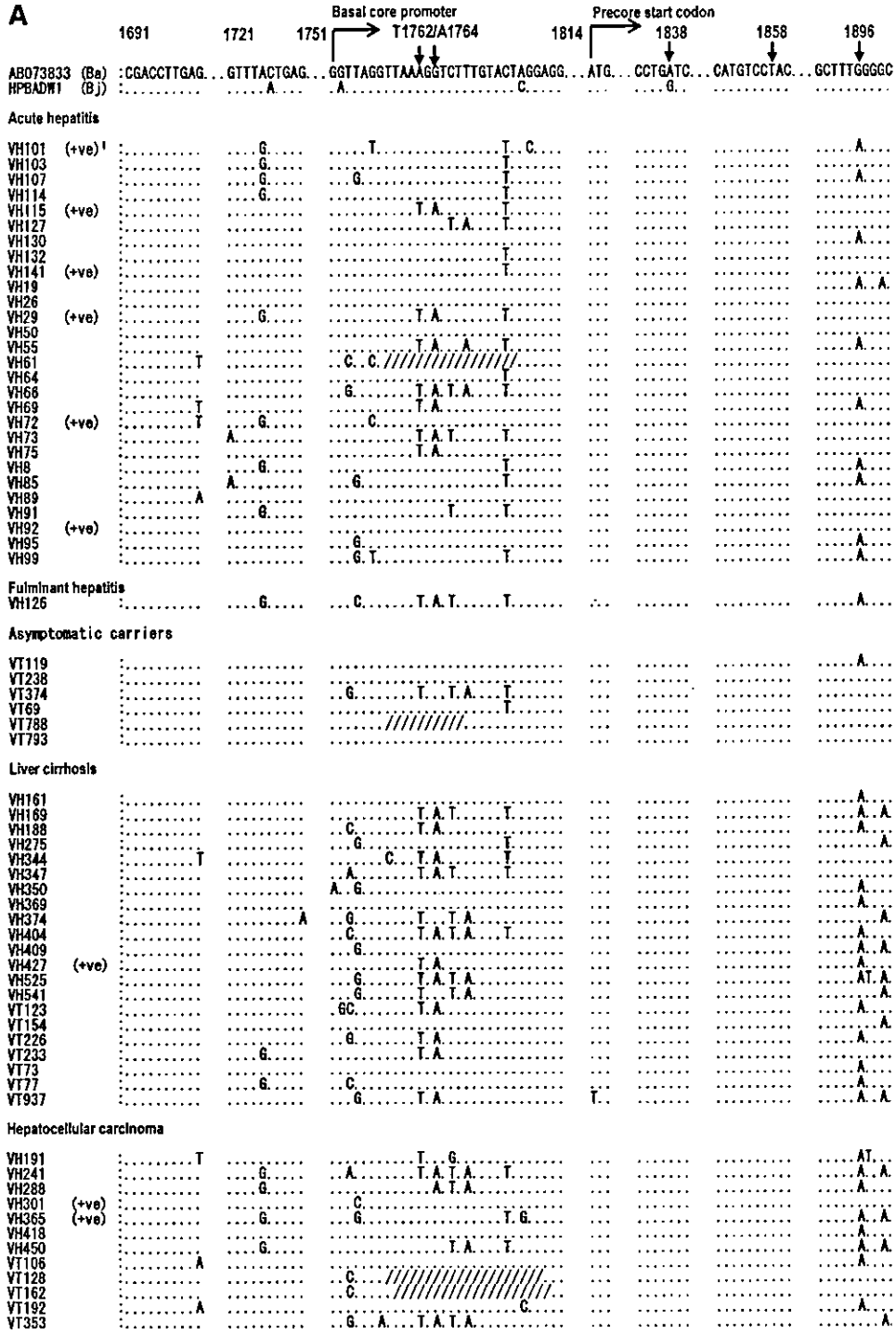

Fig. 1. Alignment of the CP/PC region (nt 1689–1970) from 68 Vietnamese genotype B (A) and 47 genotype C isolates (B). Dots represented nucleotides identical to the consensus sequences while deletions were represented as forward slashes. Isolates from each genotype were grouped based on clinical diagnosis. The top line(s) in each figure were wild type consensus sequences using nucleotide numbered by Okamoto et al. [1988]. The consensus of Ba and Bj subgroup of genotype B reported by Sugauchi et al. [2002] were shown (Accession No. AB073833 and D50521, respectively). The consensus of genotype C was the one reported in Japan (Accession No. D50517). Specific changes from G to A in nucleotide 1721 and 1727 were found in genotype C detected in Vietnam.

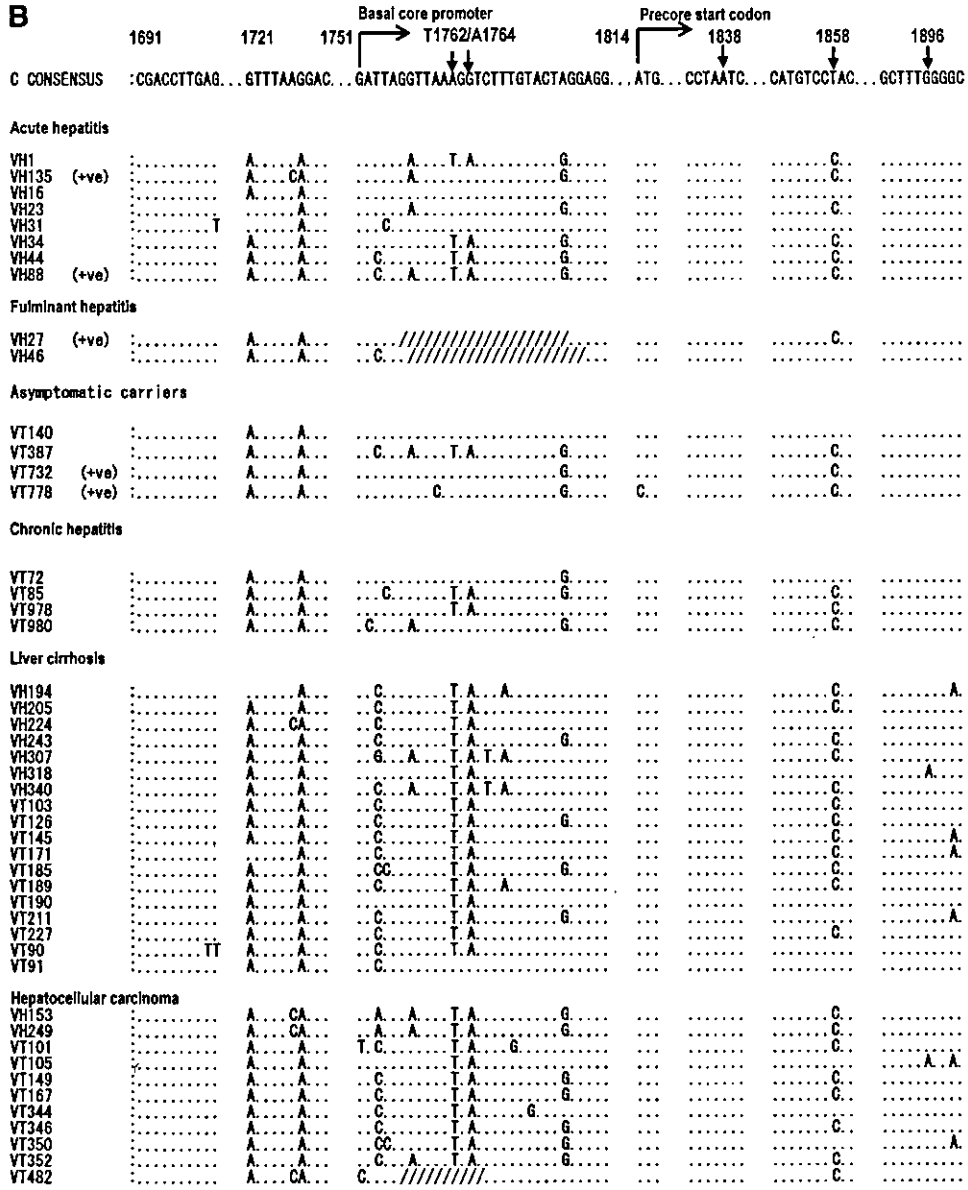

Fig. 1. (*Continued*)

1994]. C-1858 was also thought to correlate with the CP mutants [Chan et al., 1999]. In the present study, about 70% of genotype C isolates in Vietnamese patients possessed this variant. The result was remarkably different from a recent study in Japan [Kobayashi et al., 2004], in which no C-1858 variant was found in either genotype B or C. One of the possibly explanations is that genotypes B and C in Southeast Asia belong to subgroups that have a distinctive phylogenetic entity [Sugauchi et al., 2002; Huy et al., 2004]. In fact, the consensus nucleotide sequence at nt 1838 (genotype B, Ba specific) [Sugauchi et al., 2003] or nt 1721 and nt 1727 (genotype C) supports the notion that HBV genotypes B and C detected in Vietnam belong to subgroups of genotype B and C. In addition, phylogenetic tree analysis showed that C-1858 isolates have been grouped to each other, but cannot form a unique subgroup of C-1858 in genotype C (Fig. 2) as mentioned previously [Alestig et al., 2001]. In the present study, the analysed region is rather short (282 nucleotides) and partly covered the region of genotype B and C recombination in Southeast Asia (genotype Ba), which is spanned from nt 1740 to 2443 [Sugauchi et al., 2003]. In fact, all genotype B in this study were confirmed as subgroup Ba (data not shown). The resulting sub-branches reflect the recombination of genotype B and C in this region;

TABLE III. Core Promoter and Precore Mutant in Chronic HBV Infected Patients

	Core promoter (CP) region[a]			Precore region	
	T1762/A1764	T1762 alone	Deletion in CP	C-1858	A1896
Genotype B (n = 39)	13 (33.3)*	4 (10.2)	3 (7.6)	0***	23 (58.9)**
ASC (n = 6)	0	1 (16.6)	1 (16.6)	0	1 (16.6)
LC (n = 21)	11 (52.3)	2 (9.5)	0	0	14 (66.6)†
HCC (n = 12)	2 (16.6)	1 (8.3)	2 (16.6)	0	8 (66.6)†
Genotype C (n = 37)	30 (81.0)*	0	1 (27.0)	26 (70.2)***	2 (5.4)**
ASC (n = 4)	1 (25.0)	0	0	3 (75.0)	0
CH (n = 4)	2 (50.0)	0	0	3 (75.0)	0
LC (n = 18)	17 (94.4)****	0	0	12 (66.6)	1 (5.5)
HCC (n = 11)	10 (90.9)****	0	1 (9.0)	8 (72.7)	1 (9.0)

[a]Number of cases with percentage in parenthesis.
*$P < 0.001$.
**$P < 0.001$.
***$P < 0.0001$.
****$P < 0.01$.
†$P < 0.05$.

therefore it was associated with the low bootstrap values to differentiate the two genotypes; as well as the C-1858 variant.

Seven cases of deletion mutants, which spanned the TA-rich regions of the CP region, were found. These deletions have been known to result in a frame-shift and/or truncation of the X protein at the C terminal end [Kidd-Ljunggren et al., 1997]. Although two out of three cases of fulminant hepatitis had a deletion in the CP region, this mutant was also found in other diagnoses, and might have no significant role [Kramvis and Kew, 1999]. G1899A, a mutant that changes glycine at codon 29 to aspartic acid, has been linked to G1896A [Yuan et al., 1995]. However, in the present study, G1899A occurred independently with G1896A in 5/13 cases (38.4%) of genotype B and 5/6 cases (83.3%) of genotype C, respectively. Therefore, its role is not clearly identified in Vietnamese isolates.

In this study, the correlation between the HBV DNA level and CP/PC mutant was unclear in both the acute and chronic groups, although the HBV DNA level was found to be insignificantly lower in CP mutant isolates; and higher in the PC mutant isolates. In addition, the HBV DNA level was not significantly different between the acute and chronic states; and was in a lower range than in the previous studies, in which the level was usually around 10^8 copies/ml in patients with HBeAg +ve [Lindh et al., 1999]. One explanation relates to the time of collecting serum samples from these patients in the acute group. In the present study, up to 10 cases of acute group had A1896. These low viral titer samples might be approaching the period of seroconversion [Parekh et al., 2003] at the time of investigation.

In conclusion, mutants in the HBV CP/PC regions prevailed in chronic and acute hepatitis B patients in Vietnam. In chronic infection, CP mutants, especially

TABLE IV. Characteristic of CP/PC Mutant in Both Acute and Chronic Forms

	Core promoter		Precore	
	WT	MUT	WT	MUT
Acute forms				
Age[a]	34.7 (14.4)	36 (10.1)	33.6 (13.7)	39.0 (9.8)
HBeAg (+ve/−ve)	5/19**	4/11**	8/21	1/9
HBV DNA	5.27 (1.44)	5.78 (1.17)	5.34 (1.29)	5.81 (1.46)
ALT	1,171 (966)	959 (772)	1,120 (975)	1,011 (667)
Chronic Forms				
Age	46.8 (18.0)*	52.9 (11.9)*	50.5 (15.4)	51.7 (12.2)
HBeAg (+ve/−ve)	4/21***	1/50***	3/48	2/23
HBV DNA	5.50 (1.22)****	4.77 (1.15)****	4.69 (1.08)†	5.42 (1.29)†
ALT	91 (177)	81 (81)	69 (60)‡	116 (190)‡

[a]Age, ALT, HBV DNA were denoted in mean with the standard deviation in parenthesis; HBeAg was denoted in number of cases.
*$P = 0.465$.
**$P = 0.734$.
***$P < 0.05$.
****$P = 0.605$.
†$P = 0.490$.
‡$P = 0.606$.

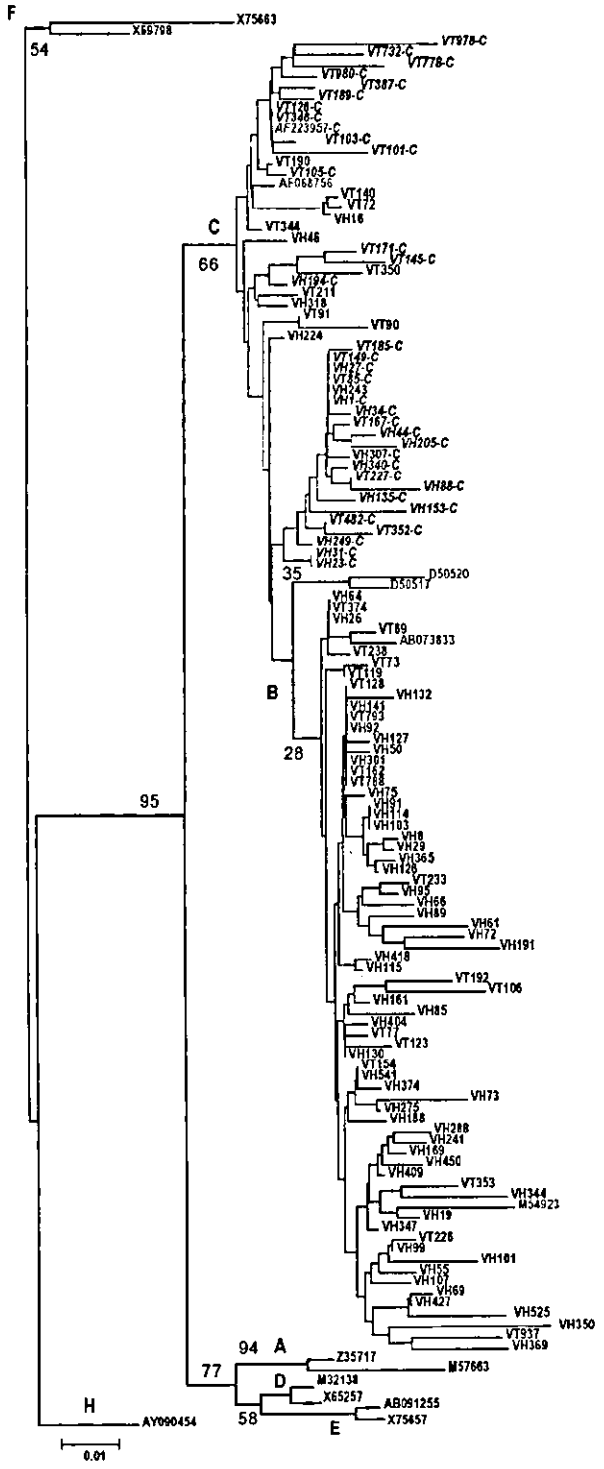

Fig. 2. Phylogenetic tree. This tree was constructed by the neighbour-joining method based on the partial nucleotide sequence of the CP/PC gene (282 nt, from nt 1691–1972) of 115 HBV Vietnamese isolates (VH and VT) and 15 reported isolates from genotype A–H in database. Bootstrap values were indicated in major branches and sub-branches of genotype B and C. Genotype G was excluded from this analysis due to their common 36-nucleotide insertion in the core gene. Sequences of genotype C written in italic-C were those with C-1858.

the T1762/A1764 double mutant, were linked to genotype C of HBV, which had a high rate of C-1858 variants and could be associated with the more severe diseases. In acute infection, the influence of HBV genotypes on CP mutants was not clear, although genotype B, possessing a higher rate of the A1896 mutant, was linked to acute hepatitis manifestation in Vietnam.

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＜速　報＞

C型慢性肝炎における遺伝子発現－cDNAマイクロアレイを用いて－

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はじめに：C型慢性肝炎では，リンパ球を主体とする免疫反応の関与が想定されている．我々は，C型慢性肝炎患者の末梢血リンパ球の網羅的な遺伝子発現プロファイルの検討を行った．

方法：各施設の倫理委員会の承認下に，文書で同意を得たC型慢性肝炎10例を検討した．男女比7：3，平均年齢50±9歳(35～61歳)で，ALT平均139±97 IU/l(33～309 IU/l)，HCV RNA平均237±258 KIU/ml (0.8～850＜KIU/ml)，HCV血清型はgroup 1，2それぞれ5例だった．肝生検8例の組織所見は，F 0～1，2，3がそれぞれ2，4，2例，A 1，2，3がそれぞれ3，3，2例だった．cDNAマイクロアレイはClontech社製のAtlas™ Human 1.2 Arraysで，1枚に1,176個の遺伝子cDNAがプロットされている汎用チップである．患者末梢血リンパ球と対照のmRNAをそれぞれCy 3，Cy 5で蛍光標識して反応させ，両者の蛍光強度比(発現比)を求めた．対照の2倍以上を活性化された遺伝子，0.5倍以下を抑制された遺伝子と見做した．なお，対照mRNAは健常者30例の末梢血を混合したリンパ球から抽出した．

結果と考察(表1)：活性化された遺伝子のうち共通性が最も高いのはmigration inhibitory factor-related protein 14(MRP-14)で10例中6例に認めた．次いで，interleukin-8(IL-8)precursorと，puromycin-sensitive amino-peptidase(PSA)とを10例中5例に認めた．MRP-14は乾癬や関節炎などの慢性炎症に関係し[1]，IL-8はC型慢性肝炎患者血中で高値を示し[2]，PSAは細胞増殖への関与が知られている[3]．抑制された遺伝子で最も共通性が高いのはplatelete basic protein(PBP)precursorで10例中7例に認めた．次いで，monoamine oxidase(MAO-A)が10例中5例に認められた．PBPは血小板α顆粒から放出後に活性化されてneutrophil activating peptide 2(NAP 2)となり[4]，MAO-Aはセロトニンなどの代謝に関わる[5]．以上の結果は，C型慢性肝炎の肝組織を用いたHondaらの報告とは共通点がなかった[6]．彼らは肝生検組織を検体とし健常人肝組織を対照とした点，自製のcDNAマイクロアレイを用いた点で我々と異なっていた．次に，症例2－5，10は遺伝子発現プロファイルが類似しているが，これらの症例のHCV血清型やRNA量，ALT値，肝組織など臨床的背景には共通点がなかった．今後さらに，遺伝子発現プロファイルと臨床経過などとの関連を検討する必要がある．

索引用語：C型慢性肝炎，cDNAマイクロアレイ，遺伝子発現

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表1　対照に比して遺伝子発現の異なる遺伝子と発現比

(1) 発現比が2倍以上(網かけ)の遺伝子										
	症例1	2	3	4	5	6	7	8	9	10
MRP-14	2.89	2.42	2.31	2.94	4.50	1.81	0.87	0.76	0.32	3.02
IL-8 prec.	1.13	17.54	3.81	5.84	4.90	1.37	0.95	0.48	1.23	4.60
PSA	0.96	2.30	5.10	2.04	1.18	2.26	1.94	0.83	1.19	2.26
(2) 発現比が0.5倍以下(下線)の遺伝子										
	症例1	2	3	4	5	6	7	8	9	10
PBP prec.	1.50	0.46	0.40	0.40	0.44	0.43	0.83	0.69	0.31	0.42
MAO-A	1.16	0.46	0.11	0.24	0.69	0.40	1.87	0.67	1.24	0.39

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Changes in virus loads and precore mutations in chronic hepatitis B patients treated with 4 weeks of daily interferon alfa-2a therapy

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Abstract

Interferon (IFN) alfa-2a was administered to 23 patients with chronic hepatitis B daily for 4 weeks and the relation between the efficacy of the treatment and changes in total hepatitis B virus (HBV) DNA and precore mutant levels was investigated. At 6 and 12 months after the completion of IFN therapy, 39.1% (9/23) and 36.8% (7/19) of patients, respectively, showed alanine transaminase (ALT) normalization; 31.3% (5/16) and 50.0% (7/14), respectively, became negative for HBe-antigen (HBeAg); and 42.1% (8/19) and 41.2% (7/17), respectively, became undetectable for HBV DNA. All 18 of the patients who were positive for HBeAg at baseline nevertheless had the precore mutation. The level of precore mutant as a proportion of the total HBV DNA level was constant at baseline, and 3 and 6 months after the completion of therapy. Thus, the investigation showed that in chronic hepatitis B, the precore mutation occurs at a constant proportion beginning in the HBeAg-positive phase, and IFN therapy inhibits the growth of the wild-type and precore mutant viruses equally.

Keywords: Chronic hepatitis B; rIFNα-2a; HBV DNA; Precore mutant

1. Introduction

Drugs that have been used to treat chronic hepatitis B include interferon (IFN), propagermanium, and steroids. Recently, lamivudine has been introduced and its use in combination with IFN has attracted interest. IFN therapy was first reported by Greenberg et al. [1] in 1976, whose work shows that IFN inhibits viral growth. In the US and Europe, IFN monotherapy generally consists of long-term administration of 5–10MU per day three times per week for 4–6 months [2,3]. In Japan, in 1986, the National Health Insurance coverage established 4 weeks as the standard treatment period. Consequently, in the present study, rIFN alfa-2a was administered daily for 4 weeks at a dose of 9MU per day for the first 3 days and 18MU per day thereafter.

The efficacy of IFN therapy is estimated by seroconversion from HBe-antigen (HBeAg) to HBe-antibody (HBeAb), undetectable response for hepatitis B virus (HBV) DNA, and normalization of alanine transaminase (ALT). Factors reported to be associated with response to IFN therapy are the baseline levels of HBV DNA and ALT [2,3]. Moreover, the clinical significance of infection with the precore mutant virus, which does not produce HBeAg, has recently drawn attention. We therefore, quantitatively analyzed precore mutant levels and examined the changes in these levels with IFN monotherapy.

2. Materials and methods

The subjects were 23 patients with chronic hepatitis B, 16 males and 7 females, with a mean age of 36.3 ± 9.8 years. Eighteen of the patients were positive for HBeAg and five were negative. Although all 23 patients were positive for HBV DNA in the polymerase chain reaction (PCR) assay, two patients were below detection limits by the bDNA probe assay. The precore mutant level was not less than 10^7 copies/ml in 12 patients and less than 10^7 copies/ml in 11

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Table 1
Baseline characteristics of patients

Patients number	Sex	Age	Grading[a]	Staging[a]	Interval from prior IFN (Month)	ALT (IU/l)	HBeAg (index)	HBeAb (%)	HBVDNA bDNA-p (Meq/ml)	HBVDNA PCR (copy/ml)	Precore mutant (copy/ml)
1	Female	35	–	–	38	287	0.8	96.4	96	8×10^7	8×10^7
2	Male	46	A1	F1	–	67	0.8	95.1	<0.7	5×10^3	<100
3	Female	52	A2	F1	10	310	179.8	0	1900	3×10^8	3×10^8
4	Female	29	–	–	24	304	3.6	57.8	3.4	3×10^6	3×10^6
5	Male	34	A1	F0	–	74	53.5	0	44	3×10^7	8×10^6
6	Female	22	A1	F1	–	259	407.7	0	78	3×10^7	3×10^7
7	Female	34	A1	F1	7	116	385.4	0	190	7×10^7	4×10^7
8	Male	43	A2	F2	14	206	113.3	0	1800	4×10^8	7×10^8
9	Male	56	A3	F2	–	148	7.2	58.0	1.6	9×10^5	9×10^5
10	Male	33	A1	F0	–	142	271.9	0	350	1×10^8	1×10^5
11	Male	33	–	–	41	87	292.2	0	150	3×10^7	3×10^7
12	Male	40	A2	F0	–	995	451.8	0	2100	4×10^8	4×10^8
13	Male	28	A1	F1	–	42	0.8	82.1	710	8×10^8	8×10^8
14	Male	44	A2	F2	49	57	1.8	17.2	2.8	5×10^6	8×10^5
15	Female	30	A2	F1	–	211	183.4	0	3800<	8×10^8	8×10^8
16	Female	27	A2	F2	–	87	9.2	23.3	<0.7	9×10^4	5×10^3
17	Male	36	–	–	10	448	231.8	0	25	1×10^7	1×10^6
18	Male	33	A3	F3	7	44	5.2	54.7	1	3×10^6	1×10^6
19	Male	27	A1	F2	5	184	53.9	0	400	3×10^7	3×10^7
20	Male	58	A3	F3	13	367	0.8	87.6	74	3×10^7	3×10^7
21	Male	26	A1	F3	8	115	172.8	0	1000	2×10^8	1×10^8
22	Male	28	A2	F0	10	338	174.7	0	490	1×10^7	7×10^6
23	Male	42	–	–	96	191	2.2	78.6	2.1	1×10^5	5×10^3

[a] Five cases were not measured.

patients, 1 of whom had a precore mutant level of less than 10^2 copies/ml. Fourteen patients had previously received IFN therapy for intervals from 5 to 96 months, during which they had been administered 477MU daily for 4 weeks. Baseline ALT was 34 to 66 IU/l in three patients and not less than 67 IU/l in 20 patients. Eighteen patients underwent liver biopsy, of whom four had a fibrosis score of F0, six a score of F1, five a score of F2, and three a score of F3 (Table 1).

rIFN alfa-2a was initially administered at a dose of 9MU per day for three consecutive days and 18MU per day for the subsequent 25 days (total dose, 477MU). Excluded were patients who had received an antiviral agent or immunomodulator within 3 months before the study; those who had received an injectable agent containing glycyrrhizin/cysteine/glycine or shosaiko-to (Chinese herbal medicine) within 1 month before the study; and those with a white blood cell (WBC) count of less than 3000/mm^3 or a platelet count of less than 100,000/mm^3.

The virological tests performed were the total amount of HBV DNA, using a bDNA probe assay (Quantiplex, Chiron) and competitive polymerase chain reaction assay (nested-PCR, Otsuka Assay), and the HBV precore mutant levels, using a quantitative mutation-site specific polymerase chain reaction assay (PCR-MSSA, Otsuka Assay). Using PCR-MSSA assay, precore point mutation (G–A, 83rd base of precore region) was examined using a mutation-trapped oligonucleotide primer, which yields a polymerase chain reaction amplification product only with precore mutantsand within the detection limits of 10^2 to 10^9 copies/ml [4]. Each measurement was performed immediately before treatment initiation, at treatment completion, and 6 months after treatment completion. HBeAg and HBeAb levels were measured immediately before treatment initiation, at treatment completion, and 3, 6, and 12 months after treatment completion. They were measured by radioimmunoassay (RIA), and a cutoff index higher than 2.1 for HBeAg was judged to be positive, and an inhibition percent higher than 50 for HBeAb was judged to be positive. Liver histology findings were assessed according to the Knodell histologic activity index [5] and the Desment scoring system [6].

The efficacy of the treatment was evaluated at its completion and at 6 and 12 months after completion according to ALT normalization and loss of HBeAg and HBV DNA.

The statistical analysis was performed using Fisher's exact test and the Wilcoxon 2-sample test.

3. Results

3.1. Efficacy

The rate of patients with normalized ALT levels was 4.3% (1/23) at treatment completion, 39.1% (9/23) at 6 months after completion, and 36.8% (7/19) at 12 months after completion. Although all measurement rates changed during the follow-up, there were many cases with normalized ALT levels after the treatment completion. Of the patients who were positive for HBeAg at baseline, the rate of patients who

Table 2
The rate of biochemical and virological response

	At treatment completion	3 months after treatment completion	6 months after treatment completion	12 months after treatment completion
ALT normalized	4.3%	34.8%	39.1%	36.8%
	1/23	8/23	9/23	7/19
HBeAg lost[a]	38.9%	29.4%	31.3%	50.0%
	7/18	5/17	5/16	7/14
HBV DNA cleared[b]	47.6%	40.0%	42.1%	41.2%
	10/21	8/20	8/19	7/17

Reduction of the number of patients during the follow-up is caused by without patient's consent.
[a] Five patients were excluded because negative at study initiation.
[b] Two patients were excluded because undetectable at study initiation.

became negative for HBeAg was 38.9% (7/18) at treatment completion, 31.3% (5/16) at 6 months after completion, and 50.0% (7/14) at 12 months after completion. Thus, the highest negative rate was at 12 months after the treatment completion. The rate of patients who became undetectable for HBV DNA (bDNA probe assay) was 47.6% (10/21) at treatment completion, 42.1% (8/19) at 6 months after completion, and 41.2% (7/17) at 12 months after completion. A rate of more than 40% undetectable was maintained after the treatment completion. The inability to obtain consent resulted in a reduction in the number of patients followed (Table 2).

3.2. *Efficacy according to patient baseline characteristics*

Examination of baseline patient characteristics, ALT normalization, and loss of HBeAg and HBV DNA at 6 months after treatment completion revealed a trend toward greater efficacy with respect to the rate of ALT normalization and

Table 3
Efficacy according to baseline characteristics of patients

Features			6 Months after treatment completion		
		n	ALT normalized ($n = 23$)	HBeAg lost[a] ($n = 16$)	HBV DNA cleared[b] ($n = 19$)
Sex	Male	16	25.0% (4/16)	20.0% (2/10)	38.5% (5/13)
	Female	7	71.4% (5/7)	50.0% (3/6)	50.0% (3/6)
Age	<40	15	40.0% (6/15)	25.0% (3/12)	38.5% (5/13)
	40≦	8	37.5% (3/8)	50.0% (2/4)	50.0% (3/6)
Prior IFN therapy	Yes	14	35.7% (5/14)	40.0% (4/10)	53.8% (7/13)
	No	9	44.4% (4/9)	16.7% (1/6)	16.7% (1/6)
Staging	F0,F1	10	50.0% (5/10)	28.6% (2/7)	25.0% (2/8)
	F2,F3	8	37.5% (3/8)	20.0% (1/5)	66.7% (4/6)
	Non-perform	5	20.0% (1/5)	50.0% (2/4)	40.0% (2/5)
ALT (IU/ml)	67≦	20	35.0% (7/20)	33.3% (5/15)	37.5% (6/16)
	34–66	3	66.7% (2/3)	0% (0/1)	66.7% (2/3)
HbeAg (index)	100–1000	11	27.3% (3/11)	22.2% (2/9)	33.3% (3/9)
	2.1–100	7	42.9% (3/7)	42.9% (3/7)	50.0% (3/6)
	<2.1	5	60.0% (3/5)	–	50.0% (2/4)
HBeAb (%)	50–100	8	62.5% (5/8)	50.0% (2/4)	42.9% (3/7)
	0–50	15	26.7% (4/15)	25.0% (3/12)	41.7% (5/12)
HBV DNA (Meq./ml)	100≦	11	27.3% (3/11)	25.0% (2/8)	33.3% (3/9)
	0.7–100	10	40.0% (4/10)	42.9% (3/7)	50.0% (5/10)
	<0.7	2	100% (2/2)	0% (0/1)	–
HBV DNA (copies/ml)	10^7–10^9	16	31.3% (5/16)	27.3% (3/11)	35.7% (5/14)
	10^2–10^7	7	57.1% (4/7)	40.0% (2/5)	60.0% (3/5)
	<10^2	0	–	–	–
Precore mutant (copies/ml)	10^7–10^9	12	41.7% (5/12)	37.5% (3/8)	45.5% (5/11)
	10^2–10^7	10	30.0% (3/10)	25.0% (2/8)	37.5% (3/8)
	<10^2	1	100% (1/1)	–	–

[a] Five patients were excluded because negative at study initiation.
[b] Two patients were excluded because undetectable at study initiation.

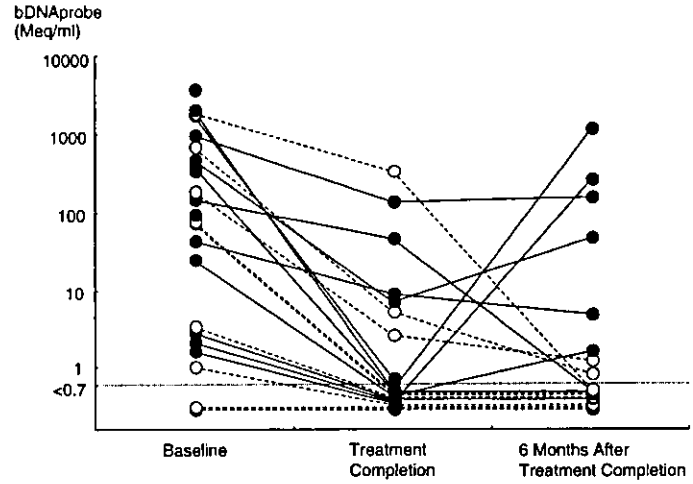

Fig. 1. Changes in Serum HBV DNA levels on interferon (IFN) therapy. And normalization of ALT in each patients at 6 months after the treatment completion. HBV DNA levels were significantly lower in patients with normalized ALT than with abnormal ALT at 6 months after the treatment completion. Open circles with dotted lines showed the changes in serum HBV DNA levels in normalized alanine transaminase (ALT) at 6 months after treatment completion, and closed circles with solid lines showed the abnormal alanine transaminase (ALT) at the same time. It was performed by the bDNA probe assay, since the changes in total hepatitis B virus (HBV) DNA levels were clearer than by the polymerase chain reaction (PCR) assay.

loss of HBeAg and HBV DNA in female patients than in male patients. Moreover, patients who had undergone previous IFN therapy showed greater efficacy, as indicated by the negative rate for HBeAg and HBV DNA, than patients who had not undergone previous therapy. In addition, the lower the baseline viral load, the greater was the efficacy with respect to the rate of ALT normalization and loss of HBeAg and HBV DNA. However, there were no significant differences between baseline characteristics and efficacy (Table 3).

3.3. *Efficacy based on changes in viral markers*

Regardless of its level at baseline, HBV DNA tended to decrease from the initiation of IFN therapy to its completion. After treatment completion, this decrease continued in five patients, while HBV DNA levels increased in the remaining patients. Two of the patients who exhibited more than 100 Meq/ml of the virus at treatment completion had ALT normalization at 6 months after treatment completion; conversely, seven patients who had less than 100 Meq/ml of the virus or were undetectable for the virus at treatment completion did not show ALT normalization at 6 months after treatment completion. Thus, there was no relation between the virus level at treatment completion and efficacy at 6 months after completion. However, HBV DNA levels at 6 months after treatment completion were significantly lower in patients with normalized ALT than with abnormal ALT at the same time (the ALT normalization rate of positive HBV

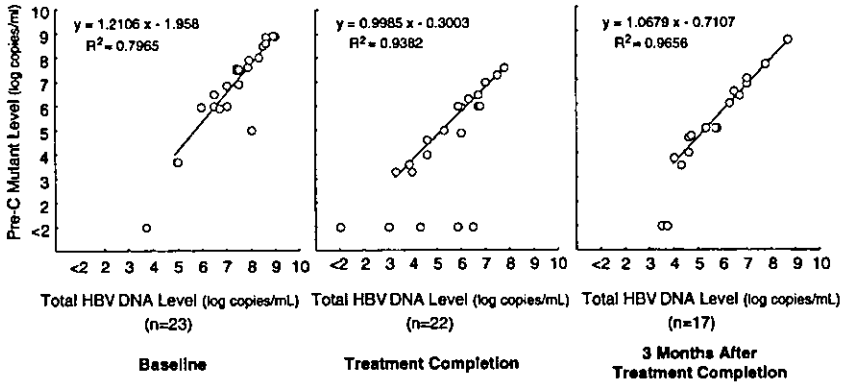

Fig. 2. Total HBV DNA Levels and Precore Mutant Levels on interferon (IFN) Therapy. Total hepatitis B virus (HBV) DNA and precore mutant were measured in: 23 patients at baseline, 22 patients at the treatment completion and 17 patients at 3 months after the treatment completion. Correlation coefficients are shown at each point. From the point of sensitivity of detection limit during the treatment, hepatitis B virus (HBV) DNA was performed by the polymerase chain reaction (PCR) assay.

DNA patients was 25%, and 70% for negative HBV DNA patients; $P = 0.0359$). It performed by the bDNA probe assay, since the changes in total HBV DNA levels were clearer than by the PCR assay (Fig. 1).

At baseline, only one patient had a precore mutant level of less than 10^2 copies/ml and was thus considered to have the wild-type virus. A correlation was seen between the total HBV DNA level and the precore mutant level at baseline, with no consistent trend seen between the proportion of precore mutant in relation to total HBV DNA. A correlation between total HBV DNA and precore mutant level was also seen at treatment completion and 3 months after completion, with no trend seen between the proportion of precore mutant at these timepoints. At all timepoints, the slope of the curve was equal, and no change was seen in the mutant proportion. However, there were exceptions in four patients: only the precore mutant level decreased to the limit of detection at treatment completion, indicating that inhibition of precore mutant growth exceeded inhibition of wild-type growth. From the point of sensitivity of detection limit during the treatment, HBV DNA was performed by the PCR assay (Fig. 2).

4. Discussion

Although no drugs have shown adequate efficacy in chronic hepatitis B, lamivudine has recently been introduced. Lamivudine has potent anti-HBV activity, but long-term lamivudine treatment frequently produces the YMDD mutation and increases HBV DNA and ALT levels [7]. As treatment discontinuation in this case may produce a rebound effect and result in acute exacerbation of the hepatitis, strategies such as continued lamivudine therapy with concurrent IFN use have been adopted [8]. Combined therapy with lamivudine and IFN reportedly increases the negative rate for HBeAg [9].

The first results of 4 weeks of IFN monotherapy in Japan were reported in 1983 by Matsumura et al. [10]. Since then, there have been occasional reports on topics such as the virological and immunological changes seen during IFN monotherapy, but there have been few reports on the efficacy of IFN therapy. The criteria for evaluating the efficacy of IFN therapy are not clearly defined. Generally, negative response for HBeAg, seroconversion to HBeAb, and normalization of ALT are assessed. Recently, however, the results of clinical studies using liver carcinogenesis or survival as the endpoint have been reported. Factors such as IFN therapy, age, histological progression in the liver, and post-treatment negative for HBeAg, HBV DNA, and HBsAg and normalization of ALT, play significant roles in efficacy after treatment completion [11–13].

In the present study, treatment with rIFN alfa-2a for 4 weeks resulted in, at 6 months and 12 months after treatment completion, normalization of ALT and almost the same undetectable HBV DNA rate, however the negative HBeAg rate rose from about 30 to 50%. Thus, in this study, treatment with rIFN alfa-2a did not show its effectiveness at each timepoint. It will be necessary to perform long-term follow-up observations of these patients, using liver carcinogenesis or survival as the endpoint.

The close relation between HBV mutation and disease type has been shown, and there have been numerous studies on this topic. Mutation at nucleotide 1896 of the precore region is considered particularly important, because it is thought to be related to the pathophysiology of fulminant and other hepatitis. In this study, we evaluated the frequency of the occurrence of precore mutants using MSSA for the detection of point mutations at the 83rd base in the precore region for the mutant HBV genome. MSSA can be used for the detection of 10^2 copies/ml of precore mutants in the presence of 10^7 copies/ml of wild-types, its sensitivity is considered to be at least 0.001%. With the use of this MSSA, even if only wild-type genomes are present, precore mutant-type can be identified on electrophoresis [4]. There have been numerous reports in the US and Europe indicating that this precore mutant is resistant to IFN [14,15]. In Japan, however, IFN efficacy in patients positive for HBeAb was first reported by Muraoka et al. [16], and since then other groups have also reported inhibition of HBV growth and ALT normalization with IFN therapy [17,18]. Possible factors in this discrepancy include the fact that, in the US and Europe, negative responses, not only for HBeAg and HBV DNA, but also for HBsAg, are the objectives of treatment and baseline characteristics such as the period of infection and HBV genotype differ greatly. Therefore, an examination of the response to IFN in which there is uniformity with respect to these factors is needed. In the present study, the total HBV DNA level and precore mutant level were correlated before and after IFN treatment. Among the 18 patients who were positive for HBeAg at baseline, none was negative for the precore mutant. Thus, in chronic hepatitis B, the precore mutation occurred at a constant proportion beginning from the HBeAg-positive phase, and IFN therapy inhibited growth of the wild-type virus and the precore mutant virus equally in most of the patients. In some patients, inhibition of the precore mutant growth exceeded inhibition of the wild-type growth; there were no cases in which wild-type growth was inhibited. Shindo et al. [19] reported that the precore wild-type and mutant have similar sensitivities to IFN. However, because they used the restriction fragment length polymorphism (RFLP) assay [20] to determine mutant virus levels and did not perform a quantitative examination, and because the IFN was administered intermittently (three times per week for 17 weeks) and the efficacy rate was 26.1% (6/23 patients; defined as showing seroconversion to HBeAb, loss of HBV DNA and normalization of ALT), the results of their study cannot be adequately compared with those of our investigation.

In an investigation in patients with type B cirrhosis, Ikeda et al. [21] reported high rates of carcinogenesis when the precore mutant is present at high concentrations. They

further report that high precore mutant concentrations correlate with a high total HBV DNA level in such patients, indicating that the inflammation associated with the hepatitis is severe, and acts to indirectly promote carcinogenesis. In addition, it is reported that a primary infection by HBV with a gene mutation contributes to the infection becoming fulminant and severe [22]. However, it is also reported that mutation of the precore region during the natural course of chronic hepatitis B is related to quiescence of the hepatitis and a decrease in the virus level [23].

The time required to reach the true endpoint of the present study, carcinogenesis or survival, makes it difficult to provide conclusions regarding whether the hepatic lesions in patients will progress or whether the hepatitis will become quiescent and the patients will become asymptomatic carriers. However, it is already evident that IFN treatment inhibits the growth of both the wild-type and precore mutant viruses seen in chronic hepatitis B and that it is also effective in patients who are positive for HBeAg and have a predominance of the wild-type virus.

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The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan

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Abstract

One hundred seventy-one patients with chronic hepatitis C were included in this study (genotype1 and high viral loads (1H), $n = 130$; non-1H, $n = 37$; N.D., $n = 4$). The combination therapy of interferon and ribavirin for 24 weeks with an additional 24 weeks of interferon monotherapy (48-week treatment) was undergone by 42 1H patients and 5 non-1H patients. The combination therapy of interferon and ribavirin was administered for 24 weeks in 67 1H patients and 22 non-1H patients. Among the 1H patients, the HCV relapse rate was significantly higher in those receiving 24-week combination treatment than in those receiving 48-week treatment (78% versus 42%, $P = 0.003$). Among the non-1H patients, no significant difference was found between them. Sustained virological response (SVR) rates were observed to decrease as the timing of HCV RNA disappearance was delayed. In spite of the small rate (16%), SVR was obtained from the patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment. In 1H patients, 24-week combination treatment followed by interferon monotherapy for 24 weeks was concluded to be the treatment offering the most hope among those that the medical insurance can be applied in Japan.

Keywords: Chronic hepatitis C; Interferon and ribavirin combination therapy; Combination therapy followed by IFN monotherapy

1. Introduction

Interferon is the only available treatment for patients with chronic hepatitis C since HCV was discovered in 1989 [1–4]. Thirty percent of patients with chronic hepatitis C achieved SVR by interferon therapy but the efficacy was not satisfactory. Furthermore, in the patients considered to be the most treatment-resistant, that is, the 1H patients, only 5–8% showed SVR. In Japan, 40–50% of the patients with chronic hepatitis C belong to the 1H group. Therefore, finding how to eradicate the HCV RNA

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of 1H patients is most important for the treatment of chronic hepatitis C.

Recently, ribavirin, a nucleic acid analogue, exhibiting in vitro activity against various kinds of DNA and RNA viruses has been developed. The combination therapy of ribavirin and interferon has been shown to be very useful in the eradication of HCV in patients with chronic hepatitis C [5–7], although the mechanism of action of ribavirin remains speculative and ribavirin monotherapy led to no significant decrease of the amount of HCV RNA in the patients with chronic hepatitis C [8]. Most recent studies, performed with large numbers of naïve patients, have shown that the combination therapy of interferon and ribavirin can increase the SVR rate two-fold compared with interferon monotherapy for patients with chronic hepatitis C [9–12]. Especially, in the 1H patients, the combination therapy of interferon and ribavirin was more useful than in the other patients. Furthermore, Poynard et al. [10] showed that 1H patients treated by combination therapy for 48 weeks had a higher SVR rate than those treated for 24 weeks (28% versus 8%). Therefore, the combination therapy of interferon and ribavirin for 48 weeks is recommended as the standard therapy for 1H patients in Europe and the United States [13,14].

In Japan, the combination therapy of interferon and ribavirin was approved in 2001. However, the duration of the combination therapy is limited to 24 weeks in the medical insurance. As mentioned above, the SVR rate in 1H patients treated by the combination therapy for 24 weeks was clearly lower than those treated for 48 weeks. Furthermore, prolonged interferon monotherapy was reported to suppress relapse after cessation of therapy and to achieve a higher SVR rate in patients with chronic hepatitis C [15]. This study assessed the efficacy of the combination therapy of interferon and ribavirin for 24 weeks with an additional 24 weeks of interferon monotherapy compared with that of the combination therapy for 24 weeks.

2. Patients and methods

2.1. Patients

The current study was conducted at Osaka University Hospital and the institutions of the Osaka Liver Disease Study Group. The 171 patients included in this study had HCV RNA detectable in serum by the polymerase chain reaction (PCR) method, had elevated ALT (above the upper limit of the normal) and had been histologically proven to have chronic hepatitis. No patients were positive for hepatitis B surface antigen and anti-human immunodeficiency virus antibody or had other forms of liver disease (such as alcoholic liver disease and autoimmune liver disease). This study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

2.2. Determination of HCV RNA levels and HCV genotype

Serum HCV RNA levels were quantified using branched DNA (bDNA) probe assay (version 2; Chiron, Dai-ichi Kagaku, Tokyo) [16,17] or combined PCR assay (Amplicor-HCV monitor assay) [18]. In this study, a high viral load, as described previously [16,18,19], was designated as the condition of a serum HCV RNA level of more than 10^6 equivalents/ml by bDNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay. HCV genome typing was classified by serological genotyping assay [20].

2.3. Treatment schedule

Of the 171 patients with chronic hepatitis C enrolled in this study, 130 had HCV RNA with genotype 1 and high viral loads (1H group), which were difficult to eradicate by anti-viral therapy. Of the remaining 41 patients, 37 had HCV RNA with genotype 2 or low viral loads (non-1H group); genotype or viral levels could not be determined for four. One hundred thirty-six patients in whom treatment had been done without the discontinuation of interferon till the end of the scheduled duration were studied (1H, $n = 109$; non-1H, $n = 27$).

The combination therapy of interferon-α-2b and ribavirin was administered for 24 weeks in 67 patients of the 1H group and 22 patients of the non-1H group. In this protocol, interferon-α-2b was given intramuscularly every day for the first 2 weeks and then three times a week for the following 22 weeks in combination with ribavirin at a daily dose of 600 or 800 mg, depending on body weight (<60 or ≥60 kg, respectively). The combination therapy of interferon-α-2b and ribavirin for 24 weeks, followed by interferon-α-2b monotherapy three times a week for a further 24 weeks, was administered to 42 patients of the 1H group and 5 patients of the non-1H group. The pretreatment characteristics of the patients were similar (Table 1).

The starting doses of interferon-α-2b were 10 MU per day for 38, 6 MU per day for 127, and 3 MU per day for 6 patients. With ribavirin, 800 mg per day was started in 92, 600 mg per day in 77, and 400 mg per day in 2 patients. Among the 171 patients, the interferon dose was decreased in six patients during the treatment, and the interferon was stopped along with ribavirin in 33 patients (19%) due to side effects. The ribavirin dose was decreased in 43 patients (25%) during the treatment, and stopped without discontinuance of interferon in six patients. Eighty-seven patients (51%) completed treatment without discontinuance or dosage decrease of both drugs.

After the sufficient informed consent at the end of the combination therapy of interferon and ribavirin, the patients themselves decided whether to be treated for 24 or 48 weeks. The information included the results of clinical trials of the combination therapy for 24 and 48 weeks in other countries, such as the SVR rate, HCV relapse rate.

Table 1
Baseline characteristics of patients according to therapeutic protocol

	24-week treatment		48-week treatment
	1H group	Non-1H group	1H group
	67	22	42
Age (yo)	55.8 ± 10.9	55.7 ± 12.8	54.0 ± 11.7
M/F	40/27	15/7	28/14
ALT (IU/L)	107 ± 71	102 ± 45	103 ± 58
Fibrosis	1.9 ± 0.9	1.9 ± 1.2	1.8 ± 1.1
History of IFN treatment			
Naïve	34	11	17
Relapser	21	7	17
Non-responder	11	4	8
Unknown	1	0	0

Note: All comparisons are not significant. Twenty-four-week treatment, interferon plus ribavirin treatment for 24 weeks; 48-week treatment, interferon plus ribavirin treatment for 24 weeks followed by interferon monotherapy for 24 weeks. 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than 1H group. Fibrosis, Knodell's histological score (category 4).

Also, side effects were presented and the combination therapy of interferon-α-2b and ribavirin for 48 weeks was explained as not being covered by medical insurance in Japan. In the 47 patients who agreed to receive the additional 24 weeks of interferon monotherapy, the starting doses of interferon-α-2b were 10 MU per day for 10, 6 MU per day for 35, and 3 MU per day for 2 patients. All patients completed the additional treatment although interferon was decreased only in one patient from 10 to 6 MU per day.

2.4. Statistical analysis

Age, histological scores before interferon therapy, and serum ALT levels are expressed as mean ± S.D. The chi-squared test was used for statistical analysis of the comparison between group frequencies. When appropriate, the clinical and laboratory features of the two groups were compared by Student's *t*-test. Histological evaluation was substituted as a variable for Knodell's histological scores [21].

3. Results

3.1. Results of interferon and ribavirin combination therapy

Seventy-five percent of all of the patients of 1H group (82/109), including not only patients who received 24-week treatment but also those who received 48-week treatment, had no detectable HCV RNA at 24 weeks after the beginning of combination therapy of interferon and ribavirin. This was also the case for 100% of the non-1H patients (27/27). In patients given 24-week treatment of combined interferon and ribavirin, 45 out of 67 of the 1H group were negative for HCV RNA at the end of therapy, but only 22% of the patients (10/45) showed no detectable HCV RNA at 24 weeks after cessation of therapy. On the other hand, HCV RNA was negative in all non-1H patients at the end of the 24-week treatment, and the SVR rate was 86% (19/22) (Fig. 1). In patients with 48-week treatment (24-week combination treatment, followed by 24-week interferon monotherapy), HCV RNA reappeared during interferon monotherapy (break through) in 11 out of 37 patients (30%) who were negative for HCV RNA at the end of 24-week combination therapy: SVR was finally reached in 15 out of 26 patients who continued to be sero-negative for HCV RNA at the end of 48-week treatment. On the other hand, HCV RNA was not cleared even by 48-week treatment in all five patients who were positive for HCV RNA at the end of 24-week treatment (Fig. 2). In the non-1H patients who received 48-week treatment, HCV RNA was negative in all five patients at the end of the 24-week treatment, and SVR was attained by 80% (4/5).

The HCV RNA relapse rate after treatment was compared according to the duration of treatment. In all patients, 57% of those receiving 24-week treatment (38/67) had HCV RNA relapse, as compared with 39% of those receiving 48-week treatment (12/31). Among the 1H patients, a significant dif-

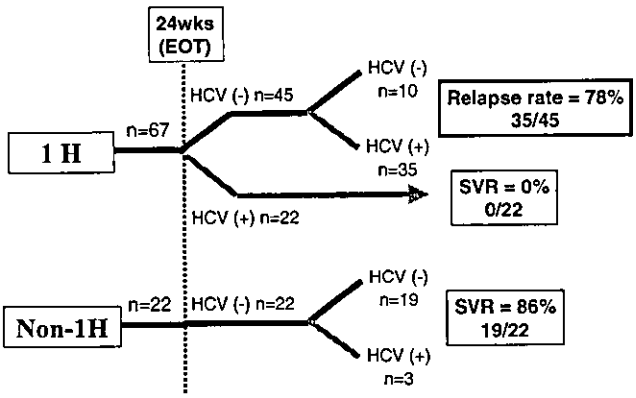

Fig. 1. Efficacy of the combination therapy (24-week treatment). 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than those of the 1H group. EOT, end of treatment. HCV, serum HCV RNA positivity by polymerase chain reaction.

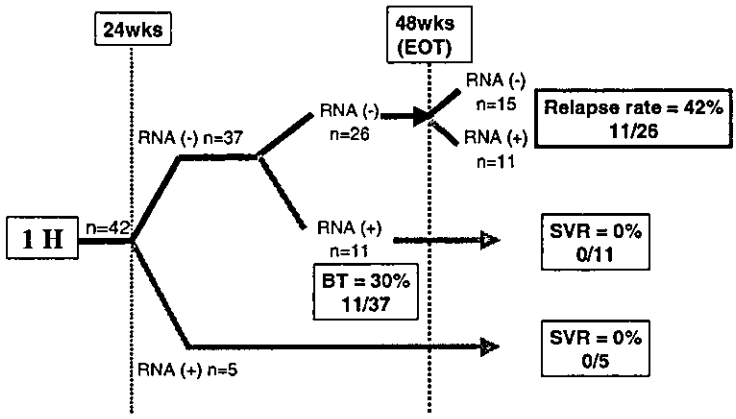

Fig. 2. Efficacy of the combination therapy followed by interferon monotherapy (48-week treatment). 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than those of the 1H group. EOT, end of treatment. HCV, serum HCV RNA positivity by polymerase chain reaction. BT, break through.

ference was found in HCV relapse rate between those receiving 24-week treatment and those receiving 48-week treatment (78% versus 42%, $P = 0.003$). Among the non-1H patients, HCV RNA relapsed in 14% (3/22) of those receiving 24-week treatment (Fig. 1) and 20% (1/5) of those receiving 48-week treatment.

3.2. Timing of HCV RNA disappearance and efficacy of treatment

The relationship between the timing of HCV RNA disappearance and SVR rate according to the duration of treatment was evaluated. As shown in Fig. 3A, in all patients receiving 24-week treatment, 71% (12/17) of the patients who had no detectable HCV RNA by week 4, 61% (11/18) by week 8 (beyond week 4), and 21% (4/19) by week 12 (beyond week 8) had SVR. Although 11 patients became negative for HCV RNA by week 24 (beyond week 12), none of them attained SVR. A tendency for a decrease in the SVR rate was observed as the timing of the HCV RNA disappearance was delayed. In the patients receiving 48-week treatment, 86% (6/7) of those who had no detectable HCV RNA by week 4, 100% (6/6) by week 8 (beyond week 4), 40% (4/10) by week 12 (beyond week 8), and 16% (3/19) by week 24 (beyond week 12) attained SVR.

Among the 1H patients, the same tendency was also observed (Fig. 3B). In the patients receiving 24-week treatment, 50% (3/6) of those who had no detectable HCV RNA by week 4, 40% (4/10) by week 8 (beyond week 4), and 18% (3/17) by week 12 (beyond week 8) attained SVR. None of the 10 patients who became negative for HCV RNA by week 24 (beyond week 12) showed SVR. In the patients receiving 48-week treatment, 80% (4/5) of those who had no detectable HCV RNA by week 4, 100% (5/5) by week 8 (beyond week 4), 38% (3/8) by week 12 (beyond week 8), and 16% (3/19) by week 24 (beyond week 12) had SVR. In spite of the small rate (16%), SVR was obtained from the patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment.

Fig. 4 shows the relationship between the timing of HCV RNA disappearance and the prediction value in 1H patients who received the combination therapy of interferon and ribavirin for 24 weeks. As the timing of the HCV RNA disappearance was late, the positive prediction value decreases and the negative prediction value increases. In particular, the negative prediction value at week 12 was 100%, that is, none

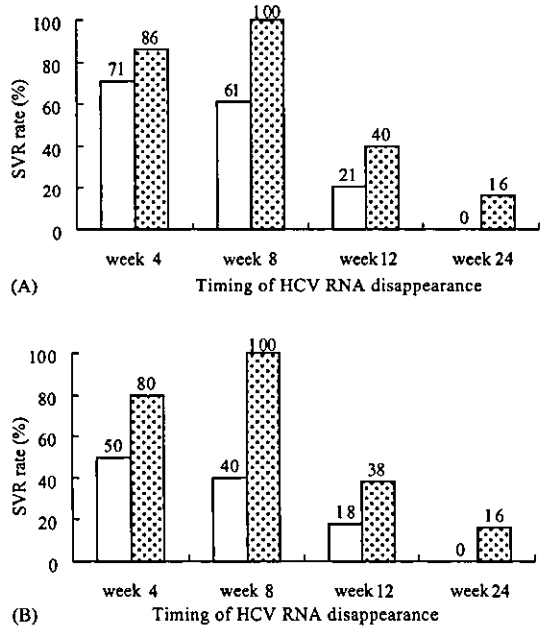

Fig. 3. Timing of HCV RNA disappearance and SVR rate (A) all patients, (B) patients with genotype 1 and high viral loads. (▭) Combination therapy of interferon and ribavirin (24-week treatment); (▨) combination therapy followed by interferon monotherapy (48-week treatment).

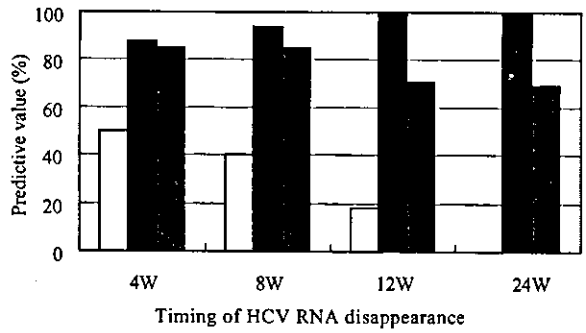

Fig. 4. Timing of HCV RNA disappearance and prediction value in patients with genotype 1 and high viral loads who received the combination therapy of interferon and ribavirin for 24 weeks. (□) Positive prediction value; (■) negative prediction value; (■) predictive accuracy.

of the patients who were positive for HCV RNA at week 12 attained SVR.

4. Discussion

In Japan, randomized control studies were performed on the combination therapy of interferon and ribavirin for 24 weeks in patients with chronic hepatitis C, and the combination therapy was approved in November 2001. However, the duration of the combination therapy is limited to 24 weeks in the medical insurance because of the lack of clinical mega-trial evidence for the combination therapy for 48 weeks in Japan. From the results of international trials, the SVR rate in 1H patients treated by the combination therapy for 48 weeks has been shown to be higher than that of those treated for 24 weeks [10]. Moreover, for interferon monotherapy, prolonged interferon treatment was reported to suppress relapse after cessation of therapy and to lead to a higher SVR rate in patients with chronic hepatitis C [15]. Our strategy, the interferon and ribavirin combination therapy with an additional 24 weeks of interferon monotherapy, was conducted against this background.

Poynard et al. [22,23] evaluated the HCV RNA relapse rates after cessation of the combination therapy in naïve patients with chronic hepatitis C. Among patients with genotype 1, the relapse rates were 62% in those treated by interferon and ribavirin combination therapy for 24 weeks and 26% in those treated for 48 weeks: among patients with genotype 2/3, 21% in those for 24 weeks and 15% in those for 48 weeks. Among patients with genotype 1, the SVR rate increased due to suppression of the relapse rate by the combination therapy for 48 weeks. On the other hand, the patients with genotype 2/3 require only 24 weeks of therapy. In our study, patients with genotype 1 and high viral load (1H group) were evaluated, distinguishing them from others (non-1H group) since the efficacy of anti-viral therapy for the 1H patients has been known to be remarkably low. Among the 1H patients, the HCV relapse rate was significantly higher in those receiving 24-week combination treatment than in those receiving 48-week treatment, 24-week combination treatment followed by interferon monotherapy for 24 weeks (78% versus 42%, $P = 0.003$). Among the non-1H patients, no significant difference was found between those receiving 24-week treatment and those receiving 48-week treatment (14% versus 20%). These results indicate that our strategy of 48-week treatment is useful for the 1H group; the non-1H group seems to require only 24 weeks of therapy, similar to the patients with genotype 2/3 in the above-mentioned.

In the 1H patients receiving 48-week treatment, HCV RNA reappeared during interferon monotherapy in 11 out of 37 patients (30%) who were negative for HCV RNA at the end of 24-week combination therapy. The break through phenomenon should be taken into account when the efficacy of this treatment is evaluated. The SVR ratio in 1H patients receiving 48-week treatment can be calculated from the prevalence of undetectable HCV RNA at 24 weeks after the beginning of combination therapy of interferon and ribavirin (75%, 82/109), of break though (30%, 11/37) and of HCV relapse rate (42%, 11/26); the expected SVR is 30% ($(82/109) \times (1 - (11/37)) \times (1 - (11/26)) \fallingdotseq 0.30$). In the same manner, the SVR ratio in 1H patients receiving 24-week treatment is expected to be 17% $(82/109) \times (1 - (35/45)) \fallingdotseq 0.17$. In 1H patients, 48-week treatment, 24-week combination treatment followed by interferon monotherapy for 24 weeks, may be the useful treatment which can be actually performed in Japan.

The relationship between the timing of HCV RNA disappearance and the SVR rate according to the duration of treatment was evaluated. SVR rates decreased with a delay in the timing of HCV RNA disappearance in patients receiving 24-week treatment; the negative prediction value at week 12 was 100%, that is, none of the patients who were positive for HCV RNA at week 12 had SVR. In spite of the small rate (16%), SVR was attained for patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment. Accordingly, treatment withdrawal should be offered to patients who remain HCV RNA-positive after 12 weeks of therapy if the patient cannot continue treatment for 48 weeks for reasons including side effects and social issues. The patients who were positive for HCV RNA at week 24 should stop treatment because additional interferon monotherapy for 24 weeks could not clear HCV RNA in all five patients who were positive for HCV RNA at week 24.

Pol et al. [24] have reported the synergistic effect of ribavirin and interferon in 343 patients with the genotype 1b. In the study, ribavirin was administered for 4, 6, 12 months in combination with interferon-α for 12 months. A 12-month course of ribavirin achieved significantly greater virological efficacy than 6 or 4 months at the end of the 12-month course of interferon-α (59, 49, and 29%), the same trend seen at the end of follow-up duration (43, 36, and 21%). These results indicate that the maximum efficacy can be obtained