not observe any production of endogenous type I interferons in cells exposed to either PS-ON or PO-ON.

The inhibitory activity of PS-ON appears to target the postbinding and prereplication stage and possibly at the fusion step of HCV infection. The fusion process appears to be structurally conserved among many enveloped viruses and can be classified into types I and II.[34] The type I membrane fusion is exemplified by the influenza and HIV-1 via hemagglutinin and gp41, respectively. The type II fusion includes the alpha-viruses and flaviviruses.[34–36] It has been proposed that HCV uses a type II fusion process because of its similarity to flaviviruses.[37] Our recent study suggests that HCV and flaviviruses are indeed structurally similar.[38] It is conceivable that the fusion process of HCV may be susceptible to inhibition by the amphipathic structure of PS-ON, but further confirmation is necessary. HCV entry has been shown to occur via receptor-mediated endocytosis and is sensitive to lysosomotropic agents and inhibitors of vacuolar ATPases.[39] The finding that PS-ON acts at the postbinding step like concanamycin A and bafilomycin A1, which are potent inhibitors of the vacuolar ATPases, supports this hypothesis. Furthermore, all HCV genotypes appeared to be susceptible to the APs equally, suggesting that the process involved is highly conserved.

HCV entry involves multiple cellular factors, such as CD81, SR-B1, Claudin-1, heparin sulfate, DC-SIGN, and L-SIGN, and possibly LDL receptor.[5–10,31] CD81 and Claudin-1 have been postulated to act on the postbinding step.[6,40] SR-B1 is likely involved in an early viral entry step to the cells. Its interaction with apolipoproteins and cholesterol transfer property appear to be important for viral entry,[41] possibly at the level of membrane fusion.[42] The overall mechanism of HCV entry is complex and involves multiple factors and steps. The APs likely interact with 1 of these essential steps to abort HCV entry. The unique inhibitory effect of the APs on HCV infection makes it a valuable reagent to study the molecular pathway of HCV entry. The APs can also be developed as a molecular probe to image and dissect biochemically this complex process.

Our study demonstrates that APs are potent inhibitors of HCV infection. APs are equally effective against all HCV genotypes and can inhibit de novo HCV infection in the human hepatocyte-transplanted uPA/SCID mouse model. This approach has the advantage of a novel and highly conserved target mechanism that is distinct from the small molecule inhibitors being developed clinically as well as the well-established pharmacology of antisense-based nucleic acid molecules in clinical trials. The effectiveness of this class of compounds in blocking de novo HCV infection supports its value in liver transplantation to prevent reinfection, which occurs invariably and presents a major problem for the management of these patients.[43] So far, prophylactic reagents based on neutralizing antibodies have been disappointing in clinical trials of liver transplantation.[44] Our studies illustrate the promise of this class of compounds as a potent antiviral against HCV and support its further development in the therapy of hepatitis C.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.04.048.

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Conflicts of interest

The authors diclose the following: J.-M.J. and A.V. are employees of REPLICor, Inc. The remaining authors disclose no conflicts.

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Randomized Trial of High-Dose Interferon-α-2b Combined With Ribavirin in Patients With Chronic Hepatitis C: Correlation Between Amino Acid Substitutions in the Core/NS5A Region and Virological Response to Interferon Therapy

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The aim of this study was to compare the efficacy of high-dose interferon (IFN)-α-2b with standard dose of IFN-α-2b in combination with ribavirin (RBV) for patients with chronic hepatitis C virus (HCV) infection, and to investigate the predictive factors associated with virological response. Two hundred Japanese patients with high HCV viral load (>100 KIU/ml) were randomized to 6 or 10 mega units (MU) of 24-week IFN-α-2b with RBV. Predictive factors were investigated; including pretreatment amino acid (aa) sequences of the core region and the IFN-sensitive determining region (ISDR). The sustained virological response rate was not different in the two groups (24% vs. 30%) but the incidence of depression was significantly higher in the 10 MU group than 6 MU group (7% vs. 0%, $P=0.02$). Younger age (<60) and HCV genotype (2a/b) were significant predictors of sustained virological response. In patients infected with genotype 1b, substitutions of core aa 70 and/or 91 were predictive for non-virological response ($P<0.001$), and substitutions in the ISDR was observed frequently in virological responders. Early viral kinetics study showed that serum HCV core antigen decreased more slowly in both patients with aa 70 and/or 91 substitutions in the core and with absence of substitutions in the ISDR. In conclusion, the use of a higher dose of IFN-α-2b in combination with RBV did not improve virological response but resulted in higher incidence of depression. Amino acid substitutions in the core and ISDR are predictive of virological response to the therapy in patients with genotype 1b and high viral load. ***J. Med. Virol. 81:640–649, 2009.***

KEY WORDS: HCV; interferon; ribavirin; core region; ISDR

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma [Kiyosawa et al., 1990; Niederau et al., 1998]. Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection. The most effective therapy available at present is the combination therapy of pegylated (PEG)-IFN and ribavirin (RBV) [Manns et al., 2001; Fried et al., 2002; Hoofnagle et al., 2003]. Among HCV genotypes, genotype 1b is the most resistant genotype to IFN therapy [Fried et al., 2002]. The limitation of use of the combination therapy for HCV infection with genotype 1b is due to the low response rate during therapy and high relapse rate after the therapy [McHutchison et al., 1998]. Several studies have evaluated the potential benefits of a larger dose of IFN with varying results [Lindsay et al., 1996; Fried et al., 2000; Ferenci et al., 2001; Hadziyannis et al., 2001; Di Marco et al., 2002; Brouwer et al., 2004]. Although treatment has been

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switched to the combination of PEG-IFN and RBV in recent years, it is important to know if a larger dose of IFN is beneficial to patients with chronic hepatitis C.

Many molecular mechanisms through which HCV evades host innate immunity have been reported to date. HCV core, E2 and NS5A proteins have been reported to inhibit the IFN signaling system [Gale et al., 1997; Taylor et al., 1999; Blindenbacher et al., 2003; Bode et al., 2003; Foy et al., 2003; Lin et al., 2006; Ciccaglione et al., 2007]. Variations of amino acid (aa) sequences in the E2 and the NS5A region have been reported to correlate with the effect of IFN therapy [Enomoto et al., 1996; Chayama et al., 1997, 2000; Polyak et al., 1998, 2000; Hashimoto et al., 1999; Puig-Basagoiti et al., 2001; Pascu et al., 2004; Gaudy et al., 2005; Brillet et al., 2007; Torres-Puente et al., 2008]. Recently, Akuta et al. [2005, 2006, 2007a, b] reported that substitution of aa 70 and/or 91 in the core region is an independent and significant predictor of non-virological response.

The aim of the present study was to evaluate the therapeutic efficacy and safety of a large dose of IFN-α-2b combined with RBV. For this purpose, a randomized trial was conducted to compare the therapeutic effects of high-dose (10 MU) versus standard dose (6 MU) of IFN-α-2b combined with RBV in patients with high HCV viral titers. The second endpoint of this study was to analyze the predictive factors associated with virological response including aa substitutions in the core region and the NS5A region.

PATIENTS AND METHODS

Patient Selection

Two hundred adult patients enrolled into the study. The inclusion criteria were positivity for antibody to HCV, HCV RNA levels higher than 100 KIU/ml, and the diagnosis of chronic hepatitis C was confirmed by liver biopsy. The liver biopsy specimens were evaluated as described by Desmet et al. [1994], and classified into F0 to F3. None of the patients included in this study had liver cirrhosis (F4). Other exclusion criteria included leukocytopenia (leukocyte $<4{,}000/mm^3$) and anemia (hemoglobin concentration <10 g/dl). Patients with human immunodeficiency or hepatitis B super infection, previous organ transplantation, other causes of liver disease, poorly controlled diabetes, de-compensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, hemophilia or auto-immune type diseases were also excluded.

Study Design

The double-blind, multi-center randomized clinical trial was conducted in 23 centers in Hiroshima city (The Hiroshima Liver Study Group). The study was approved by the Ethics Committee of Hiroshima University. Written informed consent was obtained form all participants. Eligible patients were assigned randomly into either of the two groups without further stratification using sequentially numbered cards in sealed envelopes. Patients were randomized to treatment with combination of IFN-α-2b (Intron A, Shering Plough, Kenilworth, NJ) at a dose of 6 MU (Group A) or 10 MU (Group B) plus RBV (Rebetol, Shering Plough). IFN-α-2b was administered intramuscularly daily over the initial 2 weeks and three times weekly in the remaining 22 weeks. The dose of RBV was adjusted according to body weight (600 mg/day for ≤60 kg, 800 mg/day for >60 kg). Adverse events were monitored clinically by careful interview and hematological examination throughout the study. The dosage of RBV was reduced in patients who experienced a decrease in hemoglobin concentration to <10 g/dl.

Blood samples were taken 2 and 4 weeks after the beginning of therapy and every 4 weeks thereafter. Biochemical and hematological tests were performed in each center, including alanine amino transferase (ALT). Part of the serum samples were kept frozen at −80°C until further analysis. Viral genotypes were determined by phylogenetic analysis after reverse transcription (RT)-polymerase chain reaction (PCR) and direct sequencing.

Assessment of Efficacy

Serum HCV RNA was detected by nested PCR assay (Cobas Amplicor HCV test v 2.0, Roche Diagnostics, Tokyo, Japan; limit of detection, 50 IU/ml) at weeks 2, 4 and every 4 weeks during treatment and 24 weeks after the cessation of therapy. Positive samples were analyzed further by quantitative assay (Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics; limit of detection, 500 IU/ml).

The primary endpoint of this study was sustained virological response, defined as undetectable serum HCV RNA by qualitative PCR test and normalization of ALT 24 weeks after the treatment. Non-virological response was applied to those patients with positive qualitative HCV RNA PCR tests in all examinations. Virological response was used to define the remaining patients who became PCR negative at least once during the treatment.

Nucleotide Sequencing of the Core and NS5A Gene

The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR] [Enomoto et al., 1996]) sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: The first-round PCR was performed with primers CC11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and the second-round PCR with primers CC9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse) as described by Akuta et al. [2005, 2006, 2007a, b]. After denaturation at 95°C for 5 min, 35

cycles of amplification were set as follows; denaturation for 30 sec at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. (b) Nucleotide sequences of ISDR in NS5A: PCR was performed with IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5OA2KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′). After denaturation at 98°C for 30 sec, 35 cycles of amplification were set as follows; denaturation for 10 sec at 98°C, annealing of primers for 30 sec at 66°C, and extension for 15 sec at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated in a 2% agarose gel and purified by GENECLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [Kato et al., 1990].

Quantitation of HCV Core Antigen

HCV core antigen levels were measured using stored serum samples just before and 4 weeks after the start of the therapy as described previously [Aoyagi et al., 1999].

Statistical Analysis

The baseline characteristics of the patients in the two groups were compared and the differences were assessed by Chi-square test with Yate's correction and Mann–Whitney *U*-test. To assess the sustained virological response rates, an intention-to-treat (ITT) analysis and a per-protocol (PP) analysis were conducted. The response rates and substitutions in the core region and the ISDR were compared by Fisher's exact test. All *P* values reported are two-sided and those less than 0.05 were considered significant. To determine the predictors of sustained virological and non-virological responses, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors included the following variables: age, sex, alcohol consumption, past history of IFN monotherapy, body mass index, ALT, hemoglobin, platelets, HCV RNA level, genotype, liver histology, total RBV dose (adjusted for body weight [mg/kg]) and total dose of IFN-α-2b. The odds ratio and 95% confidence intervals (95% CI) were also calculated. Variables with statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

Patient Demographics

Patient enrollment started in January 2002, and the trial ended in March 2005. The disposition of patients throughout the trial is shown in Figure 1. A total of 200 patients were randomized to treatment, and 198 patients met the eligibility criteria and underwent

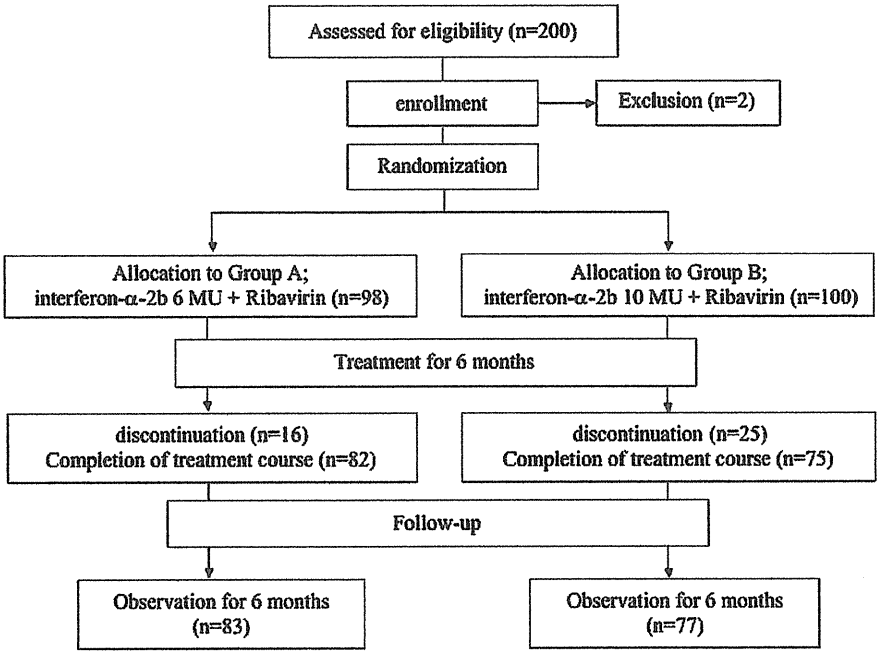

Fig. 1. Flow chart of number of patients throughout the trial. A total of 200 patients were included in this study. One hundred ninety-eight patients met the eligibility criteria and they underwent randomization, 98 patients in Group A and 100 patients in Group B.

TABLE I. Baseline Characteristics of the Patients

Characteristic	Group A (n = 98)	Group B (n = 100)	P
Age (years)[a]	55 ± 10.3	55 ± 11.0	0.43
Male sex (%)	63	75	0.07
Alcohol consumption (%)[b]	23	20	0.61
Past history of IFN monotherapy (%)	33	35	0.72
Body-mass index (kg/m^2)[a]	23.3 ± 2.9	24.2 ± 3.6	0.05
ALT (IU/L)[a]	79.2 ± 45.3	109.4 ± 111.2	0.31
Hemoglobin (g/dl)[a]	14.2 ± 1.4	14.5 ± 1.2	0.02
Platelets (×10^4/mm^3)[a]	14.8 ± 4.8	16.5 ± 5.0	<0.05
HCV RNA (KIU/ml) (%)			
100–850	49	47	
≥850	51	53	0.80
Genotype (%)			
1b	82	72	
2a/2b	17	28	0.32
3a/3b	1	0	
Liver histology[a,c]	2.0 ± 0.84	1.8 ± 0.82	0.05

ALT, alanine aminotransferase.
[a]Values are mean ± SD.
[b]Percentage of patients who consumed alcohol at >30 g/day.
[c]Liver fibrosis was scored 0 (F0), no fibrosis; 1 (F1), periportal expansion; 2 (F2), portoportal septa; 3 (F3), portocentral linkage or bridging fibrosis.

randomization. Ninety-eight patients were assigned to Group A and 100 patients to Group B. Patients were observed for 24 weeks after the treatment. Sixteen patients of Group A and 25 patients of Group B discontinued the treatment because of adverse events. Table I lists the baseline characteristics of the patients. Hemoglobin concentrations and platelet counts were higher in group B patients. The other parameters were similar between the two groups.

Overall Sustained Virological Response

The effect of therapy in the two groups is summarized in Table II. The sustained virological response rate was lower significantly in patients of group B with genotype 2a/b relative to those of group A (ITT analysis). This reflects the fact that a larger number of patients dropped out form the protocol because of the adverse effects (1 [6%] of 16 in group A and 10 [43%] of 23 in group B, $P = 0.02$). All patients who stopped treatment did not achieve sustained virological response. Patients with genotype 1b had a lower sustained virological response rate than those with genotype 2a/b (33/124 [27%] vs. 26/39 [67%], $P < 0.01$).

Dose Reduction or Discontinuation and Adverse Events

Table III summarizes the laboratory abnormalities and the dose reduction and discontinuation of IFN-α-2b and RBV due to adverse events. The overall discontinuation rate was 16% for group A and 25% for group B (not significant). The most frequent adverse event associated with dose reduction was anemia. A larger number of patients of group B developed depression ($P = 0.02$).

Predictive Factors Associated With Sustained Virological Response

Univariate analysis identified three parameters that correlated with sustained virological response: age (<60 years, $P = 0.007$); genotype (2a/b, $P < 0.001$); and platelet count (>15 × 10^4/mm^3, $P = 0.01$). Multivariate analysis including the above variables identified two parameters that independently predicted sustained virological response: age ($P = 0.02$) and genotype ($P < 0.001$) (Table IV).

TABLE II. Rates of Sustained Virological Response According to Adherence

Genotype	Group A	Group B	P
1b	n = 68	n = 56	
ITT	16/68 (24%)	17/56 (30%)	0.39
PP	16/53 (30%)	17/41 (41%)	0.25
2a/b	n = 16	n = 23	
ITT	15/16 (94%)	11/23 (48%)	0.005
PP	15/15 (100%)	11/13 (85%)	0.21

ITT, intention to treatment analysis; PP, per protocol analysis; IFN, interferon; RBV, ribavirin.

TABLE III. Dose Reduction or Discontinuation and Adverse Events

	Group A (n = 98) % (n)	Group B (n = 100) % (n)	P
Discontinuation	16 (16)	25 (25)	0.13
Dose reduction or discontinuation of			
IFN	20 (20)	41 (41)	0.002
RBV	36 (35)	50 (50)	0.04
IFN and/or RBV	37 (36)	55 (55)	0.01
Depression	0 (0)	7 (7)	0.02

IFN, interferon; RBV, ribavirin.

TABLE IV. Factors Associated With Sustained Virological Response to Combination Therapy of Interferon Plus Ribavirin by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	*P*
Age (years)	0: ≥60	1	
	1: <60	2.420 (1.173–5.002)	0.020
Genotype	0: 1b	1	
	1: 2a/b	5.301 (2.401–11.702)	<0.001

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown.

Analysis of aa Sequences in the Core Gene in Genotype 1b Patients

The relationship between aa substitutions in the core region and the viral response to therapy was investigated in patients with genotype 1b using 93 available serum samples. Figure 2 shows the sequences of aa 61–110 of the HCV core region in 93 patients just before commencement of treatment. Table V summarizes the relationship between the response to IFN therapy and

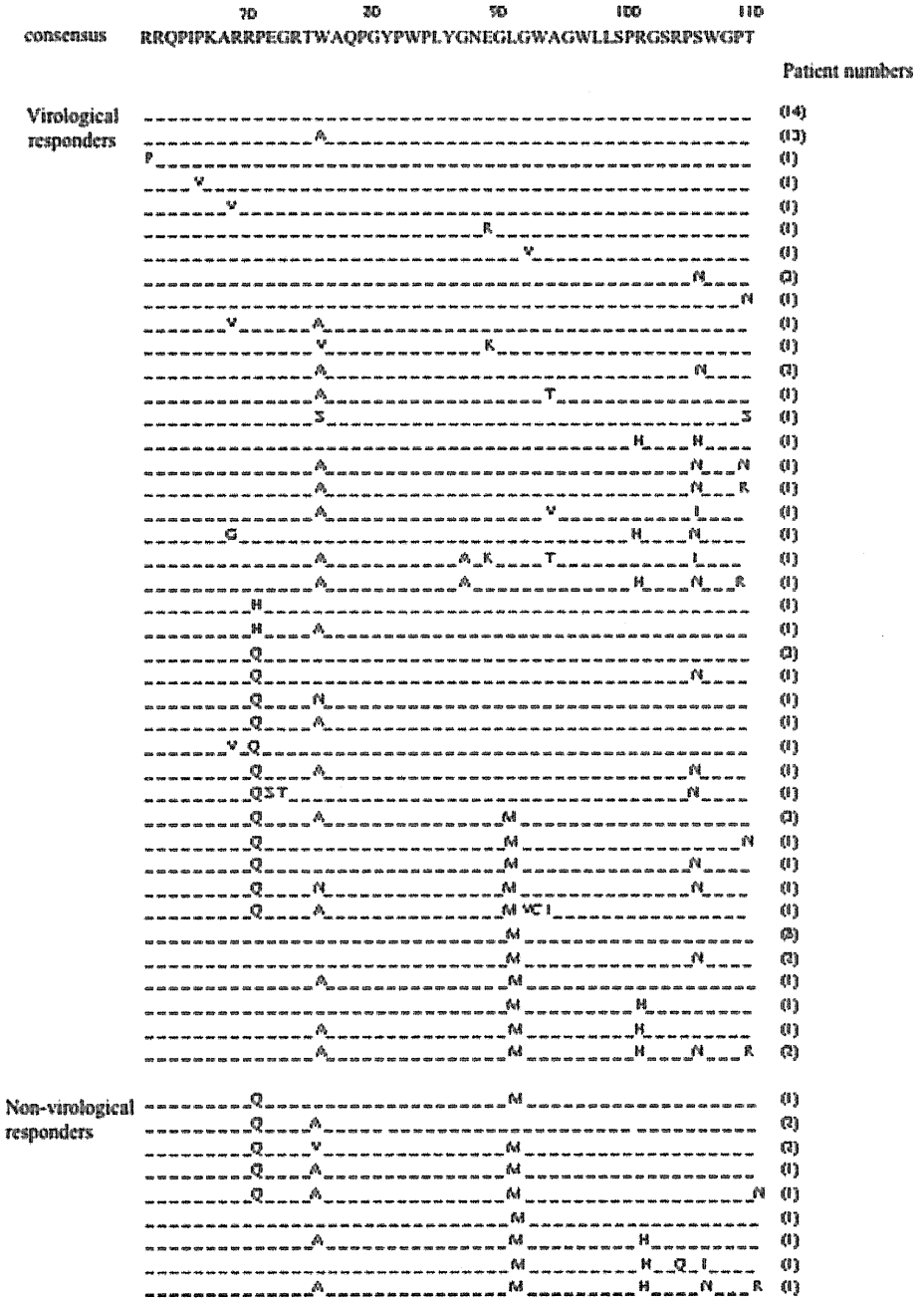

Fig. 2. Sequences of amino acids 61–110 in the core region at commencement of combination therapy in 93 patients infected with hepatitis C virus genotype 1b. Dashes indicate amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids are shown by standard single-letter codes.

TABLE V. Amino Acid Substitutions in the Core Region in Non-Virologic Responders and Virological Responders in 93 Patients With HCV Genotype 1b

Presence of substitution site	Non-virological response (n = 11) % (n)	Virological response (n = 82) % (n)	*P*
aa 70	64 (7)	23 (19)	0.01
aa 75	73 (8)	45 (37)	0.11
aa 91	82 (9)	30 (25)	0.001
aa 106	27 (3)	31 (26)	1.0
aa 110	18 (2)	12 (10)	62
aa 70 and 91	45 (5)	10 (8)	0.006
aa 70 and/or 91	100 (11)	44 (36)	<0.001

aa, amino acid.

substitutions of aa. Among aa substitutions, only substitutions of aa 70 and 91 were associated with non-virological response. All non-virological responders had aa substitutions at 70 or 91, or both substitutions. In contrast, only 36 of 82 (44%) virological responders had these substitutions ($P < 0.001$, Table V). In contrast to non-virological response, these substitutions were not predictive for sustained virological response ($P = 0.11$–0.82).

Next, the effect of substitutions of aa 70 and 91 in the core region on early viral kinetics was analyzed by dividing patients into four groups according to the pattern of aa substitutions. As shown in Figure 3, the most rapid decrease in core antigen was noted in patients where both aa 70 and 91 were wild-type (double-wild). In contrast, the poorest reduction was noted in patients with both of aa 70 and 90 substitutions (double-mutant). Patients with either of the two aa substitutions (mutant/wild or wild/mutant) showed decrease in between the above two groups. HCV core antigen decreased below the detectable limit (20 fmol/L) at week 4 in 37 of 40 (93%) patients who had neither aa 70 nor aa 90 substitutions. In contrast, it decreased below the detectable limit in only 5 of 12 patients (42%) who had both aa 70 and 91 substitutions ($P = 0.031$).

Analysis of Nucleotide Sequence of the NS5A Gene

The aa sequences of ISDR in the NS5A gene were determined in 40 patients where PCR for this region was positive. Seventeen of 40 patients had no aa

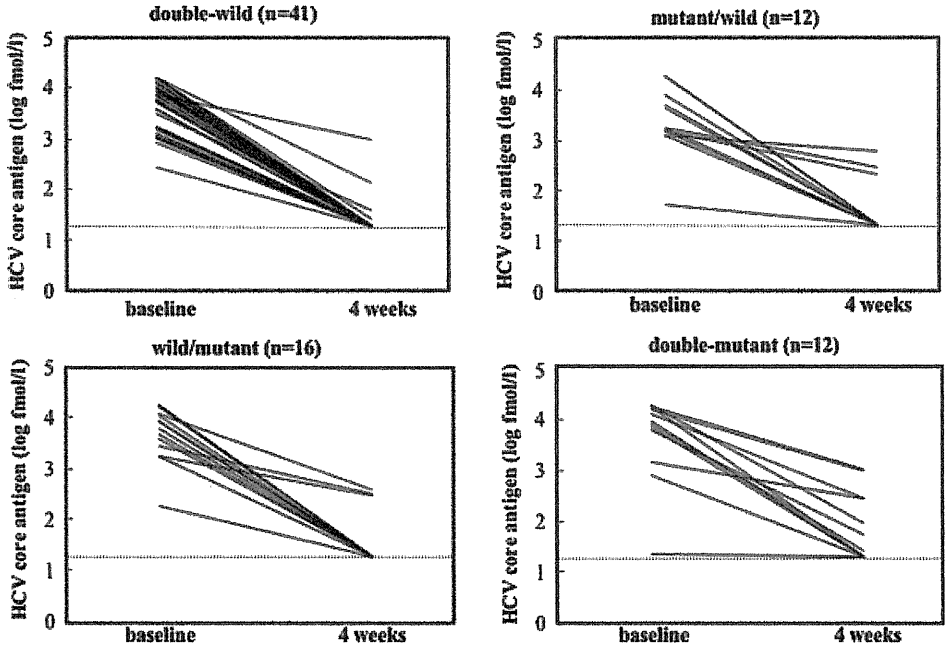

Fig. 3. Reduction of amount of HCV core antigen based on the presence of substitutions at amino acid 70 or 91. Eighty-one patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. The response was divided into four patterns based on the presence of substitution(s) at aa 70 and/or 91. Double-wild; no substitution, neither at aa 70 nor aa 91, mutant/wild; substitution only at aa 70, wild/mutant; substitution only at aa 91, double-mutant; substitutions at both aa 70 and 91. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VI. Amino Acid Substitutions in the IFN-Sensitive Determining Region (ISDR) in Non-Virologic Responders and Virological Responders in 40 Patients With HCV Genotype 1b

ISDR[a]	Non-virological response (n = 8) % (n)	Virological response (n = 32) % (n)	*P*
Wild-type (n = 17)	36 (6)	64 (11)	
Mutant-type (n = 23)	9 (2)	91 (21)	0.012

aa, amino acid.
[a]Absence of amino acid substitutions was evaluated as wild-type, and presence of one or more amino acid substitutions as mutant-type.

substitutions in ISDR (wild-type), while the remaining 23 patients had one or more substitutions (mutant-type). The relationship between aa substitutions of ISDR and effects of treatment was analyzed. The existence of aa substitution in the ISDR was not predictive for sustained virological response ($P = 0.137$), however, such substitution was observed frequently in virological responders compared to non-virological responders (66% vs. 25%, $P = 0.012$) (Table VI). The use of a different categorization based on the number of substitutions in the ISDR (0/1 vs. ≥2) yielded similar results, that is, not predictive for sustained viral response but predictive for virological responders (data not shown).

HCV core antigen decreased more rapidly in patients with ISDR mutant-type compared to those with wild-type (Fig. 4). HCV core antigen decreased below the detectable limit at week 4 in only 6 of 17 (35%) patients with wild-type. In contrast, it decreased below the detectable limit in 19 of 23 (83%) in patients with ISDR mutant-type ($P = 0.006$).

Predictive Factors Associated With Sustained Virological Response and Non-Virological Response in Patients With Genotype 1b

Finally, the predictive factors associated with sustained virological response and non-virological response were analyzed in patients with genotype 1b, including aa substitutions in the core region and ISDR. Univariate analysis showed two parameters correlated with sustained virological response: age (<60 years, $P = 0.004$) and presence of aa substitutions in the core (aa 70 and/or 91, $P = 0.04$). However, multivariate analysis, including the above variables, identified no parameters that influenced sustained virological response independently (age, $P = 0.89$; core, $P = 0.07$). Univariate analysis showed two parameters correlated with non-virological response: age (<65 years, $P = 0.02$) and aa substitutions in the core (double-mutant, $P = 0.01$). Multivariate analysis including the above variables identified aa substitutions in the core as an independent factor that influenced non-virological response (age, $P = 0.40$; core, $P = 0.03$) (Table VII).

DISCUSSION

Treatment of patients with chronic HCV infection had improved by the advent of PEG-IFN and RBV combination therapy. However, a substantial number of patients do not respond to the combination therapy [Taliani et al., 2006]. Several studies described attempts to improve the sustained virological response rate in such patients. Recent trials showed that a longer treatment period results in a higher sustained virological response rate [Berg et al., 2006; Sánchez-Tapias et al., 2006]. However, there are no conclusive studies that compared a larger dose of IFN with standard dose. Although the treatment had shifted in recent years to PEG-IFN and

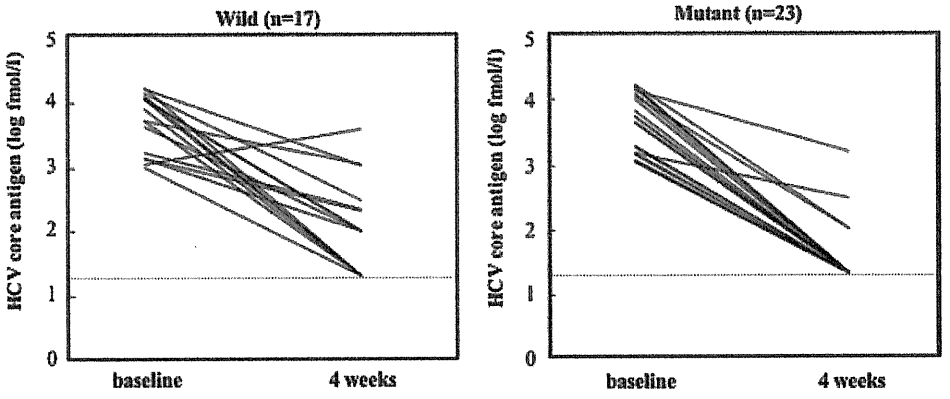

Fig. 4. Reduction of amount of HCV core antigen based on the presence of substitutions in the ISDR. Sixty-five patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. Patients were divided into two groups based on the presence of amino acid substitution(s) in the ISDR. Wild-type; absence of substitutions, mutant-type; presence of one or more substitutions. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VII. Factors Associated With Non-Virological Response to Combination Therapy of Interferon Plus Ribavirin Identified by Multivariate Analysis in Patients With Genotype 1b

Factor	Category	Odds ratio (95% CI)	P
Amino acid substitutions in the core region[a]	0: No double-mutant	1	0.028
	1: Double-mutant	7.000 (1.238–39.566)	

Only the variable that achieved statistical significance ($P < 0.05$) on multivariate logistic regression is shown.
[a]The mutant aa 70 and 91 pattern was evaluated as double-mutant, and other patterns as non-double-mutant.

RBV combination therapy, a different dose of IFN was used in the present study to test whether a larger dosage of IFN improves the outcome of IFN therapy.

In this study, the larger dose did not increase sustained virological response nor decrease non-virological response. Instead, the dose reduction of IFN and/or RBV was significantly higher in the higher dose group (Table III). Furthermore, the incidence of depression was significantly higher in the high-dose group (Table III). These results suggest that a high dose of IFN is not beneficial to patients who receive IFN and RBV combination therapy, and probably who will receive the PEG-IFN and RBV combination therapy.

The predictive factors for sustained virological response and non-virological response to the combination therapy for patients with genotype 1b were analyzed. Logistic regression analyses identified pretreatment substitutions at both aa 70 and 91 in the core region (double-mutant) as a singular predictive factor for non-virological response (Table VII). Furthermore, the existence of aa substitution in the ISDR was significantly more frequent in virological responders compared to non-virological responders (Table VI), in agreement with previous reports [Puig-Basagoiti et al., 2001; Pascu et al., 2004]. It has been reported that the numbers of aa substitutions in the ISDR correlate with serum HCV RNA levels [Enomoto et al., 1996]. However, no apparent correlation was observed in this study. As shown in Figures 3 and 4, patients who had substitutions of aa 70 and/or 91 in the core region or no aa substitutions in ISDR had poor initial reduction in the HCV core antigen. These results are consistent with recent studies that have shown the importance of a rapid initial decline of the viral load in obtaining a better response rate [Fried et al., 2002; Davis et al., 2003]. These results suggest that aa substitution analysis should provide important information on treatment of patients with genotype 1b.

The core protein of the HCV has been reported to disturb the IFN signaling by interacting with STAT1 SH2 domain [Lin et al., 2006] or repressing IRF1 [Ciccaglione et al., 2007]. These studies did not analyze the effect of aa substitutions in the core region. Further study is necessary to clarify the effect of aa substitutions in the core region and to identify a molecular target to improve the therapy.

Although aa substitution in the core region was identified as an important predictor in patients with genotype 1b in this study, aa substitutions of the core region and ISDR in patients with genotype 2a/b infection were not analyzed. Although the sustained virological response rate in patients who completed the therapy was high (26/28 [93%], per protocol analysis), few patients were unable to achieve sustained virological response. Furthermore, a significant number of patients could not complete the treatment course because of adverse effects. A more effective and easy to complete therapy should be developed to treat such patients. The predictive factors in such patients should also be clarified.

The recent development of a new type of drug targeting NS3/4 protease may improve the outcome of treatment in patients with chronic hepatitis C [Reesink et al., 2006; Forestier et al., 2007; Kieffer et al., 2007; Sarrazin et al., 2007a,b]. However, drug resistant mutants might emerge against such a small molecule therapy targeting viral enzyme(s). The functions of virus proteins that resist IFN including core, ISDR and PePHD should be clarified further to develop a better therapy that can achieve a higher sustained virological response rate with fewer and milder side-effects.

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Journal of Gastroenterology

Epidemiology of hepatocellular carcinoma in Japan

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Primary liver cancer, 95% of which is hepatocellular carcinoma (HCC), is ranked third in men and fifth in women as a cause of death from malignant neoplasms in Japan. The number of deaths and death rate of HCC began to increase sharply in 1975. These numbers peaked at 34510 and 27.4/100000, respectively, in 2004, but decreased to 33662 annual deaths and a 26.7/100000 death rate in 2006. Although hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are both major causes of HCC, HCV-related HCC represents 70% of all cases. The incidence of HCC without hepatitis B surface antigen (HBsAg) or antibodies to HCV (anti-HCV) accounts for 8%–15% of HCC patients nationwide. Geographically, HCC is more frequent in western than eastern Japan, and death rates of HCC in each prefecture correlate with anti-HCV, but not HBsAg, prevalence. Interferon therapy for chronic hepatitis C reduces the risk of development of HCC, especially among patients with sustained virological response. Further research should focus on the mechanisms of carcinogenesis by HCV and HBV, development of more effective treatments, and establishment of early detection and preventative approaches. Better understanding of HCC unrelated to HCV and HBV, possibly caused by steatohepatitis and diabetes, should also be a major concern in future studies.

Key words: HCC, HCV, HBV, nonalcoholic steatohepatitis (NASH), interferon

Introduction

The three leading causes of death in Japan since 1981 are malignant neoplasms, cardiovascular diseases, and cerebrovascular diseases. For the past 30 years, liver cancer has been the third leading cause of death from malignant neoplasms in men, following lung and stomach cancer. In women, liver cancer has ranked fifth as a cause of death during the past decade, following colon, stomach, lung, and breast cancer. Primary liver cancer can be classified into three types according to the cell from which the cancer originated, namely, hepatocellular carcinoma (HCC), cholangiocellular carcinoma, and other. As HCC accounts for up 95% of all primary cancer cases, the term "liver cancer" usually means HCC.[1]

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two major causes of HCC in Japan.[2,3] The increase in incidence of HCC in Japan, however, is largely attributable to the increase of HCV infection in the general population during the past 50 to 60 years.[2]

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Changes in deaths and death rates of primary liver cancer

Changes in annual deaths from primary liver cancer among different age groups between 1958 and 2006 are shown in Fig. 1. The total number of deaths from HCC was stable at fewer than 10000 persons/year until 1975 before showing a sharp increase. The spike in 1995 resulted from a change in the International Classification of Disease (ICD) code from ICD 9 to ICD 10, which included intrahepatic bile duct cancer, accounting for approximately 5% of HCC deaths.

The majority of HCC mortalities were in patients below the age of 69 until 1999, when this age reached 70 years. In 2006, 66% of patients with HCC were over 70. The number of deaths from HCC reached 34510 in 2004, but decreased to 33662 in 2006.

The death rates of liver cancer by sex (Fig. 2) are consistently higher in men than in women. A sharp rise in the death rate of primary liver cancer in men began

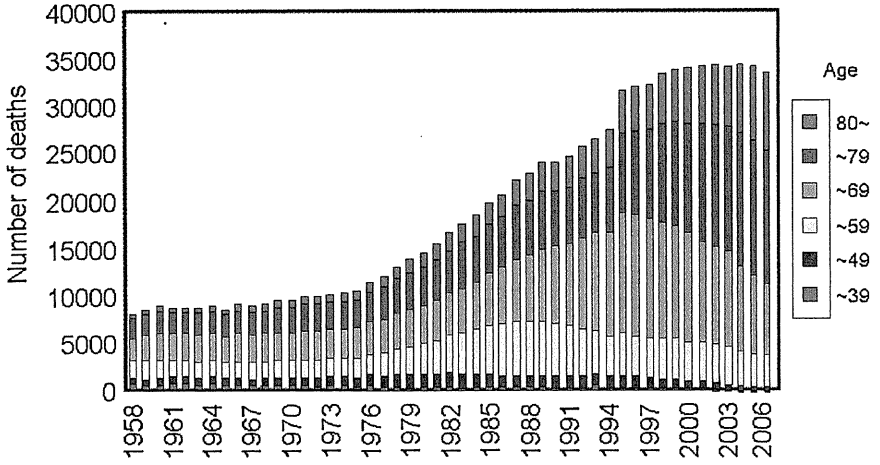

Fig. 1. Changes in annual deaths of patients (by age, in years) with primary liver cancer between 1958 and 2006. (Taken from the Vital Statistics of Japan, released every year by the Ministry of Health, Labour, and Welfare)

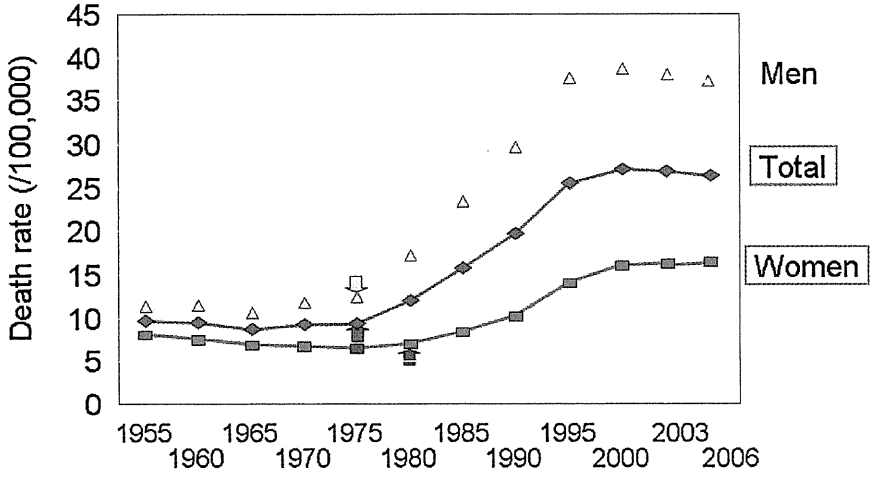

Fig. 2. Changes in the death rate of primary liver cancer in men (*triangles, yellow*), women (*rectangles, pink*), and in total (*diamonds, blue*)

in 1975, and a more gradual rise in women commenced in 1980. The total age-adjusted death rate peaked in 2002 (27.5/100000 persons in 2002), and decreased to 27.0 in 2003. In 2006, the total age-adjusted death rate stood at 26.7/100000, which is caused by a decrease in death rate (36.7) in men, but offset by an increase in women to 17.2.

Age and sex in HCC

Changes in the mean age of HCC patients and male/female ratio every 2 years between 1984 and 2003 are shown in Fig. 3. In that period, the mean age of female HCC patients was higher than that of males, and the mean ages of both sexes progressively increased. As reported previously, however, HBV-related HCC was stable from 1982 to 2003, implying that this change originated from HCV-related HCC patients. The male/female ratio was 4.5 in 1984–1985 and 2.5 in 2002–2003 (see Fig. 3), showing that the proportion of female patients with HCC had increased. This increase in female patients is also considered as a consequence of increased HCV-related HCC.

Changes in etiology of HCC in Japan

A nationwide survey on primary liver cancer has been conducted every 2 years since 1968 by the Liver Cancer Study Group of Japan.[1,4–9] Five serological surveys performed between 1990 and 2001 have documented that most patients with HCC are positive for either HBsAg or antibodies to HCV (anti-HCV). Tests for HBsAg became available in 1975 and those for anti-HCV in 1990. HBsAg-positive cases of HCC constituted 42% of patients in 1977–1978, but only 15.5% in 2002–2003 (Fig. 4). In contrast, anti-HCV-positive cases of HCC accounted for more than 70% of cases diagnosed until 2000–2001. However, this number dipped to 69.6% in 2002–2003, and has since remained at less than 70%. In contrast, HCV of unknown origin and other cases of HCC have been increasing gradually, and constituted 14.9% of all cases in 2002–2003.

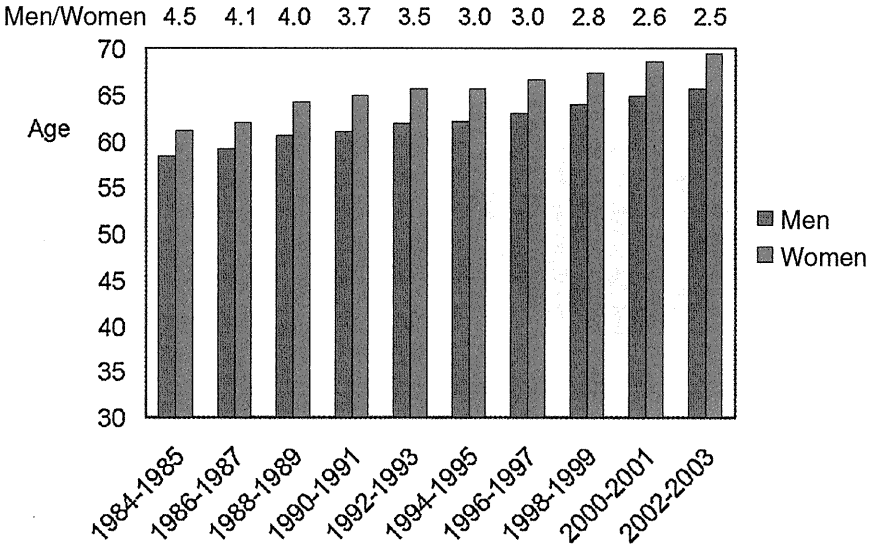

Fig. 3. Changes in the mean age (in years) of men (*blue bars*) and women (*pink bars*) patients with hepatocellular carcinoma (HCC) between 1984 and 2003

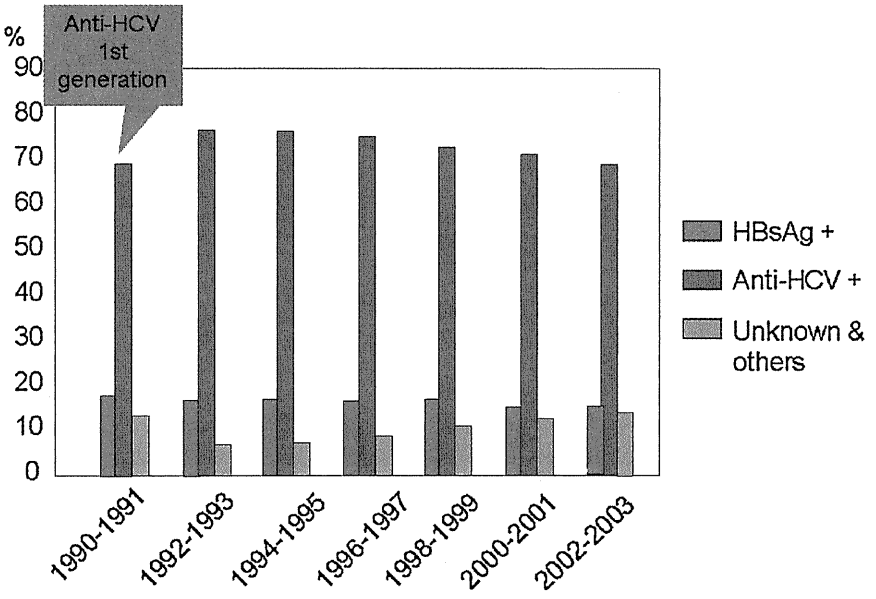

Fig. 4. Changes in the etiology of HCC between 1990 and 2003: hepatitis B surface antigen (*HBsAg+, pink*), antihepatitis C virus (*anti-HCV+, blue*), and *unknown* and *others* (*green*)

In cross-sectional studies conducted at Shinshu University Hospital, HCV-related HCC was found in the majority of cases (72%) (Fig. 5). Non-B non-C HCC (NBNC-HCC) accounted for 10% of cases in 2002–2007. In these 28 patients, nonalcoholic steatohepatitis (NASH) accounted for 14%.

Geographic variation of liver cancer and HBV/HCV infection

Although Japan is a relatively small country with a homogeneous population, the incidence of HCC varies greatly among different regions. The Vital Statistics of Japan for 2005 published in 2007 by the Japanese Ministry of Health, Labour, and Welfare on the incidence of deaths as a result of HCC in its 48 prefectures shows a steady increase in death rates of HCC from east to west in Japan. The average age-adjusted death rate of HCC among 48 prefectures was 27.2 per 100000 persons in 2005 (Fig. 6). Furthermore, nationwide health screening for HBsAg and anti-HCV in citizens over 40 years of age has been performed since 2002, and the prevalence rates of these markers have been analyzed for each prefecture in Japan. In 2006, the average HBsAg and anti-HCV prevalences were 1.0% and 0.7%, respectively, in this group (see Fig. 6). There was a highly significant association between the death rate of HCC and prevalence of anti-HCV in each prefecture (Fig. 7; correlation coefficient = 0.66; $P < 0.001$,

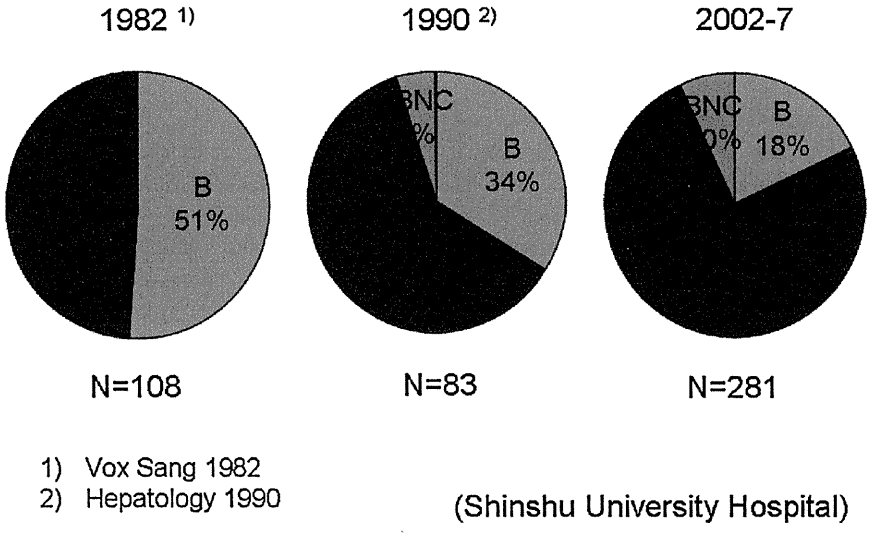

Fig. 5. Clinical features of hepatitis B (*B*) virus (HBV)- and hepatitis C (*C*) virus (HCV)-related HCC in 1982, 1990, and 2002–2005 at Shinshu University Hospital. *NBNC*, non-B non-C

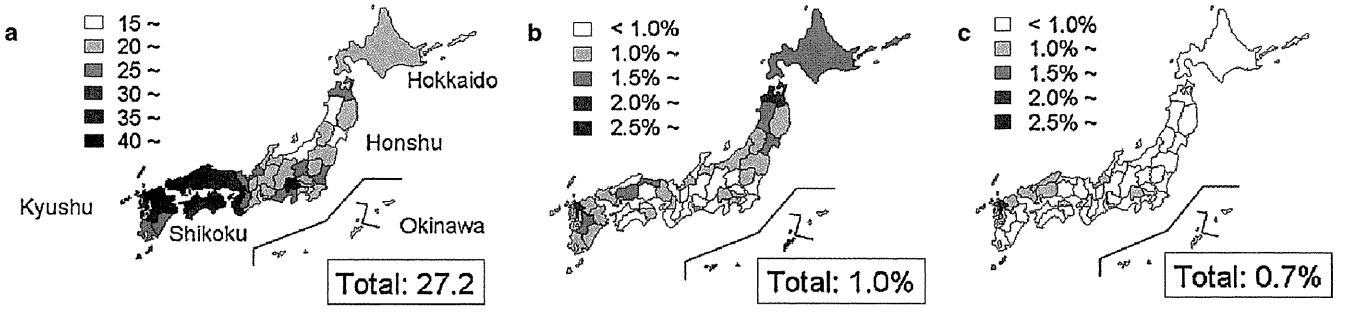

Fig. 6. **a** Death rate of primary liver cancer was 27.2 per 100000 in 2005 among people over 40 years of age in 48 prefectures in Japan. In the same group in 2006, HBsAg prevalence was 1.0% (**b**) and anti-HCV prevalence was 0.7% (**c**)

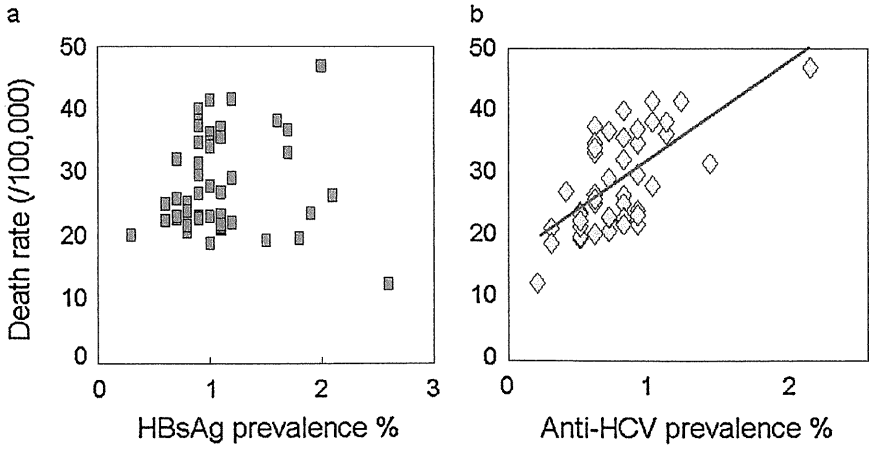

Fig. 7. Relationship between the death rate of primary liver cancer and prevalence of (**a**) HBsAg ($r = 0.02$, P = NS) and (**b**) anti-HCV ($r = 0.66$, $P < 0.001$, $y = 16.3x + 16.1$) among the general population over 40 years of age in 2006

$y = 16.3x + 16.1$), but no correlation with the prevalence of HBsAg was seen (Fig. 6). For instance, although Okinawa Prefecture had the highest prevalence of HBsAg (2.6%), its HCC death rate was the lowest (12.5/100000 persons). A possible explanation for this discrepancy is that the HBV genotype Bj, which shows good clinical prognosis,[10,11] is the dominant HBV genotype in Okinawa. In contrast, areas with high rates of anti-HCV, especially in western Japan, had high death rates from HCC. HCV appears to be the major contributor to primary liver cancer in these regions; Saga Prefecture shows both the highest HCC death rate (46.9/100000) and highest prevalence rate of anti-HCV (2.1%) in Japan.

Table 1. Summary of findings in representative studies on the incidence of hepatocellular carcinoma (HCC) among patients with chronic hepatitis C virus (HCV) infection treated with interferon alone in Japan

	Treated							
	Untreated		Non-SVR		SVR		Total	
Author	No. HCC/no. cases	%	No. HCC/no. cases	%	No. HCC/no. cases	%	No. HCC/no. cases	%
Kasahara[12]			41/709	5.8	5/313	1.6	46/1022	4.5
Imai[13]	19/140	13					18/419	4.3
Ikeda[14]	67/452	15	23/730	3.2	5/461	1.1	28/1191	2.4
Yoshida[15]	67/395	17	214/1556	13.8	27/836	3.2	241/2392	10.1
Okanoue[16]			119/849	14.0	8/397	2.0	127/1246	10.2
Ikeda[17]	59/352	17	34/171	19.9	1/53	1.9	94/576	16.3
Total	212/1339	16	432/4015	10.8	46/2060	2.2	554/6846	8.1

SVR: sustained virological response

Antiviral therapy suppresses the incidence of HCC

As described in prior sections, HCV infection is the major cause of HCC in Japan, suggesting that eradication of HCV may decrease the incidence of HCC. A summary of different studies on the incidence of HCC among patients with chronic hepatitis C who were treated with interferon in Japan can be found in Table 1.[12–17] These studies show a moderate decrease in the risk of HCC in patients with chronic hepatitis C treated with interferon, especially in patients with sustained virological response as compared with nonresponders and nontreated patients.

Recently, Ikeda et al. prospectively studied patients with chronic HCV infection and evidence of occult HBV infection [negative results for HBsAg and HBV DNA but positive results for antibodies to hepatitis B core antigen (anti-HBc) in serological testing].[17] Patients with HCV-related cirrhosis and positive results for anti-HBc were at high risk for HCC, even in patients with a sustained virological response to interferon (IFN) therapy. Thus, anti-HBc positivity is a marker of high risk for HCC among patients with HCV-related cirrhosis.

Between 1992 and 2001, approximately 300000 patients with chronic hepatitis C received IFN monotherapy in Japan. As shown in Fig. 1, it is remarkable that the number of deaths and the death rate of HCC began to decrease in 2005. These phenomena suggest that antiviral treatment indeed reduces the risk of HCC in patients with HCV infection.

Conclusion

The number of deaths and death rate of HCC showed a sharp increase from 1975 onward but had begun to decrease in 2006. Although both HBV and HCV infection play a major role in HCC in Japan, HCV-related HCC represents 70% of all cases. The incidence of HCC without HBsAg or anti-HCV accounts for 7%–15% in Japan, and half of NBNC-HCC cases are of unknown origin. Geographically, HCC is more frequent in western than eastern Japan, and the death rates of HCC in each prefecture correlate with anti-HCV, but not HBsAg, prevalence. IFN therapy for chronic hepatitis C reduces the risk of development of HCC, especially in patients with sustained viral response.

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