associated with genotype and viral load as well as viral mutation in genotype 1b infection [4]. An important question is whether IFN therapy is effective in reducing the incidence of HCC in the patients with chronic hepatitis C. Kasahara et al. [5] reported that the incidence of HCC was reduced by IFN in sustained responders; thus, improving the response rate is an essential issue to reduce the incidence of HCC.

Rivabirin and IFN combination treatment has been used in patients with chronic hepatitis C, which showed improvement of the sustained response rate from IFN monotherapy [6]. In Japan, this combination therapy is allowed for the treatment of patients with chronic hepatitis C at a limited duration of 24 weeks; however, the sustained response has been shown to improve especially in genotype 1b infection.

In the present study, the incidence and risk factors of the development of HCC after interferon therapy were examined. The reduction of occurrence in HCC was predicted after 24 weeks' treatment with ribavirin and IFN combination therapy.

Table 1. Clinical characteristics of the patients who received IFN monotherapy

Gender	
Male	282
Female	213
Age (mean ± SE)	52.3±0.57
Genotype	
1b	249
2a	63
2b	39
Unknown	141
HCVRNA level, kIU/ml (median 470)	1.1 to >850
Liver biopsy	
F1	132
F2	184
F3	123
F4	35
Total dose of IFN, MU (mean ± 53)	498±53
Outcome of IFN therapy	
SVR	155
NR	312
Development of HCC	
Yes	30
No	464

Patients and Methods

IFN Monotherapy Study

The first IFN monotherapy study included 495 consecutive patients with chronic hepatitis C in whom 24 weeks of IFN monotherapy was carried out from January 1994 to December 2001. The clinical characteristics of the patients are shown in table 1. The mean age is 52.3 years, and the HCV genotype was examined using the mixed-primer method [7]. Plasma level of HCVRNA was measured by amplicore monitor (version 2, Roche, Basel). The histological findings were classified according to established international criteria [8]. The median dosage of administered IFN was 640 MU, and sustained virological response (SVR) was defined as negative HCVRNA 6 months after interferon therapy and 155 patients achieved SVR. Otherwise, the patients were defined as non-responders. This study was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and written informed consent was obtained from all the patients included in this study.

The diagnosis of HCC was established by CT scan during hepatic arteriography (CTHA) and arterio-portography via the superior mesenteric artery as well as needle biopsy of the nodule. Development of HCC was observed in 30 patients during the observation period.

Ribavirin and IFN Combination Study

In 227 patients with chronic hepatitis C from December 2001 to November 2002, ribavirin and IFN combination therapy were carried out. Ribavirin was administered 800 mg per day in the patients having body weight 60 kg or more, and 600 mg with less than 60 kg. IFNα-2b of 6 MU was administered everyday during the initial two weeks followed by 3 times per week for remaining 22 weeks. The clinical characteristics are shown in table 2. The therapy was discontinued in 12 patients because of anemia, appetite loss, depression, and skin rash. Dose reduction of ribavirin was necessary in 21 patients because of anemia. Thus, the outcome of the combination therapy was assessed in 215 patients.

Statistical significance was assessed by Student's t test, χ^2 analysis with Yates' correction, and Kaplan-Meier method using the log-rank test as indicated. Multivariate analysis was carried out by the Cox proportional hazard model.

Results

The development of HCC was observed in 31 patients after IFN monotherapy. The clinical characteristics of the patients which developed HCC was evaluated by univariate analysis. A statistically significant difference was noted in age, gender, genotype, fibrosis of the liver and outcome of interferon therapy. The serum HCVRNA level before treatment and the serum ALT level were not different between the two groups (table 3).

The incidence of HCC after interferon therapy was compared according to the fibrosis score of the liver. The incidence of HCC was 0.3% per year in the patients with F1 and 1.1% per year in F2; however, it was 3.8% in the F3 groups. The development of HCC was significantly higher in the patients in the F3 and F4 groups than those in the F1 and F2 groups (Kaplan-Meier method, log-rank test, $p < 0.01$; fig. 1).

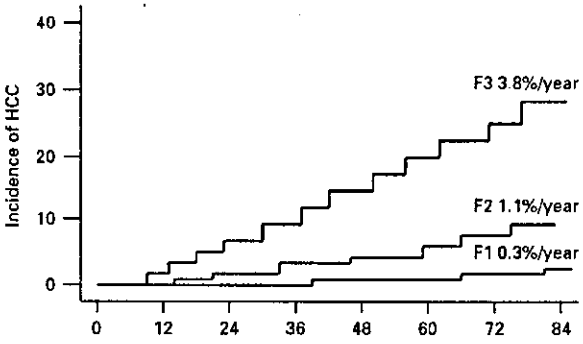

Fig. 1. Incidence of HCC was 0.1% in patients with fibrosis score F1, 1.1% in F2 and 3.8% in F3 (Kaplan-Meier method).

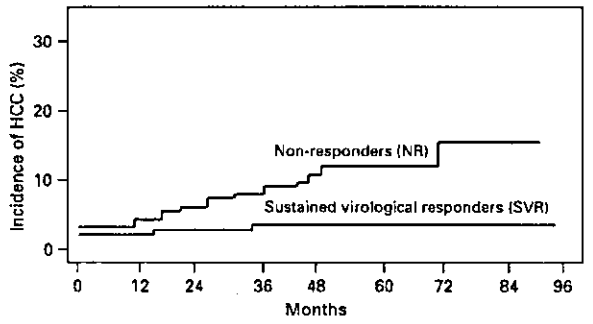

Fig. 2. Incidence of HCC was compared between sustained virological responder and non-responder patients. HCC development was significantly higher in the non-responders than in the sustained virological responders after IFN monotherapy.

Table 2. Clinical characteristics of the patients received ribavirin and IFN combination therapy

Gender	
Male	126
Female	101
Age (mean ± SE)	58.4 ± 1.2
Genotype	
1b	181
2a	30
2b	15
Mixed	1
HCVRNA level, kIU/ml (median 680)	67 to >850
Liver biopsy	
F1	86
F2	75
F3	64
F4	2
Outcome of IFN therapy	
SVR	61
NR	154
Withdrawal	12

Table 3. Comparison of the patients with or without development of HCC after IFN therapy (univariate analysis)

Development of HCC	Yes (n = 31)	No (n = 464)	p
Gender			
Male	22	260	<0.05
Female	9	204	
Age	60 ± 1.2	52 ± 0.6	<0.001
Genotype			
1b	22	228	<0.01
2a and 2b	1	102	
HCVRNA level, kIU/ml	512 ± 34	496 ± 18	n.s.
Liver biopsy			
F1 and F2	7	309	<0.001
F3 and F4	23	135	
ALT, IU/l	125 ± 8.3	118 ± 20	n.s.
Outcome of IFN therapy			
SVR	3	152	<0.01
NR	28	312	

The incidence of HCC was compared between sustained responders and non-responders. The incidence of HCC in sustained responders was 0.2% per year in the sustained responders; however, it was 3.9% per year in the non-responders. This difference was statistically significant (fig. 2).

Multivariate analysis using Cox hazard model was done. Age, gender, fibrosis of the liver and outcome of interferon were found to be independent risk factors (table 4). Among these risk factors, age, gender and fibrosis of the liver cannot be changed. Thus, to reduce the incidence of HCC, the improvement of sustained virological response is an important issue.

Since the end of 2001, ribavirin and IFN combination therapy for 24 weeks has been allowed in Japan, and 235 patients have been treated. The sustained virological response rate in genotype 1b dividing them according to their HCVRNA level before treatment. In the patient

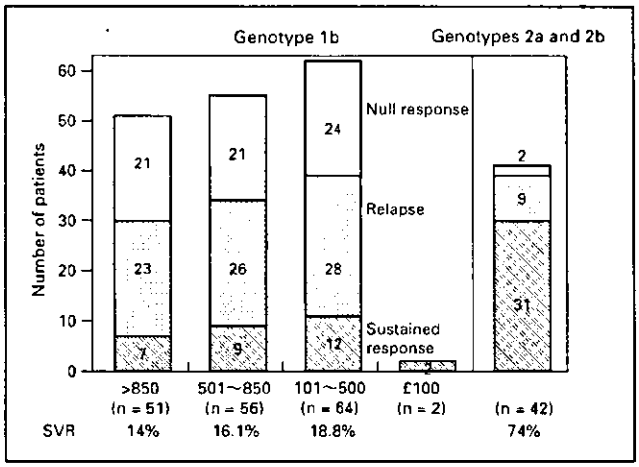

Fig. 3. Outcome of ribavirin and IFN combination therapy. ▨ = Sustained response; ▩ = relapse; □ = null response.

Table 4. Risk factors for the development of HCC after IFN monotherapy (Cox proportional hazard model)

Variable	Odds ratio	95% CI	p
Age (>56 vs. <55)	7.5	2.3–14.6	<0.005
Gender (male vs. female)	1.9	1.1–27.4	<0.05
Fibrosis (F3 and F4 vs. F1 and F2)	3.7	1.8–18.6	<0.01
Outcome of IFN (NR vs. SVR)	2.8	1.2–23.6	<0.05

group with a HCVRNA level higher than 850 kIU/ml, the sustained virological response rate was 0% by interferon monotherapy, while it was 14.0% by ribavirin and interferon combination therapy for 24 weeks. Similarly, it was 3.7% with a HCVRNA level from 500 to 850 kIU/ml on monotherapy, but it was 16.1% on combination therapy. The sustained virological response rate was 13.1% on monotherapy in those with a HCVRNA level from 100 to 500 kIU/ml, while it was 18.8% on combination therapy. However, a relapse rate, i.e. reappearance of HCVRNA after discontinuation of combination therapy, of 40–50% was observed in each group, and null response, i.e. no achievement of negative plasma HCVRNA during combination treatment, of around 30% was observed in each group. In the patients with genotype 2a and 2b infection, a sustained virological response was achieved in 74% (fig. 3).

Since the incidence of HCC reduced from 3.9% per year in non-responders to 0.2% per year in sustained virological responders, the incidence of HCC after treatment has been estimated to be reduced from 3.1 to 2.8% per year overall with 24 weeks' treatment with rivabirin and IFN combination therapy.

Discussion

HCC is the most life-threatening problem in the long-term course of chronic hepatitis C. The rising incidence of HCC has been pointed out not only in Japan but in the United States [9] and Europe [10]. Therefore, prevention of the development of HCC is an important issue in the clinical setting. In the present study, we analyzed the incidence and risk factors of HCC after IFN monotherapy in patients with chronic hepatitis C. The risk factors for the development of HCC were found to be age, male gender, fibrosis of the liver, and outcome of IFN therapy. Kasahara et al. [5] reported that the incidence of HCC was reduced by IFN in sustained responders, which is consistent with our data. They also reported that age, male gender and severe fibrosis of the liver were risk factors for the development of HCC. Imai et al. [11] reported similar risk factors for the development of HCC after IFN monotherapy in HCV-infected patients. Therefore, the liver fibrosis score is likely to be one of the most important risk factors for the subsequent development of HCC in HCV-infected patients, even following IFN therapy. Our data demonstrating that the degree of hepatic fibrosis is an independent risk factor for the development of HCC associated with HCV infection is certainly consistent with this supposition. Among these risk factors, age, male gender and fibrosis score of the liver cannot be changed before IFN therapy; thus, to reduce the incidence of HCC, improvement of the sustained response rate is an essential issue in patient care of HCV infection.

Recently, HCC-free survival could be obtained by IFN in patients with chronic hepatitis C, and the gain in HCC-free survival was greater when a patient was younger and fibrosis of the liver was more advanced [12]. The gain in HCC-free survival was calculated as difference between expected HCC-free survival with sustained virological response and that without. In this setting, improvement in achieving a sustained response is the central issue. Furthermore, the risk of death from liver-related disease was significantly reduced not only in sustained virological responders but also in biochemical responders in chronic hepatitis C [13].

Although the incidence of HCC has not been investigated after ribavirin and IFN combination therapy, HCC

development seems to be reduced by combination therapy by improving the sustained response rate, especially in genotype 1b infection. In the present study, the incidence of HCC is estimated to be reduced from 3.1 to 2.8% per year by combination therapy for 24 weeks. However, the sustained virological response rate has been shown to improve in genotype 1 infection by extended combination therapy for 48 weeks or by peginterferon-alfa-2b instead [14]. Thus, to reduce the incidence of HCC, extended treatment with ribavirin and IFN for 48 weeks is necessary in genotype 1b infection.

In the patients with HCV infection, the recurrence rate of HCC in the liver is as high as 20% per year, even after complete curative treatment was given to the primary HCC nodule [15]. The recurrence rate and prognosis was improved after elimination of hepatitis C virus RNA by IFN [16]. Furthermore, previous IFN therapy was shown to reduce the multicentric recurrence of HCC and improve the patients' survival in chronic HCV infection [17]. The rate of first recurrence of HCC was similar in patients treated with IFN and in untreated patients, but in the patients treated with IFN after curative treatment was given to the primary HCC nodule, the rate of second or third recurrence was lower than in the untreated group [15]. Moreover, IFN therapy enhanced patient survival after treatment of the HCC nodule.

From these results, it is concluded that IFN reduced the risk of the development of HCC when a sustained virological response was achieved in chronic hepatitis C. To reduce the risk of the development of HCC, it is an essential issue to improve the sustained response rate by prolonged ribavirin and IFN combination therapy.

Acknowledgement

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Rare Quasispecies in the YMDD Motif of Hepatitis B Virus Detected by Polymerase Chain Reaction with Peptide Nucleic Acid Clamping

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Key Words
Hepatitis B e antigen · Hepatitis B virus · Lamivudine · Peptide nucleic acid · YMDD mutant

Abstract
The emergence of drug-resistant mutants of hepatitis B virus (HBV) is a serious problem during antiviral therapy of patients with chronic hepatitis B. Lamivudine-resistant mutants with a mutation in the YMDD motif of reverse transcriptase of HBV emerge in approximately one half of the treated patients within 5 years. To date, the detection of YMDD mutants by polymerase chain reaction (PCR) with peptide nucleic acid (PNA) clamping is most sensitive. In this study, the performance of this method was evaluated in various clinical settings. The PCR-PNA method was able to detect the emergence of YMDD mutants 2–3 months earlier than the previously developed method involving restriction fragment length polymorphism. Further, rare quasispecies were detected by PCR-PNA in patients with chronic hepatitis B who were positive for hepatitis B e antigen (HBeAg). Many previously unrecognized mutants, such as those with YLDD and YMED, were found in them. Although precise sequence analyses of 10 patients identified YVDD and YIDD sequences in 6 of them, only 1 patient had a typical YVDD sequence that was identical with that in the reported lamivudine-resistant strain. All HBV mutants with the YIDD sequence accompanied stop codon(s) in the overlapping envelope (S) gene, suggesting that these strains would have no relevance as regards the emergence of lamivudine resistance. These results suggest that it would be difficult to detect lamivudine-resistant mutants before the therapy and that they would have a greater ability to evade the attack of antiviral drugs by frequent nucleotide substitutions than previously expected.

Introduction

Infection with hepatitis B virus (HBV) is a serious healthcare problem worldwide. Only interferon, lamivudine representing (–)-β-*L*-2′,3′-dideoxy-3′-thiacytidine and famciclovir standing for 2-[2-(2-amino-9H-purin-9-yl)ethyl]-1,3-propanediol diacetate have been approved for the treatment of chronic hepatitis B. Lamivudine reduces HBV DNA loads to almost undetectable levels in most patients who receive it and reduces inflammatory

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activity in the liver [1–5], along with marked biochemical and histological improvements. However, HBV mutants resistant to lamivudine have appeared in some patients during their treatment with this drug [6–10]. An increase in virus load during therapy is seen, reflecting a viral breakthrough that often brings about biochemical flare-ups of liver enzymes. Such lamivudine-resistant mutants have a characteristic mutation converting the 552nd amino acid for methionine, in the YMDD motif of reverse transcriptase, to isoleucine (YIDD mutant) [11–13] or valine (YVDD mutant) [11, 13]. An additional amino acid substitution (L528M) is often identified along with the YVDD mutant. A mutant with both these mutations has been reported to have the strongest drug resistance [14–16].

A method has been developed by us for detecting lamivudine-resistant HBV mutants [8]. It is based on restriction fragment length polymorphism (RFLP) of HBV DNA sequences amplified by polymerase chain reaction (PCR), and can detect the emergence of such mutants a few months before virological breakthroughs. By means of this RFLP method, the takeover of lamivudine-resistant mutants by the wild-type HBV was found to occur after the withdrawal of lamivudine [8]. No lamivudine-resistant mutants were detected, however, in any patients before they were placed on lamivudine, which might be due to the sensitivity of this method, which is not high enough for detecting mutants in low concentrations. To further explore the possibility of there being drug-resistant mutants even before the commencement of therapy, we have developed a more sensitive method for detecting rare mutants amongst a vast majority of the wild-type HBV [17].

The method involved PCR with peptide nucleic acid (PNA) clamping [18–20], and could detected 10^2 copies of YMDD mutants in the presence of 10^6 copies of the wild-type HBV [17]. By means of this PCR-PNA method, YMDD mutants were detected in 1 of the 62 (1.6%) serum samples from patients with acute or chronic HBV infection positive for antibody to hepatitis B e antigen (anti-HBe) in serum who had not received lamivudine [17]. In addition, many YMDD mutants were found that had not been reported before [17]. In the present study, the analysis for YMDD mutants was conducted in serum samples from hepatitis B e antigen (HBeAg)-positive patients, and many rare mutants were identified, some of which have not been documented previously.

Materials and Methods

Serum Samples

A total of 85 serum samples from 72 adult Japanese patients with HBV infection were studied, of whom 10 (14%) were positive for HBeAg and 62 (86%) for the corresponding antibody (anti-HBe) in serum. Lamivudine was given to 3 of the 10 (30%) HBeAg-positive patients and 19 of the 62 (31%) anti-HBe-positive patients. All serum samples were stored frozen at –80° until molecular biological examinations. All patients were negative for serum markers of hepatitis C virus (HCV) or human immunodeficiency virus type 1 infection.

Serological Markers of HBV Infection

Hepatitis B surface antigen (HBsAg) was determined by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland), and HBeAg as well as anti-HBe by radioimmunoassay (Abbott Diagnostics, Abbott Park, Ill., USA). HBV DNA was determined by transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per milliliter. The lower detection limit of this assay is 3.7 LGE/ml, which is equivalent to 5,000 copies/ml. The activity of HBV DNA polymerase was determined by a radioassay (Perkin Elmer, Boston, Mass., USA) that has a lower detection limit of 30 cpm. The antibody against HCV (anti-HCV) was tested for by the third-generation enzyme immunoassay (Roche Diagnostics).

Detection of YMDD Mutants by PCR with PNA Clamping

HBV DNA was extracted from 100 µl of serum by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 µl of H_2O. The first-round of PCR was performed with an outer primer set (PLF1: 5′-GGT ATG TTG CCC GTT TGT CC-3′ and BR123: 5′-TTC CAA TTA CAT ATC CCA T-3′) and a second-round PCR with an inner primer set (PLF2: 5′-CCT ATG GGA GTG GGC CTC AG-3′ and PLR2: 5′-CCA ATT ACA TAT CCC ATG AAG TTA AGG GA-3′). The PNA used was an 18-mer (PNA 552: H_2N-CAC ATC ATC CAT ATA ACT-CON_2H) that exactly matches the original YMDD sequence (fig. 1). The PNA-mediated PCR clamping method was optimized by changing the annealing temperature of PNA from 68 to 75°, and the PNA concentration from 1.25 to 10 µ*M*. The optimized PCR with PNA clamping was performed in a total volume of 25 µl, consisting of a reaction buffer [100 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl and 15 m*M* $MgCl_2$], 0.2 m*M* each of dNTPs, 1 µl of the DNA solution, 12.5 pmol each of primers, 150 pmol PNA 552 and 1 U Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo) along with 0.2 µg of anti-Taq high (Toyobo, Osaka, Japan). The optimized amplification conditions included initial denaturation at 95° for 4 min and 25 cycles of amplification (denaturation at 95° for 45 s, PNA annealing at 73° for 2 min, annealing and extension of primer at 63° for 50 s), followed by the final extension at 63° for 7 min. YVDD and YIDD mutants were detected by primer sets, PLF2 and TMApaII: 5′-CAG ACT TGG CCC CCA ATA CCA CAT CGT GCA-3′ as well as YNSsp I: 5′-TTT CCC CCA CTG TTT GGC TTTC AGT AAT AT-3′ and BR109: 5′-AAG GGA GTA GCC CCA ACG TT-3′, as described previously [8]. YVDD and YIDD mutants can be detected after they had been digested by restriction enzyme *Alw*44I (Toyobo, Osaka, Japan) and *Ssp*I (Takara Shuzo, Otsu, Japan), respectively (fig. 1). The error rate of the Taq DNA polymerase was estimated to be 1.76×10^{-5} per site when amplifying about 10^2 copies of plasmid under the same conditions as above and cloning and sequencing [17].

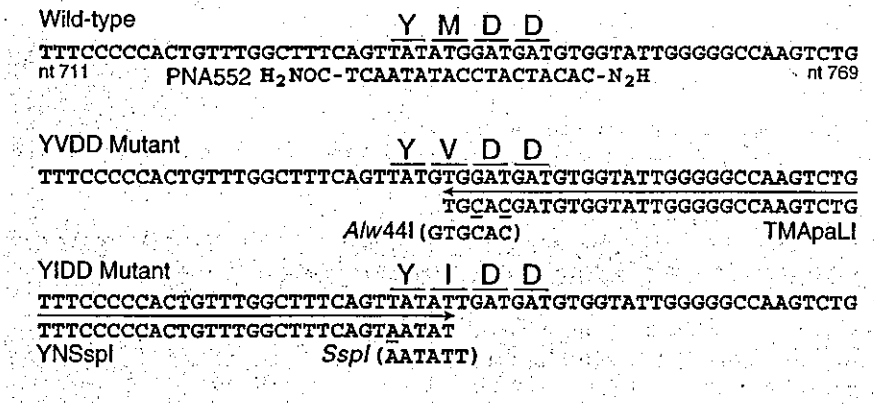

Fig. 1. The experimental design. PNA is an 18-mer oligonucleotide of antiparallel polarity, and exactly matches the YMDD sequence. The amplified HBV DNA sequences from YVDD mutants only become sensitive to digestion with *Alw*44I by a change from GAT to CAC triplet. Consequently, the amplified HBV DNA sequences from YIDD mutants alone become sensitive to digestion with *Ssp*I by a change from TAT to AAT triplet. Sequence positions are in accord with those of Ono et al. [23].

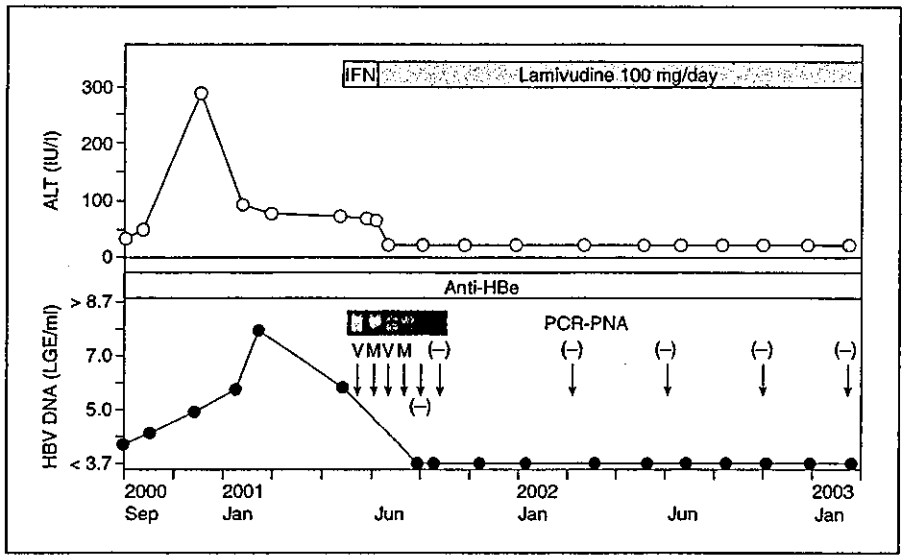

Fig. 2. Clinical and virological course of an anti-HBe-positive patient with chronic hepatitis B who possessed the YVDD mutant before the start of lamivudine therapy. The YVDD mutant was detected by PCR-PNA [17] in 2 of 4 serum samples obtained before lamivudine therapy. The patient responded to lamivudine completely. IFN = Interferon; V = YVDD mutant; M = wild-type with YMDD motif.

Cloning and Sequencing Rare YMDD Mutants

Products (1 µl) of the second-round of PNA-PCR were subjected to PCR with primers PLF2 and BR109 for 35 cycles (94°, 1 min; 58°, 1 min; 72°, 1.5 min) after initial denaturation at 94° for 4 min and followed by the final extension at 72° for 7 min. Amplicons were purified by electrophoresis on 2% (wt/vol) agarose gel and cloned into pGEM-T Easy Vector (Promega, Madison, Wisc., USA) with the standard method, and then transformed into *Escherichia coli* JM 109 (Takara Shuzo, Otsu, Japan). Sequencing was performed in the ABI PLISMTM 310NT Genetic analyzer (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Independent clones from 10 patients, including 5 with HBeAg positive and 5 with anti-HBe, were sequenced for analysis.

Results

Detection of YMDD Mutants in a Patient before the Start of Lamivudine Therapy

Only 1 of 72 patients (1.4%) tested positive for YVDD mutants before they received lamivudine therapy. The detection of YMDD mutants was comparatively infrequent as in our previous study in which they were found in only 1 of the 62 (1.6%) patients with chronic hepatitis B with anti-HBe in serum [17]. It was much less, however, than the detection by others [21, 22] of YMDD mutants in patients before the commencement of lamivudine. The single patient in the present study was positive for anti-HBe and responded completely to lamivudine without any breakthroughs as illustrated in figure 2. When HBV DNA was extracted from serial frozen serum samples and analyzed, YVDD mutants were detected only twice; lami-

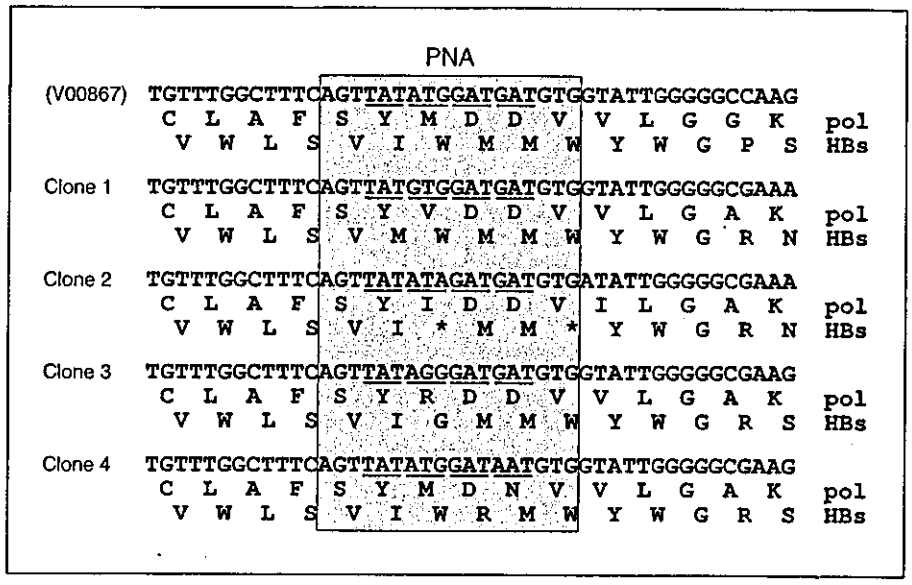

Fig. 3. Nucleotide and amino acid sequences of clones from a patient including the YMDD motif, a clone having a YMDD sequence. The sequence of clone 1 with YVDD mutation was the same as that reported with lamivudine-resistant YVDD mutants. The YIDD sequence in clone 2, however, was different from the reported lamivudine-resistant YIDD mutant in that it possessed TAT ATA in place of TAT and ATT. These substitutions induce a TAG stop codon in the overlapping envelope gene (shown by an asterisk). Other variants, such as YRDD and YMDN, were also found in this patient (data not shown).

vudine suppressed HBV very well in this patient. The patient was negative for HBV DNA by PCR during therapy, and he experienced no breakthroughs (fig. 2).

To confirm the presence of YVDD mutants, HBV DNA clones were propagated from his pretreatment sera and sequenced. Many unusual mutants with mutations in the YMDD motif were recovered. Only 1 of the 43 (2%) clones had the YVDD sequence. The wild-type sequence (YMDD) was detected in 6 of the 43 clones (14%). Notably, 26 of these 43 clones (61%) possessed a stop codon in the overlapping envelope (S) gene. None of the 16 clones that had been obtained by PCR without PNA clumping disclosed any nucleotide mutations or amino acid substitutions in the YMDD motif (table 1, fig. 3). Only a few mutations were detected in the sequences where PNA does not anneal.

Table 1. Amino acid sequences of YMDD variants in HBV DNA clones from a patient before lamivudine therapy tested with or without PNA

Mutation	With PNA (n = 43)	Without PNA (n = 16)
YMDD	6 (14)	16 (100)
YVDD	1 (2)	0
YIDD	13 (30)[a]	0
YRDD	3 (7)	0
YMDN	10 (23)[b]	0
YIND	3 (7)[a]	0
YINN	7 (16)[a]	0

Figures in parentheses represent percentage.
[a] Created a stop codon in the overlapping envelope (S) gene in the YMDD motif.
[b] Included the three clones with a stop codon in the overlapping envelope gene.

Detection of YMDD Mutants in a Patient with Chronic Hepatitis B Who Rebounded after the Withdrawal of Lamivudine

Serial serum samples from 1 patient who rebounded after the withdrawal of lamivudine therapy were examined. The activity of HBV-DNA polymerase started to increase soon after the patient was taken off lamivudine (fig. 4). Both YIDD and YVDD mutants were detected with the RFLP method [8] while he was receiving lamivudine. However, the YIDD mutant was detected about 2 months earlier with PCR with PNA clamping than with the RFLP method. After the withdrawal of lamivudine, both YIDD and YVDD mutants became undetectable with the RFLP method, but they were still detectable with PCR with PNA clamping immediately after the rebound. Neither of these mutants, however, was detectable by either detection method 3 years thereafter. These results indicate that PCR with PNA clamping is useful for monitoring the appearance and disappearance of YMDD mutants during and after lamivudine therapy.

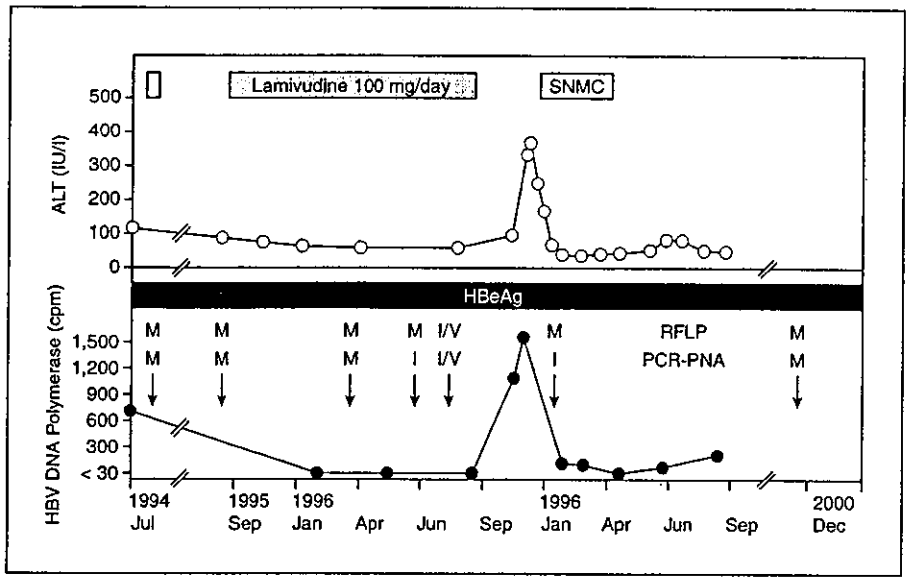

Fig. 4. Clinical and virological course of an HBeAg-positive patient with chronic hepatitis B who developed YMDD mutants during lamivudine therapy. The results of detecting YMDD mutants with the RFLP method of Chayama et al. [8] without PNA clamping and the PCR with PNA clamping [17] are compared. SNMC = Stronger Neo-Minophagen C (Minophagen Pharmaceutical Co., Tokyo, Japan); M = wild type with YMDD motif; I = YIDD mutant; V = YVDD mutant.

Table 2. Amino acid sequences of YMDD variants in HBV DNA clones from nine patients who tested negative for them by PCR with PNA clamping before or without lamivudine therapy

	Clone	YMDD	YIDD	YRDD	YLDD	YMDN	YMND	YMNN	YMED	YMHD	YIND	YINN
Anti-HBe												
Patient 1	10	4 (40)	2 (20)[a]	0	0	0	3 (30)[a]	0	0	0	0	1 (10)[a]
Patient 2	10	0	5 (50)[a]	0	0	0	3 (30)[a]	1 (10)[a]	0	0	0	1 (10)[a]
Patient 3[b]	11	10 (91)	0	0	0	1 (9)	0	0	0	0	0	0
Patient 4	10	10 (100)	0	0	0	0	0	0	0	0	0	0
HBeAg												
Patient 5	11	9 (82)	0	0	1 (9)	0	0	0	1 (9)	0	0	0
Patient 6	11	3 (27)	8 (72)[a]	0	0	0	0	0	0	0	0	0
Patient 7	11	5 (45)	0	1 (9)	0	2 (18)	2 (18)[a]	0	0	1 (9)	0	0
Patient 8	10	2 (20)	3 (30)[a]	0	0	0	3 (30)[a]	0	0	0	2 (20)[a]	0
Patient 9[c]	11	1 (9)	3 (27)[a]	0	3 (27)	1 (9)	0	1 (9)[a]	0	0	1 (9)[a]	1 (9)[a]
Total	95	44 (47)	21 (22)	1 (1)	4 (4)	4 (4)	11 (12)	2 (2)	1 (1)	1 (1)	3 (3)	3 (3)

Figures in parentheses represent percentage.

[a] Created a stop codon in the overlapping envelope (S) gene.

[b] Developed viral breakthrough after 21 months on lamivudine.

[c] Developed viral breakthrough after 7 months on lamivudine.

Detection of YMDD Mutants in Patients with Chronic Hepatitis B

Although the YVDD mutant was detected in only 1 of 72 patients with PCR-PNA and restriction enzyme digestion analyses, we went on to analyze quasispecies of HBV in this region in 9 additional patients by cloning and sequencing. Multiple clones from 4 patients with anti-HBe and 5 with HBeAg were examined for YMDD mutants. Only 1 patient, who tested positive for anti-HBe in serum, did not exhibit YMDD mutants (patient 4 in table 2). However, their YMDD clones had a unique nucleotide sequence (TAC ATG GAT GAT); the biological relevance of these substitutions is unknown.

YIDD sequences were detected in 5 patients, with the incidence of YIDD mutants in 60% of the 10 patients examined (including the patient positive for YVDD mutant). However, all these YIDD sequences were different from the typical lamivudine-resistant strain (TAT ATT GAT GAT) and they all possessed a stop codon in the overlapping envelope (S) gene (clone 2 in fig. 3). This suggests that these clones with YIDD mutation would represent defective that were unrelated to typical lamivudine resistance.

Discussion

Although lamivudine is effective in suppressing viral replication and lowering alanine aminotransferase levels in serum [1–5], a frequent emergence of YMDD mutants detracts enormously from its effects [6–10]. It is not known, however, if such mutants exist before the therapy. In an attempt to detect YMDD mutants, we previously developed a PCR-based RFLP method that can sensitively identify YVDD and YIDD mutants [8]. The RFLP method is not sensitive, however, in detecting a minor population of YMDD mutants that coexist with the vast majority of the wild-type HBV without mutations in the YMDD motif. Only 10^2 copies of YMDD mutants were detected when mixed with 10^4 copies of the wild-type HBV, even when wild-type sequences were digested by restriction enzyme in the products of the first-round PCR. Hence the sensitivity was not sufficient for detecting very small amounts of mutants that exist among overwhelming numbers of the wild-type virus. We developed, therefore, a more sensitive detection method of mutants by using PCR with PNA clamping [17].

Although the PCR-PNA method could detect YMDD mutants 10- to 100-fold more sensitively than the previous PCR-RFLP method [17], such mutants were not detected in sera from patients before the start of lamivudine, even though they later developed a viral breakthrough within 1 year on lamivudine (patient 9 in table 2). This suggests that it would be very difficult to detect YMDD mutants before the commencement of therapy by increasing the sensitivity of the methods. It does need to be pointed out that the patients who tested positive for YVDD mutant did not develop a breakthrough during at least 23 months and showed a good response to the therapy. This suggests that the YMDD mutant detected in our patient might have been an incompetent virus that would have a mutation in the other part of the genome not analyzed so far. Combined, these observations suggest that increasing the sensitivity of the assay per se would be inappropriate for detecting mutants before therapy. Different approaches should be considered in selecting patients suitable for antiviral treatments.

Previous studies have found YMDD mutants in pretreatment sera from the patients who tested positive for anti-HBe [17, 21, 22]. The detection goes along with the fact that HBV sequences with multiple substitutions exist in the patients who are mounting active immune responses against HBV with anti-HBe immune responses. In fact, 5 patients with HBeAg were found to harbor many YMDD mutants in the present study. This suggests that mutants would not be exclusive for the patients who are under immune pressure against HBV with anti-HBe in serum. Many mutants identified in this study and a previous study [17] underscore the production of a number of mutants during viral genome synthesis. Only a part of these mutants might be able to survive and produce their progeny and evolve under the immunological pressure. Further study should be directed to gain a full profile of rare quasispecies of HBV mutants, which would help understand the mechanism for the emergence of drug-resistant HBV mutants. Detection of rare quasispecies by PCR with PNA clamping will be a useful tool in monitoring for YMDD mutants arising before and after lamivudine therapy.

Acknowledgments

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Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan

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***Background/Aims*: The mortality due to hepatocellular carcinoma (HCC) has ranged widely in various areas of Japan since 30 years ago and the incidence was particularly high in once *Schistosoma japonicum* (*Sj*)-endemic areas. Our aim was to estimate the spread time of hepatitis C virus (HCV) infection in the past with possible relevance to a higher incidence of HCC in once *Sj*-endemic than *Sj*-nonendemic areas.**

***Methods*: During 2001, 131 strains of HCV-1b were obtained from patients in three previously *Sj*-endemic areas, as well as *Sj*-nonendemic areas in Japan and a cross-sectional study was conducted on them with molecular evolutionary analyses.**

***Results*: A phylogenetic tree reconstructed on HCV-1b sequences in the NS5B region disclosed 2 independent clusters for *Sj*-positive and -negative groups with a high bootstrap value. The estimated effective number of HCV-infections indicated a transition from quiescence to rapid exponential growth in the 1920s among patients with schistosomiasis, which is 20 years earlier than that among patients without schistosomiasis.**

***Conclusions*: The estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* since 1921. A high incidence of HCC there would be attributed to a long duration of HCV infection since 1920s.**

***Keywords*: Hepatitis C virus; *Schistosoma japonicum*; Molecular evolutionary analysis; Hepatocellular carcinoma**

1. Introduction

Recently, the molecular clock has been successfully applied to long-term serial serum samples containing hepatitis C virus (HCV) from the US and Japan and estimated the spread time of HCV in the 1930s in Japan, which is 30 years earlier than that in the US in the 1960s [1]. Insofar as a long duration of HCV infection is the most important factor for the development of hepatocellular

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Abbreviations HCV, hepatitis C virus; Anti-HCV, antibody to HCV; HCC, hepatocellular carcinoma; *Sj*, *Schistosoma japonicum*.

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carcinoma (HCC), it can be predicted that the incidence of HCC will increase in the US over the next 2–3 decades. Thus, a combination of classical epidemiological approaches and molecular evolutionary analyses would be particularly useful in the study of contagious diseases, typified by HCV infection.

The way how individuals contracted HCV infection has remained unclear in Japan. Recently, a Japanese report (Ministry of Health, Labour and Welfare: Distribution of age-adjusted mortality rate from liver cancer by prefecture between 1971 and 1975, Tokyo, 2001) indicated that the mortality due to HCC has already varied widely in various areas of Japan since 30 years ago; the incidence of HCC was much higher in Saga/Fukuoka, Hiroshima and Yamanashi Prefectures, which were once endemic for schistosomiasis japonica in the long past. Hence, a high incidence of HCC in the 1970s would be related to HCV transmitted by injection treatment for *Schistosoma japonicum* (*Sj*) conducted since 1921 in these areas. In fact, shared needles and syringes for intravenous injection treatment with antimonyl potassium tartrate or sodium antimony tartrate posed a significant risk for HCV transmission in endemic areas [2]. Indeed, the prevalence of antibody to HCV (anti-HCV) is high (36.5; 95% CI=28.1–44.9%) in patients with chronic schistosomiasis [2] and therefore, HCV infection is considered responsible for the development of HCC in patients with chronic schistosomiasis.

Since, once popular intravenous injection for schistosomiasis was a risk factor for HCV transmission, the spread time of HCV in the areas once endemic for *Sj* in Japan would deserve determination. In this study, molecular evolutionary analyses using principles of both population genetics and mathematical epidemiology [3] were applied to HCV-infected patients with and without a past history of chronic schistosomiasis in once *Sj*-endemic areas.

2. Meterials and methods

2.1. Sample collection

In Japan during 2001, 181 random serum samples positive for anti-HCV were obtained from patients with chronic liver disease in widely separated areas previously endemic for *Sj*, including Kofu in Yamanashi ($n=75$), Katayama in Hiroshima ($n=50$) and Chikugo in Saga/Fukuoka Prefectures ($n=56$). Schistosomiasis was diagnosed by ultrasonographic (US) and/or computer tomographic (CT) modalities or serological examinations [4]. Two kinds of serological tests, which can detect past history of schistosomiasis, were available in this study. In brief, IgG antibodies binding to two different *schistosome antigens, Sj* adult worm antigen and *Sj* egg antigen, were detected using an enzyme-linked immunosorbent assay (ELISA). As it is now accepted that ELISA titer of egg antigen-specific IgG is reliable for case-detection rather than IgG for adult worm antigen [4–6], the results based on the egg antigen-specific IgG were accepted in this study. Samples of more than 0.25 of optical density at 415 nm were determined to be positive, as previously confirmed [4–6]. The serum samples were tested for anti-HCV by Lumipulse II Ortho HCV (Ortho-Clinical Diagnostics K.K., Tokyo, Japan). As patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. For a cross-sectional study, 30 serum samples were obtained from patients infected with HCV in Aichi Prefecture where *Sj* has not been endemic. The age- and sex- matced patients were also selected from the *Sj*-nonendemic areas excluding influece of these factors on HCC incidence. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by Ethic Committees of institutions. Every patient gave a written informed consent to participate in the virological research of HCV. Information of injection treatment for *Sj* was obtained by means of self-administrated questionnaires or structured interviews. None had been treated with interferon therapy for HCV infection. HCC incidence was estimated by historical information from patients themselves and/or medical records during 2001. HCC was diagnosed by liver biopsy or combination of imaging modalities such as US, enhanced CT and angiography.

2.2. Genotyping and sequencing

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. They were reverse-transcribed to cDNA using SuperScript II Rnase H^- Reverse Transcriptase (Invirogen Corp., Carlsbad, California, USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) by the method described previously [7].

A sequence spanning 339 nucleotides (nt) in the NS5B region was amplified by polymerase chain reaction (PCR) with primers described previously [1]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, California, USA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in PCR, PLATINUM Pfx DNA Polymerase (Invirogen Corp.) with a very high fidelity was used. The sequences determined were utilized to confirm HCV genotypes and construct phylogenetic trees.

2.3. Test for clustering between Sj-positive and -negative groups

The phylogenetic tree was first constructed to examine the evolutionary history for *Sj*-positive and *Sj*-negative groups by the neighbor joining method [8]. Furthermore, to test whether either *Sj*-positive or *Sj*-negative group have evolved independently or not, we conducted an interior branch test for the neighbor-joining tree [9]. Thereafter, a *t*-test was conducted for the interior branch length and its standard error, which is computed using the bootstrap procedure.

2.4. Demographic model

A reconstructed tree was built on the NS5B sequence of 339 nt by a heuristic maximum-likelihood topology search with stepwise-addition and the nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85 with the molecular clock enforced by PAUP version 4.0b8.

As estimates of the demographic history, a nonparametric function $N(t)$, known also as the skyline plot, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time [3,10]. A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences [10]. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood ratio tests of the parametric maximum-likelihood estimates [11,12].

2.5. Statistical method

Data for continuous variables were demonstrated as the mean± standard deviation. The Fishers' exact test, Chi square test with Yates' correction and one-way ANOVA followed by the Scheffe's multiple comparison test were used to evaluate differences in the mean age, sex ratio

and incidence of HCC between groups, respectively. Differences with P values less than 0.05 were considered significant.

3. Results

Of 181 anti-HCV positive samples, 113 were classified into HCV genotype 1b (HCV-1b), which is predominant in Japan. Fifty-two of 181 samples (29%) were negative for HCV RNA or incomplete for sequencing and the remaining 16 samples (9%) of genotype 2a were excluded in this study due to a minor population. Of the HCV-1b strains, 47 were recovered from patients in Yamanashi, 31 in Hiroshima and 35 in Saga/Fukuoka Prefectures. Along with 18 HCV-1b strains in Aichi Prefecture serving as controls, a cross-sectional study was conducted on them with molecular evolutionary analyses. The patients in areas previously endemic for *Sj* revealed a significantly higher prevalence of chronic schistosomiasis [24/47 (51%) in Yamanashi (Kofu area), 21/31 (68%) in Hiroshima (Katayama area) and 19/35 (54%) in Saga/Fukuoka (Chikugo area)] than that in Aichi Prefecture (0/18 [0%], $P<0.0001$). There were no significant differences in the mean age or sex ratio among patients from these four areas (Fig. 1). Although the mean age of *Sj*-positive patients was just higher than that of *Sj*-negative patients in once *Sj*-endemic areas or matched-control patients in Aichi Prefecture, there were also no significant differences between these groups (Table 1).

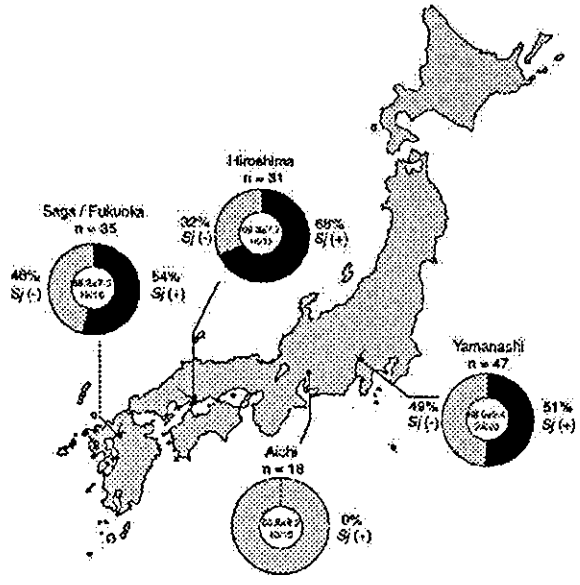

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie graphs include the age (mean± standard deviation) and sex ratio (male/female).

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls
	Positive ($n=64$)	Negative ($n=49$)	(Aichi) ($n=18$)
Mean age			
Total	69.9±7.7	67.4±8.7	66.5±9.2
Yamanashi	69.9±7.2	67.3±11.2	
Hiroshima	71.2±8.7	67.6±6.5	
Saga/Fukuoka	69.0±7.7	67.5±7.1	
Sex (male/female)			
Total	34/30	24/25	9/9
Yamanashi	13/11	11/12	
Hiroshima	10/11	5/5	
Saga/Fukuoka	11/8	8/8	
Incidence of HCC	25/55 (45%)	11/48 (23%)	3/18 (17%)

The incidence of HCC in *Sj*-positive patients was significantly higher than that in *Sj*-negative patients ($P=0.0226$) or controls ($P=0.0488$).
Abbreviations: HCC, hepatocellular carcinoma.

For cross-sectional study on the viral population size between HCV-infected patients with and without a past history of schistosomiasis, a phylogenetic tree for HCV-1b strains in the *Sj*-positive and -negative patients was constructed with use of the maximum-likelihood method enforced by the molecular clock as introduced in our previous report [1] and an independent study by Pybus et al. [3]; a substitution rate of 5.3×10^{-4} per site per year [1,3] was assumed for HCV. The phylogenetic tree disclosed 2 independent clusters for *Sj*-positive and -negative groups, with a high bootstrap value (81%) by the interior branch testing (Fig. 2), which is comparative with past epidemiological backgrounds in Japan. From distinct evolutionary histories in the two populations, the effective number of HCV-1b infections through time, $N(t)$, were assessed by the skyline plot. The parameters for several models in Genie v3.5 [3,10] were also examined. Time t was then transformed to year using the same rate, assuming the collecting time (year 2001) as the present. Fig. 3 shows the skyline plots and population growth for *Sj*-positive and -negative patients, according to a specific demographic model in Genie v3.5 with three parameters, piecewise expansion growth model, that was evaluated by the likelihood ratio testing [11,12]. Molecular evolutionary results thus obtained supported our previous study in which the divergence time of the most recent common ancestor of HCV-1b in each area in Japan was estimated before 1850 [1]. Our estimates of the effective number of HCV-infections showed a transition from constant size to rapid exponential growth in the 1920s among patients with chronic schistosomiasis in endemic areas, which is 20 years earlier than that among patients without schistosomiasis in the 1940s. Information on HCC was available for 121 of the 131 patients with HCV-1b. Although they were relatively small in number, the incidence of HCC was significantly higher in *Sj*-positive than -negative patients ($P=0.0226$) or controls ($P=0.0488$) (Table 1).

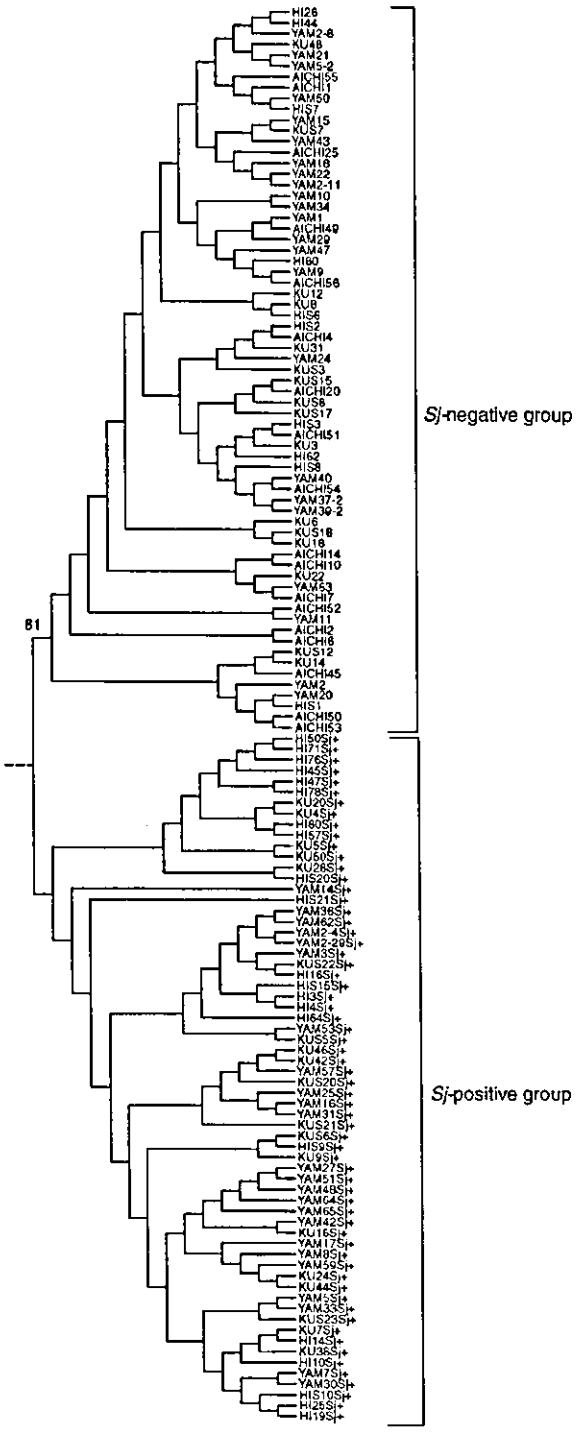

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS; Hiroshima, KU/KUS; Saga/Fukuoka, Aichi; control strains.

4. Discussion

The specific demographic model based on the neutral theory [3,11,12], which has a constant size in the past and changes to exponential growth until the present, is applied to investigate the Japanese endemic of HCV. By means of the molecular evolutionary analyses, the spread time of HCV in *Sj*-positive patients was estimated 20 years earlier than that in *Sj*-negative patients from three areas in Japan where *Sj* was previously endemic (Yamanashi, Hiroshima, Saga/Fukuoka Prefectures). The spread time of HCV much earlier in *Sj*-positive than -negative patients indicates that the previous intravenous injection treatment with antimony compounds (antimonyl potassium tartarate or antimony sodium tartarate) on patients with schistosomiasis since 1921 [2] would have been a significant risk factor for HCV transmission in endemic areas through re-used needles and syringes. Indeed, it might be possible that HCV transmission from *Sj*-positive patients to *Sj*-negative patients occurs in the once *Sj*-endemic areas, but we could not find such strains in this study. One of the reasons is that residents in the village around the river, where schistosomiasis had been the most prevalent, might have been isolated from those in the other areas of the same Prefecture in the past due to the endemic disease 'schistosomiasis'. Interestingly, most Japanese strains from *Sj*-nonendemic areas in the database clustered with the *Sj*-negative group of the present study. Hence, factors other than the injection treatment for *Sj*, such as intravenous stimulants popular during and after World War II [13] and medical treatments including transfusion with blood units from paid donors in the past, would have imposed the risk for HCV transmission in most areas in Japan [14]. In addition, there would have been opportunities for HCV transmission through inadequately sterilized needles and syringes in general practices, which have contributed to a large reservoir of chronic HCV infection in Japan during the 1950s [13]. Such inadequately sterilized medical injections were still common in the less-developed world in the 20th century. WHO estimates that unsafe injections result in 2.3–4.7 million new HCV infections worldwide every year [15].

Although the spread time of HCV in *Sj*-positive group was earlier than that in *Sj*-negative group, there was no significant difference of mean age between the 2 groups. Two possibilities are considered. One is a sampling bias; as patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. Second, the ages that patients had been infected with HCV were different between the 2 groups; the treatments for *Sj* in Japan were mainly conducted among relatively younger people including school children after screening of *Sj* [4,16,17], while the

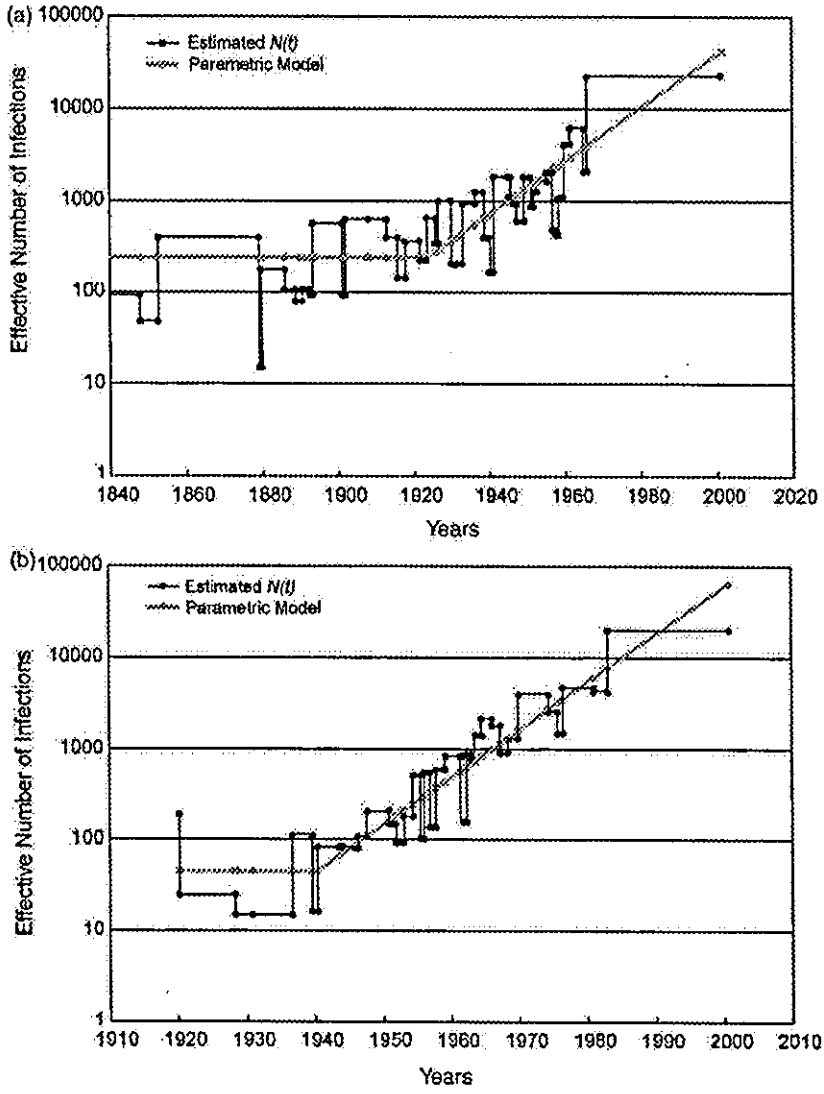

Fig. 3. The maximum-likelihood estimates of $N(t)$ on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and stepwise plots by the black line that represents corresponding nonparametric estimates of $N(t)$ (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region.

other risk factors such as blood transfusion were found in older people excluding at least children.

A disease possibly caused by schistosomal infection in Japan is documented in a book written some 300 years ago. In 1847, the clinical picture of this disease was precisely described by Yoshinao Fujii in the book 'Katayama-ki' that documented an endemic disease in Katayama area as Katayama's disease (equivalent to schistosomiasis). Water-borne epidemics of schistosomiasis prevailed in inhabitants around rivers (the tributaries of the Fuji river in Yamanashi, the Takaya river in Hiroshima and the Chikugo river in Saga/Fukuoka) in Japan, mediated by small shellfish (Miyairi-kai) serving as the natural host. More than 200,000 individuals were estimated to have been infected with *Sj* in Yamanashi Prefecture alone during 1965 through 1990 [16] and approximately 1,000,000 patients in the entire Japan since 1920s [17]. To cope with these endemics, more than 10 million intravenous injections with antimony compounds had been given in Japan since 1921 [17]. Thus, Japan would have started ahead of any other countries, in terms of HCV spread in association with schistosomiasis, wherein intravenous drugs were invented. Although acute schistosomal infection has disappeared in Japan since long ago, there are still elderly people with

chronic schistosomiasis in previously endemic areas, some of whom are developing HCC [2,14]. Substantial transmission among regions is supported by the lack of regional clustering of HCV sequences in this study.

A similar situation is reported in the Nile delta in Egypt where schistosomiasis once prevailed mediated by small shellfish [18] and the national campaigns for injection treatment with antimonyl potassium tartarate (tartar emetic) from the 1961 until 1986 are suspected to have given rise to the highest endemicity of HCV in the world ever, involving >20% of the national population there [19]. The prevalence of anti-HCV is extremely high (>70%) in patients with schistosomiasis there [18,20,21]. Highly prevalent HCV infection in the general Egyptian population accounts for most HCC cases in Egypt [22]. A question may arise whether schistosomiasis alone is responsible for the development of HCC. Patients co-infected with HCV and *Schistosoma mansoni* (*Sm*) may have a high incidence of viral persistence, accelerated fibrosis and development of HCC [23,24]. A recent population-based study between two large populations with district histories of *Sm* and hepatitis C infections, however, failed to indicate any interaction between *Sm* infection and the prevalence or severity of hepatitis C [25]. Moreover, no significant histological differences were found between anti-HCV-positive Egyptian patients with and without schistosoma [26]. Hence, the long duration of persistent HCV infection would be a more important factor for the development of HCC than the pathogeneticity of *Sm* itself.

Estimating the effective number of HCV infections has been very informative in looking back epidemic spreads of HCV infection in the Unites States [1] and Egypt [12,27]. In addition, it would also be useful in predicting the population size and extent of HCV infection. Studies to foresee future spreads of HCV would be required to cope with and prevent healthcare problems where de novo infections are increasing. The advantage of molecular evolutionary analyses, its ability to accurately estimate the dynamics of HCV based on a limited number of isolates in particular [3], will extend its application anywhere in the world where clinical sequelae of persistent HCV infection pose increasing burdens on the public health of nations.

In conclusion, the evolutionary analyses indicated that the estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* conducted since 1921. The high incidence of HCC in *Sj*-endemic areas is most likely attributed to long duration of HCV infection there transmitted through injection treatments.

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Influence of Age, Sex, and Degree of Liver Fibrosis on the Association Between Serum Alanine Aminotransferase Levels and Liver Inflammation in Patients with Chronic Hepatitis C

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In 504 patients with chronic hepatitis C who underwent liver biopsy, the correlation between serum ALT levels and histologic liver inflammation was investigated, and the effects of age, sex, and degree of histologic liver fibrosis on this correlation were analyzed. Serum ALT levels were significantly higher in male than in female patients only when activity of hepatitis was mild. In contrast, ALT levels were significantly higher in younger (≤50 years old) than in older patients only when activity of liver inflammation was severe. Fibrosis was closely associated with activity of hepatitis and, also, serum ALT level. More importantly, a significant number of older patients and patients with severe hepatic fibrosis had severe hepatic inflammation even when their serum ALT level was not markedly elevated (≤70 IU/L). Age, sex, and degree of liver fibrosis independently influenced serum ALT levels in patients with chronic hepatitis C.

KEY WORDS: chronic hepatitis C; serum alanine aminotransferase; age; sex; fibrosis.

Aminotransferase levels are sensitive indicators of liver-cell injury (1). The serum alanine aminotransferase (ALT) level, in particular, is the most commonly used variable in assessments of liver disease; it is used as an indicator of the presence of chronic liver disease and the severity of liver damage. In patients with chronic viral hepatitis, the serum ALT level is sometimes considered in the decision to initiate antiviral therapy (2), although this is now controversial (3). Furthermore, ALT levels may be associated with the risk of hepatocellular carcinoma in patients with cirrhosis (4). However, precise correlation between the serum ALT level and activity of hepatitis (liver inflammation) has not been sufficiently established, especially as it relates to the degree of liver fibrosis. In addition, the influences of patient age and sex on the correlation between serum ALT level and activity of hepatitis have not been well analyzed. Better interpretation of serum ALT levels in routine follow-up for patients with chronic hepatitis C would be important for physicians to make more appropriate clinical decisions. In the present study, therefore, we investigated the influence of patient age, sex, and degree of liver fibrosis on the correlation between serum ALT level and histologically evaluated activity of hepatitis in patients with chornic hepatitis C.

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METHODS

Between 1990 and 1998, a total of 504 patients with chronic hepatitis C underwent ultrasonography-guided needle liver biopsy. These patients comprised 332 men and 172 women (mean age, 49.0 ± 10.4 years). None of them had received interferon therapy before liver biopsy. In all patients, infection with hepatitis C virus (HCV) had been confirmed by detection of anti-HCV antibody by second-generation assay (Dinabot; Tokyo) and of serum HCV RNA by reverse-transcription polymerase chain reaction (PCR) assay (5). PCR with genotype-specific primers on a core region (6) showed HCV genotype 1b in 304 patients, 2a in 152 patients, and 2b in 25 patients; the test showed mixed genotypes in 8 patients, and genotype was not determined in 15 patients.

Activity of hepatitis and liver fibrosis were evaluated by two independent liver pathology specialists who were blinded to the clinical data. Evaluation was according to the classification of Desmet *et al.* (7). Activity of hepatitis was graded as minimal (A0), mild (A1), moderate (A2), or severe (A3). The degree of fibrosis was scored as none (F0), mild (F1), moderate (F2), or severe (F3). Serum ALT level was measured by the routine method on the day of liver biopsy.

Data are shown as mean ± SD. Differences in proportions were analyzed by chi-square test. Differences in mean quantitative values were analyzed by Student's *t*-test. Multivariate analysis was done using logistic regression (8); the variables analyzed were age at biopsy (≤50 vss > 50 years), sex, and degree of liver fibrosis (F0/F1 vs. F2/F3). Statistical analyses were performed with the SAS statistical package (SAS Institute, Cary, NC) (9). All *P* values were two-tailed, and values less than 0.05 were accepted as statistically significant.

RESULTS

Association Among Serum ALT Level, Activity of Hepatitis, and Degree of Liver Fibrosis. The mean serum ALT level for all patients was 91.7 ± 67.5 IU/L. Among the 504 patients, activity scores were A0 ($n = 42$), A1 ($n = 286$), A2 ($n = 165$), and A3 ($n = 11$). Fibrosis scores were F0 ($n = 92$), F1 ($n = 225$), F2 ($n = 138$), and F3 ($n = 49$). Serum ALT levels increased according to the degree of activity (Figure 1). Also, ALT levels increased in association with the progression of liver fibrosis (Figure 2).

Distribution of the activity of hepatitis is shown according to the degree of liver fibrosis in Table 1. The proportion of patients with high activity (A2 or A3) markedly increased in patients with a high degree of fibrosis (F2 or F3). Thus, activity of hepatitis and degree of liver fibrosis together were associated with an elevation in serum ALT.

Association Between Serum ALT Level and Patient Age and Sex. Serum ALT levels were analyzed according to age and sex of patients. Serum ALT levels were significantly higher in male patients than in female patients (males, 98.1 ± 69.9 IU/L, vs. females, 80.1 ± 63.1 IU/L; $P = 0.0048$). However, serum ALT levels were similar between younger patients (≤50 years old; $n = 246$) and older patients (>50 years old; $n = 258$) (younger, 95.8 ± 69.5 IU/L, vs. older, 88.2 ± 66.8 IU/L; $P = 0.2148$).

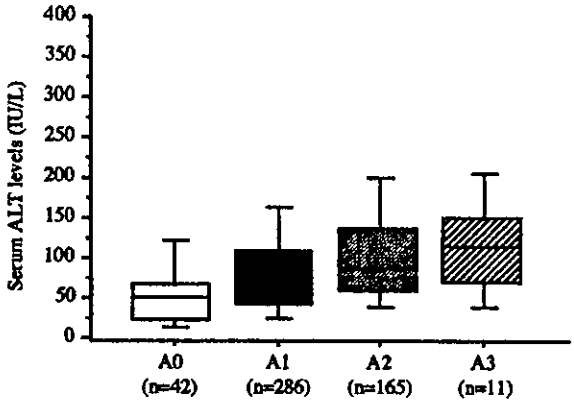

Fig 1. Serum alanine aminotransferase (ALT) levels per activity of hepatitis. ALT levels increase in association with the grade of the activity. ALT levels are significantly higher in patients with A1 activity than in those with A0 activity ($P = 0.0475$) and in patients with A2 activity than in those with A1 activity ($P = 0.0003$).

When patients were stratified by low activity of hepatitis (A0/A1) and high activity of hepatitis (A2/A3), we found that serum ALT levels did not differ by sex among patients with A2/A3 (males, 111.9 ± 70.4 IU/L, vs. females, 104.9 ± 72.2 IU/L; $P = 0.5362$); however, we found a marked difference between the sexes in patients with A0/A1 (males, 90.9 ± 68.8 IU/L, vs. females, 66.0 ± 52.8 IU/L; $P = 0.0010$) (Figure 3). In contrast, we found a significant difference in serum ALT levels between younger and older patients with A2/A3 (younger, 128.6 ± 75.7 IU/L, vs. older, 98.2 ± 65.8 IU/L; $P =$

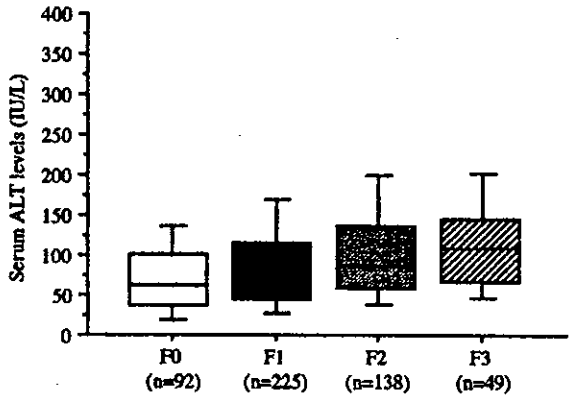

Fig 2. Serum alanine aminotransferase (ALT) levels per degree of liver fibrosis. ALT levels increase in association with the degree of fibrosis. ALT levels are significantly higher in patients with F2 fibrosis than in those with F1 fibrosis ($P = 0.0097$).