Fig. 2 Cumulative incidence of development of HCC according to the effectiveness of treatment (MVR vs. non-MVR). **a** All cases; **b** chronic hepatitis; **c** cirrhosis. *Solid lines* MVR, *dotted lines* non-MVR. *MVR* Maintained viral response

The cumulative carcinogenesis rate for MVR-positive patients was 8% at 3 years, 14% at 5 years, and 14% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 18% at 3 years, 40% at 5 years, and 44% at 7 years. MVR during LAM significantly suppressed the cumulative HCC incidence compared with non-MVR in the cirrhosis patients ($p = 0.029$).

Relationship between IVR and MVR

Maintained viral response (MVR) was achieved by 142 (48%) of the 293 patients enrolled in this study. IVR was achieved by 204 (79%) of the 259 patients who were examined for IVR. The relationship between IVR and MVR is shown in Fig. 3; 60% (122/204) of the IVR-positive patients achieved an MVR, while only 16% (9/55) of the IVR-negative patients achieved an MVR ($p < 0.001$). The LAM-resistant YMDD mutant virus was found in 149 (51%) of all patients during follow-up, and in 52% (107/204) of the IVR-positive patients, a finding which was nearly equal to that for the IVR-negative patients (51%, 28/55). Among the IVR-positive patients, the MVR rate was lower in patients with the YMDD mutation, compared with that in those without the YMDD mutation (44%, 47/107 vs. 77%, 75/97, $p = 0.016$), while the MVR rates were low in the IVR-negative patients, irrespective of their YMDD mutation status (with and without the mutation, 15 vs. 18%, respectively). ADV was added to LAM treatment for 73 (68%) of the 107 IVR-positive patients with the YMDD mutation and 20 (36%) of the 55 IVR-negative patients with the YMDD mutation. However, MVR was only achieved at the low rates of 33% (24/73) for the former patients and 20% (4/20) for the latter.

Discussion

Lamivudine treatment has been shown to improve the liver histological findings in patients with HBV-infected liver disease by reducing the HBV load and stabilizing inflammatory activity [16–18]. One report has shown that LAM effectively reduced the incidence of HCC in patients with chronic hepatitis B, but the study only compared LAM-treated patients with non-treated patients in a matched case-controlled study [19]. However, there have been few detailed reports about the relationship between virological response and HCC development in HBV-infected patients during LAM treatment. In the present study, we retrospectively examined the incidence of HCC to clarify the indicators of LAM therapy, including median HBV-DNA levels, for reducing the risk of HCC in HBV-infected patients.

Fig. 3 Relationship between IVR and MVR. *IVR* Initial viral response, *MVR* maintained viral response, *N.S.* not significant
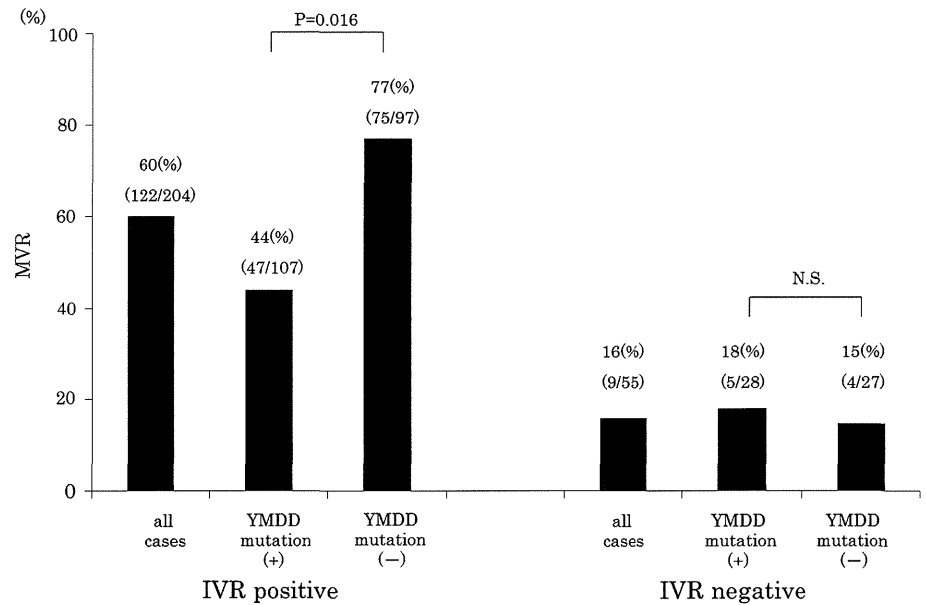
Many investigators have reported that serum HBV DNA levels higher than 4.0–4.5 log copies/ml before HBV treatment serve as a strong risk predictor of HCC [23–25]. Di Marco et al. [26] have reported that the incidence of HCC was higher in patients with serum HBV levels of more than 5.0 log copies/ml, at least once, during LAM therapy than in those in whom serum HBV levels were maintained at 5.0 log copies/ml or less. However, the add-on ADV therapy had not been adopted when the study of Di Marco et al. was reported. When the use of ADV is possible, an evaluation method is needed to measure the antiviral effects of nucleoside/nucleotide analogues against HBV-related liver disease. In the present study, we set the cut-off value for HBV-DNA at 4.0 log copies/ml. The basis of this cut-off value is that a serum HBV DNA level higher than 4.0 log copies/ml before HBV treatment was reported to serve as a strong risk predictor of HCC [23]. MVR, defined as a median HBV-DNA level of less than 4.0 log copies/ml measured every 6 months during therapy, was adopted as an indicator of viral replication, and non-MVR (median HBV-DNA >4.0 log copies/ml) during LAM therapy was shown to be significantly associated with the development of HCC in HBV-infected patients. We also found that a median HBV-DNA level of >4.0 log copies/ml during LAM therapy was a risk factor for HCC development. On the other hand, IVR, defined as HBV-DNA of <4.0 log copies/ml in the first 6 months of the follow-up period after the initiation of therapy, was not associated with the development of HCC in HBV patients in this study. As shown in Fig. 3, 84% of the IVR-negative patients could not achieve an MVR, suggesting that it is crucial to achieve an IVR in order to achieve an MVR. The reason why IVR was not a significant factor for MVR seemed to be the appearance of the YMDD mutation, which reduced the antiviral effect of

LAM for HBV in IVR-positive patients. The LAM-resistant YMDD mutant virus was found in 52% of the IVR-positive patients. Although ADV was added to LAM treatment for 73 patients, only 33% of these patients could achieve an MVR. We speculate that the antiviral effect of ADV is not very strong [27] and it takes time to reduce the YMDD mutant virus, which may explain the low MVR rate (33%) in patients with the add-on ADV therapy. The immediate administration of ADV when the LAM-resistant YMDD mutant virus appears can be important [28]. A switch to ETV, which induces resistant virus less frequently, could also raise MVR rates among IVR-positive patients without the YMDD mutant virus.

As the duration of the add-on ADV therapy was included in this study, we compared the cumulative incidence of HCC in patients receiving LAM monotherapy with that in patients who also received the add-on ADV therapy. Sixteen (10%) of the 164 patients who received the LAM monotherapy developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 10% at 5 years, and 15% at 7 years. On the other hand, 16 (12%) of the 129 patients who received LAM plus ADV developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 14% at 7 years. No significant difference was found between these two groups ($p = 0.986$). In addition, we examined the cumulative incidence of HCC development according to the effectiveness of treatment (MVR vs. non-MVR) in patients for whom the observation period was terminated when ADV was added, and the same results were obtained (data not shown).

Older age (≥ 50 years), cirrhosis, and low platelet count ($<14 \times 10^4/mm^3$) were shown to be significantly associated with the development of HCC in patients with HBV infection. These results were consistent with those of

previous reports [29–31], suggesting that patients of older age with advanced fibrosis should be followed up carefully for longer periods in order to detect early stages of HCC even if LAM therapy does effectively suppress HBV. Of note, in the present study we estimated the cumulative HCC incidence according to the initial diagnosis of chronic hepatitis or cirrhosis. In the chronic hepatitis patients, older age (≥ 50 years), HBe Ag negativity, and low platelet count ($<14 \times 10^4/mm^3$) were significant risk factors for the development of HCC, but this was not the case in the cirrhosis patients. Because liver biopsies had not been performed, the liver fibrosis stage could not be evaluated with respect to the risk factors for HCC in this study. Instead, the factors of age, HBe Ag status, and platelet count may reflect the degree of liver fibrosis in chronic hepatitis patients. In fact, cirrhotic patients, in comparison with chronic hepatitis patients, were of older age (chronic hepatitis vs. cirrhosis: 46.3 ± 10.7 vs. 51.9 ± 9.8 years, $p < 0.001$), had higher rates of HBe Ag negativity (chronic hepatitis vs. cirrhosis: 39 vs. 51%, $p = 0.065$), and had lower platelet counts (chronic hepatitis vs. cirrhosis: 15.6 ± 4.9 vs. $9.3 \pm 3.8 \times 10^4/mm^3$, $p < 0.001$). This seems to explain why none of these factors were significant risk factors for HCC in cirrhotic patients. On the other hand, in the chronic hepatitis patients, MVR was not a significant factor for HCC development, while MVR was a significant factor for HCC development in the cirrhotic patients. We speculate that HBV suppression induced by LAM therapy could reduce the incidence of HCC in patients infected with HBV, especially those with cirrhosis, who displayed higher malignant potential. Investigation over a longer period is needed to clarify the effect of HBV suppression on the development of HCC in chronic hepatitis patients.

In conclusion, the present study shows that the attainment of an MVR induced by LAM therapy has a significant beneficial effect on the clinical course of HBV-infected patients by decreasing the incidence of HCC. The newer nucleotide analogues, such as ETV and tenofovir, should be able to further reduce the incidence of HCC, given their greater potency.

Conflict of interest The authors declare that they have no conflict of interest.

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Reducing Peg-IFN doses causes later virologic response or no response in HCV genotype 1 patients treated with Peg-IFN alfa-2b plus ribavirin

Tsugiko Oze · Naoki Hiramatsu · Changho Song · Takayuki Yakushijin · Sadaharu Iio · Yoshinobu Doi ·
Masahide Oshita · Hideki Hagiwara · Eiji Mita · Toshifumi Ito · Yoshiaki Inui · Taizo Hijioka ·
Shinji Tamura · Harumasa Yoshihara · Atsuo Inoue · Yasuharu Imai · Eijiro Hayashi · Michio Kato ·
Masanori Miyazaki · Atsushi Hosui · Takuya Miyagi · Yuichi Yoshida · Tomohide Tatsumi ·
Shinichi Kiso · Tatsuya Kanto · Akinori Kasahara · Norio Hayashi · Tetsuo Takehara

Abstract

Background The timing to the first undetectable hepatitis
C virus (HCV) RNA level is strongly associated with
sustained virologic response in pegylated interferon (Peg-
IFN) plus ribavirin combination therapy for patients with
chronic hepatitis C (CH-C) with genotype 1. This study
was conducted to clarify the impact of drug exposure to
Peg-IFN on the timing of HCV RNA negativity in Peg-IFN
plus ribavirin combination therapy for CH-C patients with
genotype 1.

Methods A total of 1409 patients treated with Peg-IFN
alfa-2b plus ribavirin were enrolled and classified into four
categories according to the Peg-IFN dosage. Furthermore,
100 patients were extracted from each Peg-IFN dosage
category to adjust for characteristic factors, using the
propensity score method.

Results Peg-IFN exposure was dose-dependently associ-
ated with the timing of HCV RNA negativity ($p \leq 0.001$).
The HCV RNA negative rate at week 4 decreased from
12% with a Peg-IFN dose of >1.5 µg/kg/week to 1–3%
with a dose of <1.5 µg/kg/week ($p \leq 0.001$), and at week
12 the rate had decreased from 44% with a dose of

T. Oze and N. Hiramatsu contributed equally to this work.

T. Oze · N. Hiramatsu (✉) · C. Song · T. Yakushijin ·
M. Miyazaki · A. Hosui · T. Miyagi · Y. Yoshida ·
T. Tatsumi · S. Kiso · T. Kanto · A. Kasahara · T. Takehara
Department of Gastroenterology and Hepatology,
Osaka University Graduate School of Medicine, 2-2,
Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: hiramatsu@gh.med.osaka-u.ac.jp

S. Iio
Higashiosaka City Central Hospital, Higashiosaka, Japan

Y. Doi
Otemae Hospital, Osaka, Japan

M. Oshita
Osaka Police Hospital, Osaka, Japan

H. Hagiwara · N. Hayashi
Kansai Rosai Hospital, Amagasaki, Japan

E. Mita
National Hospital Organization Osaka National Hospital,
Osaka, Japan

T. Ito
Osaka Koseinenkin Hospital, Osaka, Japan

Y. Inui
Hyogo Prefectural Nishinomiya Hospital, Nishinomiya, Japan

T. Hijioka
National Hospital Organization Osaka Minami Medical Center,
Kawachinagano, Japan

S. Tamura
Minoh City Hospital, Minoh, Japan

H. Yoshihara
Osaka Rosai Hospital, Sakai, Japan

A. Inoue
Osaka General Medical Center, Osaka, Japan

Y. Imai
Ikeda Municipal Hospital, Ikeda, Japan

E. Hayashi
Kinki Central Hospital of Mutual Aid Association
of Public School Teachers, Itami, Japan

M. Kato
National Hospital Organization Minami
Wakayama Medical Center, Tanabe, Japan

≥ 1.2 µg/kg/week to 18% with a dose of <1.2 µg/kg/week ($p = 0.001$). Treatment failure (patients without a 1-log decrease of HCV RNA at week 4 or a 2-log decrease of HCV RNA at week 12, or positive at week 24) was found in 54–66% of patients given <1.2 µg/kg/week ($p \leq 0.001$), and these patients accounted for 64% of the non-responders.

Conclusions The timing of HCV RNA negativity depends significantly on the Peg-IFN dose. Reducing the Peg-IFN dose can induce a later virologic response or non-response in HCV genotype 1 patients treated with Peg-IFN plus ribavirin.

Keywords Chronic hepatitis C · Pegylated interferon plus ribavirin · Drug adherence · HCV RNA negativity · Propensity score matched study

Introduction

The timing to the first undetectable hepatitis C virus (HCV) RNA level during pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) genotype 1 is strongly associated with a sustained virologic response (SVR), defined as undetectable HCV RNA at 24 weeks after the finishing of the treatment. The SVR rate was 87–100% in patients with undetectable HCV RNA at week 4, 73–81% at week 12, and 14–44% between weeks 12 and 24 in patients receiving the standard 48-week treatment [1–8]. These results suggest that HCV RNA negativity should be achieved as soon as possible during treatment in order to attain a higher SVR rate.

Previous studies have revealed that many factors affect the complete virologic response (c-EVR) and SVR, such as age, gender, degree of liver fibrosis, HCV genotype, HCV viral load, and the amount of drug exposure [1–3, 9–16]. Of these factors, only the amount of drug exposure can be controlled in order to try to improve the antiviral effect, as the other factors are fixed for individual patients. Also, recently, the single-nucleotide polymorphisms (SNPs) of the *IL28B* gene have been revealed to be associated with the antiviral effects of pegylated interferon-alpha and ribavirin therapy [17–19].

Peg-IFN has been reported to be dose-dependently correlated with c-EVR [13]. Patients' characteristic factors can be related to drug adherence, as aged patients, female patients, and patients with progression of liver fibrosis have a tendency to show low drug adherence. This suggests that patients with low drug adherence could be those who are difficult to treat. Therefore, patients with similar characteristic factors should be compared in order to precisely assess the actual impact of drug exposure on the timing to the first undetectable HCV RNA level.

Only a few randomized controlled trials (RCTs) have examined the relationship between drug dose reduction and antiviral effect with Peg-IFN plus ribavirin combination therapy [1–3, 20–23], and the findings are controversial. Manns et al. [1] reported that the SVR rate was significantly lower in patients given 0.5 µg/kg/week of Peg-IFN than in those given 1.5 µg/kg/week of Peg-IFN (34 vs. 42%, $p < 0.05$). McHutchison et al. [3] reported that the SVR rate did not differ between groups given 1.0 and 1.5 µg/kg/week of Peg-IFN (38 vs. 40%, $p = 0.20$). No detailed study of the relationship between dose reduction and delay of HCV RNA negativity or the relationship between dose reduction and an increase of non-responders to the treatment has been reported, and the real impact of drug exposure on the anti-viral effect remains unclear.

In this present work, we conducted a matched study in which characteristic factors other than drug exposure were adjusted using propensity scores. We investigated the impact of drug exposure to Peg-IFN on the timing to the first undetectable HCV RNA level.

Patients and methods

Patients

The present study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 1409 Japanese patients with CH-C treated with a combination of Peg-IFN alfa-2b plus ribavirin were enrolled in this study between December 2004 and July 2008.

Patients eligible for this study were those who were infected with HCV genotype 1 and had a viral load of $\geq 10^5$ IU/ml, but were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcoholic liver disease, autoimmune hepatitis). Informed consent was obtained from each patient included in this study, which was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

Treatment

All patients received Peg-IFN alfa-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough). Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 µg based on body weight (body weight 35–45 kg, 60 µg; 46–60 kg, 80 µg; 61–75 kg, 100 µg; 76–90 kg, 120 µg; 91–120 kg, 150 µg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight

(body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients.

Dose reduction

As a rule, dose modification, which was performed according to the intensity of the adverse hematologic effects, was done by following the manufacturer's drug information. The dose of Peg-IFN alfa-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to <1500/mm^3, the neutrophil count declined to <750/mm^3, or the platelet (Plt) count declined to <8 × 10^4/mm^3, and was discontinued if the WBC count declined to <1000/mm^3, the neutrophil count declined to <500/mm^3, or the Plt count declined to <5 × 10^4/mm^3. Ribavirin was also reduced from 1000 to 600 mg, or from 800 to 600 mg, or from 600 to 400 mg if the hemoglobin (Hb) level decreased to <10 g/dl, and was discontinued if the Hb level decreased to <8.5 g/dl.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL). The HCV RNA level was evaluated every 4 weeks during treatment. A rapid virologic response (RVR) was defined as undetectable serum HCV RNA at week 4, a c-EVR as undetectable serum HCV RNA at week 12, and a late virologic response (LVR) as detectable HCV RNA at week 12 but undetectable at week 24. Patients with <a 1-log decrease in the HCV RNA level at week 4 or <a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 were considered to have experienced treatment failure (non-response, NR) and had to stop treatment. If patients discontinued the treatment due to adverse events, without HCV RNA negativity being attained, they were also regarded as having had treatment failure.

Assessment of drug exposure

The amounts of Peg-IFN alfa-2b and ribavirin actually taken were evaluated by reviewing the medical records and calculating the amount taken from the start until the timing of the first undetectable HCV RNA level for the patients achieving HCV RNA negativity, and calculating the amount taken throughout the treatment for the patients not attaining HCV RNA negativity. For patients who discontinued the treatment, if their HCV RNA had become negative before discontinuation, the drug amount data were calculated from the start of treatment until the timing of the first undetectable HCV RNA level, and if HCV RNA had not become negative before discontinuation, the data throughout the treatment before discontinuation were used. The amounts of both drugs were divided individually on the basis of body weight at baseline as the average: Peg-IFN alfa-2b was expressed as µg/kg/week and ribavirin as mg/kg/day.

Evaluation of impact of drug exposure on HCV RNA negativity

We evaluated the relationship between the exposure to both drugs and HCV RNA negativity at week 24 by univariate and multivariate analyses for the patients who completed 24 weeks of treatment, using the mean administration doses of both drugs during the first 24 weeks and the characteristic factors other than drug exposure at baseline.

The patients were divided into four categories according to the Peg-IFN dose: up to 0.9 µg/kg/week of Peg-IFN; from 0.9 to less than 1.2 µg/kg/week; from 1.2 to less than 1.5 µg/kg/week; and from 1.5 µg/kg/week. The propensity score matching method was used to adjust the patients' characteristic factors among these categories. This score was calculated for each patient by logistic regression analysis, with four patient characteristic factors as independent variables; age, gender, Plt values, and history of IFN treatment. We then performed 1:1 nearest neighbor matching within a caliper of 0.15 standard deviation of the propensity score: one patient in each group with 0.9–1.2 µg/kg/week, 1.2–1.5 µg/kg/week, and ≥1.5 µg/kg/week to one patient with <0.9 µg/kg/week, and extracted 100 patients from each category.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD or median values. Factors associated with HCV RNA negativity at week 24 were assessed by univariate analysis using the Mann–Whitney U-test or the χ^2 test, and by multivariate analysis using logistic regression analysis. To analyze the difference between baseline data among the four Peg-IFN groups, analysis of variance (ANOVA) or the χ^2 test was performed. The significance of trends in values for the timing to the first undetectable HCV RNA level was determined with the Mantel–Haenszel χ^2 test. A two-tailed p value of <0.05 was considered significant. Statistical analysis was conducted with SPSS version 15.0J (SPSS, Chicago, IL, USA).

Table 1 Baseline characteristics of patients before matching

Factor	All patients	<0.9 µg/kg/week of Peg-IFN	0.9–1.2 µg/kg/week of Peg-IFN	1.2–1.5 µg/kg/week of Peg-IFN	≥1.5 µg/kg/week of Peg-IFN	p value
Number	1409	153	159	670	427	
Age (years)	56.3 ± 10.4	58.0 ± 9.9	57.3 ± 10.2	55.9 ± 10.6	56.3 ± 10.4	0.069
Sex: male/female	722/687	70/83	69/90	376/294	207/220	0.004
History of IFN treatment: naïve/experienced	862/547	98/55	96/63	408/262	260/167	0.894
White blood cells (/mm^3)	5060 ± 1532	4325 ± 1419	4566 ± 1394	5246 ± 1562	5215 ± 1456	<0.001
Neutrophils (/mm^3)	2578 ± 1073	2129 ± 1049	2258 ± 949	2699 ± 1080	2667 ± 1052	<0.001
Red blood cells (×10^4/mm^3)	440 ± 46	424 ± 42	429 ± 45	445 ± 46	441 ± 45	<0.001
Hemoglobin (g/dl)	14.0 ± 1.4	13.6 ± 1.2	13.7 ± 1.5	14.1 ± 1.4	14.1 ± 1.4	<0.001
Platelets (×10^4/mm^3)	16.3 ± 5.6	11.9 ± 3.9	13.2 ± 4.8	17.4 ± 5.7	17.4 ± 5.2	<0.001
ALT (IU/l)	78 ± 61	93 ± 64	87 ± 68	75 ± 58	73 ± 60	0.001
Serum HCV RNA (KIU/ml)[a]	1450	1300	1900	1700	1900	0.176
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	810/186 (17%)	78/34 (30%)	70/30 (30%)	392/77 (16%)	270/45 (14%)	<0.001
Activity, 0–1/2–3	527/468	43/67	38/62	259/210	187/129	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 413 patients

[c] Percent of patients with 3–4

Results

Clinical characteristics of all patients according to Peg-IFN dosage before matching

A total of 1409 patients were enrolled in this study, and the baseline characteristics of the patients are shown in Table 1. Based on the Peg-IFN dosage, these patients were classified into four categories. With the decrease of Peg-IFN dosage, the ratio of female-to-male patients increased, the peripheral blood cell count decreased, and the number of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) increased significantly ($p < 0.001$). Patients with a lower Peg-IFN dosage tended to be older ($p = 0.07$).

Next, we analyzed the factors associated with HCV RNA negativity at week 24 for the 1226 patients who completed 24 weeks of treatment, using the baseline characteristic variables, excluding liver histology, shown in Table 1 and the mean doses of both drugs during the first 24 weeks. The HCV RNA negative rate at week 24 was 68% (829/1226). The results of univariate analysis are shown in Table 2. The factors evaluated by multivariate analysis were those for which the p value was <0.10 by univariate analysis for HCV RNA negativity at week 24: age, gender, history of IFN treatment, WBC, neutrophils, red blood cells (RBC), Hb, Plt, alanine aminotransferase, and the mean doses of Peg-IFN and ribavirin during the first 24 weeks. By the multivariate analysis, in addition to the RBC value ($p = 0.02$), Plt value ($p < 0.001$), and

Table 2 Univariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Negative	Positive	p value
Number	829	397	
Age (years)	55.1 ± 10.5	57.6 ± 10.1	<0.001
Sex: male/female	437/392	189/208	0.094
History of IFN treatment: naïve/experienced	523/306	229/168	0.069
White blood cells (/mm^3)	5175 ± 1498	4566 ± 1394	<0.001
Neutrophils (/mm^3)	2665 ± 1087	2429 ± 1059	<0.001
Red blood cells (×10^4/mm^3)	445 ± 44	434 ± 47	<0.001
Hemoglobin (g/dl)	14.1 ± 1.4	13.9 ± 1.4	0.004
Platelets (×10^4/mm^3)	17.2 ± 5.6	14.8 ± 5.3	<0.001
ALT (IU/l)	73 ± 55	83 ± 63	0.001
Serum HCV RNA (KIU/ml)	1750	1800	0.673
Mean Peg-IFN dose (µg/kg/week)	1.39 ± 0.24	1.25 ± 0.32	<0.001
Mean ribavirin dose (mg/kg/day)	10.6 ± 1.8	9.7 ± 2.2	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

history of IFN treatment ($p = 0.04$), the factor of Peg-IFN exposure was an independent factor for HCV RNA negativity at week 24 ($p < 0.001$) (Table 3). The mean dose of ribavirin did not show a significant correlation with HCV RNA negativity at week 24 ($p = 0.07$).

Clinical characteristics of patients extracted from each Peg-IFN dosage category after matching

Patients in the four Peg-IFN categories were matched by the propensity score method and 100 patients were extracted from each category. The c-statics for the propensity score model between the patients with <0.9 μg/kg/

Table 3 Multivariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Category	Odds ratio	95% CI	p value
Age	1 year	–	–	NS
Sex	Male/female	–	–	NS
History of IFN treatment	Naïve/experienced	0.756	0.581–0.984	0.037
White blood cells	$1 \times 10^3/mm^3$	–	–	NS
Neutrophils	$1 \times 10^3/mm^3$	–	–	NS
Red blood cells	$1 \times 10^4/mm^3$	1.004	1.001–1.007	0.02
Hemoglobin	1 g/dl	–	–	NS
Platelets	$1 \times 10^4/mm^3$	1.054	1.026–1.083	<0.001
ALT	1 IU/l	–	–	NS
Mean Peg-IFN dose	0.1 μg/kg/week	1.096	1.045–1.149	<0.001
Mean ribavirin dose	1 mg/kg/day	1.060	0.994–1.130	0.074

ALT alanine aminotransferase, *CI* confidence interval, *IFN* interferon, *NS* not significant, *Peg-IFN* pegylated interferon

week of Peg-IFN and those given different levels of Peg-IFN were 0.62 for 0.9–1.2 μg/kg/week of Peg-IFN, 0.82 for 1.2–1.5 μg/kg/week of Peg-IFN, and 0.82 for ≥1.5 μg/kg/week of Peg-IFN.

The baseline characteristics of the patients extracted according to the Peg-IFN dosage category are shown in Table 4. There was no significant difference among the four Peg-IFN categories in any of the factors, indicating that the extracted cohort of 400 patients was well matched according to propensity score methods.

Timing to the first undetectable HCV RNA level according to Peg-IFN dosage

We evaluated the relationship between the virologic response during the treatment and the drug exposure to Peg-IFN using our matched cohort of 400 patients (Fig. 1). Of the 400 patients, 23 had discontinued treatment due to adverse events by week 24 (<0.9 μg/kg/week, $n = 5$; 0.9–1.2 μg/kg/week, $n = 4$; 1.2–1.5 μg/kg/week, $n = 5$; ≥1.5 μg/kg/week, $n = 9$). The proportion of patients with treatment failure increased according to the decrease in the dose of Peg-IFN: 66% among patients with <0.9 μg/kg/week of Peg-IFN, 54% among those with 0.9–1.2 μg/kg/week of Peg-IFN, 35% among those with 1.2–1.5 μg/kg/week of Peg-IFN, and 32% among those with ≥1.5 μg/kg/week of Peg-IFN ($p < 0.001$). Additionally, the timing to the first undetectable HCV RNA level tended to shift to an earlier time

Table 4 Baseline characteristics of patients after matching

Factor	All patients	<0.9 μg/kg/week of Peg-IFN	0.9–1.2 μg/kg/week of Peg-IFN	1.2–1.5 μg/kg/week of Peg-IFN	>1.5 μg/kg/week of Peg-IFN	p value
Number	400	100	100	100	100	
Age (years)	56.9 ± 9.6	57.4 ± 10.0	56.6 ± 9.9	56.8 ± 9.5	56.7 ± 9.1	0.941
Sex: male/female	190/210	47/53	47/53	46/54	50/50	0.948
History of IFN treatment: naïve/experienced	286/114	70/30	71/29	73/27	72/28	0.970
White blood cells (/mm³)	4557 ± 1344	4331 ± 1310	4532 ± 1372	4642 ± 1399	4725 ± 1280	0.186
Neutrophils (/mm³)	2261 ± 955	2070 ± 855	2200 ± 883	2416 ± 1068	2357 ± 972	0.054
Red blood cells (×10⁴/mm³)	429 ± 43	423 ± 40	427 ± 42	432 ± 44	434 ± 46	0.300
Hemoglobin (g/dl)	13.8 ± 1.5	13.7 ± 1.3	13.7 ± 1.6	13.9 ± 1.4	14.0 ± 1.5	0.300
Platelets (×10⁴/mm³)	12.1 ± 3.7	11.8 ± 3.9	12.0 ± 3.8	12.1 ± 3.5	12.4 ± 3.6	0.625
ALT (IU/l)	89 ± 67	98 ± 65	92 ± 70	86 ± 61	80 ± 69	0.254
Serum HCV RNA (KIU/ml)[a]	1700	1400	1800	1700	1750	0.742
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	186/89 (32%)	47/22 (32%)	46/22 (32%)	48/21 (30%)	45/24 (35%)	0.958
Activity, 0–1/2–3	115/159	25/42	26/42	32/37	32/38	0.585

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 125 patients
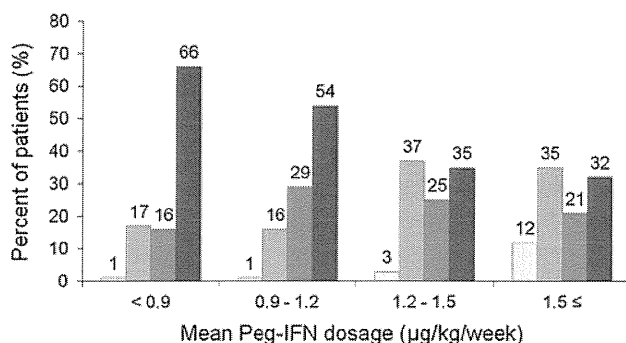
[c] Percent of patients with 3–4

Fig. 1 Timing to the first undetectable hepatitis C virus (HCV) RNA level according to pegylated interferon (*Peg-IFN*) dosage. *Light gray bars* patients with undetectable HCV RNA at week 4. *Medium gray bars* patients with undetectable HCV RNA during 5 to 12 weeks. *Dark gray bars* patients with undetectable HCV RNA during 13–24 weeks. *Black bars* patients with treatment failure (patients with less than a 1-log decrease in HCV RNA level at week 4 or less than a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 and those with treatment discontinuance without HCV RNA negativity). Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$)
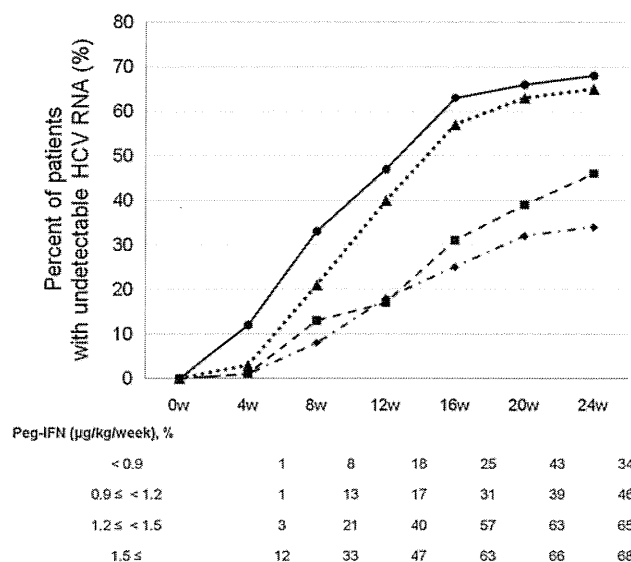

Peg-IFN (µg/kg/week), %						
	0w	4w	8w	12w	16w	20w	24w
< 0.9		1	8	18	25	43	34
0.9 ≤ < 1.2		1	13	17	31	39	46
1.2 ≤ < 1.5		3	21	40	57	63	65
1.5 ≤		12	33	47	63	66	68

Fig. 2 Longitudinal negative HCV RNA rates from the start to 24 weeks of the treatment. *Filled circles* Peg-IFN ≥1.5 µg/kg/week, *filled triangles* Peg-IFN 1.2–1.5 µg/kg/week, *filled squares* Peg-IFN 0.9–1.2 µg/kg/week, *filled diamonds* Peg-IFN <0.9 µg/kg/week. The HCV RNA negative rate at week 4 was significantly higher among the patients with Peg-IFN ≥1.5 µg/kg/week than among those with Peg-IFN <1.5 µg/kg/week ($p \leq 0.001$). The HCV RNA negative rates at weeks 12 and 24 were significantly higher among the patients with Peg-IFN ≥1.2 µg/kg/week than among those with Peg-IFN <1.2 µg/kg/week ($p = 0.001$, $p = 0.002$, respectively). *w* week

during the treatment according to the increase in the Peg-IFN dose ($p \leq 0.001$).

Figure 2 shows the longitudinal data of the HCV RNA negative rate. The data for patients with treatment failure were included until the end of each patient's treatment. The

percentage of patients with undetectable HCV RNA at week 4 decreased from 12 to 1–3% if they were given <1.5 µg/kg/week of Peg-IFN ($p \leq 0.001$). As for the HCV RNA negative rates at week 12 and week 24, there was no significant difference between patients with 1.2–1.5 µg/kg/week and those with >1.5 µg/kg/week of Peg-IFN. The two groups with <1.2 µg/kg/week of Peg-IFN showed significantly lower HCV RNA negative rates than the other two groups given ≥1.2 µg/kg/week of Peg-IFN (week 12, 18 vs. 44%, $p = 0.0001$, week 24, 40 vs. 67%, $p = 0.0002$). The patients with <0.9 µg/kg/week tended to show a decreased HCV RNA negative rate at week 24 compared to the patients given 0.9–1.2 µg/kg/week (34 vs. 46%, $p = 0.08$).

Figure 3 shows the proportion of patients, according to Peg-IFN exposure, among those with undetectable HCV RNA at week 4 ($n = 17$) and at week 12 ($n = 122$), as well as the proportion of patients with detectable HCV RNA at week 24 ($n = 213$). The patients given ≥1.5 µg/kg/week of Peg-IFN accounted for 70% of the patients with undetectable HCV RNA at week 4, and those given ≥1.2 µg/kg/week of Peg-IFN accounted for 71% of the patients with undetectable HCV RNA at week 12. On the other hand, the patients given <1.2 µg/kg/week of Peg-IFN accounted for 64% of the patients with detectable HCV RNA at week 24.

Discussion

The association between drug exposure and HCV RNA negativity has been reported [9–13]. However, most studies have shown only the fixed-point relationship at week 12, and it remains unclear whether dose modification accelerates or delays the timing to the first undetectable HCV RNA level. The present study is the first to clarify this. Induction regimens in which a high dose (360 µg/week) of Peg-IFN alfa-2a was administered for the first 12 weeks failed to improve SVR rates compared to treatment with a standard dose of Peg-IFN in the CHARIOT [22] and PROGRESS [23] RCTs. In contrast, the adherence study of McHutchison et al. revealed that patients who received ≥80% of the planned dose of Peg-IFN and ribavirin for ≥80% of the full 48 weeks of treatment had a significantly higher SVR rate (51%) than those who received <80% of the planned dose of one or both drugs for ≥80% of the full 48 weeks of treatment (34%) ($p = 0.011$) [10]. These apparently paradoxical results for the relationship between drug dosage and antiviral effect imply that the dose-dependent increase of the antiviral effect was observed up to a certain dose and the antiviral effect then reached a plateau above the regular dose. This paradoxical effect could explain the impact of Peg-IFN reduction from a regular dose on the timing to the first undetectable HCV
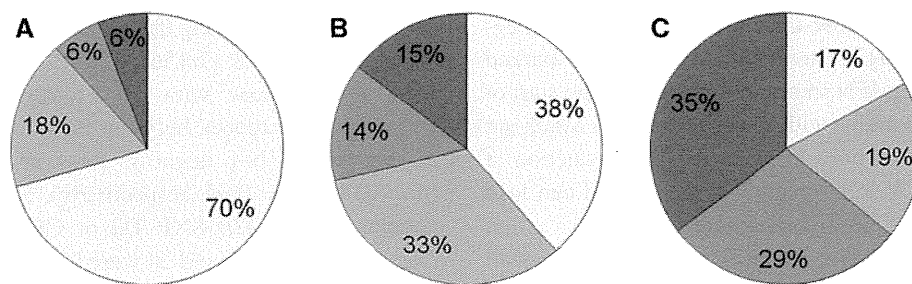
Fig. 3 Proportions of patients according to Peg-IFN exposure among patients with undetectable HCV RNA at weeks 4 and 12 and those with detectable HCV RNA at week 24. **a** Week 4 ($n = 17$), **b** week 12 ($n = 122$), **c** week 24 ($n = 213$). *Light gray segments* Peg-IFN <0.9 µg/kg/week. *Medium gray segments* Peg-IFN 0.9–1.2 µg/kg/week. *Dark gray segments* Peg-IFN 1.2–1.5 µg/kg/week. *Black segments* Peg-IFN ≥1.5 µg/kg/week

RNA level in the present study differing from that of the induction therapy with a high Peg-IFN dose in the above two studies.

In the present study, characteristic matched patients were extracted from a large retrospective cohort to examine the impact of Peg-IFN dosage on viral dynamics. The reason for using a matched cohort was that performing an RCT according to Peg-IFN doses poses an ethical problem, because a low dose of Peg-IFN is known to show little efficacy. The reason for our focusing on Peg-IFN dosage was based on the finding that ribavirin was indeed a significant factor for HCV RNA negativity at week 24 on univariate analysis, but not on multivariate analysis, and Peg-IFN was significantly correlated with HCV RNA negativity at week 24 in an independent manner in this study cohort. Our previous report that Peg-IFN, but not ribavirin, was correlated with c-EVR supports this [13].

To calculate the propensity score, we chose four covariates as candidates for adjustment: age, gender, Plt values, and history of IFN treatment, because there was a need to match universal features such as age, gender, and factors associated with HCV RNA negativity at week 24, such as Plt values and the history of IFN treatment. As shown in Table 4, the baseline characteristic factors in the different Peg-IFN patient categories were well matched after propensity score adjustment. That is, c-statics, the hallmark of application to logistic regression analysis, was regarded as adequate for random assignment. Only the c-statics for the patients given <0.9 µg/kg/week of Peg-IFN and the patients given 0.9–1.2 µg/kg/week of Peg-IFN showed a low value (0.62), because the number of patients in the Peg-IFN category of 0.9–1.2 µg/kg/week ($n = 153$) was not very large. However, the patient characteristic factors in two categories after extraction were well matched and were considered to be adequate for further analysis. In this study, the populations extracted after matching were composed of patients with relatively advanced liver fibrosis compared to the original population; the mean Plt value was lower and the proportion of patients with

progression of liver fibrosis (METAVIR fibrosis score 3 or 4) was higher in the extracted population than in the original one (mean Plt value $12.1 \times 10^4/mm^3$ vs. $16.3 \times 10^4/mm^3$, proportion of patients with progression of liver fibrosis, 32 vs. 19%, respectively). The patients with <0.9 µg/kg/week of Peg-IFN, which was the smallest population among the four Peg-IFN categories and included more patients with advanced liver fibrosis, were used as the control for the propensity score matching.

Recently, the usefulness of extended therapy has been revealed for patients with LVR, defined as HCV RNA negativity between week 12 and week 24 (or week 36). In addition, we have reported that, even with extended treatment of 72 weeks, the timing of HCV RNA disappearance showed a strong correlation with relapse after treatment [24]. Accordingly, at present, it is necessary to verify how reducing drug doses affects the delay of the timing to the first undetectable HCV RNA level or treatment failure, and in the present study we demonstrated the appropriate dose of Peg-IFN required to attain HCV RNA negativity by 24 weeks. As shown in Fig. 1, Peg-IFN dose-dependently affected the timing to the first undetectable HCV RNA level during the treatment. These results indicate that dose reduction of Peg-IFN can cause a shift from c-EVR to LVR and a shift from LVR to HCV RNA-positivity at week 24. The proportion of patients with treatment failure among those given <0.9 µg/kg/week of Peg-IFN (66%) was decreased by half among the patients given ≥1.2 µg/kg/week of Peg-IFN (32–35%). Considering that the effectiveness of extended treatment for patients with LVR is obvious, if patients without a c-EVR were to attain HCV RNA negativity by 24 weeks, those patients would have the potential to attain an SVR with extended treatment. However, if patients do not attain HCV RNA negativity, those patients must discontinue the treatment. Therefore, causing patients to shift from HCV RNA negativity by week 24 to being HCV RNA-positive at week 24 would be missing the chance to obtain SVR even with extended treatment. As shown in Fig. 2, the longitudinal negative

rate of HCV RNA was dose-dependently affected by Peg-IFN at all points during the treatment. Therefore, a marked dose reduction of Peg-IFN should not be done at the start of treatment even for patients with lower Plt values (which are indicative of advanced fibrosis), because dose reduction of Peg-IFN before HCV RNA negativity is attained can lead to an increased possibility of treatment failure.

Next, as shown in Fig. 3, 70% of the patients with undetectable HCV RNA at week 4 were given ≥1.5 μg/kg/week of Peg-IFN, 71% of those with undetectable HCV RNA at week 12 were given ≥1.2 μg/kg/week, and 64% of those with detectable HCV RNA at week 24 were given ≤1.2 μg/kg/week. Therefore, in HCV genotype 1 patients treated with Peg-IFN plus ribavirin, the treatment goal for c-EVR or non-NR should be to maintain a Peg-IFN dose of ≥1.2 μg/kg/week, and that for RVR should be to maintain a Peg-IFN dose of ≥1.5 μg/kg/week. Using granulocyte–macrophage colony-stimulating factor for patients who develop a severe decrease of blood cells and are forced to decrease Peg-IFN can be beneficial, as long as HCV RNA is positive.

A limitation of the present study is that the actual SVR rate could not be compared among the four Peg-IFN categories because some patients with LVR were treated for 72 weeks and some were treated for 48 weeks; actual SVR rates were 20% in patients with Peg-IFN <0.9 μg/kg/week, 18% in those with 0.9–1.2 μg/kg/week, 36% in those with 1.2–1.5 μg/kg/week, and 48% in those ≥1.5 μg/kg/week. On the assumption that the SVR rate for patients with RVR is 90%, the SVR rate for those with c-EVR without RVR is 75% for 48-week treatment, and the SVR rate for those with LVR is 60% for 72-week treatment, the SVR rate of response-guided therapy was calculated to be 23% for patients given <0.9 μg/kg/week of Peg-IFN, 30% for those given 0.9–1.2 μg/kg/week of Peg-IFN, 45% for those given 1.2–1.5 μg/kg/week of Peg-IFN, and 50% for those given ≥1.5 μg/kg/week of Peg-IFN in the matched cohort in the present study. Thus, dose reduction of Peg-IFN can reduce the SVR rate even if response-guided therapy is done. Another limitation of this study is that the IL28B SNP, which is known to be a host factor affecting the antiviral effect, could not be examined in all cases, because the characteristic matched patients were extracted from a large retrospective cohort. However, we had the result of the IL28B SNP (rs8099917) for 290 patients; 214 patients had TT and 76 had TG or GG. The proportions of patients with the IL28B SNP TT were similar among the four Peg-IFN categories (≤0.9 μg/kg/week, 76%, 31/41; 0.9–1.2 μg/kg/week, 71%, 27/38; 1.2–1.5 μg/kg/week, 67%, 99/147; ≥1.5 μg/kg/week, 77%, 57/74, p = 0.853). Therefore, it would appear that there was no bias for any cases. Among the patients with the IL28B SNP TT, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/58), 33% (19/58),

and 69% (40/58) among the patients with <1.2 μg/kg/week of Peg-IFN and 4% (7/156), 62% (97/156), and 82% (128/156) among those with ≥1.2 μg/kg/week. There were significant differences between these two Peg-IFN groups in the HCV RNA negative rates at weeks 12 and 24 (p = 0.002, p = 0.04, respectively). Similarly, among the patients with IL28B SNP TG or GG, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/21), 0% (0/21), and 10% (2/21) among the patients with <1.2 μg/kg/week of Peg-IFN and 2% (1/55), 9% (5/55), and 27% (15/55) among those with ≥1.2 μg/kg/week. The HCV RNA negative rates at weeks 12 and 24 tended to be higher in the patients with ≥1.2 μg/kg/week of Peg-IFN (p = 0.06, p = 0.13, respectively). From the above-mentioned results, it appears that the dose-dependent effect of Peg-IFN on the timing of HCV RNA negativity could be considered regardless of the IL28B SNP.

In conclusion, this matched study has demonstrated that, in patients with CH-C with genotype 1 receiving Peg-IFN plus ribavirin combination therapy, Peg-IFN dose-dependently affects the timing to the first undetectable HCV RNA level and the failure to attain HCV RNA negativity. Dose reduction of Peg-IFN to <1.2 μg/kg/week before HCV RNA negativity is attained delays HCV RNA clearance dose-dependently and increases the rate of treatment failure. Maintaining the Peg-IFN dose at ≥1.2 μg/kg/week, and preferably at ≥1.5 μg/kg/week, can accelerate the timing to the first undetectable HCV RNA level for CH-C genotype 1 patients treated with Peg-IFN plus ribavirin.

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ORIGINAL ARTICLE

Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

Yoko Tamada,[1,2] Hiroshi Yatsuhashi,[1,2] Naohiko Masaki,[3] Makoto Nakamuta,[4]
Eiji Mita,[5] Tatsuji Komatsu,[6] Yukio Watanabe,[7] Toyokichi Muro,[8] Masaaki Shimada,[9]
Taizo Hijioka,[10] Takeaki Satoh,[11] Yutaka Mano,[12] Toshiki Komeda,[13]
Masahiko Takahashi,[14] Hiroshi Kohno,[15] Hajime Ota,[16] Shigeki Hayashi,[17]
Yuzo Miyakawa,[18] Seigo Abiru,[1,2] Hiromi Ishibashi[1,2]

For numbered affiliations see
end of article.

Correspondence to
Professor Hiroshi Yatsuhashi,
Clinical Research Center, NHO
National Nagasaki Medical
Center and Department of
Hepatology, Nagasaki University
Graduate School of Biomedical
Sciences, Address: 2-1001-1
Kubara, Omura, Nagasaki
856-8562, Japan;
yatsuhashi@nmc.hosp.go.jp

ABSTRACT
Objective To examine recent trends of acute infection with hepatitis B virus (HBV) in Japan by nationwide surveillance and phylogenetic analyses.
Methods During 1991 through 2009, a sentinel surveillance was conducted in 28 national hospitals in a prospective cohort study. Genotypes of HBV were determined in 547 patients with acute hepatitis B. Nucleotide sequences in the preS1/S2/S gene of genotype A and B isolates were determined for phylogenetic analyses.
Results HBV genotype A was detected in 137 (25% (accompanied by genotype G in one)) patients, B in 48 (9%), C in 359 (66%), and other genotypes in the remaining three (0.5%). HBV persisted in five with genotype A including the one accompanied by genotype G; another was co-infected with HIV type 1. The genotype was A in 4.8% of patients during 1991−1996, 29.3% during 1997−2002, and 50.0% during 2003−2008 in the capital region, as against 6.5%, 8.5% and 33.1%, respectively, in other regions. Of the 114 genotype A isolates, 13 (11.4%) were subgenotype A1, and 101 (88.6%) were A2, whereas of the 43 genotype B isolates, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. Sequences of 65 (64%) isolates of A2 were identical, as were three (23%) of A1, and five (18%) of B2, but none of the B1, B3 and B4 isolates shared a sequence.
Conclusions Acute infection with HBV of genotype A, subgenotype A2 in particular, appear to be increasing, mainly through sexual contact, and spreading from the capital region to other regions in Japan nationwide. Infection persisted in 4% of the patients with genotype A, and HBV strains with an identical sequence prevailed in subgenotype A2 infections. This study indicates the need for universal vaccination of young people to prevent increases in HBV infection in Japan.

Significance of this study

What is already known about this subject?
▶ In Japan, a national prevention programme was started in 1986 with selective vaccination of babies born to mothers who carry hepatitis B virus (HBV). Since then, the prevalence of hepatitis B surface antigen among younger generations has decreased sharply.
▶ However, retrospective studies indicate that the frequency of HBV genotype A is increasing among patients with acute hepatitis B (AHB) within the capital region of Japan.
▶ Infection with genotype A more often persists than infection with other genotypes.
▶ Because there is no reliable and comprehensive surveillance system for AHB in Japan, the incidence of AHB and factors responsible for changes over many years are not known.

What are the new findings?
▶ This is a prospective cohort study for surveillance of AHB throughout Japan in a national research programme.
▶ The incidence of AHB in Japan has not decreased, because genotype A infections have increased over time.
▶ Genotype A infections started to increase in the capital region of Japan, and then spread to other regions 5−6 years later.
▶ About 90% of genotype A found in AHB patients in Japan is subgenotype A2.
▶ Subgenotype A2 isolates from patients with AHB tend to preserve sequence identity over time, indicating that particular subgenotype A2 strains have been transmitted without undergoing mutations.

Hepatitis B virus (HBV) has been classified into 10 genotypes, designated A−J, based on a >8% divergence in the full-genome sequence.[1−7] Different genotypes are associated with distinct clinical manifestations, such as severity and progression of liver disease, as well as response to antiviral treatments.[8−10] Some genotypes are subclassified: genotype A into at least two subgenotypes, A1 (Asian/African type) and A2 (European type)[11−13];

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Significance of this study

How might it impact on clinical practice in the foreseeable future?

▶ It needs to be noted that subgenotype A2 infections are spreading among sexually active generations in Japan.

▶ Although selective vaccination has prevented mother-to-baby transmission of HBV since 1986, it does not contain sporadic infections in Japan.

▶ Herd vaccination of younger generations needs to be considered in Japan.

B into B1 (Japanese type) and B2 (Asian type)[14 15]; and C into C1 (Southeast-Asian type) and C2 (East-Asian type).[16] Subgenotypes also influence the replication of HBV and clinical manifestation.[15 17 18]

According to a report from Japan in 2001,[19] genotype C was the most prevalent (84.7%), followed by genotype B (12.2%) and A (1.7%), among patients with chronic hepatitis B. In 2002, genotype A became the most prevalent in patients with acute hepatitis B (AHB) around Tokyo, the capital region of Japan.[20 21] Several reports have shown that infection with HBV genotype A is associated with particular sexual behaviours, such as homosexual activity and promiscuous sexual contacts, and tends to persist longer than that with HBV genotype C.[22 23] These reports have raised concerns about the horizontal HBV infection in adults, which, in general, is considered to resolve spontaneously. However, adult-acquired HBV infection may result in chronic HBV infection in some instances.

Information on changes in genotype distribution over time, as well as genotype-specific clinical manifestations, may help in planning preventive measures and antiviral therapy strategies. Therefore it is important to examine how genotype A infection has spread in Japan, and what clinical and virological characteristics it possesses.

We have been conducting a nationwide, sentinel surveillance on acute viral hepatitis for more than 30 years. As part of this surveillance, a prospective cohort study has been conducted on 547 patients with AHB in 28 medical centres over the 19 years from 1991 to 2009. Geographical and longitudinal distributions of HBV genotypes/subgenotypes were surveyed, and their influence on clinical outcome was evaluated.

PATIENTS AND METHODS

Patients

A total of 681 patients with sporadic AHB were enrolled consecutively in a survey carried out by the Japan National Hospital Acute Hepatitis Study Group (JNHAHSG). They were admitted to 28 national hospitals from January 1991 to the end of December 2009. They were grouped geographically into two areas: the capital region (Gunma, Saitama, Tokyo and Kanagawa) and other regions (figure 1). Patients were also longitudinally categorised into three periods: 1st (1991–1996), 2nd (1997–2002) and 3rd (2003–2008). In addition, the year 2009 provided the most recent data. Of the 681 patients, 547 (80.3%) entered the study, for whom serum samples were available on admission and had been stored at −20°C.

The diagnosis of AHB was based on the following criteria: (1) acute onset of liver injury without a history of liver dysfunction; (2) detection of hepatitis B surface antigen (HBsAg) in the
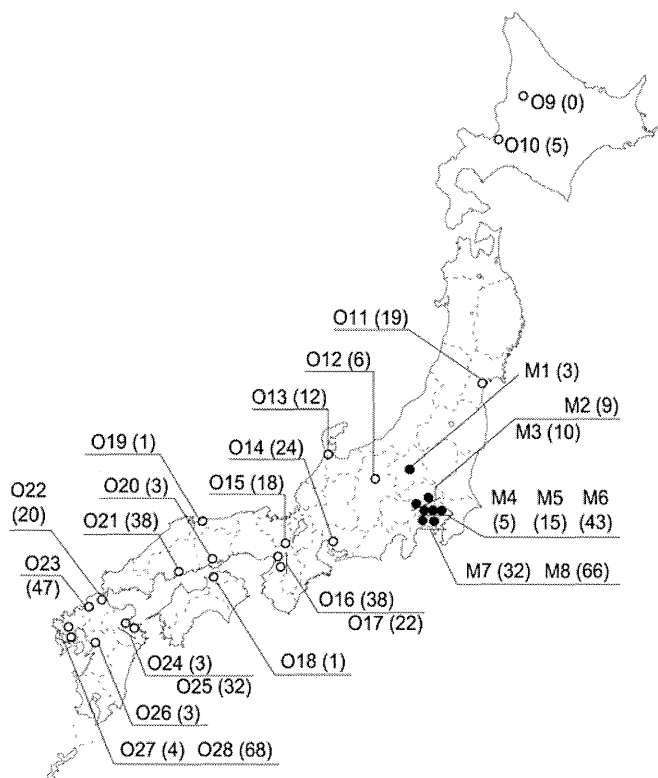

Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1—M8) are indicated by eight closed circles, and those in other regions (O9—O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma Hospital, Gunma; M2, Nishisaitama-Chuo Hospital, Saitama; M3, National Disaster Medical Center, Tokyo; M4, Tokyo Hospital, Tokyo; M5, Tokyo Medical Center, Tokyo; M6, National Center for Global Health and Medicine, Tokyo; M7, Sagamihara Hospital, Kanagawa; M8, Yokohama Medical Center, Kanagawa; O9, Asahikawa Medical Center, Hokkaido; O10, Hokkaido Medical Center, Hokkaido; O11, Sendai Medical Center, Miyagi; O12, Matsumoto Medical Center, Nagano; O13, Kanazawa Medical Center, Ishikawa; O14, Nagoya Medical Center, Aichi; O15, Kyoto Medical Center, Kyoto; O16, Osaka National Hospital, Osaka; O17, Osaka-Minami Medical Center, Osaka; O18, Zentsuji Hospital, Kagawa; O19, Yonago Medical Center, Tottori; O20, Okayama Medical Center, Okayama; O21, Kure Medical Center and Chugoku Cancer Center, Hiroshima; O22, Kokura Medical Center, Fukuoka; O23, Kyushu Medical Center, Fukuoka; O24, Beppu Medical Center, Oita; O25, Oita Medical Center, Oita; O26, Kumamoto Medical Center, Kumamoto; O27, Ureshino Medical Center, Saga; and O28, Nagasaki Medical Center, Nagasaki.

serum; (3) positivity for IgM antibody to HBV-core antigen (IgM anti-HBc) in high titres (detectable in sera diluted 10-fold); and (4) absence of past or family history of chronic HBV infection. Severe acute hepatitis (SAH) was defined as prothrombin time (PT) ≤40% and hepatic encephalopathy of grade ≤I. Fulminant hepatitis (FH) was diagnosed from PT ≤40% and hepatic encephalopathy of grade ≥II. Patients in whom HBsAg remained in the serum for >6 months after onset were considered to have acquired chronic HBV infection. The following information was collected from each patient: year and age at onset, gender, residential area, HBsAg, IgM anti-HBc, alanine aminotransferase, total bilirubin, PT, severity of liver disease, mortality, routes of transmission, sexual behaviours, travelling abroad in recent past, HBV genotype, mutations in precore (PreC) and core promoter (CP) regions, and RNA of hepatitis D virus. Antibody to HIV type 1 (anti-HIV) was

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determined in patients who were at high risk and gave consent to testing.

Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the Ministry of Education, Culture, Sports Science and Technology of Japan, and was approved by the ethics committee of each institution.

Extraction of HBV DNA

HBV DNA was extracted from serum (100 μl) by the SMITEST EX-R&D Nucleic Acid Extraction Kit (MBL Co, Nagoya, Japan) and used for genotyping/subgenotyping and detecting mutations in PreC and CP regions.

HBV genotypes

Genotypes were determined in Nagasaki Medical Center with the SMITEST HBV Genotyping Kit (MBL) by hybridisation with type-specific probes immobilised on a solid-phase support.[24]

Determination of HBV subgenotypes

For subgenotyping, HBV DNA was amplified by PCR with TaKaRa Ex Taq (Takara Bio, Shiga, Japan). PCR was performed with appropriate nested primers to amplify a ~1.2 kb sequence in the preS1/S2/S gene (nucleotides 2854–835 in the reference isolate (AB116077)). PCR products were purified, subjected to cycle sequencing reaction with the BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan), and applied to the DNA sequencer (3100-Avant; Applied Biosystems).

Mutations in the PreC and CP regions

The A1896 mutation in the PreC region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA; Roche Diagnostics, Tokyo, Japan), and mutations in the CP region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit; Roche Diagnostics). The results were recorded as 'wild-type' and 'mutant types' dominantly expressed by HBV isolates.[25]

Phylogenetic analyses

Nucleotide sequences were aligned, and phylogenetic trees were constructed by the CLUSTAL W program v1.83 (DDBJ homepage: http://clustalw.ddbj.nig.ac.jp/top-j.html). The statistical validity was assessed by bootstrap resampling with 1000 replicates. Reference HBV strains were retrieved from the GenBank database.

Statistical analysis

Results were expressed as percentage or mean±SD. Statistical differences were evaluated by χ^2 and Fisher exact tests for categorical variables, and analysis of variance and Scheffe's test for quantitative variables, using the SPSS software. The 95% CI, for the difference in means, was calculated in analyses for quantitative variables. $p < 0.05$ was considered significant.

RESULTS

Distribution of HV genotypes

HBV genotypes were determined in the 547 patients with AHB. The genotype was A in 137 (25.0%) patients (accompanied by G in one (0.2%)), B in 48 (8.8%), C in 359 (65.6%), D in one (0.2%), E in one (0.2%), and H in one (0.2%). Because HBV genotype G is a defective virus and cannot replicate by itself,[26 27] the single patient with mixed genotypes A and G was included in the 137 patients with genotype A in further analyses. RNA of hepatitis

D virus was detected in three of the 453 (0.7%) patients. Anti-HIV was examined in patients at high risk of infection and detected in 14 of the 53 (26.4%) who gave consent to testing.

Demographic and clinical differences among patients infected with HBV of distinct genotypes

Demographic and clinical characteristics of patients with different genotypes are compared in table 1. There was no difference in mean age among patients with genotypes A, B and C. The proportion of men was higher in patients with genotype A than B or C (94.2% vs 79.2%, p<0.05; or 56.0%, p<0.0001), and in those with genotype B than C (79.2% vs 56.0%, p<0.05).

Maximum levels of total bilirubin were higher in patients with genotype A than C (9.6±7.6 vs 7.1±6.2 mg/dl, p<0.05), with a difference of 2.5 mg/dl (95% CI 0.93 to 4.08), whereas the highest alanine aminotransferase activity and lowest PT values did not differ among patients with distinct genotypes.

SAH developed in four (2.9%) patients with genotype A, four (8.3%) with genotype B, and 26 (7.2%) with genotype C. FH developed in one (2.1%) patient with genotype B and eight (2.2%) with genotype C; no patients with genotype A developed FH. Eight (1.5%) patients died, including one with genotype B and seven with genotype C. There were no significant differences among patients with different genotypes in the frequency of SAH or FH or mortality.

The outcome of AHB was traceable in 514 of the 547 (94.0%) patients. Chronic infection with persistence of HBsAg for >6 months developed in five of the 123 (4.1%) patients with genotype A (including the one accompanied by genotype G), none of the 46 (0%) with genotype B, and none of the 342 (0%) with genotype C; it was more common in patients with genotype A than C (p<0.05). HBV infection persisted exclusively in the patients with genotype A, either alone (four patients) or together with genotype G (one).

Among the five patients who acquired chronic HBV infection, four (three with genotype A and one with mixed genotypes A and G) were examined for anti-HIV, and one with genotype A was found to be positive. HBV infection persisted in three (including the one with anti-HIV) of the five patients for >1 year after the onset, and the remaining two (both without anti-HIV) cleared HBsAg from the serum after retaining it for >6 months.

Mutations in the PreC and/or CP region were detected in 3.7% (4/109) of patients with genotype A, 15.4% (6/39) of those with genotype B, and 25.5% (79/310) of those with genotype C. They were significantly less common in patients with genotype A than B or C (A vs B, p<0.05; A vs C, p<0.0001). The only patient with genotype A who had the PreC mutation was simultaneously infected with genotype G.

Routes of transmission were identifiable in 275 of the 547 (50%) patients, and the main route was heterosexual contacts; those in the remaining patients could not be disclosed. The frequency of heterosexual activity did not differ among patients with distinct genotypes. However, homosexual activity was more common in patients with genotype A than B or C (21.2%, 0% and 0.8%, respectively (A vs B, p<0.001; A vs C, p<0.0001)). Among the 32 homosexual men, HBV genotype A was detected in 29 (91%). Consent to anti-HIV testing was given by 10 of the 29 patients, and four of these (40%) were positive.

Longitudinal changes in the distribution of genotypes

Figure 2 illustrates changes in the distribution of HBV genotypes through three 6-year periods over 18 years (1991–2008). In addition, data from 2009 are shown. HBV genotype A accounted

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Table 1 Demographic and clinical characteristics of patients with acute hepatitis who were infected with HBV of different genotypes (1991–2009)

Feature	Total (n=547)	HBV genotypes A (n=137)† (25.0%)	B (n=48) (8.8%)	C (n=359) (65.6%)	Others (n=3)‡ (0.5%)
Age (years)	35.6±14.8	35.2±12.2	39.6±15.6	35.1±15.5	49.7±13.6
Male	367 (67.1%)	129 (94.2%)¶ * †† ***	38 (79.2%)†† *	201 (56.0%)	3 (100%)
ALT (IU/l)§	2553±1563	2289±1069	2557±1412	2342±1728	3333±2406
T-Bil (mg/dl)§	7.8±6.7	9.6±7.6††*	7.7±7.4	7.1±6.2	9.0±2.5
PT (%)§	74.6±22.6	75.2±15.9	73.8±24.5	74.7±24.5	15.8‡‡
Severe hepatitis	34 (6.2%)	4 (2.9%)	4 (8.3%)	26 (7.2%)	0 (0.0%)
Fulminant hepatitis	10 (1.8%)	0 (0.0%)	1 (2.1%)	8 (2.2%)	1 (33.3%)
Mortality	8 (1.5%)	0 (0.0%)	1 (2.1%)	7 (1.9%)	0 (0.0%)
HBsAg persisting >6 months	5/514 (1.0%)	5/123 (4.1%)†† *	0/46 (0.0%)	0/342 (0%)	0/3 (0.0%)
PreC/CP mutations					
PreC	43/461 (9.3%)	1/109 (0.9%)¶ * †† *	6/39 (15.4%)	34/310 (11.0%)	2/3 (66.7%)
CP	69/461 (15.0%)	3/109 (2.8%)†† ***	0/39 (0.0%)†† *	63/310 (20.3%)	3/3 (100%)
PreC and/or CP	92/461 (20.0%)	4/109 (3.7%)¶ * †† ***	6/39 (15.4%)	79/310 (25.5%)	3/3 (100%)
Transmission route					
Homosexual	32 (5.9%)	29 (21.2%)¶ ** †† ***	0 (0.0%)	3 (0.8%)	0 (0.0%)
Heterosexual	217 (39.5%)	52 (38.0%)	25 (52.1%)	139 (39.6%)	1 (33.3%)
Medical procedure	16 (2.9%)	2 (1.5%)	2 (4.2%)	12 (3.3%)	0 (0.0%)
Other	10 (1.8%)	1 (0.7%)	1 (2.1%)	7 (1.9%)	1 (33.3%)
Undetermined	272 (49.7%)	53 (38.7%)†† *	20 (41.7%)	198 (55.2%)	1 (33.3%)
Anti-HIV	14/53 (26.4%)	11/35 (31.4%)	0/3 (0.0%)	3/15 (20.0%)	0/0

Values are mean±SD or number (%).
†One patient with genotype A was simultaneously infected with genotype G.
‡Each patient was infected with genotype D, E or H.
§Highest values during the clinical course are shown for ALT and T-Bil, and lowest values for PT.
Statistical analysis was performed to compare genotypes A, B and C.
¶Significantly different compared with genotype B.
††Significantly different compared with genotype C.
*p<0.05, **p<0.001, ***p<0.0001.
‡‡Data from the patient with genotype E only.
ALT, alanine aminotransferase; CP, core promoter; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PreC, precore; PT, prothrombin time; T-Bil, total bilirubin.

for 6% (9/150) in the 1st period, 15.4% (19/123) in the 2nd, and 39.4% (89/226) in the 3rd, with significant differences between 1st and 2nd (p<0.05), 2nd and 3rd (p<0.0001), and 1st and 3rd (p<0.0001). Conversely, AHB associated with genotype C decreased through three periods with significant differences, while AHB associated with genotype B did not change appreciably.

On the basis of these results, the yearly incidence in each of the three 6-year periods is calculated to be: 25.0 cases including 1.5 with genotype A in the 1st period; 20.5 cases including 3.2 with genotype A in the 2nd; and 37.7 cases including 14.8 with genotype A in the 3rd. Hence, the incidence of AHB had not changed markedly over the 12 years from 1991 to 2002, but increased thereafter until 2008. Of the increment in the 3rd period of 17.2 (37.7 minus 20.5) cases, there were 11.6 (14.8 minus 3.2) with genotype A; they accounted for 67% (11.6/17.2) of the recent increase in AHB.

Regional distributions and longitudinal changes in genotype A

Among the 183 patients from the capital region, the genotype was A in 65 (35.5%), B in 22 (12.0%), C in 94 (51.4%), E in one (0.5%), and H in one (0.5%) (table 2). Of the remaining 364 (66.5%) patients from other regions, by contrast, the genotype was A in 72 (19.8%), B in 26 (7.1%), C in 265 (72.8%), and D in one (0.3%). Genotype A was significantly more common in the capital than in other regions (35.5% vs 19.8%, p<0.0001). In the capital region, genotype A accounted for 4.8% (2/42) in the 1st period, 29.3% (12/41) in the 2nd, and 50.0% (42/84) in the 3rd. There were significant differences between the 1st and 2nd periods (p<0.05), 2nd and 3rd (p<0.05), and 1st and 3rd (p<0.0001). In other regions, by contrast, genotype A accounted for 6.5% (7/108) in the 1st period, 8.5% (7/182) in the 2nd, and

33.1% (47/142) in the 3rd. For the first time in other regions, genotype A increased in the 3rd period, in comparison with the 1st and 2nd (1st vs 3rd, p<0.0001; 2nd vs 3rd, p<0.0001).
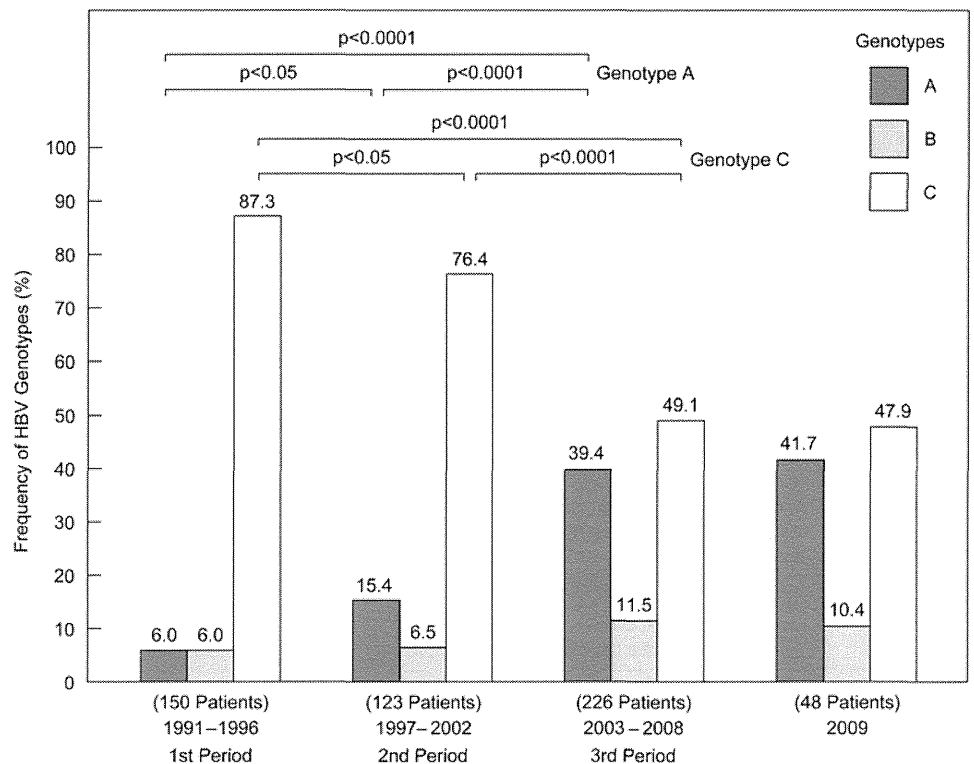
Subgenotypes of genotype A

Of the 137 genotype A isolates, amplification and sequencing of HBV DNA were feasible in 114 (83.2%); the isolate from the single patient with genotypes A and G was excluded. A phylogenetic tree was constructed, on the entire preS1/S2/S genes of ~1.2 kb, for these 114 isolates along with 34 genotype A isolates retrieved from the database (figure 3).

Of the 114 isolates in this study, 101 (88.6%) were subgenotype A2, and the remaining 13 (11.4%) were subgenotype A1. In a pair-wise comparison, the sequence divergence among the 101 subgenotype A2 isolates was 0–1.3%, and that among the 13 subgenotype A1 isolates spanned 0% to 2.3%. The sequence divergence between subgenotype A2 and A1 isolates ranged from 2.6% to 4.7%.

A sequence of 1203 nucleotides was possessed in common by three of the 101 (3%) isolates of subgenotype A2. For convenience, the group comprising these three isolates was labelled 'identical group I'. Likewise, an additional six 'identical groups' were found, and numbered from 'II' to 'VII'. They comprised 35 (35%), seven (7%), two (2%), three (3%), 12 (12%) and three (3%) of the 101 isolates of subgenotype A2. In contrast, only one identical group, designated 'VIII', was constructed by three of the 13 (23%) isolates of subgenotype A1.

Some isolates of subgenotype A1 and A2 were obtained from patients who had travelled to foreign countries in the recent past (5/13 (38.5%) patients with A1 to Africa, Philippines, Myanmar and China; and 5/101 (5.0%) patients with A2 to Europe, Thailand, Brazil and the USA).

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

Subgenotypes of genotype B

Of the 48 isolates of genotype B, subgenotyping was feasible in 43 (90.0%). A phylogenetic tree was constructed on preS1/S2/S-gene sequences from these 43 isolates, along with those from 25 isolates of genotype B retrieved from the database (figure 4). Of the 43 isolates in this study, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. In a pair-wise comparison, the sequence divergence among 10 subgenotype B1 isolates ranged from 0.4% to 1.4%, and that among 28, two and three isolates of subgenotypes B2, B3 and B4 spanned 0—1.7%, 0.5% and 0.6—0.8%, respectively. The inter-subgenotype divergence among B1—B4 ranged from 0.6% to 4.4%.

One 'identical group' made up of five isolates was detected among the 28 of subgenotype B2; it was named 'IX'. In contrast, no 'identical group' was found in 10, two or three isolates of subgenotype B1, B3 or B4.

Some isolates of subgenotypes B2, B3 and B4 were obtained from patients who had travelled to foreign countries in the recent past (7/28 (25.0%) patients with B2 to China and other countries; 1/2 (50.0%) patients with B3 to a country unknown; and 1/3 (33.3%) patients with B4 to Vietnam). However, none of the 10 subgenotype B1 isolates was associated with travel to foreign countries.

Identical groups

The proportion of isolates that shared a sequence in identical groups was higher for subgenotype A2 (64.4%) than for A1, B1, B2, B3 or B4 (23.1%, 0%, 17.9%, 0% or 0%, respectively (A2 vs A1, $p<0.001$; A2 vs B1, $p<0.0001$; A2 vs B2, $p<0.0001$)).

Homosexual activity was more common in patients belonging to the seven identical groups than the non-identical group of subgenotype A2 (17/65 (26.2%) vs 3/36 (8.3%), $p<0.05$). Among the isolates in the seven identical groups of subgenotype A2, those in groups I, III and VII clustered locally during short periods of 2—7 years. In contrast, subgenotype A2 isolates in groups II and VI were scattered widely over longer periods of 11—16 years.

DISCUSSION

In Japan, as in most Asian countries, the persistent HBV carrier state had been established mainly through perinatal transmission from mother to baby and horizontal infection during infancy. In 1986, a national prevention programme was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg). In 1995, this was extended to babies born to HBeAg-negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[28 29] However, there are an

Table 2 Changes in the distribution of genotype A compared between the capital region and other regions over three periods

Area	n	1st Period (1991—1996)	2nd Period (1997—2002)	3rd Period (2003—2008)	2009
Capital region	65/183 (35.5%)†***	2/42 (4.8%)‡* §***	12/41 (29.3%)†* §*	42/84 (50.0%)†*	9/16 (56.3%)
Other regions	72/364 (19.8%)	7/108 (6.5%)§***	7/82 (8.5%)§***	47/142 (33.1%)	11/32 (34.4%)
Total	137/547 (25.0%)	9/150 (6.0%)‡* §***	19/123 (15.4%)§***	89/226 (39.4%)	20/48 (41.7%)

Statistical analysis of the differences between the capital and other regions was performed, as well as through the 1st, 2nd and 3rd periods.
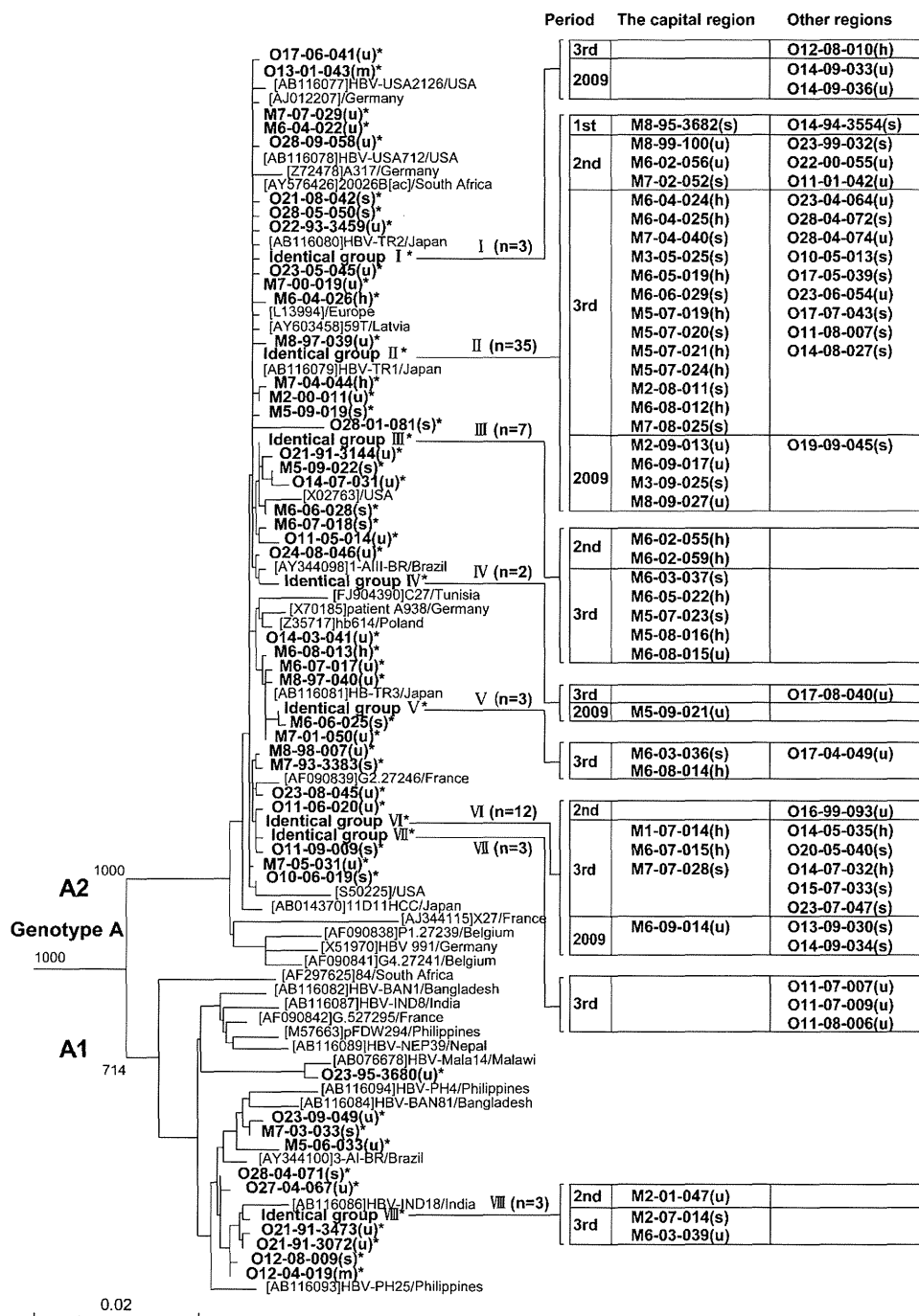†Significantly different compared with other regions.
‡Significantly different compared with the 2nd period.
§Significantly different compared with the 3rd period.
*$p<0.05$, ***$p<0.0001$.

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1—M8 and those in other regions O9—O28 (corresponding to those in figure 1). Year of onset is indicated by the last two digits after the first hyphen. Numbers after the second hyphen represent the identification numbers of patients in each year (not always consecutive). Transmission routes are shown in lower-case letters in parentheses: h, homosexual; s, heterosexual; m, medical procedure; o, others; and u, undetermined. Isolates with identical sequences are bracketed in 'Identical groups I through VIII' on the tree. Each bracket is divided by areas and periods. Reference hepatitis B virus (HBV) isolates, including 12 of subgenotype A1 and 22 of subgenotype A2, were obtained from the database and specified by their accession numbers, isolate names and countries of origin. Bootstrap values are indicated on major phylogenetic branches.


Period	The capital region	Other regions
3rd		O12-08-010(h)
2009		O14-09-033(u)
		O14-09-036(u)
1st	M8-95-3682(s)	O14-94-3554(s)
2nd	M8-99-100(u)	O23-99-032(s)
	M6-02-056(u)	O22-00-055(u)
	M7-02-052(s)	O11-01-042(u)
3rd	M6-04-024(h)	O23-04-064(u)
	M6-04-025(h)	O28-04-072(s)
	M7-04-040(s)	O28-04-074(u)
	M3-05-025(s)	O10-05-013(s)
	M6-05-019(h)	O17-05-039(s)
	M6-06-029(s)	O23-06-054(u)
	M5-07-019(h)	O17-07-043(s)
	M5-07-020(s)	O11-08-007(s)
	M5-07-021(h)	O14-08-027(s)
	M5-07-024(h)	
	M2-08-011(s)	
	M6-08-012(h)	
	M7-08-025(s)	
2009	M2-09-013(u)	O19-09-045(s)
	M6-09-017(u)	
	M3-09-025(s)	
	M8-09-027(u)	
2nd	M6-02-055(h)	
	M6-02-059(h)	
3rd	M6-03-037(s)	
	M6-05-022(h)	
	M5-07-023(s)	
	M5-08-016(h)	
	M6-08-015(u)	
3rd		O17-08-040(u)
2009	M5-09-021(u)	
3rd	M6-03-036(s)	O17-04-049(u)
	M6-08-014(h)	
2nd		O16-99-093(u)
3rd	M1-07-014(h)	O14-05-035(h)
	M6-07-015(h)	O20-05-040(s)
	M7-07-028(s)	O14-07-032(h)
		O15-07-033(s)
		O23-07-047(s)
2009	M6-09-014(u)	O13-09-030(s)
		O14-09-034(s)
3rd		O11-07-007(u)
		O11-07-009(u)
		O11-08-006(u)
2nd	M2-01-047(u)	
3rd	M2-07-014(s)	
	M6-03-039(u)	

estimated one million HBV carriers in Japan at present.[30] Furthermore, many Japanese remain at increased risk of horizontal infection with HBV, because they have not received selective vaccination and therefore do not have the antibody to HBsAg. Because AHB is extremely under-reported and no national surveillance data are available in Japan, the incidence has not been determined accurately. In the USA, the incidence of AHB has decreased markedly since the adoption of a comprehensive immunisation strategy in 1991.[31 32]

In the present study over 1991—2009, we conducted a nationwide, sentinel surveillance on AHB in Japan. In the 547 patients recruited over 19 years, genotype C was the most prevalent (65.6%), followed by genotype A (25.0%) and genotype B (8.8%). Demographic and clinical differences were observed among patients with genotypes A, B and C (table 1).

The proportion of men reached 94.2% for genotype A infection, higher than that for genotype B (79.2%) or C (56.0%) infection. In the analysis of the route of transmission, homosexual activity was reported by 21.2% of patients with genotype A; all were male. In general, sexual activity tends to be higher in men than women. The predominance of genotype A in men may be attributable to a high frequency of homosexual activity among men.

Although adult-acquired HBV infection persists at a high frequency of ~10% in European countries and the USA,[33] it rarely, if ever, becomes chronic in Japan. Recent studies suggest that the chance of a chronic outcome of AHB may differ by HBV genotype[21 34]; it is more common for genotype A than other genotypes.[22 35 36] In the present study, HBV infection persisted in 4.1% of patients with genotype A, in comparison with 0% of

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 reference isolates of subgenotype B1 and 13, two and two of those of B2, B3 and B4, respectively, were retrieved from the database; they are specified as in figure 3.
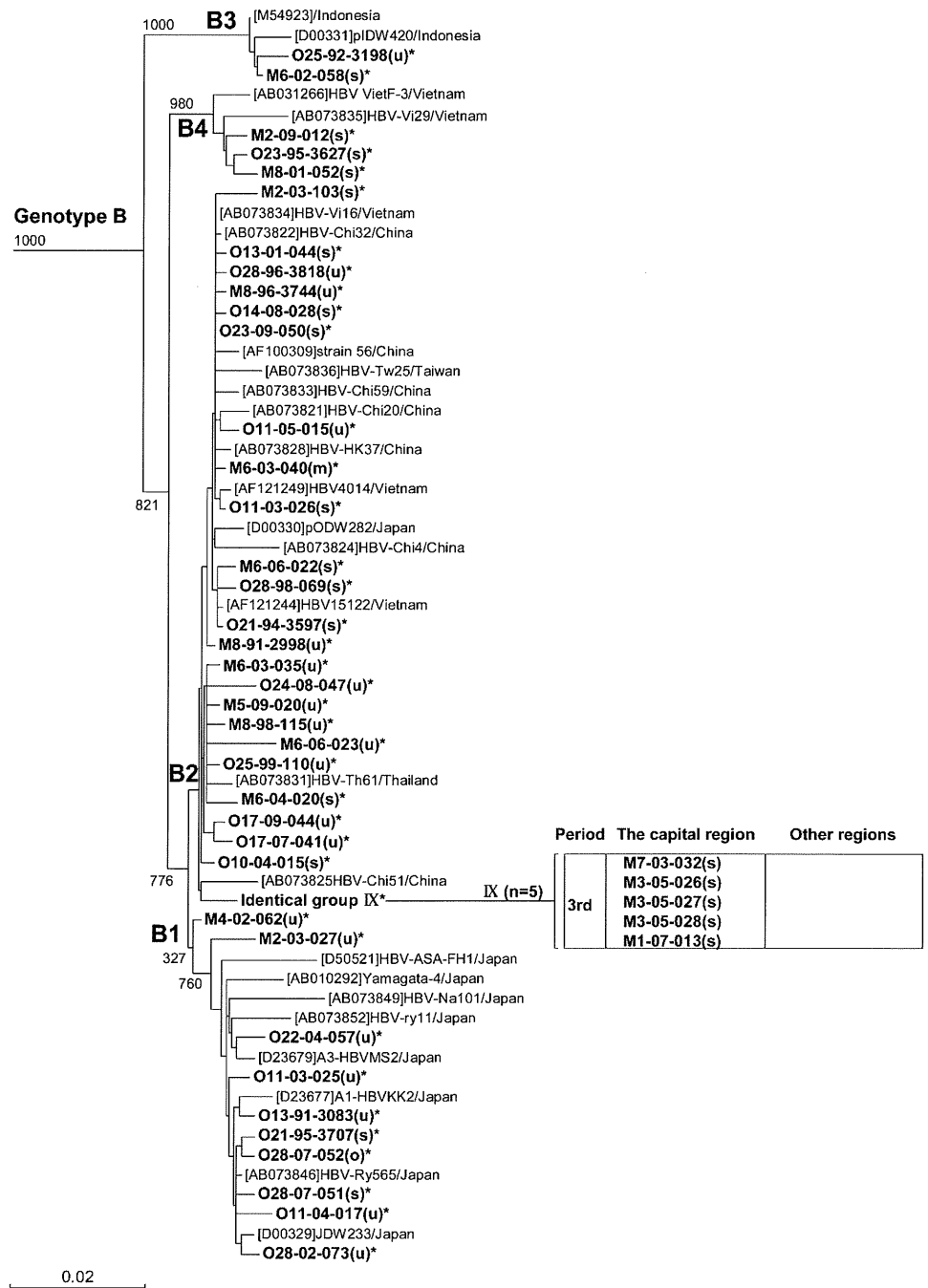
those with genotype C. Remarkably, all five patients with AHB who acquired chronic infection possessed HBV genotype A, either alone (four patients) or together with HBV genotype G (one). Increasing genotype A infections may have changed the genotype distribution in patients with AHB and those with chronic HBV infection. In Japanese patients with chronic hepatitis B, the proportion of genotype A has doubled, from 1.7% in 1999–2000 to 3.5% in 2005–2006.[37]

The genotype was A in 29 of the 32 (91%) homosexual men. Of the 29 homosexuals with genotype A, 10 gave consent to anti-HIV testing, and four of these (40%) were found to be positive. Of the five patients who acquired chronic HBV infection, anti-HIV was tested in four (three with genotype A and one with genotypes A and G), and one with genotype A was found to be positive. There is a possibility that co-infecting HIV in this patient with genotype A may have promoted chronic

HBV infection; HIV is known to prolong and aggravate HBV infection by compromising immune responses.[38]

Patients with FH in this study were infected with either HBV genotype B (1/48 (2.1%)) or C (8/359 (2.2%)); no patients with genotype A developed FH. PreC and/or CP mutations were significantly less common in genotype A (1/109 (3.7%)) than B (6/39 (15.4%)) or C (279/310 (5.5%)) infection. The single patient with genotype A who had PreC mutation was simultaneously infected with HBV genotype G. There is a possibility that the PreC mutation in this patient was from HBV genotype G.[26] FH did not develop in any patients with genotype A, which may be attributable, at least in part, to the lack of PreC mutation in genotype A infections.[39]

Previous reports have shown that genotype A is common in patients with AHB in Metropolitan Tokyo,[20 21 40] as well as around Aichi located in the middle of Mainland Japan.[22]