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Gut Online First, published on November 7, 2011 as 10.1136/gutjnl-2011-300832

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

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Revised 3 October 2011
Accepted 5 October 2011

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];

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.

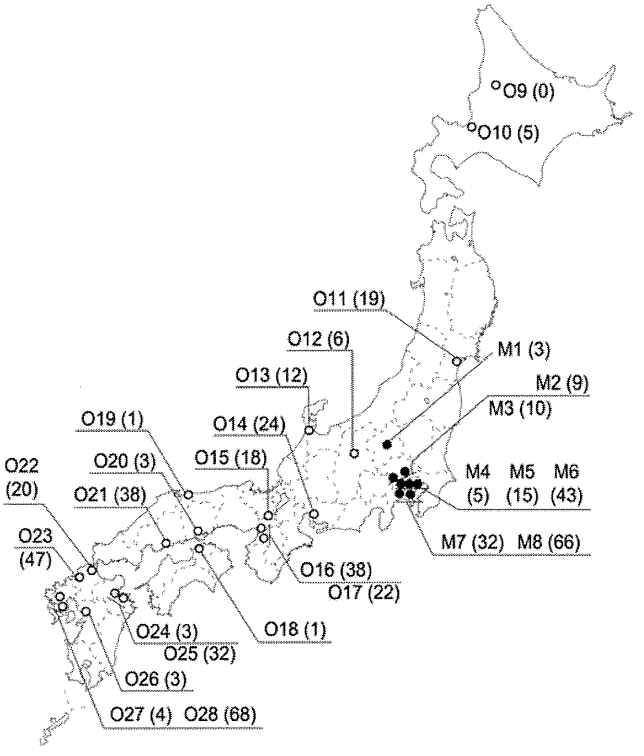

Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1—M8) are indicated by eight closed circles, and those in other regions (O9—O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma 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.

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 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

Tamada Y, Yatsuhashi H, Masaki N, *et al*. *Gut* (2011). doi:10.1136/gutjnl-2011-300832

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

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).

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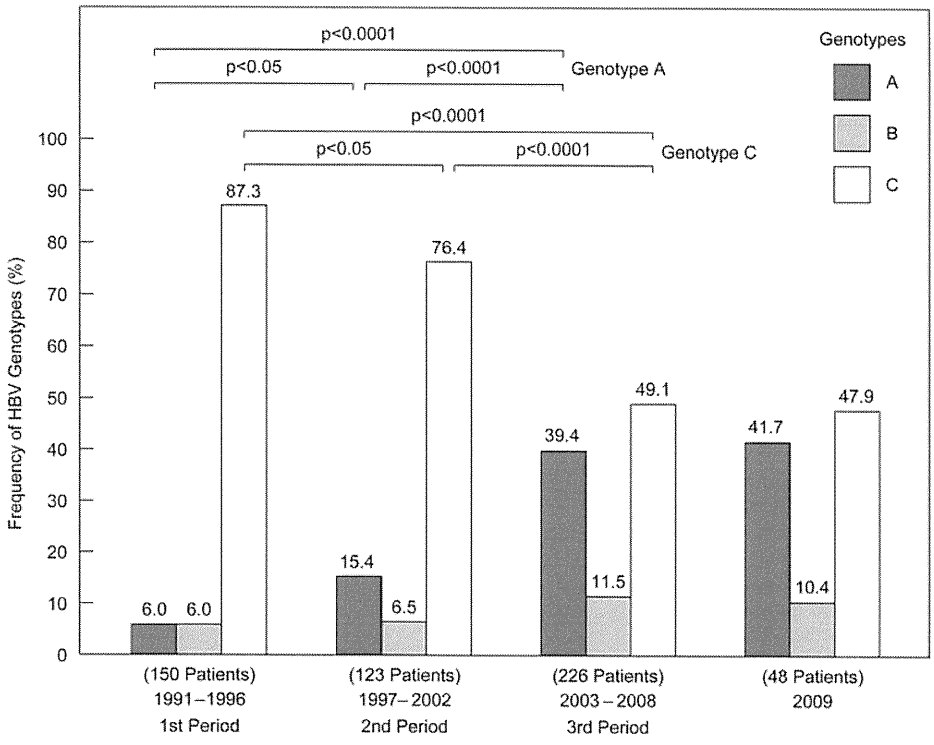

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

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.
*$p<0.05$, ***$p<0.0001$.

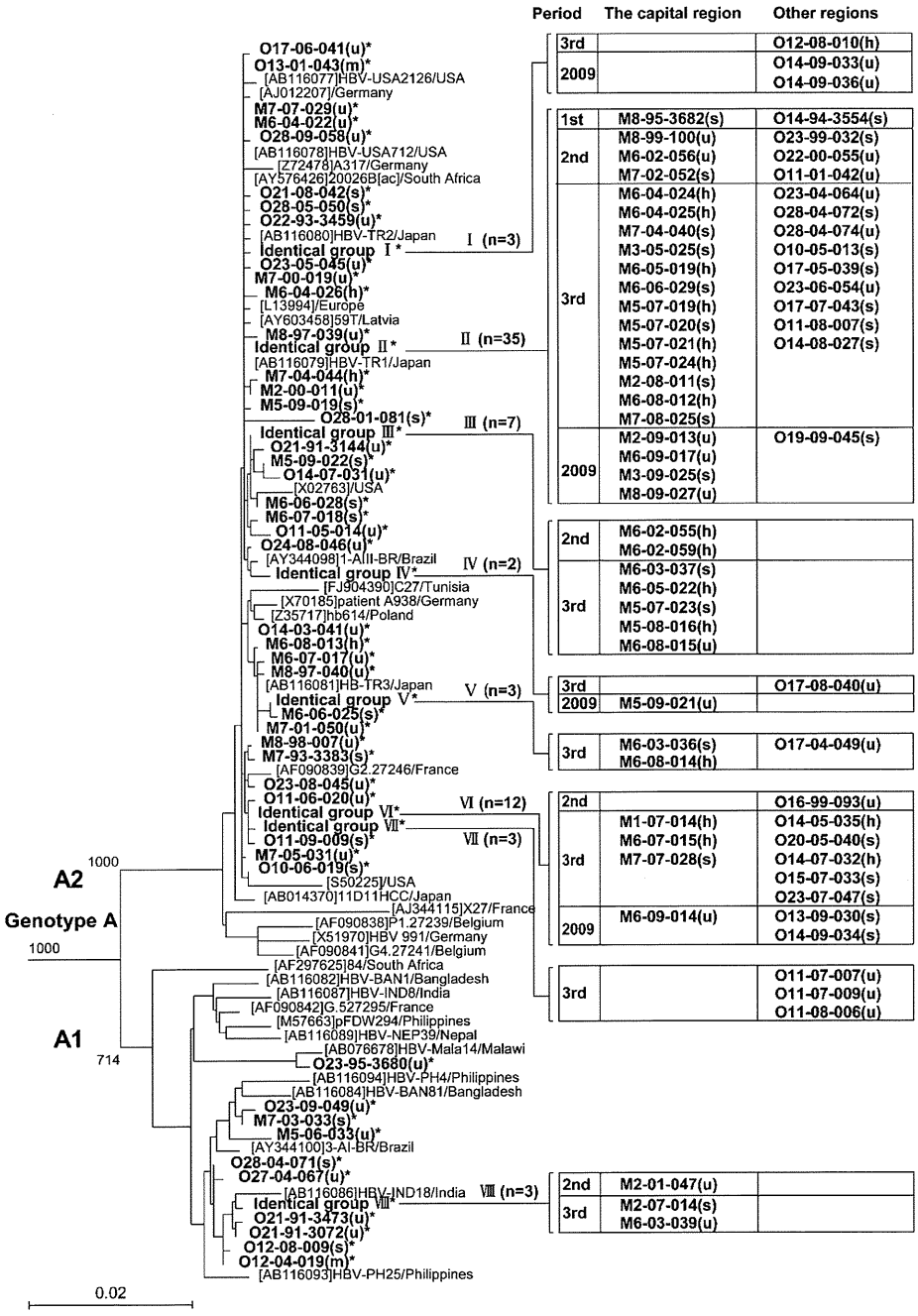

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1—M8 and those in other regions O9—O28 (corresponding to those in figure 1). Year of onset is indicated 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.

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

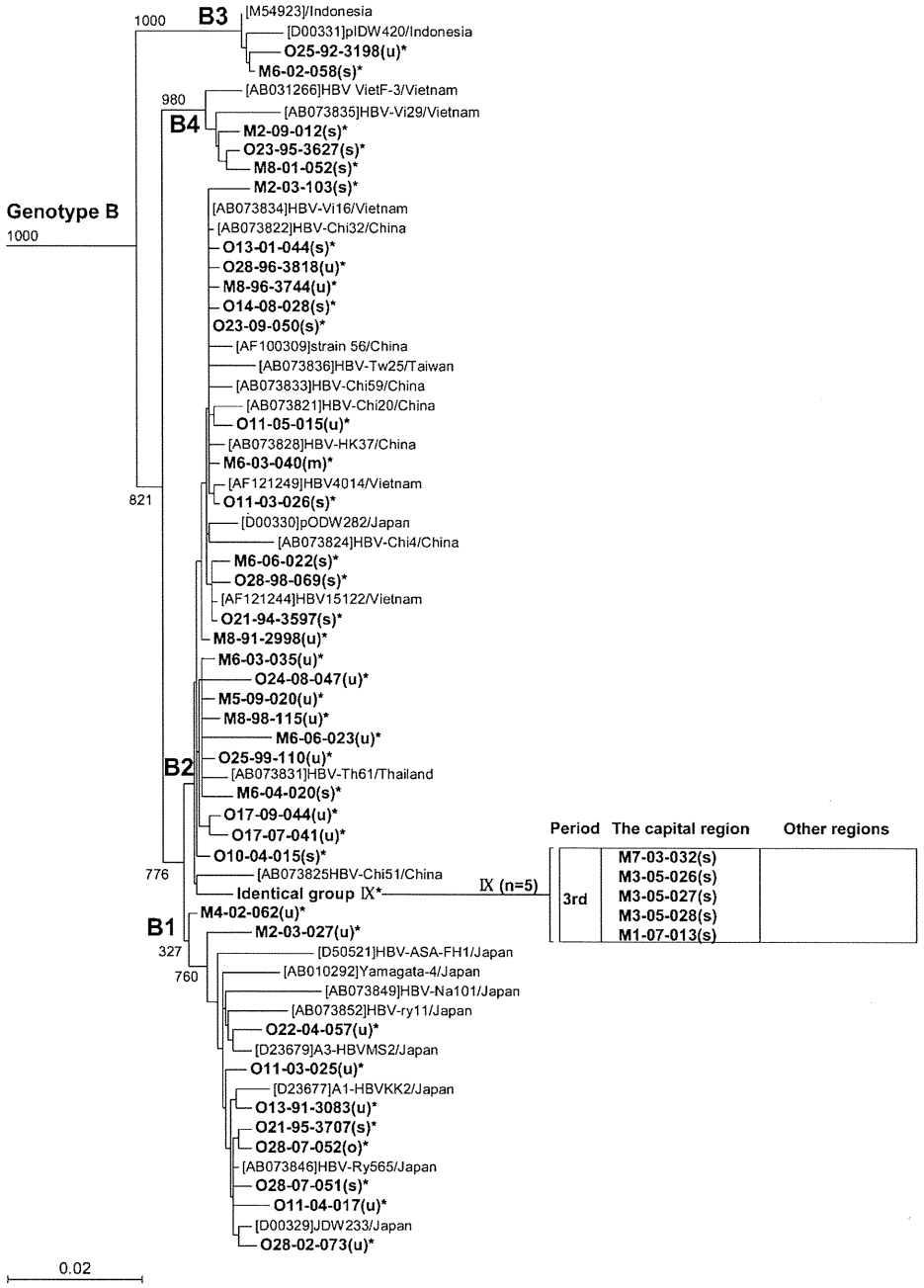

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 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]

Yotsuyanagi *et al*[23] reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi *et al*[41] found that, in patients with AHB, the proportion with genotype A has increased over time. The present study indicates that the number of patients with AHB in Japan would not have decreased. We found that the proportion of patients with genotype A infection is increasing in the 28 national hospitals in Japan (6.0% in the 1st period, 15.4% in the 2nd, and 39.4% in the 3rd (figure 2)), with the prevalence much higher in the capital than other regions (35.5% vs 19.8% (table 2)).

In this study, there was a time lag in the increase in genotype A infection between the capital region and other regions of Japan (table 2). In the capital region, the prevalence of genotype A started to increase in the late 1990s, and kept increasing through the early 2000s (4.8% in the 1st period, 29.3% in the 2nd, 50.0% in the 3rd, and 56.3% in 2009). In other regions, by contrast, the frequency of genotype A did not change during the late 1990s, and increased significantly in the 2000s (6.5% in the 1st period, 8.5% in the 2nd, 33.1% in the 3rd, and 34.4% in 2009). Thus infiltration of genotype A infection into other regions occurred 5–6 years behind the epidemic in the capital region. This indicates that genotype A infection originated in the capital region and then spread to other areas of Japan.

Some genotypes are classified into several subgenotypes, and they have distinct geographical distributions.[42] Hence, subgenotypes are useful in tracing the route of HBV infection. By phylogenetic analysis (figures 3 and 4), 88.6% of genotype A isolates had the European–American type (A2), and the remaining 11.4% possessed the Asian–African type (A1). Likewise, 76.7% of genotype B isolates had Asian types (B2–B4), and the remaining 23.3% possessed the type endemic to Japan (B1). Of the 157 HBV isolates of genotype A or B, 147 (93.6%) had subgenotypes foreign to Japan. They are thought to have been transmitted from foreign sex workers, and spread among certain populations who share particular sexual behaviours in Japan.[41]

Of note, some HBV isolates of distinct subgenotypes possessed an identical sequence in the preS1/S2/S gene. The isolates of subgenotype A2 were prominent in this regard, and more often had the same sequence than those of other subgenotypes, such as A1, B1 and B2. The high prevalence of subgenotype A2 isolates with an identical sequence would not have been caused by cross-contamination. If cross-contamination had occurred, it would have affected isolates of all subgenotypes, and not influenced subgenotype A2 isolates preferentially. As many as 35% of subgenotype A2 isolates had an identical sequence, and those with the same sequence increased to 56.3% in the recent 2009 survey in Metropolitan Tokyo. Furthermore, some subgenotype A2 isolates in groups I, III and VII clustered locally within short periods, whereas others in groups II and VI were scattered widely over a long period of time. On the basis of these results, it is tempting to speculate that some subgenotype A2 strains would have been transmitted from person to person without undergoing mutations for many years.

In summary, the present study indicates the following. (1) AHB in the 28 national hospitals in Japan has not decreased, because genotype A infections are increasing. (2) Genotype A infections started to increase in the capital region, and then spread to local areas 5–6 years later. (3) Approximately 90% of genotype A in patients with AHB is subgenotype A2. (4) Subgenotype A2 strains with an identical sequence are spreading among younger generations with high sexual activity. (5) On the basis of the results obtained, AHB in Japan is not decreasing, because HBV of subgenotype A2 is prevailing in particular subpopulations at high risk. Finally, in order to prevent further increases in AHB in Japan, universal vaccination of young people deserves consideration.

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Acknowledgements We thank participating physicians (MD) in the JNHAHSG for collaborating, with data and sample collection: Hideo Nishimura, Asahikawa Medical Center; Yukio Ohhara, Hokkaido Medical Center; Masakazu Kobayashi, and the late Takeshi Sodeyama, Matsumoto Medical Center; Fujio Makita, Nishigunma Hospital; Takeo Saoshiro, and Akira Saito, Nishisaitama-Chuo Hospital; Michiyasu Yagura, and the late Hideharu Harada, Tokyo Hospital; Keiichi Hirata, National Disaster Medical Center; Yoko Nakamura, Sagamihara Hospital; the late Hideo Morimoto, Kanazawa Medical Center; Noboru Hirashima, Higashi Nagoya National Hospital; Michio Kato, Osaka National Hospital; Tetsuo Yamamoto, Yonago Medical Center; Haruhiro Yamashita, Okayama Medical Center; Hirotaka Kouno and Eiichi Takezaki, Kure Medical Center and Chugoku Cancer Center; Toru Hayashi, and Shuji Oda, Zentsuji Hospital; Akihide Masumoto, Kokura Medical Center; Hironori Sakai, Beppu Medical Center; Koichi Honda, Oita Medical Center; Kazuhiro Sugi, Kumamoto Medical Center; Michiaki Koga, and Hiroaki Kawasoe, Ureshino Medical Center; Michitami Yano, and Manabu Daikoku, Nagasaki Medical Center. All the hospitals belong to National Hospital Organization (NHO). We appreciate the excellent technical assistance of Ms Rumiko Hamada, Ms Yuka Jiuchi, Ms Mika Fukuda and Ms Rumiko Nakao. Finally, we express our deep gratitude to the late Dr Koji Yano (Nagasaki Medical Center) for his contribution to the design and coordination of this study, and we dedicate this article to him.

Funding This study was supported by a grant-in-aid from the National Hospital Organization (grant number H21-NHO(Kan)-03).

Competing interests None.

Ethics approval Approved by the ethics committee of each institution.

Contributors YT, HY and HI designed data collection tools, monitored data collection for the whole study, wrote the statistical analysis plan, cleaned and analysed the data. YT, HY and YM drafted and revised the paper. HY, NM, MN, EM, TK, YW, TM, MS, TH, TS, YM, TK, MT, HK, HO, SH and SA collaborated in data and sample collection.

Provenance and peer review Not commissioned; externally peer reviewed.

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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

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Gut published online November 7, 2011
doi: 10.1136/gutjnl-2011-300832

J Gastroenterol
DOI 10.1007/s00535-011-0466-y

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Dynamics of regulatory T cells and plasmacytoid dendritic cells as immune markers for virological response in pegylated interferon-α and ribavirin therapy for chronic hepatitis C patients

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Received: 4 July 2011 / Accepted: 3 August 2011

Abstract
Background For the treatment of chronic hepatitis C, a combination of pegylated interferon-α (PEG-IFNα) and ribavirin has been widely used as a standard of care. Enhancement of immune response against hepatitis C virus (HCV) is known to be involved in the efficacy of the combination therapy. Our aim was to elucidate whether or not the frequency or function of blood cells is related to the outcome of the therapy.
Methods Sixty-seven chronic hepatitis C patients with high viral load of HCV genotype 1 infection who underwent 48 weeks of PEG-IFNα2b and ribavirin therapy were examined. During the treatment, frequencies of myeloid or plasmacytoid dendritic cells, Th1, Th2 cells, NK cells, and regulatory T cells were phenotypically determined.
Results Among the patients enrolled, 29 showed a sustained virological response (SVR), 18 a transient response (TR) and 17 no response (NR). The clinical and immunological markers were compared between the SVR and non-SVR patients, including TR and NR. Based on clinical, histological, immunological parameters, and cumulative dosage of PEG-IFNα2b and ribavirin, multivariate analyses revealed that higher platelet counts and higher regulatory T cell frequency at week 12 are indicative of SVR. Even in patients who attained complete early virological response at week 12, multivariate analyses disclosed that higher platelet counts and higher plasmacytoid dendritic cell frequency are indicative of SVR.
Conclusions In PEG-IFNα and ribavirin combination therapy for chronic hepatitis C patients, the increments of regulatory T cells and plasmacytoid dendritic cell frequency are independently related to favorable virological response to the therapy.

Keywords Early virological response · Plasmacytoid dendritic cells · Regulatory T cells

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Published online: 27 September 2011

Introduction

Hepatitis C virus (HCV) is one of the major causative agents of chronic liver diseases and hepatocellular

carcinoma (HCC) in the world [1, 2]. In order to prevent the development of HCV-induced liver diseases, eradication of HCV from infected patients may be required. For the treatment of chronic hepatitis C, a combination of pegylated interferon-α (PEG-IFNα) and ribavirin treatment has been used as a standard of care (SOC) [3, 4]. However, in patients with HCV genotype 1 and high viral load, approximately 50% of them are able to clear the virus by 48 weeks of SOC [5, 6]. In addition to HCV genotype and quantity, several demographic factors have been reported as therapeutic determinants in PEG-IFNα and ribavirin therapy, such as age, gender, ethnicity, and liver fibrosis [5, 6]. In addition, it is accepted that initial changes of serum HCV RNA titer from the beginning of the therapy, i.e., early virological response (EVR), correlate well with the clinical outcomes of the treated patients [5, 7]. It has been reported that the patients who fail to clear HCV at week 24 are not likely to attain SVR after 48 weeks of the therapy, suggesting that non-EVR can serve as a negative predictor of SVR [8]. Even in patients who attained EVR, 30% of them eventually relapse during the 48 weeks of therapy. Prolongation of the duration of PEG-IFNα and ribavirin therapy from 48 to 72 weeks is recommended to improve the SVR rate by decreasing relapsers [9]. Thus, identifying potential relapsers during therapy and providing additional weeks of treatment may be clinically important, because it can offer them a better chance of attaining SVR.

In chronic hepatitis C, multifaceted immune dysfunction may be implicated in the persistence of HCV including dendritic cells (DC), NK cells, and T cells [10, 11]. Some investigators have reported that the dynamics of immune cells throughout the therapy are involved in the efficacy of PEG-IFNα and ribavirin. In chronic HCV infection, the enhancement of HCV-specific Th1 response or DC function has been reported to be involved in therapeutic HCV eradication [12, 13]. We have previously demonstrated that plasmacytoid dendritic cell (PDC) frequency and DC function are involved in HCV eradication in patients who underwent 48 weeks of PEG-IFNα and ribavirin therapy [14]. These reports have supported the possibility that the enhancement of certain immune responses is a prerequisite for therapeutic HCV clearance. However, one of the limitations of these studies is that the conclusions were drawn from relatively small numbers of patients and evaluated by univariate analysis. Therefore, multivariate analyses are arguably required in order to validate the significance or independence of immune cell markers in the therapeutic efficacy.

In this study, we have extended our investigation to elucidate whether or not the dynamics of immune cells are involved in therapeutic outcomes. Consequently, the independent significance of regulatory T cell or plasmacytoid DC frequency is revisited in the efficacy of PEG-IFNα and ribavirin therapy for chronic hepatitis C patients.

Materials and methods

Subjects

Among chronic hepatitis C patients who had been followed at Osaka University Hospital, Osaka Kosei-nenkin Hospital, Higashi Osaka Municipal Hospital, and Osaka National Hospital, 67 patients who received PEG-IFNα2b and ribavirin combination therapy for 48 weeks were enrolled in the present study. The study was approved by the ethics committee of the Osaka University Graduate School of Medicine and all the hospitals listed above (approval no. 08156). Written informed consent was obtained from all patients. At enrollment, the patients were confirmed to be positive for both serum anti-HCV antibody (Ab) and HCV RNA, but were negative for hepatitis B virus and human immunodeficiency virus. All of them were infected with HCV genotype 1b with serum HCV RNA quantity of more than 100 kilo international units (KIU)/ml, as determined by methods described elsewhere [15]. All patients had shown persistent or fluctuating serum alanine aminotransferase (ALT) abnormalities at enrollment. The presence of other causes of liver disease, such as autoimmune, alcoholic, and metabolic disorders was excluded by laboratory and imaging analyses. A combination of biochemical markers and ultrasonography (US) or computed tomography scan analyses ruled out the presence of cirrhosis and tumors in the liver in all patients. Histological analyses of liver disease were performed with liver tissue obtained by US-guided biopsy. The activity and stage of the disease were assessed by two independent pathologists according to the METAVIR scoring system [16].

Treatment

All patients were treated with PEG-IFNα2b subcutaneously at a dose of 75 μg/week (body weight >40 and ≤60 kg), 105 μg/week (body weight >60 and ≤80 kg), or 135 μg/week (body weight >80 and ≤100 kg) and oral ribavirin at a dose of 600 mg/day (body weight >40 and ≤60 kg), 800 mg/day (body weight >60 and ≤80 kg), or 1000 mg/day (body weight >80 and ≤100 kg). Ribavirin was administered divided into two doses per day. All patients were treated for 48 weeks and followed for 24 weeks after the cessation of therapy.

Dose reduction of PEG-IFNα and ribavirin

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the hematological adverse effects. The dose of PEG-IFNα2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to less than 1500/mm^3, the

neutrophil count to less than 750/mm^3, or the platelet (Plt) count to less than 8×10^4/mm^3, and was discontinued if the WBC count declined to less than 1000/mm^3, the neutrophil count to less than 500/mm^3, or the Plat count to less than 5×10^4/mm^3. Ribavirin was also reduced from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg if the hemoglobin (Hb) level decreased to less than 10 g/dl, and was discontinued if the Hb level decreased to less than 8.5 g/dl. Both PEG-IFNα2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During the therapy, ferric medicine or hematopoietic growth factors, such as erythropoietin alpha or granulocyte–macrophage colony-stimulating factor were not administered.

Quantification of HCV RNA and assessment of virological response

Serum HCV RNA titers were 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 by the COBAS AMPLICOR HCV Test, version 2.0 (detection threshold 50 IU/ml).

Virological response during and after the therapy was determined according to the American Association for the Study of Liver Diseases (AASLD) practice guideline [17]. The complete early virological responders (c-EVR) were defined as those who showed a reduction in serum HCV RNA quantity to an undetectable level by qualitative PCR at week 12 of the therapy. Virological response was estimated at 24 weeks after cessation of the treatment. Sustained virological response (SVR) was defined as the maintenance of negative serum HCV RNA by PCR for more than 6 months after completion of the therapy. Transient response (TR) was defined as the reappearance of serum HCV RNA within 6 months after cessation of therapy in patients who had achieved negative serum HCV RNA at the end of the treatment. No response (NR) meant that there was persistently positive serum HCV RNA throughout the therapy period. The non-SVR group comprised TR and NR patients.

Assessment of drug exposure

The amounts of PEG-IFNα2b and ribavirin actually taken by patients during the first 12 weeks of the treatment were evaluated by reviewing the medical records as reported previously [18, 19]. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline. The dose of PEG-IFNα2b and ribavirin was expressed as micrograms per kilogram per week and milligrams per kilogram per day, respectively.

Analysis of DC subsets, helper T cells, NK cells, and regulatory T cells

For the numerical analyses of blood DC, helper T cells, NK cells, and regulatory T cells (Tregs), venous blood was drawn from patients before treatment and at weeks 8, 12, 24, and 48 during the therapy. Blood samples taken from patients in relevant hospitals were transferred to Osaka University within 6 h and were processed on the same day. Peripheral blood mononuclear cells (PBMCs) were collected by density-gradient centrifugation on a Ficoll-Hypaque cushion. After viable PBMCs had been counted, the cells were stained with combinations of various Abs for phenotypic markers. All immunological assays were performed in Osaka University.

The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA): anti-Lineage marker [Lin; CD3 (clone SK7), CD14 (clone MφP9), CD16 (clone 3G8), CD19 (clone SJ25C1), CD20 (clone L27), and CD56 (clone NCAM16.2)], anti-CD4 (clone RPA-T4), anti-CD11c (clone B-ly6), anti-CD123 (clone 7G3), anti-CD3 (clone UCHT1), anti-CD45RO (clone UCHL1), anti-CD56 (clone B159), anti-HLA-DR (clone L243), anti-CCR4 (clone 1G1). The antibodies for CD25 (clone B1.49.9) and CD4 (clone 1 3B8.2) were purchased from Beckman Coulter (Fullerton, CA, USA). Anti-CXCR3 (clone 49801) monoclonal antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Staining was performed with FITC, PE, PerCP, and APC conjugated antibodies as described previously [14]. The acquisitions and analyses of data were performed with FACS Calibur (BD Biosciences) and CellQuest software.

Blood DCs were defined as Lin$^-$ and HLA-DR$^+$ cells. Myeloid DCs (MDC) are Lin$^-$, HLA-DR$^+$, CD11c$^+$, and CD123^{low} cells, and plasmacytoid DCs (PDC) are Lin$^-$, HLA-DR$^+$, CD11c$^-$, and CD123^{high} cells. Helper T cell subpopulations were defined by the pattern of CXCR3 and CCR4; Th1 cells are CD4$^+$, CD45RO$^+$, and CXCR3$^+$, and Th2 cells are CD4$^+$, CD45RO$^+$, and CCR4$^+$. NK cells were defined as CD3$^-$ and CD56$^+$ cells. Regulatory T cells (Tregs) were defined as CD4$^+$, CD25^{high+} cells as reported previously [20]. The percentages of DC subsets and NK cells in PBMCs or Th1, Th2 cells and Tregs in CD4$^+$ T cells were determined by FACS. In order to examine the dynamics of immune cells after initiation of the treatment, we used the ratio of frequencies at each time point to those before the therapy [14].

Allogeneic mixed leukocyte reaction with DC

In some patients, we examined whether the allostimulatory ability of DCs was related to the clinical outcomes. Before, at the end of treatment, and at week 4 after completion of

the treatment, monocyte-derived DCs were generated from PBMC obtained from the patients according to methods reported previously [21]. As controls, monocyte-derived DCs were simultaneously generated from healthy donors. As responder cells in mixed lymphocyte reactions (MLR), naive $CD4^+$ T cells were isolated from PBMC of irrelevant healthy donors by using a naive $CD4^+$ T cell enrichment kit (Stemcell Technologies, Vancouver, BC). Allogeneic MLR with DC was performed as reported previously [21]. In order to compare the ability of DC among patients, we determined the MLR ratio between patients and controls as counts per minute (cpm) of [^{3}H]thymidine incorporated into CD4 T cells at the T cell/DC ratio of 10:1.

Statistical analyses

To analyze the relationship between clinical and immunological data at the baseline and virological response, univariate analysis using the Mann–Whitney *U* test or chi-squared test and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. Differences of continuous variables between groups were compared by two-way analysis of variance (ANOVA). A two-railed *P* value less than 0.05 was considered significant. These statistical analyses were performed with SPSS version 15.0 (SPSS Inc. Chicago, IL, USA).

Results

Outcome of the PEG-IFNα2b and ribavirin therapy

In 67 patients who had been treated for 48 weeks, 29 (43%) achieved SVR, 18 (27%) were TR, 17 (25%) were NR, and 3 (4%) were unknown (Fig. 1). The clinical backgrounds of these patients are summarized in Table 1. Among these cohorts, 32 patients were c-EVR and were further categorized into 24 SVR (EVR-SVR group) and 8 TR (EVR-TR group). Of the other 35 patients who were not c-EVR, 5 were SVR, 10 were TR, 17 were NR and 3 were unknown. Details of the therapeutic response in the current study are shown in Fig. 1.

Higher platelet counts and Treg increase are involved in SVR in patients who underwent PEG-IFNα2b and ribavirin therapy

In order to clarify whether the frequency and function of immune cells are involved in the outcomes of the combination therapy, we first compared these parameters between SVR and non-SVR groups. Representative dot plots of the immune cell populations are shown in Fig. 2. The identification and enumeration of immune cells were determined by FACS. The pretreatment percentages of DC in SVR were higher than those in the non-SVR group. However, those of PDC, NK cells, Th1, Th2, Treg, and DC function as judged by MLR were not different between them (Fig. 3).

As for the changes of DC subsets during the therapy, in the SVR group, the frequencies of PDC increased after the

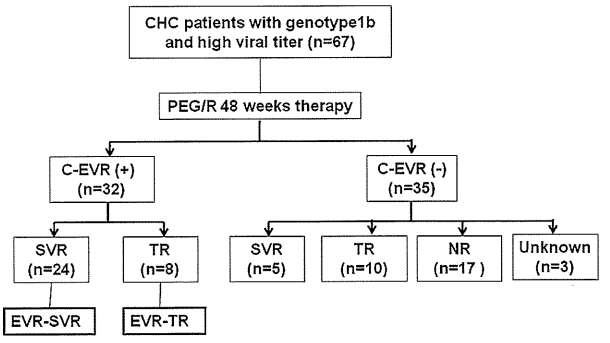


Fig. 1 Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. In 67 patients who had been treated for 48 weeks, 29 achieved SVR, 18 were TR, 17 were NR, and 3 were unknown. The complete early virological responders (c-EVR) were defined as those who show a reduction in HCV RNA quantity to an undetectable level by qualitative PCR at week 12 of the therapy. According to this criterion, 32 patients were c-EVR and were further categorized into 24 SVR (EVR-SVR) and 8 TR (EVR-TR). Of the other 35 patients who were not c-EVR, 5 were SVR, 10 were TR, 17 were NR, and 3 were unknown. *SVR* sustained virological responder, *TR* transient responder, *NR* non-responder

Table 1 Demographics and clinical backgrounds of the subjects

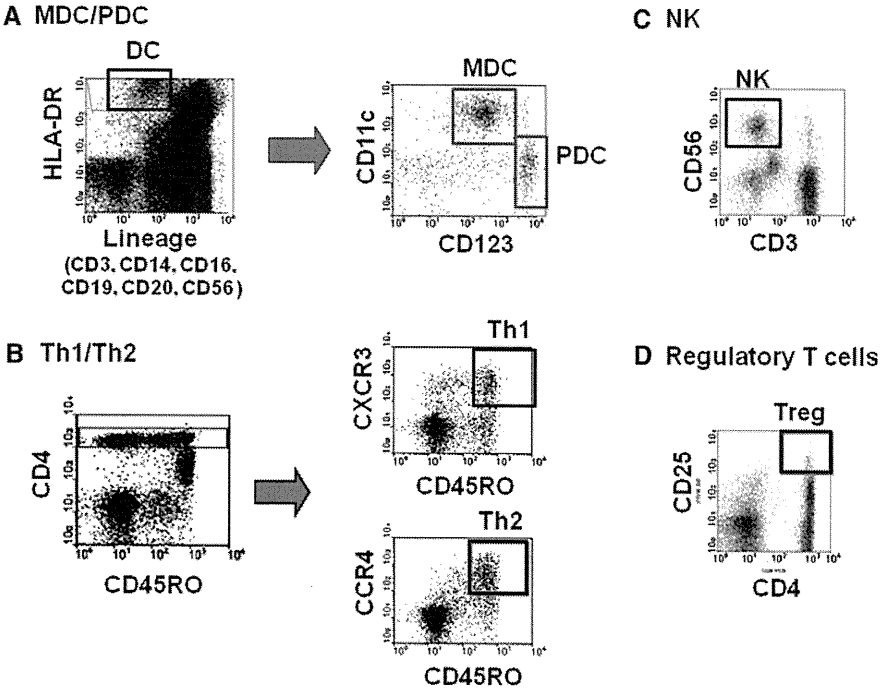
Factors	Value	Range
Number	67	
Age (years)	51.0 ± 10.3	(24–67)
Gender (M/F)	44/23	
HCV RNA (KIU)[a]	2415	
Activity: A0/1/2/3[b]	0/35/30/1	
Fibrosis: F0/1/2/3/4[b]	2/27/27/9/1	
WBC (/ml)	5229 ± 1299	(2960–9400)
Neutro (/ml)	2663 ± 826	(1077–4516)
Hb (g/dl)	14.6 ± 1.2	(12.0–18.0)
Platelets ($\times 10^4$/mm^3)	16.6 ± 4.6	(5.0–31.0)
ALT (IU/l)	83.1 ± 53.9	(14–269)
T. chol (mg/dl)	172 ± 29	(118–238)
Cr (mg/ml)	0.8 ± 0.2	(0.4–1.3)

All results are expressed as mean ± SD and range

T. chol total serum cholesterol, *Cr* creatinine

[a] Amplicore HCV monitor

[b] Ishak's histological scores



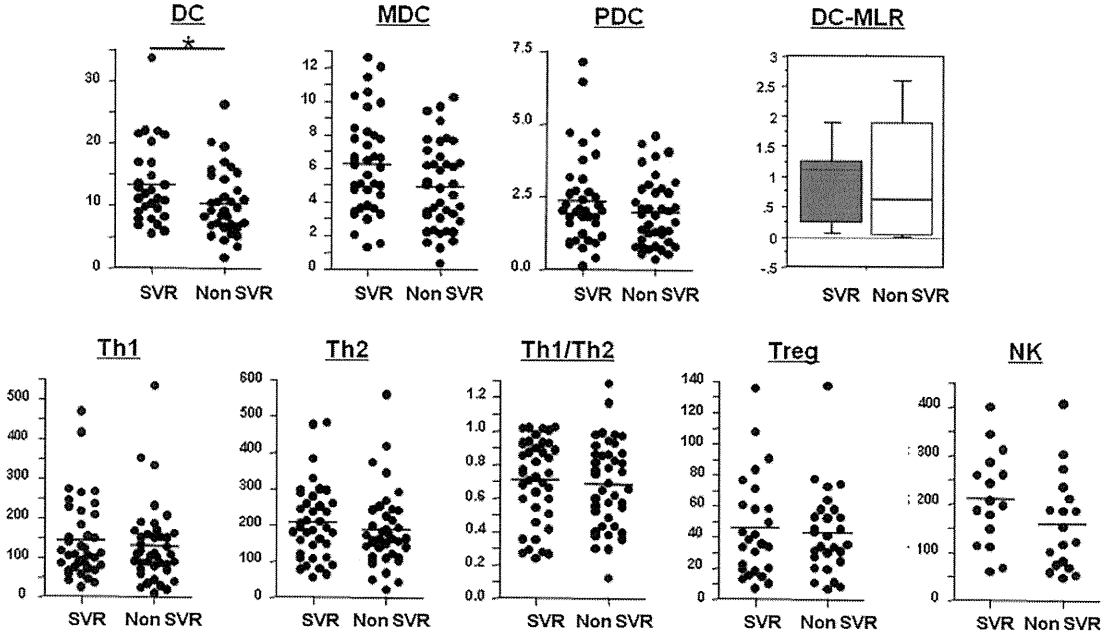


Fig. 2 Phenotypic identification of blood cells by flow cytometry. Representative analyses of myeloid and plasmacytoid dendritic cells (MDC and PDC), type 1 and type 2 helper T cells (Th1 and Th2), natural killer (NK) cells, and regulatory T cells are shown. The combination of surface molecules for the identification of cells is described in "Materials and methods"

Fig. 3 Comparison of pretreatment frequency of blood cells and allostimulatory capacity of monocyte-derived dendritic cells between SVR and non-SVR patients who had been treated with 48-week PEG-IFNα2b and ribavirin therapy. The frequencies of MDC, PDC, Th1 and Th2 cells, Th1/Th2 ratio, NK cells, regulatory T cells, and allogeneic MLR were compared between SVR and non-SVR patients. The MLR ratio between patients and controls was determined from the counts per minute (cpm) of [^{3}H]thymidine incorporated into $CD4^+$ T cells at T cell/DC ratio of 10:1. *$P < 0.05$ by Mann–Whitney U test

beginning of therapy and showed a peak at week 12 of therapy (T12W), which subsided to the end-of-treatment (EOT). Such a PDC increase at the early phase was not observed in the non-SVR group (Fig. 4a). In contrast, the MDC frequency remained at a similar level throughout the therapy, regardless of viral response (data not shown). Alternatively, in the SVR group, the percentages of Treg ($CD4^+CD25^{high+}$ cells) increased through the therapy,

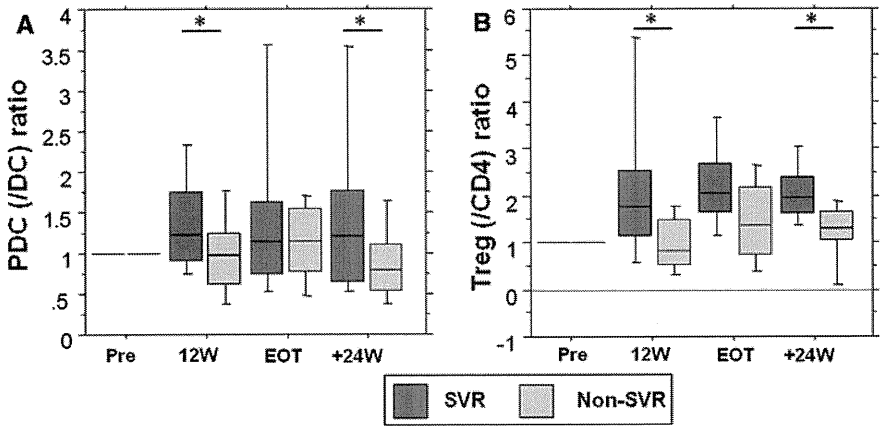


Fig. 4 Changes in frequencies of plasmacytoid dendritic cells and regulatory T cells during and after 48-week PEG-IFNα2b and ribavirin therapy in SVR and non-SVR patients. The ratios of frequencies of PDC (**a**) and Tregs (**b**) at each time point to the pretreatment values were compared between SVR and non-SVR patients. *Boxes* represent lower and upper quartiles, *solid line* within each *box* the median value, *whiskers* the minimum and maximum values. $^{*}P < 0.05$ by Mann–Whitney *U* test. *EOT* end-of-treatment (at 48 weeks of the therapy), *+24W* 24 weeks after the completion of therapy

with cell levels being higher than those in the non-SVR group (Fig. 4b). The other cells, including Th1, Th2, and NK cells, did not differ between the groups (data not shown). Univariate and multivariate analyses were performed to assess the significance of various factors, including demographic, biochemical, virological, immunological parameters, and drug adherence. The allostimulatory capacity of DC after the completion of therapy, whose significance was demonstrated in the previous paper [21], was not included in this study because the numbers of patients examined for it were limited. In univariate analyses, platelet counts, histological activity and fibrosis, dose of PEG-IFNα2b, and attainment of c-EVR were found to be significant in SVR (Table 2). As for immunological markers, pretreatment DC frequency, PDC frequency, their ratio at T12W, and Treg frequency ratio at T12W are significant (Table 3). Based on these parameters, multivariate analysis revealed that platelet counts and Treg frequency at T12W were independent factors involved in SVR (Table 4). These results show that higher platelet counts and Treg increment may be related to SVR in 48 weeks of PEG-IFNα and ribavirin treatment.

Table 2 Univariate analyses of clinical factors involved in SVR

Factors	SVR	Non-SVR
N	29	38
Age (years)	48.0 ± 11.8	53.3 ± 8.6
Gender (M/F)	20/9	24/14
WBC (/mm^3)	5361 ± 1314	5127 ± 1295
Neutro (/mm^3)	2969 ± 861	2461 ± 753
Hb (g/dl)	14.6 ± 1.2	14.5 ± 1.2
Platelets ($\times 10^4$/mm^3)	18.2 ± 4.4*	15.2 ± 4.4
ALT (IU/l)	72 ± 54	92 ± 53
HCV RNA (KIU/ml)	2103	2654
Activity: 0–1/2–3/n.d.	29/0/0$^{\#}$	27/10/1
Fibrosis: 0–2/3–4/n.d.	20/9/0*	15/22/1
PEG-IFN dose (μg/kg/day)	1.43 ± 0.14$^{\#}$	1.31 ± 0.22
Ribavirin dose (mg/kg/day)	10.6 ± 1.5	9.9 ± 1.4
c-EVR: +/−	24/5$^{\#}$	8/27

Mann–Whitney *U* test, chi-square test

n.d. not determined

$^{*}P < 0.05$, $^{\#}P < 0.01$

Higher platelet counts and PDC increase are independent factors involved in SVR after attainment of c-EVR

Next, we examined the above-mentioned immunological parameters in patients who attained c-EVR, as they were considered to be comparable with respect to the virological response to the therapy. Among 32 patients in the c-EVR group, 24 developed to SVR (EVR-SVR) and the remaining 8 to TR (EVR-TR) (Fig. 1). Univariate analysis disclosed that lower age is a characteristic of the EVR-SVR patients compared with those in the EVR-TR group (Table 5). As for immunological markers, pretreatment DC frequency, PDC frequency, and PDC ratio at T12W were higher in EVR-SVR patients than those in EVR-TR (Table 6). The pretreatment percentages of MDC, PDC, Th1, Th2, NK cells, and Tregs and those at any all points during the therapy did not differ between EVR-SVR and EVR-TR patients (data not shown). Multivariate analyses revealed that higher platelet counts and PDC increase at T12W were independent factors involved in EVR-SVR (Table 7). These results indicate that the dynamics of PDC

Table 3 Univariate analyses of immunological factors involved in SVR

Factors	SVR	Non-SVR	*P* value
N	29	38	
DC pre (/μl)	13.3 ± 6.5	10.3 ± 5.4	0.038
PDC-12W (/DC)	0.23 ± 0.09	0.18 ± 0.07	0.017
PDC-12W (/DC) ratio	1.42 ± 0.72	1.04 ± 0.63	0.028
Treg-12W (/CD4) ratio	2.49 ± 2.62	1.03 ± 0.64	0.016

Mann–Whitney *U* test, chi-square test

Only the factors that are of significance are shown

DC pre DC number before therapy, *PDC-12W (/DC)* PDC frequency in DC at T12W, *PDC-12W (/DC) ratio* the ratio of PDC frequency in DC at T12W to the pretreatment value, *Treg-12W (/CD4) ratio* the ratio of regulatory T cell frequency in CD4 at T12W to the pretreatment value

Table 4 Multivariate analyses of clinical and Immunological factors involved in SVR

Factors	Category	Odds ratio	95% CI	*P* value
Platelets		0.531	0.322–0.875	0.013
Treg-12W (/CD4) ratio	<1.2/>1.2	0.026	0.001–0.750	0.033

Logistic regression analysis, stepwise method

Table 5 Univariate analyses of clinical factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	EVR-SVR	EVR-TR
N	24	8
Age (years)	46.9 ± 12.3*	57.6 ± 6.5
Gender (M/F)	17/7	6/2
WBC (/mm^3)	5442 ± 1382	5211 ± 805
Neutro (/mm^3)	2975 ± 890	2587 ± 759
Hb (g/dl)	14.7 ± 1.1	15.1 ± 1.2
Platelets (×10^4/mm^3)	18.7 ± 4.5	15.0 ± 3.8
ALT (IU/l)	69 ± 56	91 ± 61
HCV RNA (KIU/ml)	1723	1296
Activity: 0–1/2–3/n.d.	24/0/0	6/2/0
Fibrosis: 0–2/3–4/n.d.	16/8/0	5/3/0
PEG-IFN dose (μg/kg/day)	1.43 ± 0.15	1.39 ± 0.23
Ribavirin dose (mg/kg/day)	10.8 ± 1.5	10.1 ± 2.1

Mann–Whitney *U* test, chi-square test

n.d. not determined, *EVR-SVR* SVR patients who attained complete EVR at T12W, *EVR-TR* TR patients who attained complete EVR at T12W

*$P < 0.05$

frequency during therapy serve as an independent immunological predictor for SVR in patients who attained c-EVR with PEG-IFNα and ribavirin therapy.

Table 6 Univariate analyses of immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	EVR-SVR	EVR-TR	*P* value
N		24	8	
DC pre (/μl)		13.5 ± 6.8	8.9 ± 4.5	0.030
PDC-12W (/DC) ratio	<0.8/>0.8	3/21	4/4	0.047

Mann–Whitney *U* test, chi-square test

Only the factors that are of significance are shown

DC pre, *PDC-12 (/DC) ratio*: see Table 3

Table 7 Multivariate analyses of clinical and immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	Odds ratio	95% CI	*P* value
Platelets		0.627	0.402–0.978	0.040
PDC-12W (/DC)	<0.18/≥0.18	0.028	0.001–0.787	0.036
PDC-12W (/DC) ratio	<0.8/≥0.8	0.032	0.002–0.673	0.027

Logistic regression analysis, stepwise method

PDC-12W (/DC), PDC-12W(/DC) ratio: see Table 3

Discussion

In this study, we demonstrated that the increase of Treg frequency during therapy is involved in SVR, and that of PDC is in SVR patients who attained c-EVR in 48 weeks of PEG-IFNα and ribavirin therapy. Of particular importance is that such significance is independent of viral dynamics (c-EVR), host factors (fibrosis, gender), and drug adherence.

Regulatory T cells (Treg) are immune suppressors that are supposed to alleviate HCV-induced liver inflammation. In chronic HCV infection, the increment of Tregs has been reported by several investigators, including us, although the underlying mechanisms were unspecified [20, 22]. The increase of Treg in SVR patients observed herein seems to be inconsistent with the previous reports regarding Treg as a tolerance inducer in chronic hepatitis C patients. Several controversial reports have been published with regard to the involvement of Tregs in the efficacy of PEG-IFNα and ribavirin therapy for chronic hepatitis C. Soldevila et al. [23] showed that the pretreatment frequency of Treg is higher in patients with non-response (NR) than those in the non-NR groups. Akiyama et al. [24] reported that Tregs in PBMC increased in SVR patients at earlier time points, while Tregs in liver-infiltrating lymphocytes decreased. By contrast, another group disclosed that frequency, phenotype, and function of Tregs are comparable regardless of the outcomes of PEG-IFNα and ribavirin therapy [25].

The current observation raises the possibility that the reduction of HCV load and/or liver inflammation correlates with the increment of Treg frequency, or vice versa. Recently, it was reported that liver inflammation caused by HCV induces PD-L1 on hepatocytes, which then suppress Treg proliferation in liver [26]. If such a scenario is operative as well in PEG-IFNα and ribavirin therapy, alleviation of liver inflammation may reduce PD-L1 expression on hepatocytes, thereby stimulating Treg proliferation. However, most of the TR patients, who were categorized as being in the non-SVR group, displayed normalized serum ALT levels and negative HCV RNA during treatment, of which conditions are equivalent with the SVR patients. Thus, it is still uncertain whether or not such mechanisms are applicable to the present results.

The other possibility is that phenotypically determined Tregs in this study partly consist of activated T cells. It is well known that $CD127^-$ and $FOXP3^+$ are reliable markers of Tregs [27]. In order to examine whether or not the increment of Treg frequency in this study is a contamination of activated T cells, we determined Tregs as $CD4^+CD25^{high}FOXP3^+CD127^-$ cells instead of $CD4^+CD25^{high}$ cells in some patients. In the comparison of the ratio of $CD4^+CD25^{high}FOXP3^+CD127^-$ cell frequency between the SVR and non-SVR groups at T12W, similar results were obtained with those of $CD4^+CD25^{high}$ cells (SVR vs. non-SVR, 10 patients in each group, 2.50 ± 1.20 vs. 1.54 ± 0.53, $P < 0.05$ by Mann–Whitney U test). These results suggest that the analytical results of $CD4^+CD25^{high}$ T cells reflect those of $FOXP3^+$ Tregs. Further investigation is needed to show that such Tregs are functionally suppressive and to see if the change of frequency parallels with suppressor capacity or not.

According to the AASLD practice guidelines for the treatment of chronic hepatitis C, a combination of PEG/R for 48 weeks is recommended for patients who attained c-EVR at week 12 of therapy [17]. However, in some cohorts with large numbers of patients, approximately 30% of them eventually relapse after cessation of the therapy [5]. The factors involved in post-therapeutic relapse have not been fully explored. We and others have reported that liver fibrosis, female gender, late virological response, and dosage of ribavirin (drug adherence) are critically involved in relapse [19, 28, 29]. It is well known that platelet counts in patients with chronic liver disease are well correlated with the degree of fibrosis. In the present study, multivariate analyses revealed that platelet counts but not fibrosis stage are involved in SVR. The reasons for such discrepant contributions to SVR are not clear; however, it demonstrates that the degree of fibrosis is involved in the therapeutic response in this cohort. In addition, the current study showed that the changes of PDC frequency are also somewhat involved in virological relapse in patients that once attained c-EVR.

Plasmacytoid DCs (PDC) play crucial roles in antivirus immune responses by producing IFN-β and -α [30]. In the previous study by us [14], the increment of PDC is observed in patients with SVR, of which change is more significant in those with c-EVR. No concrete explanation is available for the mechanisms of PDC increase in SVR patients. One of the possibilities is that the PDC increase is a consequence of better response to exogenous IFN-α in patients who have a higher chance of attaining SVR. IFN-α is reported to act as a regulatory factor on $CD11c^-$ DCs to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [31]. Such a possibility is supported by the findings that higher induction of IFN-stimulated genes (ISGs) in hepatocytes after PEG-IFNα and ribavirin therapy, but not higher ISG levels before therapy, is critically involved in successful outcome [32]. Thus, patients who respond well to IFN-α, as demonstrated by better PDC survival during the treatment, are likely to have better chances to eradicate HCV.

Another possible reason for the PDC increase in the periphery of SVR patients is that PDC alter their localization during the treatment. Mengshol et al. [33] reported that PDC and myeloid DC (MDC) are accumulated in inflamed liver through the interactions of chemokines and their receptors. Of particular interest is that the expression of such chemokine receptors on DCs decreased in SVR patients, but not in non-SVR ones [33]. Therefore, it is plausible that PDC may migrate from the liver to periphery/lymphoid tissue after being unleashed from chemokines in the liver. In support for this, it is reported that IFN-α alters the profiles of chemokine receptors on DC, resulting in changes of the DC migrating ability [34].

Recently, numerous other factors were reported to be involved in therapeutic response in chronic hepatitis C patients, such as mutations of HCV genome (core region) [35] or host genetic variation (single nucleotide polymorphisms near the IL28B gene) [36]. In the current study, we were unable to analyze such factors because of the limited numbers of patients. A prospective study is warranted to analyze the involvement of such factors in relation to immune cell markers, in the outcomes of SOC, or the treatment with direct-acting antiviral agents.

In summary, we demonstrated that the increase of Treg frequency is an independent factor involved in SVR in 48 weeks of SOC for chronic hepatitis C patients. In addition, the increase of PDC gains similar significance in SVR patients who attained c-EVR. The assessment of the dynamics of such cells during therapy could offer some clues to identify potential relapsers and give them a better chance of attaining SVR by rescheduling the therapy.

Acknowledgments This study was funded in part by Grant-in-Aid Ministry of Health, Labor and Welfare of Japan and Ministry of Education, Science and Culture of Japan (ID: 22590729 and 22590730).

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

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