

## Viral hepatitis

Yotsuyanagi *et al*<sup>23</sup> reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi *et al*<sup>41</sup> 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.<sup>42</sup> 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.<sup>41</sup>

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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**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.

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

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### Abstract

**Background** For the treatment of chronic hepatitis C, a combination of pegylated interferon- $\alpha$  (PEG-IFN $\alpha$ ) 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

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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-IFN $\alpha$ 2b 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-IFN $\alpha$ 2b 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-IFN $\alpha$  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

### 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- $\alpha$  (PEG-IFN $\alpha$ ) 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-IFN $\alpha$  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-IFN $\alpha$  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-IFN $\alpha$  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-IFN $\alpha$  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-IFN $\alpha$  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-IFN $\alpha$ 2b 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-IFN $\alpha$ 2b subcutaneously at a dose of 75  $\mu$ g/week (body weight  $>40$  and  $\leq 60$  kg), 105  $\mu$ g/week (body weight  $>60$  and  $\leq 80$  kg), or 135  $\mu$ g/week (body weight  $>80$  and  $\leq 100$  kg) and oral ribavirin at a dose of 600 mg/day (body weight  $>40$  and  $\leq 60$  kg), 800 mg/day (body weight  $>60$  and  $\leq 80$  kg), or 1000 mg/day (body weight  $>80$  and  $\leq 100$  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-IFN $\alpha$ 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-IFN $\alpha$ 2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to less than 1500/mm<sup>3</sup>, the

neutrophil count to less than  $750/\text{mm}^3$ , or the platelet (Plt) count to less than  $8 \times 10^4/\text{mm}^3$ , and was discontinued if the WBC count declined to less than  $1000/\text{mm}^3$ , the neutrophil count to less than  $500/\text{mm}^3$ , or the Plat count to less than  $5 \times 10^4/\text{mm}^3$ . 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-IFN $\alpha$ 2b 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-IFN $\alpha$ 2b 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-IFN $\alpha$ 2b 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 M $\phi$ P9), 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<sup>-</sup> and HLA-DR<sup>+</sup> cells. Myeloid DCs (MDC) are Lin<sup>-</sup>, HLA-DR<sup>+</sup>, CD11c<sup>+</sup>, and CD123<sup>low</sup> cells, and plasmacytoid DCs (PDC) are Lin<sup>-</sup>, HLA-DR<sup>+</sup>, CD11c<sup>-</sup>, and CD123<sup>high</sup> cells. Helper T cell subpopulations were defined by the pattern of CXCR3 and CCR4; Th1 cells are CD4<sup>+</sup>, CD45RO<sup>+</sup>, and CXCR3<sup>+</sup>, and Th2 cells are CD4<sup>+</sup>, CD45RO<sup>+</sup>, and CCR4<sup>+</sup>. NK cells were defined as CD3<sup>-</sup> and CD56<sup>+</sup> cells. Regulatory T cells (Tregs) were defined as CD4<sup>+</sup>, CD25<sup>high+</sup> cells as reported previously [20]. The percentages of DC subsets and NK cells in PBMCs or Th1, Th2 cells and Tregs in CD4<sup>+</sup> 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<sup>+</sup> T cells were isolated from PBMC of irrelevant healthy donors by using a naive CD4<sup>+</sup> 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 [<sup>3</sup>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-tailed *P* value less than 0.05 was considered significant. These statistical analyses were performed with SPSS version 15.0 (SPSS Inc. Chicago, IL, USA).

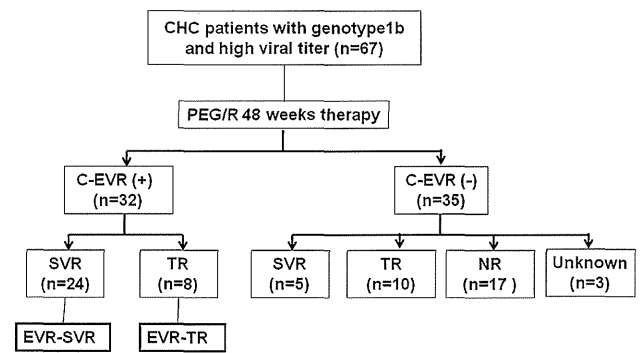
## Results

### Outcome of the PEG-IFN $\alpha$ 2b 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-IFN $\alpha$ 2b 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



**Fig. 1** Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFN $\alpha$ 2b 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 $\pm$ 10.3	(24–67)
Gender (M/F)	44/23	
HCV RNA (KIU) <sup>a</sup>	2415	
Activity: A0/1/2/3 <sup>b</sup>	0/35/30/1	
Fibrosis: F0/1/2/3/4 <sup>b</sup>	2/27/27/9/1	
WBC (/ml)	5229 $\pm$ 1299	(2960–9400)
Neutro (/ml)	2663 $\pm$ 826	(1077–4516)
Hb (g/dl)	14.6 $\pm$ 1.2	(12.0–18.0)
Platelets ( $\times 10^4$ /mm <sup>3</sup> )	16.6 $\pm$ 4.6	(5.0–31.0)
ALT (IU/l)	83.1 $\pm$ 53.9	(14–269)
T. chol (mg/dl)	172 $\pm$ 29	(118–238)
Cr (mg/ml)	0.8 $\pm$ 0.2	(0.4–1.3)

All results are expressed as mean  $\pm$  SD and range

T. chol total serum cholesterol, Cr creatinine

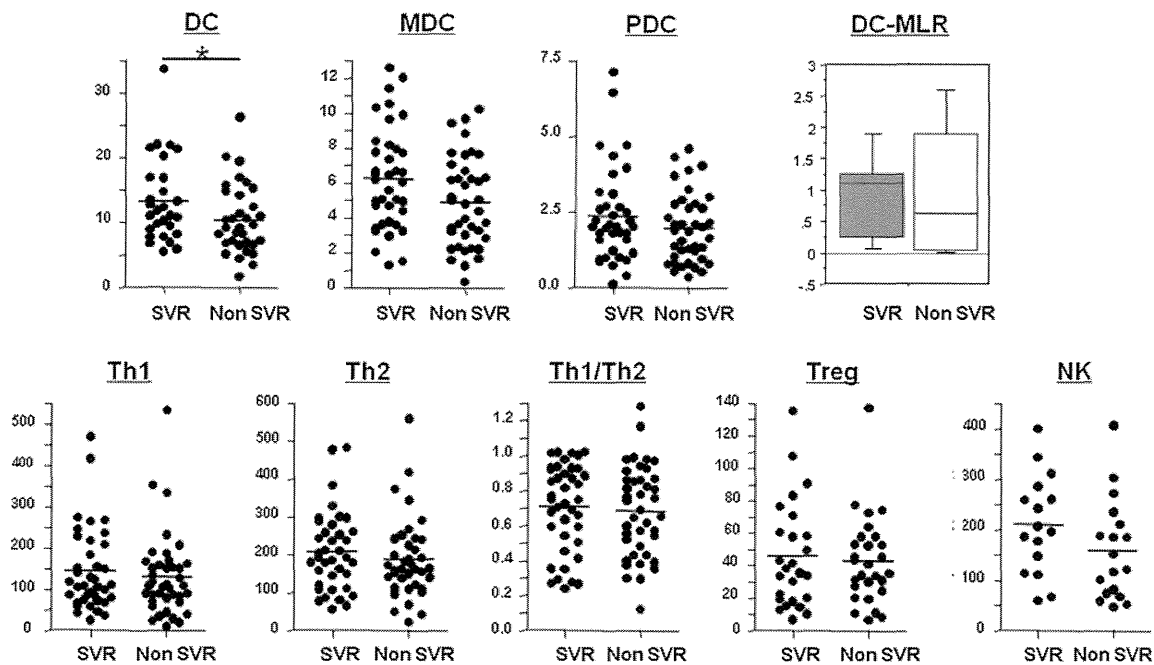
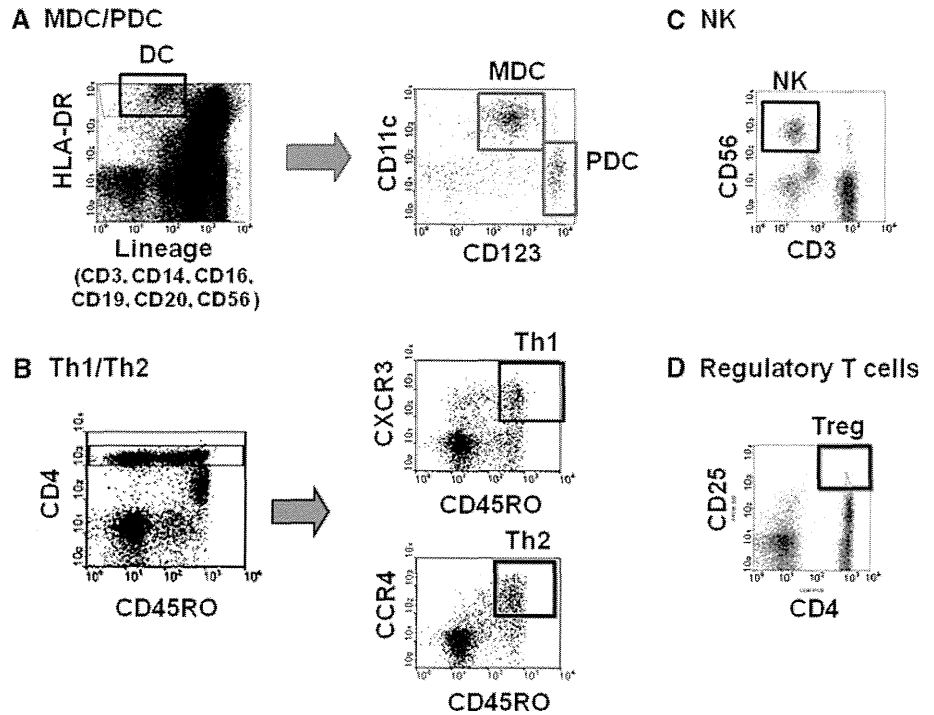
<sup>a</sup> Amplicore HCV monitor

<sup>b</sup> Ishak's histological scores

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. 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”

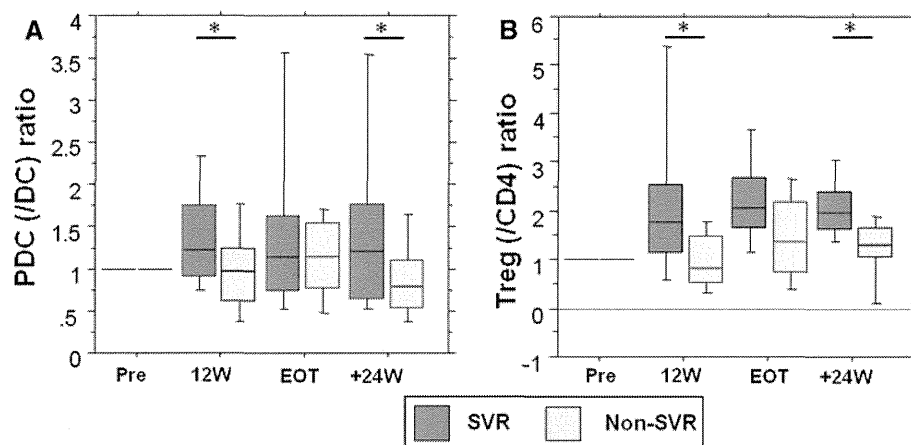


**Fig. 3** Comparison of pretreatment frequency of blood cells and allostimulatory capacity of monocyte-derived dendritic cells between SVR and non-SVR patients who had been treated with 48-week PEG-IFN $\alpha$ 2b and ribavirin therapy. The frequencies of MDC, PDC, Th1 and Th2 cells, Th1/Th2 ratio, 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 $^{\text{high}}$  cells) increased through the therapy,



**Fig. 4** Changes in frequencies of plasmacytoid dendritic cells and regulatory T cells during and after 48-week PEG-IFN $\alpha$ 2b 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

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-IFN $\alpha$ 2b, 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-IFN $\alpha$  and ribavirin treatment.

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. 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

**Table 2** Univariate analyses of clinical factors involved in SVR

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

Mann-Whitney  $U$  test, chi-square test

n.d. not determined

\* $P < 0.05$ , <sup>#</sup> $P < 0.01$

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 ( $\mu\text{l}$ )	13.3 $\pm$ 6.5	10.3 $\pm$ 5.4	0.038
PDC-12W (/DC)	0.23 $\pm$ 0.09	0.18 $\pm$ 0.07	0.017
PDC-12W (/DC) ratio	1.42 $\pm$ 0.72	1.04 $\pm$ 0.63	0.028
Treg-12W (/CD4) ratio	2.49 $\pm$ 2.62	1.03 $\pm$ 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 $\pm$ 12.3*	57.6 $\pm$ 6.5
Gender (M/F)	17/7	6/2
WBC ( $\text{mm}^3$ )	5442 $\pm$ 1382	5211 $\pm$ 805
Neutro ( $\text{mm}^3$ )	2975 $\pm$ 890	2587 $\pm$ 759
Hb (g/dl)	14.7 $\pm$ 1.1	15.1 $\pm$ 1.2
Platelets ( $\times 10^4/\text{mm}^3$ )	18.7 $\pm$ 4.5	15.0 $\pm$ 3.8
ALT (IU/l)	69 $\pm$ 56	91 $\pm$ 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 ( $\mu\text{g}/\text{kg}/\text{day}$ )	1.43 $\pm$ 0.15	1.39 $\pm$ 0.23
Ribavirin dose ( $\text{mg}/\text{kg}/\text{day}$ )	10.8 $\pm$ 1.5	10.1 $\pm$ 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-IFN $\alpha$  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 ( $\mu\text{l}$ )		13.5 $\pm$ 6.8	8.9 $\pm$ 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/ $\geq$ 0.18	0.028	0.001–0.787	0.036
PDC-12W (/DC) ratio	<0.8/ $\geq$ 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-IFN $\alpha$  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-IFN $\alpha$  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-IFN $\alpha$  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-IFN $\alpha$  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<sup>-</sup> and FOXP3<sup>+</sup> 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<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup>CD127<sup>-</sup> cells instead of CD4<sup>+</sup>CD25<sup>high</sup> cells in some patients. In the comparison of the ratio of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup>CD127<sup>-</sup> cell frequency between the SVR and non-SVR groups at T12W, similar results were obtained with those of CD4<sup>+</sup>CD25<sup>high</sup> cells (SVR vs. non-SVR, 10 patients in each group,  $2.50 \pm 1.20$  vs.  $1.54 \pm 0.53$ ,  $P < 0.05$  by Mann–Whitney  $U$  test). These results suggest that the analytical results of CD4<sup>+</sup>CD25<sup>high</sup> T cells reflect those of FOXP3<sup>+</sup> 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 antiviral immune responses by producing IFN- $\beta$  and - $\alpha$  [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- $\alpha$  in patients who have a higher chance of attaining SVR. IFN- $\alpha$  is reported to act as a regulatory factor on CD11c<sup>-</sup> 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-IFN $\alpha$  and ribavirin therapy, but not higher ISG levels before therapy, is critically involved in successful outcome [32]. Thus, patients who respond well to IFN- $\alpha$ , 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- $\alpha$  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.

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

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# ラミブジン耐性 B 型慢性肝疾患に対する アデホビル併用療法の長期成績と問題点

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## 特集II B型肝炎の抗ウイルス療法の進歩と耐性

# ラミブジン耐性 B 型慢性肝疾患に対するアデホビル併用療法の長期成績と問題点\*

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**Key Words** : hepatitis B virus (HBV), lamivudine, adefovir, hepatitis B e antigen (HBeAg), estimated glomerular filtration rate (eGFR)

### はじめに

B 型慢性肝炎の治療目標は持続的な B 型肝炎ウイルス(以下HBV)の抑制であり, 肝炎の鎮静化である。それにより肝不全への進展阻止や肝癌の発症を予防し, 最終的には生命予後の改善を目指している。

核酸アナログは B 型慢性肝炎のキードラッグであり, その中でもエンテカビル(entecavir, 以下ETV)は強い抗ウイルス活性を有し, 薬剤耐性株の出現頻度も非常に低いため, 2006年保険適応となって以降, 核酸アナログnaïve例の第一選択薬として推奨されている。しかし, ラミブジン(lamivudine, 以下LAM)は2000年に最初の核酸アナログとして登場して以来2005年までは唯一の単独で使用できる核酸アナログ製剤であった。LAMを開始した症例では耐性株が高率に出現し, いったん耐性株が出現すると肝炎のコントロールが困難で, 耐性株による肝炎の急性増悪(breakthrough hepatitis)により死に至る症例もみられたり, 耐性化をきたした症例に対して, ETVへの変更ではETV耐性ウイルスの出現頻度が

高いため, ラミブジンとアデホビル(adefovir, 以下ADV)の併用療法(LAM+ADV併用療法)が基本治療として行われている。これら核酸アナログ製剤の登場により, B 型慢性肝炎の予後は確実に改善されてきているが, 長期間使用した場合の安全性や耐性株の問題についてはいまだ明らかにはなっていない。

本稿では国立病院機構共同研究[肝疾患グループ]で集積された178例のデータを中心に, ラミブジン耐性の B 型慢性肝炎に対するLAM+ADV併用療法の長期成績について述べる。

### 抗ウイルス効果

LAM+ADV併用療法のウイルス効果を, HBV-DNAの 3 log copies/ml未満への低下と定義し, 図1にその累積ウイルス効果を示している。ウイルス効果は1年62.4%, 2年72.5%, 3年87.3%, 4年90.8%と投与期間とともに上昇した。1年後に94%の症例が 3 log copies/ml以下まで減少するETVの場合と比較するとADV追加療法は短期のウイルス効果は弱いものの, HBV-DNA量は投与期間とともに着実に低下していた。加えて長期投与を前提としている核酸アナログ製剤の一番の問題点である耐性ウイルスの出現に関しても, 本検討ではいったん低下したHBV-DNA量が上昇するviral breakthroughはいまだ2例しか認められていない。すなわちLAM+ADV併用療法

\* Long-term efficacy of lamivudine and adefovir combination therapy.

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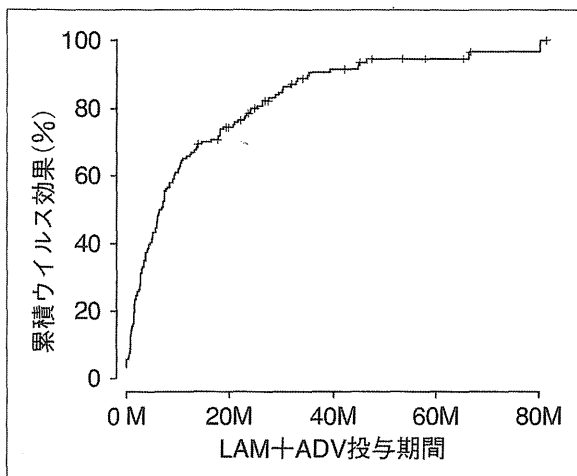


図1 経時的抗ウイルス効果

HBV-DNA 3 log copies/ml未満を抗ウイルス効果として解析した。

は短期でのウイルス効果は弱いものの耐性ウイルスの出現やbreakthrough hepatitisの出現は低く、良好な長期成績であるといえる。

LAM+ADV併用療法のウイルス効果に関与する因子をCox比例ハザードモデルによって検討すると、HBe抗原陽性例やHBV-DNA量が多い症例、慢性肝炎例が効果不良に関与する因子であった(図2)。HBe抗原陽性例やHBV-DNA量が高い場合などHBVの活動性が高い状態ではADVが効きにくいといえる。実際にADVを追加するタイミングについては、LAM耐性ウイルスが出現した時期とLAM耐性出現後のALT上昇時(耐性ウイルスが十分に上昇した後)で比較検討したLamperticoraらの報告<sup>2)</sup>において、2年でウイルスが陰性化する割合は前者で100%、後方で78%と肝炎出現後

の投与では治療効果が低かった。本邦のガイドラインでもLAM使用中にHBVのviral breakthroughが生じた場合はHBV-DNAが十分上昇する前にLAM+ADV療法を開始することが推奨されている。

### ALT正常化率と肝機能改善度

図3-aにLAM+ADV併用療法開始後のALTの推移を示す。6か月後には速やかにALT値の低下を認めており、2年後、3年後のALTの正常化率はそれぞれ75.8%、79.6%と高く、治療効果は良好といえる(表1)。

核酸アナログの長期投与によりChildスコアの改善を認めるとの報告がある<sup>3)</sup>。今回、総ビリルビン(T. Bil)、アルブミン(Alb)、プロトロンビン活性(PT)の推移を検討したところ、Alb値は図3-bに示すように有意な改善を認めた。これらのことから、長期のLAM+ADV併用療法により肝炎の鎮静化および肝予備能の改善も得られるといえる。

### HBe抗原のseroconversion

表1にHBe抗原のclearance率およびseroconversion率を示している。ADV追加前にHBe抗原が陽性であった108例の2年後、3年後のHBe抗原clearanceはそれぞれ17.6%、24.3%であり、2年後、3年後のHBe抗原のseroconversion率は10.0%、15.1%であった。LAMやETVの初回投与例でのseroconversion率は1年で16~20%といわれており<sup>4)</sup>、LAM+ADV併用療法は治療効果が低

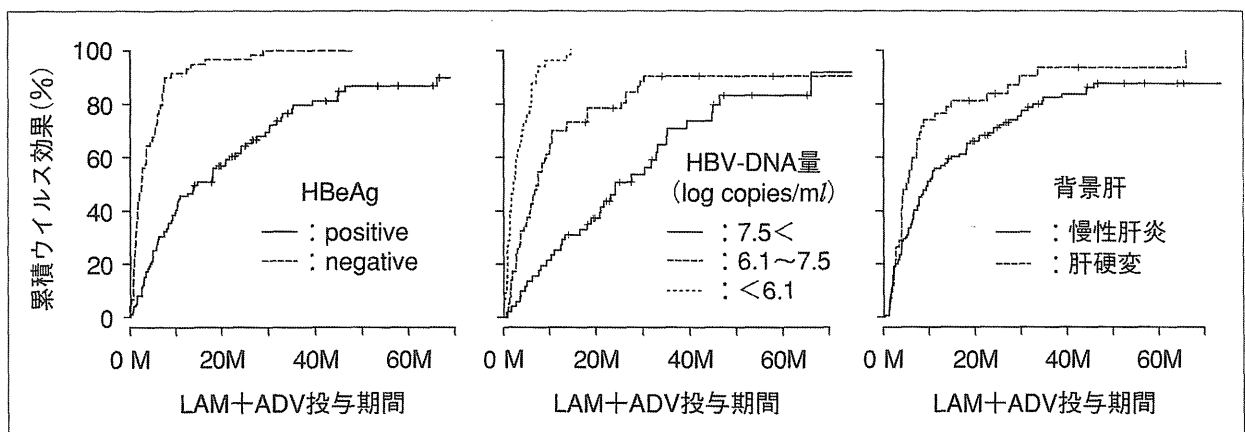


図2 HBeAg, HBV-DNA量, 背景肝別の抗ウイルス効果

いものの、経時的にはseroconversion率は増加しており、breakthroughが少ないことから、長期投与によりさらにseroconversion率は上昇するものと考えられる。またHBe抗原のclearanceに関する因子を検討したところ、ほかの報告と同様に、ADV追加前のALT値が高い症例がHBe抗原消失に至る可能性が高かった<sup>5)6)</sup>。

### 腎機能障害

ADVの注意すべき副作用に、腎機能障害がある。ADVは、近位尿細管上皮細胞に存在するトランスポーターによって尿細管細胞内に取り込まれ、細胞内濃度が高くなることにより、尿細管障害を発現させると考えられている。したがって、本剤の投与中には腎機能障害や腎不全があらわれる可能性があるため、定期的な腎機能検

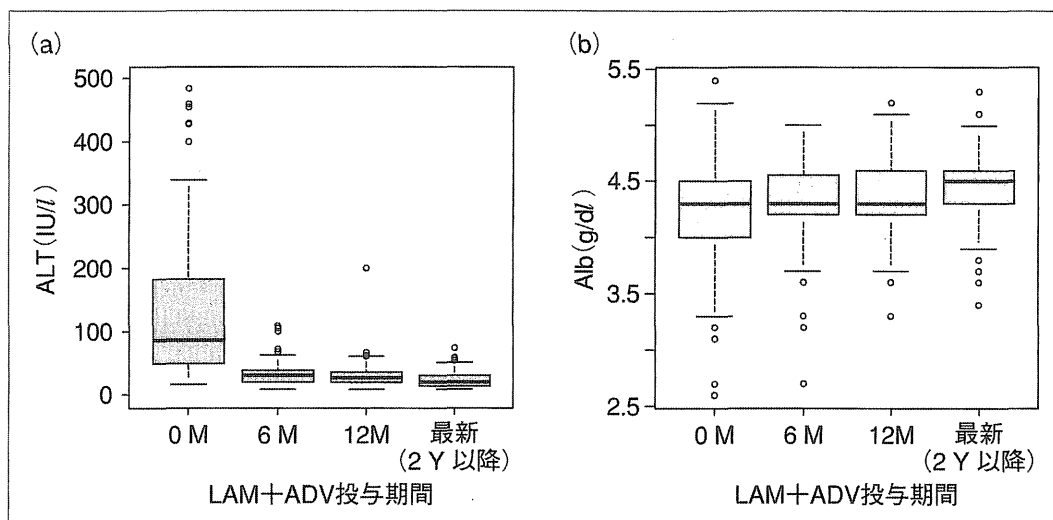


図3 ALT値, Alb値の経時的推移

表1 LAM+ADV併用療法の抗HBV効果とALT正常化

	month 24	month 36	at the end of follow-up
HBeAg clearance	17.6%	24.3%	36.6%
HBe seroconversion	10.0%	15.1%	18.2%
ALT normalization	75.8%	79.6%	83.3%

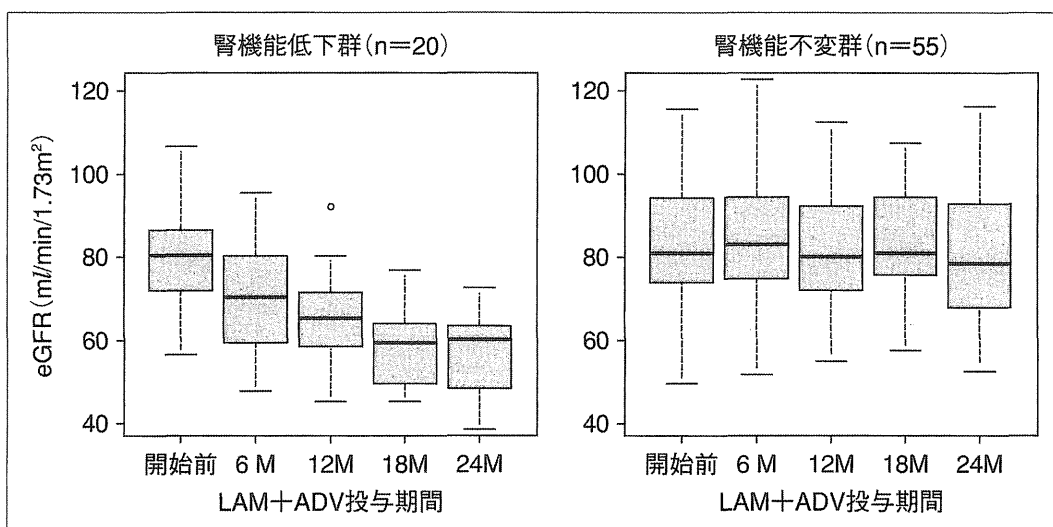


図4 LAM+ADV投与期間とeGFRの推移



査を行う必要がある。われわれの検討でも腎機能をフォローしえた75症例のうち20症例で20%以上の推定糸球体濾過量(以下eGFR)の低下を認めた。腎機能低下症例のeGFRは投与初期より徐々に低下し(図4), 腎機能低下がADVの血中濃度の上昇を導き, さらなる尿細管障害につながるため, 投与間隔の調節など調整が必要となる。

高血圧や腎機能低下例などが腎機能悪化に関与していると海外では報告されているが, 本検討では年齢, 特に50歳以上の症例が腎機能悪化に関与した。これは小関らの報告<sup>7)</sup>と合致しており, 本邦では50歳以上の症例に投与する場合は腎機能に注意する必要があると考えられた。

### おわりに

B型慢性肝疾患に対する核酸アナログ治療は長期投与になるケースが多い。各薬剤の特性を理解した診療が重要である。特にLAM+ADV併用療法では定期的な腎機能チェックが重要であることを認識すべきである。

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- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

## Prediction of early HBeAg seroconversion by decreased titers of HBeAg in the serum combined with increased grades of lobular inflammation in the liver

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<b>Background:</b>	Hepatitis B e antigen (HBeAg) seroconversion is an important hallmark in the natural course of chronic hepatitis B. This study was designed to predict early HBeAg seroconversion within 1 year, by not only biochemical and virological markers, but also pathological parameters in patients with chronic hepatitis B.
<b>Material/Methods:</b>	In a retrospective cohort study, 234 patients with HBeAg were reviewed for demographic, biochemical, virological and pathological data at the time of liver biopsy. Then, the patients who accomplished HBeAg seroconversion within 1 year thereafter were compared with those who did not, for sorting out factors predictive of early HBeAg seroconversion.
<b>Results:</b>	Early HBeAg seroconversion occurred in 58 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: alanine aminotransferase (ALT) (p=0.002), IP-10 (p=0.029), HBsAg (p=0.003), HBeAg (p<0.001), HBV DNA (p=0.001), HBcrAg (p=0.001), core-promoter mutations (p=0.040), fibrosis (p=0.033) and lobular inflammation (p=0.002). In multivariate analysis, only serum HBeAg levels <100 Paul Ehrlich Institute (PEI) U/ml and grades of lobular inflammation ≥2 were independent factors for early HBeAg seroconversion (odds ratio 8.430 [95% confidence interval 4.173–17.032], p<0.001; and 4.330 [2.009–9.331], p<0.001; respectively).
<b>Conclusions:</b>	HBeAg levels < 100 PEIU/ml combined with grades of lobular inflammation ≥2 are useful for predicting early HBeAg seroconversion. In patients without liver biopsies, high ALT levels (≥200 IU/L) can substitute for lobular inflammation (grades ≥2).
<b>key words:</b>	alanine aminotransferase • chronic hepatitis • hepatitis B virus • hepatitis B e antigen • lobular inflammation • seroconversion
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## BACKGROUND

Worldwide, an estimated 350 million people are infected with hepatitis B virus (HBV) persistently [1,2]. HBV infection is a major global concern, because up to 40% of patients can develop grave complications, such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [3]. In the natural course of chronic hepatitis B, HBeAg seroconversion, defined by the loss of HBeAg and development of the corresponding antibody (anti-HBe), is an important hallmark, because it is highly correlated with a favorable long-term outcome. Seroconversion is usually followed by sustained suppression of HBV DNA, normalization of alanine aminotransferase (ALT) levels, and clinical remission accompanied by ameliorated necro-inflammatory activities in the liver [4–6].

To date, a number of factors have been found to predispose patients to spontaneous HBeAg seroconversion [7–19]. However, few studies have evaluated pathological factors for predicting early HBeAg seroconversion. In a small series of patients from Spain, the Knodell's index of histological activity was one of the independent predictors of early HBeAg seroconversion [14]. Recently, novel markers of the replication of HBV were introduced, such as levels of HBsAg, HBeAg and HBcrAg (HBV core-related antigen), which can replace HBV DNA levels. These serological markers of HBV replication have been evaluated for sensitive and reliable prediction of early HBeAg seroconversion [20–23]. In the present study, an attempt was made to select factors predictive of early HBeAg seroconversion, from among many biochemical, virological and pathological parameters, based on the data of 234 HBeAg-positive patients with chronic hepatitis B.

## MATERIAL AND METHODS

### Patients and study design

This is a retrospective cohort study with use of stored sera and liver biopsy specimens from patients with chronic hepatitis B who were taken care of in the Hepatology Department, Nagasaki Medical Center, Japan, during 1991 through 2005. The clinical database was reviewed to identify consecutive patients who underwent liver biopsies and had been followed for longer than 1 year. The inclusion criteria were presence of hepatitis B surface antigen (HBsAg) for 6 months or longer, positivity for HBeAg at the time of liver biopsy, and lack of antiviral treatments before receiving liver biopsies. The exclusion criteria were co-infection with hepatitis C virus (HCV) or human immunodeficiency virus type-1, serological markers suggestive of autoimmune disease, daily intake of alcohol >50 g, recent exposure to hepatotoxic drugs, and no stored sera available. They were followed every 3 months or more frequently, if indicated clinically, and their serum samples were monitored for liver biochemistry and serologic markers of HBV infection, including HBsAg, HBeAg, anti-HBe, HBV DNA and HBcrAg. Serum samples had been stored at  $-20^{\circ}\text{C}$  until use.

Antiviral therapy was commenced immediately in the patients with: (1) significant fibrosis/cirrhosis detected by liver biopsy; and (2) evidence of decompensation, such as ascites, varices and hepatic encephalopathy.

To identify predictors of early HBeAg seroconversion, clinical, biological, virological and pathological data at the time

of liver biopsy were compared between patients who did and who did not achieve early HBeAg seroconversion, within 1 year after receiving liver biopsies, by univariate and multivariate analyses. Further, patients were stratified by independent factors for HBeAg seroconversion, and the cumulative incidence of HBeAg seroconversion was compared between groups using the Kaplan-Meier method. The study protocol complied with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the review board of the institution. Each patient gave a written informed consent before participating in this study.

### Routine laboratory tests for HBV markers

Quantitative measurements of HBsAg and HBeAg were carried out using commercial enzyme-linked immunosorbent assay (ELISA) kits in the ARCHITECT ANALYSER i2000 (Abbott Japan Co., Ltd., Tokyo, Japan) in accordance with the manufacturers' instructions in Nagasaki Medical Center. The sensitivity of HBsAg assay ranged from 0.05 to 250 IU/ml. Sera with HBsAg >250 IU/ml were serially diluted 100-fold so as to include them within the dynamic range. HBeAg was quantified by a two-step immunoassay with use of chemiluminescence microparticles. Briefly, undiluted samples were mixed with paramagnetic beads coated with anti-HBe. After a washing step, conjugate and reactants were added for exciting emission of the light that is proportional to the concentration of HBeAg. The result was expressed by the ratio of relative light unit (RLU) of the sample to the cut-off RLU (S/CO). Samples with S/CO values >1.0 were regarded positive for HBeAg. Then, serial dilutions of the reference standard of PE HBeAg (Paul Ehrlich Institute, Langen, Germany) were used to define the linear range of the assay and create a reference curve for linear regression. The linear range was 0.024–100 PEIU/ml. A standard curve was produced, and linear regression was used to convert assay results into appropriate units (PEIU/ml). For samples that fell outside the linear range of the assay, the assay was performed on serial dilutions to ensure the linearity.

### HBV DNA and HBcrAg

HBV DNA was determined by the COBAS Taqman HBV test (Roche Diagnostics K.K., Tokyo, Japan). Values under or over the detection range were recorded as 2.1 or 9.1 log copies/ml. HBcrAg was measured by the CLEIA HBcrAg assay kit (Fujirebio, Inc., Tokyo, Japan) in a fully automated analyzer (Lumipulse system, Fujirebio, Inc.). Values under or over the detection range were recorded as 3.0 or 7.0 log copies/ml. Assays for HBV DNA and HBcrAg were performed in a commercial clinical laboratory (SRL, Inc., Tokyo, Japan). Sera with values over the detection range were diluted to include them within the dynamic range.

### Interferon-inducible protein 10 (IP-10)

IP-10 was quantified by the Invitrogen Human IP-10 ELISA (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol in Nagasaki Medical Center.

### HBV genotyping

HBV DNA was extracted from serum (100  $\mu\text{l}$ ) with use of the SMITEST EX R&D extraction kit (MBL Co., Ltd., Nagoya, Japan). It was amplified for determination of genotypes by

CR

**Table 1.** Histological evaluation of liver biopsy specimens.

<b>(A) Fibrosis staging</b>			
<b>Stage</b>		<b>Fibrosis</b>	
0	None		
1	Enlarged, fibrotic portal tracts		
2	Periportal or portal-portal septa but intact architecture		
3	Fibrosis with architectural distortion without obvious cirrhosis		
4	Probable or definite cirrhosis		
<b>(B) Inflammation grading</b>			
<b>Grade</b>	<b>Portal/periportal activity</b>		<b>Lobular inflammation</b>
	<b>Piecemeal necrosis</b>	<b>Lymphocyte aggregation</b>	
0	None or minimal	None	None
1	Inflammation only	< 1/3 in portal triad	Inflammation alone
2	Mild	1/3–2/3 in portal areas	Focal necrosis or acidophil bodies
3	Moderate	> 2/3 in portal areas	Severe focal cell damages
4	Severe	Entire portal triad	Damage with bridging necrosis

the SMITEST HBV Genotyping Kit (MBL Co., Ltd.) based on hybridization with type-specific probes immobilized on a solid-phase support [24].

#### **Precore stop codon (G1896A) and core promoter (A1762T/G1764A) mutations**

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

#### **Histological examination**

Liver biopsy was taken by fine-needle aspiration (16G sonopsy) guided by ultrasonography. Biopsy specimens were fixed in 10% neutral formalin, cut at 3- to 4- $\mu$ m thickness, and stained with Hematoxyline-Eosin and Azan-Mallory, as well as for silver to visualize reticulin fibers. Tissue sections were examined independently by two senior liver pathologists. For each biopsy specimen, a protocol was filled out for grading necro-inflammation and staging fibrosis by the criteria of Desmet et al. [26] and Scheuer [27] (Table 1). As for the portal activity, not only piecemeal necrosis, but also lymphocytic aggregation was categorized into 5 (0–4) grades in the respective area involved.

#### **Statistical analysis**

Continuous variables were compared between groups by the Mann-Whitney *U* test, and categorical variables by  $\chi^2$  and Fisher's exact tests. The cumulative incidence of HBeAg seroconversion was calculated using the Kaplan-Meier

method, and the difference was evaluated by the log-rank test. Multiple logistic regression analysis was performed to identify independent factors in significant association with early HBeAg seroconversion. A *p* value <0.05 was considered significant. Statistical analyses were performed using the SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA).

## **RESULTS**

#### **Baseline characteristics of patients**

Among the 673 patients with HBsAg who had received liver biopsies in our hospital during 1991 through 2005, 234 (34.8%) patients who met the inclusion criteria were enrolled in this study. Demographic and laboratory characteristics at the time of liver biopsy are listed in Table 2. They had a median age of 37 years (range: 12–74), and 161 (69%) were men. Of them, 231 (99%) were infected with HBV of genotype C. The median serum ALT level at the baseline was 141 IU/l (range: 13–2644 IU/l), and the median duration of follow-up was 86.5 months (range: 12.0–213.0 months). During the follow-up, 91 (39%) received antiviral treatment, with interferon (IFN) or lamivudine, or the combination thereof.

#### **Comparison of clinical features between patients with and without early HBeAg seroconversion**

Early HBeAg seroconversion, within 1 year after receiving liver biopsies, was achieved by 58 of the 234 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: ALT (*p*=0.002), IP-10 (*p*=0.029), HBsAg (*p*=0.003), HBeAg (*p*<0.001), HBV DNA (*p*=0.001), HBcrAg (*p*<0.001), CP mutations (*p*=0.040), fibrosis (*p*=0.033) and lobular inflammation (*p*=0.002). Other factors including age, albumin, platelets, AFP, PreC mutation, cell infiltration and