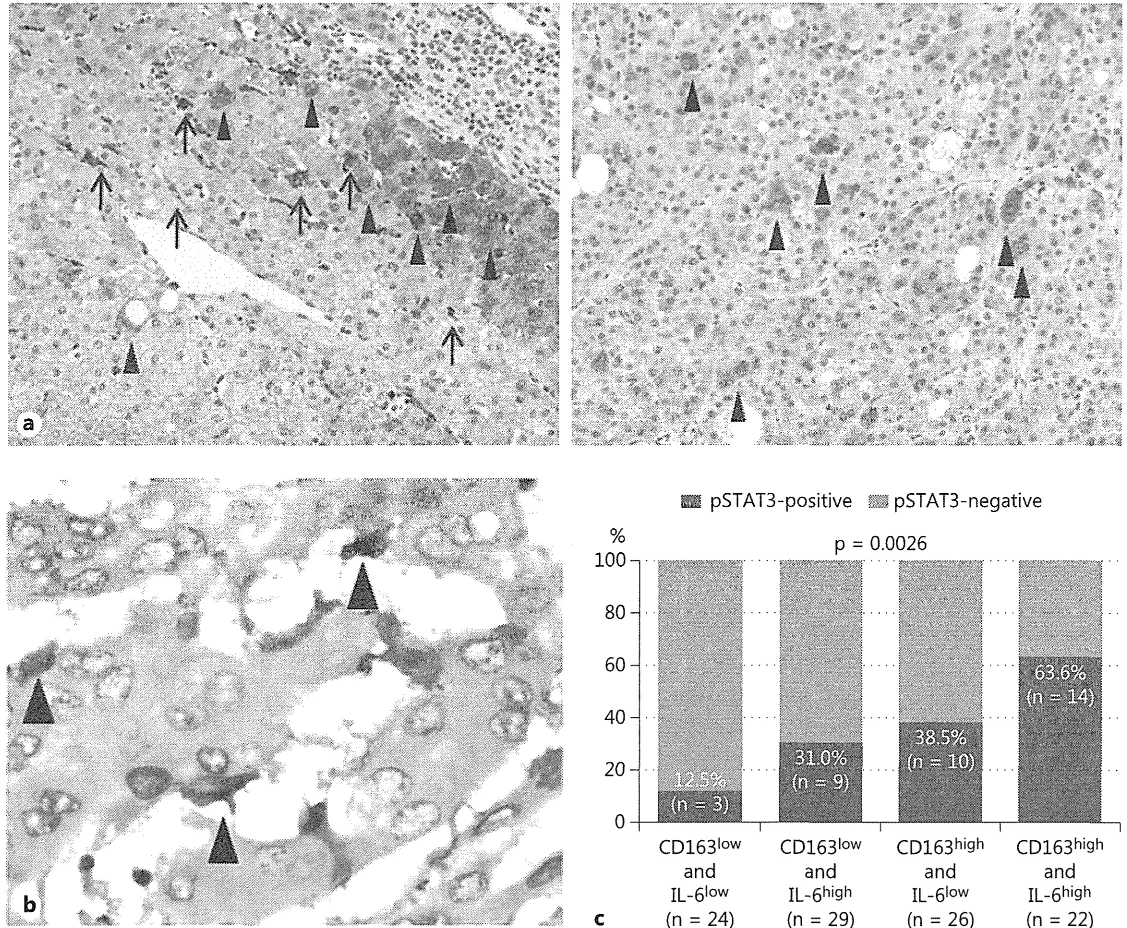

Fig. 3. Tumor-associated macrophages expressed IL-6. **a** Immunohistochemical staining of IL-6 in HCC. ×200. Not only macrophages (arrows, left panel) but also some hepatocytes (arrowheads, left panel) and some tumor cells (arrowheads, right panel) showed immunoreactivities for IL-6. **b** Double staining of IL-6 (red) and CD163 (brown). ×400. Double-positive cells (arrowheads) were frequently seen in the tumor. **c** Correlation between pSTAT3-positive and IL-6/CD163-positive staining.

tive cells in areas where pSTAT3-positive and pSTAT3-negative HCC cells existed in the pSTAT3-positive group (n = 36), and figure 2c shows the boxplots of the analyses (mean ± SD: pSTAT3-negative area, 27.7 ± 17.9; pSTAT3-positive area, 42.6 ± 26.6). In the pSTAT3-positive group, CD163-positive cells in areas where pSTAT3-positive HCC cells existed were statistically higher than in those where pSTAT3-negative HCC cells existed ($p = 0.0064$; fig. 2c).

Cytokine Expression of Macrophages

IL-6 was stained in some macrophages, HCC cells and normal hepatocytes (fig. 3a). According to the double staining of CD163 and IL-6, CD163-positive cells (tumor-associated macrophages) expressed IL-6 (fig. 3b). We divided them into two by the median values of positive macrophages of IL-6 and CD163, and thereby classified the 101 cases into four groups such as CD163^{low} and IL-6^{low}, CD163^{low} and IL-6^{high}, CD163^{high} and IL-6^{low}, and CD163^{high} and IL-6^{high}. HCCs containing high infiltration of IL-6- and CD163-positive macrophages exhibited a significantly higher rate of positive staining for pSTAT3 (fig. 3c).

IL-6 Stimulates Cell Proliferation and Migration of Human HCC Cell Lines

IL-6 stimulation increased the levels of pSTAT3 in both PLC/PRF/5 and Huh7 HCC cell lines (fig. 4a). IL-6 stimulation resulted in higher proliferation (fig. 4b) and greater migration distance (fig. 4c) versus control. S3I-

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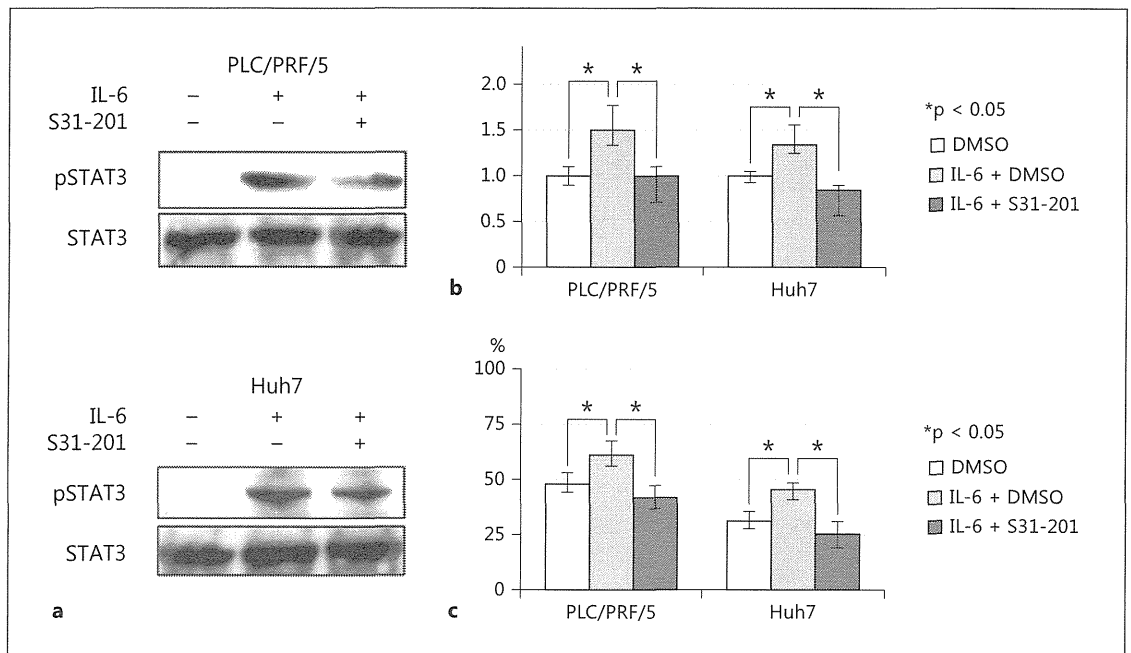

Fig. 4. IL-6 stimulation activated STAT3 signaling and promoted cell proliferation and migration in HCC cell lines. **a** PLC/PRF/5 (upper panel) and Huh7 (lower panel) were treated with 25 ng/ml IL-6 for 30 min. **b** PLC/PRF/5 and Huh7 were cultured with 25 ng/ml IL-6, 100 nM S3I-201 and conditioned medium (1% FBS); cell proliferation was evaluated 48 h after IL-6 stimulation. Control set at 1. **c** PLC/PRF/5 and Huh7 were cultured with 25 ng/ml IL-6, 100 nM S3I-201 and conditioned medium (1% FBS), and wound-healing assay was evaluated 48 h thereafter.

201, a STAT3 inhibitor, inhibited IL-6-induced STAT3 phosphorylation (fig. 4a) and decreased proliferation and migration of HCC cell lines (fig. 4b, c).

Discussion

Our results suggest that macrophage infiltration into HCC tissue stimulates tumor cells via STAT3 signaling. In the present study, pSTAT3-positive HCCs show malignant behavior and confer poor prognosis because of their high abilities of cell proliferation and migration. We found that high pSTAT3 expression was significantly correlated with larger tumor size, higher Ki-67 LI, higher Bcl-XL expression and greater frequency of IMs, and higher pSTAT3 expression was observed in the lesions of IMs and PVIs than in the primary lesions. STAT3 activation upregulates cell cycle-related, antiapoptotic and invasion genes [8–13, 26, 27]. In our results, large tumor size and high Ki-67 LI indicated cell cycle progression, high Bcl-XL expression indicated antiapoptotic function, and frequent IMs indicated invasive capacity. Furthermore, high pSTAT3 expression in the lesions of IMs and PVIs suggests that the tumor cells with STAT3 activation in the primary lesions tended to invade the vessels and metastasize to the other liver sites. Xie et al. [26, 27] reported that activated STAT3 regulated tumor invasion of melanoma cells by regulating the gene transcription of matrix metalloproteinase 2. These results suggest that pSTAT3 expression plays an important role for cell survival and migration in HCC, consistent with previous studies in HCC [14, 17, 28, 29] and other tumors [5, 8–12, 26, 27, 30].

In recent years, it has been recognized that the balance between tumor immunity and tumor progression is important [31]. The present study revealed that tumor-associated macrophages are important for pSTAT3 expression of tumor cells. First, CD163-positive cells around pSTAT3-positive HCC cells were statistically more prevalent than around pSTAT3-negative HCC cells. Some of the CD163-positive cells expressed IL-6 in HCC tissue, and STAT3 was phosphorylated by IL-6 stimulation in vitro. These results suggest that tumor-associated macrophages can activate HCC cells via STAT3 signaling by IL-6 expression. However, CD-163-positive cells were detected not only in the pSTAT3-negative tumor area but

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also in the pSTAT3-positive tumor area and in noncancerous liver tissue. IL-6-secreting tumor-associated macrophages may be part of the CD163-positive cells, and the CD163-positive cells in the pSTAT3-positive tumor area were more stained by IL-6 than in the pSTAT3-negative tumor area and normal liver tissue (data not shown). Tumor-associated macrophages express immunosuppressive cytokines including IL-4, IL-6, IL-10, IL-17, and IL-23 [32, 33]. These cytokines activate immunosuppressive inflammatory cells such as other tumor-associated macrophages, helper T cells and regulatory T cells and suppress antitumor inflammatory cells such as lymphocytes, natural killer cells and dendritic cells [34–36]. Kuang et al. [32] showed that tumor-associated macrophages expressed IL-6 in vitro, whereas Ding et al. [21] reported that tumor-associated macrophage was correlated with poor prognosis in HCC. Our results are consistent with these previous reports.

Both proliferation and migration of PLC/RPF/5 and Huh7 were increased following IL-6 stimulation and STAT3 phosphorylation. On the other hand, IL-6 was expressed in not only macrophages but also in HCC cells. STAT3 can be activated through autocrine signaling in HCC cells; moreover, other cytokines and growth factors might activate STAT3 of tumor cells [22–24]. It is very difficult to exclude activation of STAT3 by the autocrine manner. In our data, STAT3 activation of HCC cells was not correlated with surrounding IL-6-positive normal hepatocytes and HCC cells but it was correlated with the infiltration of CD-163-positive cells (fig. 2). Thus, we thought that the IL-6 secretion of tumor-associated macrophages is more important for STAT3 activation of HCC cells than the IL-6 secretion of other cells.

Recently, STAT3 phosphorylation inhibitors such as S3I-201 have been investigated in vitro and in vivo [28–30]. Avella et al. [37] reported that STAT3 can be one of the targets of chemoimmunotherapies. In our study, S3I-201 inhibited IL-6-induced STAT3 phosphorylation in vitro and decreased cell proliferation and migration. The inhibition of tumor-associated macrophages as therapeutic strategy of malignancy has been investigated, too [38–41]. Therefore, it is very important to suppress tumor-associated macrophage activation and STAT3 signaling in the treatment of HCC. Furthermore, tumor-associated macrophage activation requires STAT3 signaling [22]. We consider that the STAT3 inhibitor may suppress STAT3 activation in both tumor cells and tumor-associated macrophages, release antitumor immune systems from suppression by tumor-associated macrophages and thereby control tumor progression of HCC. Therefore, STAT3 signaling is a feasible therapeutic target for HCC.

In conclusion, STAT3 activation is one of the prognostic factors in HCC. Tumor-associated macrophage expresses IL-6 and can activate STAT3 signaling of HCC cells, resulting in their cell proliferation, antiapoptosis and migration. In the future, HCC may be suppressed by inhibition of STAT3 signaling of tumor cells and tumor-associated macrophages.

Disclosure Statement

The authors have no conflicts of interest.

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Journal of Medical Virology 85:987–995 (2013)

Impact of Peginterferon Alpha-2b and Entecavir Hydrate Combination Therapy on Persistent Viral Suppression in Patients with Chronic Hepatitis B

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The ideal approach to treat chronic hepatitis B remains controversial. This pilot study aimed to evaluate the effectiveness of peginterferon (PEG-IFN) α-2b and entecavir hydrate (ETV) as a combination therapy for patients with chronic hepatitis B, particularly in the context of virological response and the reduction of intrahepatic covalently closed circular DNA (cccDNA). A total of 17 patients with hepatitis B virus (HBV) genotype C were enrolled in this study. All subjects were treated with this combination therapy for 48 weeks and observed for an additional 24 weeks. All patients underwent liver biopsy before and after the therapy period. Changes in cccDNA levels and liver histology were monitored between biopsies. Among the 11 patients who exhibited pretherapy hepatitis B e antigen (HBeAg), 8 (73%) showed evidence of HBeAg seroconversion by the end of the follow-up period. Serum HBV DNA levels decreased by 5.2 and 3.3 log copies/ml (mean) by the end of the therapy and follow-up periods, respectively. In addition, intrahepatic cccDNA decreased significantly to 1.4 log copies/μg (mean) by the end of the therapy period. Among the 11 patients who did not experience viral relapse, only 2 (18%) exhibited high levels of cccDNA (>4.5 log copies/μg) by the end of the treatment period. In contrast, all relapsed subjects exhibited significantly higher levels of cccDNA than subjects who did not relapse ($P = 0.027$). The combination regimen is a promising approach to treat chronic hepatitis B and may achieve significant reduction in serum HBV DNA and intrahepatic cccDNA. ***J. Med. Virol. 85:987–995, 2013.*** © 2013 Wiley Periodicals, Inc.

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KEY WORDS: hepatitis B virus; peginterferon α-2b; entecavir hydrate; combination therapy; covalently closed circular DNA

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) occurs commonly and is associated with increased risk of cirrhosis and the development of hepatocellular carcinoma [Lai et al., 2003]. This type of hepatitis is a worldwide health problem, but achievement of sustained suppression of HBV replication by conventional antiviral agents is sometimes difficult because of the unique nature of HBV replication. For example, after it infects hepatocytes, linear HBV DNA transforms into covalently closed circular DNA (cccDNA), which represents the intracellular HBV template [Newbold et al., 1995; Arase et al., 2002]. Various nucleotide analogues, such as lamivudine (LVD) [Dienstag et al., 1995; Lai et al., 1998; Leung

Additional supporting information may be found in the online version of this article.

Conflicts of interest: The authors declare no conflicts of interest.

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et al., 2001], adefovir dipivoxil (ADV) [Hadziyannis et al., 2003; Marcellin et al., 2003], entecavir (ETV) [Chang et al., 2006; Lai et al., 2006], and tenofovir disoproxil fumarate have been approved as treatments to suppress HBV replication. However, the mechanism of action of nucleotide analogues is limited to reverse transcription and does not decrease the quantity of cccDNA; the cessation of this type of treatment frequently results in viral relapse. In addition, long-term use of nucleotide analogues is hampered by considerable emergence of resistant mutants [Yuen et al., 2001, 2007; Lok et al., 2003].

On the other hand, peginterferon (PEG-IFN) is known to reduce the quantity of cccDNA, presumably by inducing cytotoxic T lymphocytes (CTL), which destroy infected hepatocytes [Wursthorn et al., 2006]. Despite its rapid anti-viral effects, PEG-IFN monotherapy alone is less effective than nucleotide analogues [Wu et al., 1990]. From this perspective, a combination approach of immune modification (PEG-IFN) and blockade of reverse-transcription (nucleotide analogues) conceivably may compensate for the antiviral shortcomings inherent to each as a monotherapy, and thus, appears promising for achieving long-term suppression of viral replication that continues after the completion of antiviral therapy. However, a relatively low amount of data has been generated on the combined use of PEG-IFN and nucleotide analogues to treat chronic hepatitis B.

The present study evaluated prospectively the effectiveness of combined PEG-IFN α-2b and ETV treatment in patients with chronic hepatitis B. ETV was selected among several nucleotide analogue options because it exerts the strongest antiviral activity and has the lowest incidence of resistant mutation [Chang et al., 2006; Lai et al., 2006]. A systematic and comprehensive analysis was conducted to establish an HBV profile based on several related markers, which included serial measurements of HBeAg, anti-HBe antibody, serum HBV DNA and RNA, and intrahepatic cccDNA, and histological evaluations throughout the clinical course. This report offers profound insight on the antiviral impact of PEG-IFN and ETV combination therapy in patients with chronic hepatitis B.

METHODS

Patient Characteristics and Study Design

A total of 17 patients with chronic hepatitis B received combination therapy of PEG-IFN α-2b (PegIntron, Schering-Plough; Kenilworth, NJ) and ETV (Baraclude, Bristol-Myers Squibb; Princeton, NJ) between February 2008 and April 2010 in Kinki University Hospital or Osaka Red Cross Hospital. All patients were serum-positive for hepatitis B surface antigen (HBsAg) for at least 6 months. Additional inclusion criteria included serum HBV DNA levels greater than 5 log copies/ml at a measurement obtained 4 weeks before the first biopsy, serum alanine aminotransferase (ALT) levels greater than 31 IU/ml, and no treatment with nucleic acid analogues or IFN within 3 years prior to study initiation. Subjects with hepatitis C virus, hepatitis D virus, human immunodeficiency virus, a history of hepatocellular carcinoma, autoimmune hepatitis, primary biliary cirrhosis, or decompensated cirrhosis were excluded from the study. Patient characteristics are listed in Table I.

After patients provided informed consent, both drugs were administered throughout the 48-week treatment phase. Treatment consisted of daily doses of oral ETV (0.5 mg) and weekly subcutaneous injection of PEG-IFN α-2b (1.5 μg/kg body weight). PEG-IFN α-2b was selected for the IFN component of therapy because its dosing strategy is adjusted for body weight. For histological analysis and assessment of intrahepatic viral DNA, liver biopsy samples were obtained before and after the 48-week treatment period. All biopsies were performed percutaneously. The 48-week treatment phase was followed by a 24-week treatment-free phase. The protocol included ETV monotherapy after the 24-week follow-up phase for subjects who had relapsed after they received the full combination treatment of PEG-IFN α-2b and ETV. The schematic representation of schedule is shown in Supplementary Figure 1. The Medical Ethics Committee of Kinki University School of Medicine and Osaka Red Cross Hospital approved this study.

Response to Therapy

The virological response to combination therapy was defined as a decrease in serum HBV DNA

TABLE I. Characteristics of the Patients at Baseline

	HBeAg-positive (n = 11)	HBeAg-negative (n = 6)	Overall (n = 17)
Age (year, mean ± SD)	45 ± 12	50 ± 11	47 ± 12
Gender (male, no.; %)	9 (82)	4 (67)	13 (76)
Serum HBV DNA (log copies/ml, mean ± SD)	7.8 ± 1.3	6.8 ± 1.3	7.5 ± 1.4
ALT (IU/l, mean ± SD)	191 ± 161	93 ± 77	157 ± 143
Necroinflammation score (mean ± SD)	5.9 ± 2.3	5.5 ± 3.5	5.8 ± 2.7
No. of cases with F score >3 (%)	9 (82)	3 (50)	12 (71)
cccDNA (log copies/μg, mean ± SD)	5.8 ± 1.1	4.8 ± 0.5	5.4 ± 1.0

All patients analyzed were Asian with HBV of genotype C.
SD, standard deviation; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; IU, international unit.

4.0 log copies/ml or less. The biological response was defined as a decrease in serum ALT to normal levels of 40 IU/L or less. A complete response was defined as the achievement of HBeAg seroconversion, which was indicated by the loss of HBeAg and appearance of anti-HBe antibody, and evidence of positive virological and biological response throughout the follow-up phase. For HBeAg-negative patients, a virological and biological response that did not include the appearance of HBeAg was considered a complete response.

Virological Analyses

Serum levels of HBsAg, anti-HBs, HBeAg, and anti-HBe were measured using the chemiluminescent immunoassay (CLIA) from the ARCHITECT kit (Abbott Japan; Tokyo, Japan). The serum hepatitis B core-related antigen (HBcrAg) was quantified using the chemiluminescent enzyme immunoassay (CLEIA) with Lumipulse HBcrAg (Fujirebio, Tokyo, Japan). Luminescence was detected using Lumipulse f (Fujirebio) [Kimura et al., 2002]. The HBV DNA genotype was determined by PCR-restriction fragment length polymorphism. Serum HBV DNA was quantified using TaqMan PCR (Roche Diagnostics, Mannheim, Germany); the lower limit of quantification for HBV DNA was 2.1 log copies/ml [Allice et al., 2007].

Quantitation of HBV RNA and Intrahepatic cccDNA

HBV RNA was quantified by the method reported by Rokuhara et al. [2006].

DNA was extracted from liver biopsy specimens using the QIAamp DNA mini kit (Qiagen, Hilder, Germany) according to the manufacturer's instructions. To quantify HBV cccDNA, real-time PCR on 500 ng DNA was performed. The DR1 and DR2 regions of HBV cccDNA were amplified using forward and reverse primer sequences that is 5'-CTGCTGTGC CTTCTCATCTGC-3' and 5'-GCTCAGCTTGGAGGCTT-GAC-3', respectively. The sequence of the TaqMan probe was 5' FAM-AACAATTTATGCCTACAG-MGB 3'. The quantitative real-time PCR procedure was performed in an ABI 7700 or 7900 system (Applied Biosystems, Foster City, CA). The real-time PCR procedure included 40 cycles, each of which began with 40 sec of denaturation at 95°C and 90 sec of annealing and polymerization at 60°C. The amplified gene was used as an endogenous control for quantification purposes. The standard curve was generated by serial dilution of plasmid DNA that contained HBV genotype C DNA.

Immunostaining of Hepatitis B Virus Core Antigen

Hepatitis activity was evaluated in liver biopsies by determining the Knodell necroinflammatory score [Knodell et al., 1981]. Fibrosis was scored according to the Ishak fibrosis scoring system [Ishak et al., 1995]. Formalin-fixed, paraffin-embedded sections were subjected to immunohistological staining with antiserum specific for hepatitis B virus core antigen (HBcAg) (Dako Cytomation, Carpinteria, CA). For quantification, the mean percentages of HBcAg-positive hepatocytes were determined. Five fields of images were obtained for each sample and stain-positive nuclei were enumerated by Image J software (NIH, Bethesda, MD).

Evaluation of Safety

Hematological and biochemical examinations were performed weekly during the first 12 weeks of the treatment phase. Thereafter, blood-based examinations were performed every 4 weeks until week 28, every 8 weeks until the end of the treatment (48 weeks), and 4, 8, 12, and 24 weeks after the treatment ended. The WHO Toxicity Grading Scale was used to assess adverse reactions.

Statistical Analyses

To determine significant differences between groups, the Fisher's exact test and paired t-test were used as appropriate. A P-value of less than 0.05 was considered significant. All analyses described above were performed using the SPSS program (version 11.5, SPSS, Inc.; Chicago, IL).

RESULTS

Virological and Biological Response to Combination Therapy

All patients enrolled in the study completed the combination therapy regimen. The dose of PEG-IFN α-2b was reduced to 40 μg for three patients, and in one case, PEG-IFN α-2b was suspended at week 46 because of adverse side effects. All patients received regular, oral doses of ETV (0.5 mg) every day for 48 weeks. Baseline levels of mean ALT and HBV DNA ($\pm$SD) were 157 $\pm$ 143 IU/ml and 7.5 $\pm$ 1.4 log copies/ml, respectively, and the mean necroinflammatory score was 5.8. An advanced F score of 3 or 4 was observed in 12 of 17 patients (71%) (Table I).

At the end of the 48-week treatment period, the mean level of serum HBV DNA in all patients was 2.3 log copies/ml, and the mean decrease in HBV DNA was 5.2 log copies/ml ($P < 0.01$). A virological response was achieved in 14 patients (14/17, 94%). By the conclusion of the follow-up phase, the mean level of serum HBV DNA was 4.1 log copies/ml and the mean decrease in HBV DNA was 3.3 log copies/ml less than the baseline ($P < 0.01$); 12 of 17 cases (71%) achieved virological response (Fig. 1A; Table II). Biological response was achieved in 13 patients (76%) by the end of the treatment period and in 14 patients (82%) by the end of the follow-up phase (Fig. 1B). Of the 11 HBeAg-positive patients, HBe seroconversion was observed in four patients (36%) by the end of the treatment period and in eight

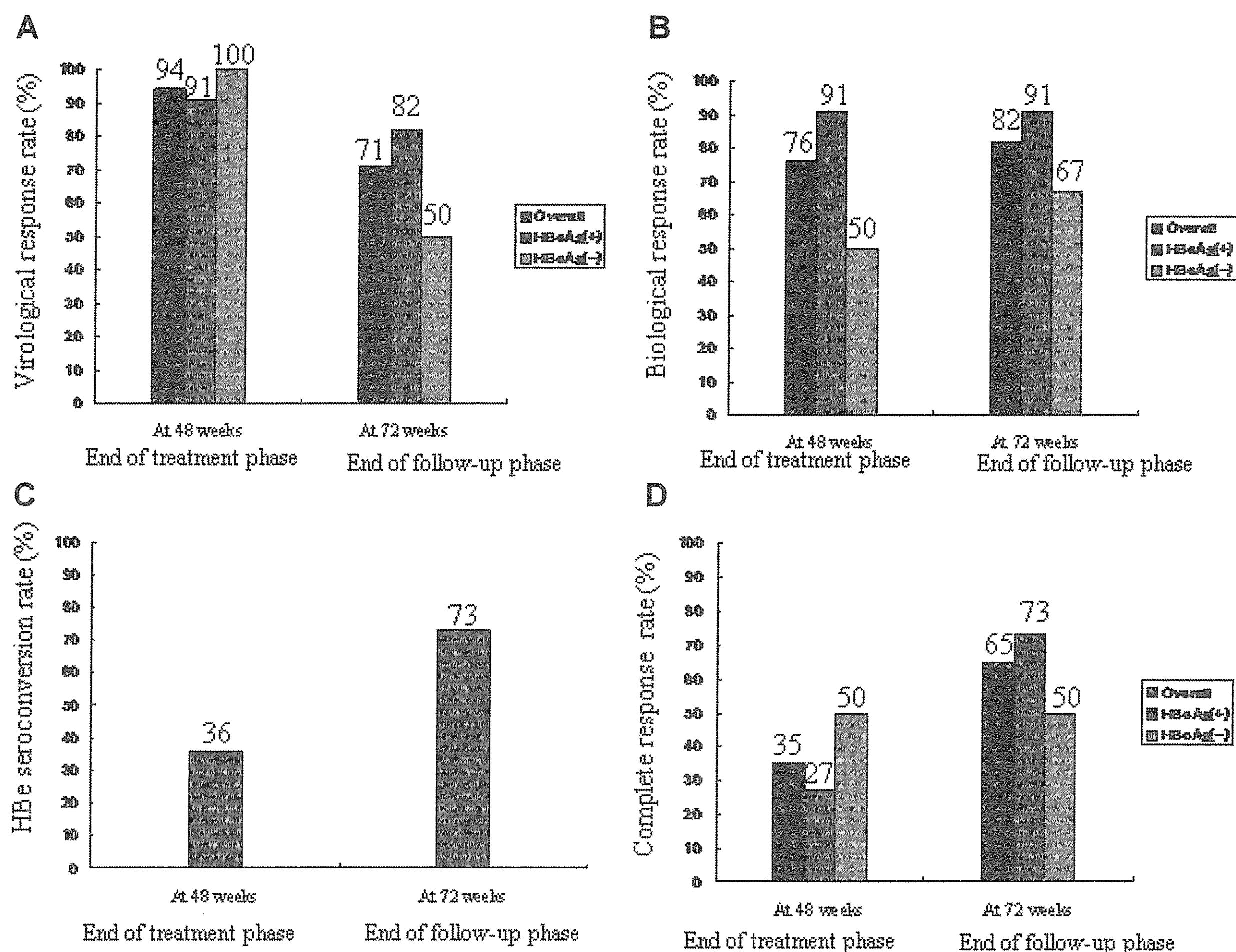

Fig. 1. Virological and biological response during and after combination therapy. **A**: Comparison of effective virological response (<4.0 log copies/ml) at the end of the treatment period with the response 24 weeks after treatment ended. **B**: Comparison of effective biological response (<40 IU/L) at the end of the treatment period with the response 24 weeks after treatment ended. **C**: Comparison of HBeAg seroconversion at the end of the treatment period with seroconversion 24 weeks after treatment. **D**: CR rates 24 weeks after the treatment period ended.

TABLE II. Summary of Therapy Effects at 48 and 72 Weeks

Serological and histological parameters	At 48 weeks	At 72 weeks
Serum HBV DNA reduction (log copies/ml, mean)	−5.2	−3.3
Serum HBV DNA <2.1 log copies/ml	14/17 (82%)	5/17 (29%)
Serum HBV DNA <4.0 log copies/ml	16/17 (94%)	12/17 (71%)
Serum HBsAg reduction (log IU/ml, mean)	−0.4	N.E.
Serum HBcrAg reduction (log U/ml, mean)	−0.7	N.E.
Serum HBV RNA reduction (log copies/ml, mean)	−2.0	N.E.
HBsAg seroconversion	0/17 (0%)	1/17 (6%)
HBeAg seroconversion	4/11 (36%)	8/11 (73%)
ALT improvement	14/17 (82%)	14/17 (82%)
ALT normalization	13/17 (76%)	14/17 (82%)
cccDNA reduction (log copies/μg, mean)	−1.4	N.E.
Inflammation score improved	14/17 (82%)	N.E.
Fibrosis score improved	5/17 (29%)	N.E.

HBsAg, hepatitis B surface antigen; IU, international unit; HBcrAg, hepatitis B core-related antigen; U, unit; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; N.E., not evaluable.

patients (73%) by the end of the follow-up phase (Fig. 1C); 65% achieved complete response (Fig. 1D).

Improved Histology and Decreased HBcAg-Positive Hepatocytes and cccDNA After Combination Therapy

Scores improved for inflammation in 14 patients (82%; Fig. 2A) and for fibrosis in five patients (29%; Fig. 2B) after combination treatment. These biopsies were used for simultaneous immunohistochemical analyses of HBcAg (Fig. 3). The median values and ranges of HBcAg-positive hepatocytes in each patient are listed in Supplementary Table I. The mean percentage of HBcAg-positive hepatocytes was 11.2% at baseline and 0.9% after the 48-week treatment phase. A decrease in hepatic cccDNA was observed in 14 of 17 patients (82%). The mean level of hepatocyte cccDNA decreased from 5.4 to 4.0 log copies/μg after the 48-week treatment period ($P = 0.007$, Fig. 4). In two patients, the level of cccDNA dropped dramatically by more than 3 log copies/μg after combination therapy. Each patient's chronic hepatitis B profile, including altered serum markers, HBV DNA, HBV RNA, and histological assessments, is listed in Supplementary Table I.

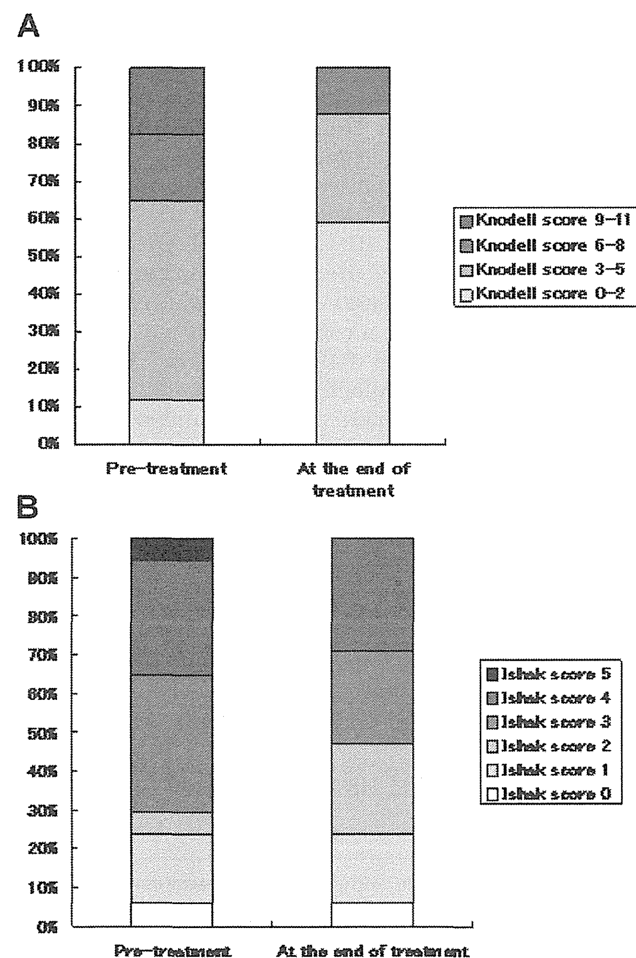

Fig. 2. Improved histology after combination therapy. Histology was evaluated before treatment initiation and at the end of the 48-week treatment period. Improved inflammation scores were evident in 14 patients (82%) (**A**), and improved fibrosis scores were evident in five patients (29%) (**B**).

Associations Among cccDNA, Relapse, and Serum Viral Markers at the End of the 48-week Treatment Period

The 14 patients who exhibited a decrease in serum DNA to less than 2.1 log copies/ml by the end of the 48-week treatment period were analyzed subsequently for associations between intrahepatic cccDNA and post-treatment relapse. Among the 14 patients with undetectable levels of serum HBV DNA at the end of treatment, 3 did not achieve virological response by the end of the follow-up phase; they were considered relapse cases of hepatitis. All three patients exhibited high levels of intrahepatic cccDNA (>4.5 log/copies/μg) by the end of the treatment. In contrast, only 2 of the 11 non-relapsed patients exhibited high levels of cccDNA by the end of the treatment period ($P = 0.027$, Table III). These data suggest that high levels of intrahepatic cccDNA can be interpreted as a risk factor for the relapse of hepatitis. However, by the end of the 48-week treatment period, cccDNA did not correlate significantly with serum viral markers (HBsAg, HBV RNA, and HBcrAg; date not shown).

Safety

The adverse effects in each patient are summarized in Supplementary Table I. During week 4 of the treatment period, patient No. 4 displayed obvious grade 3 neutropenia and the dose of PEG-IFN α-2b was reduced from 80 to 40 μg. During week 20 of the treatment period, patient No. 5 displayed obvious grade 3 thrombocytopenia and the dose of PEG-IFN α-2b also was reduced from 80 to 40 μg. Both patients were able to continue the treatment until week 48. During week 46, patient No. 10 experienced a grade 3 transient ischemic attack. Because the symptom potentially was caused by PEG-IFN α-2b, PEG-IFN α-2b administration was suspended in this patient and ETV was continued for the 2 weeks that remained in the treatment period. All adverse effects improved after the treatment was discontinued and no deaths occurred.

DISCUSSION

Thus far, several reports have described the effectiveness of combination therapies using PEG-IFN and either LVD or ADV to treat chronic hepatitis B [Chan et al., 2005; Lau et al., 2005; Wursthorn et al., 2006; Flink et al., 2007; Papadopoulos et al., 2009]. However, the therapeutic outcomes of the described approaches remain unsatisfactory. The objective of the present study was to evaluate the effectiveness of

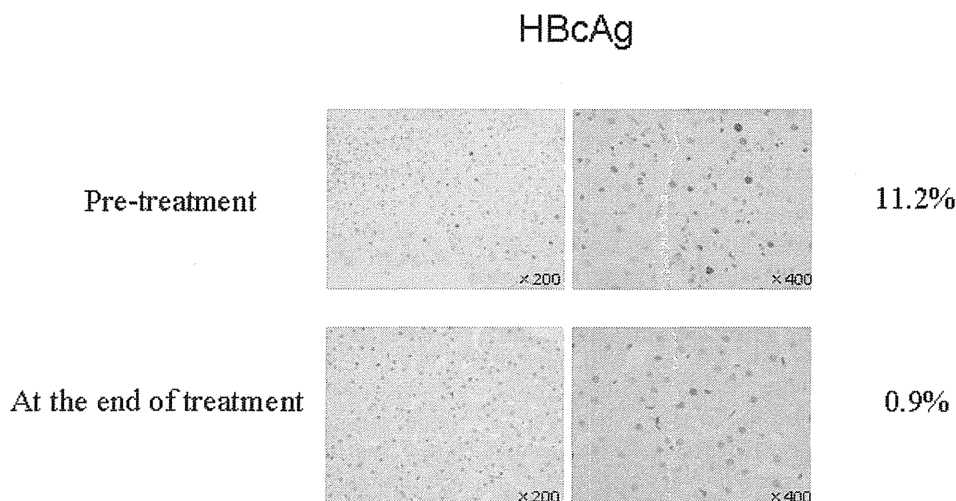

Fig. 3. Reduced quantity of HBcAg-positive hepatocytes after combination therapy. Liver biopsy samples were collected before and after the 48-week period of combination therapy; the data from a representative patient are shown. Liver sections were fixed with formalin, embedded in paraffin, and subjected to immunohistological staining with antiserum specific for HBcAg.

PEG-IFN α-2b and ETV combination therapy. The results indicated that combination therapy with ETV may bring about greater levels of HBV suppression than combination therapies that use other nucleotide analogues.

Among several potential nucleotide analogues, ETV was selected because reports have shown that it possesses stronger anti-viral activity and is less prone to resistant mutations than the other analogues [Chang et al., 2006; Colonno et al., 2006; Lai et al., 2006]. Thus, it is speculated that a combination approach with ETV presented significant promise for achieving persistent viral suppression after the completion of therapy, which is the ultimate goal of chronic hepatitis B treatment. In addition, the levels of intrahepatic cccDNA were evaluated because they may reflect the sustained antiviral effects of a drug directly, to determine the effects of combination therapy.

By the end of the treatment period, 94% of the patients achieved virological response. In addition, 76% (13/17) of the patients achieved biological response. Among the 11 patients who presented with HBeAg initially, 10 (91%) exhibited virological and biological response by the end of the therapy period. In addition, four patients (36%) exhibited HBe seroconversion, and thus, a complete response.

At the end of the follow-up phase, 12 patients (71%) exhibited a virological response, 82% exhibited a biological response rate, and the number of patients

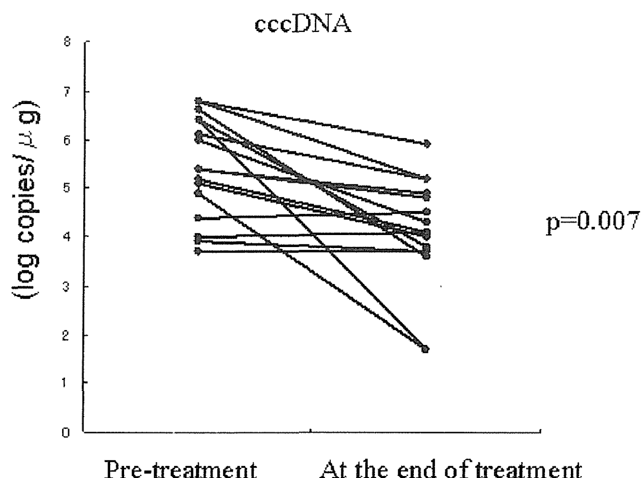

Fig. 4. Reduced levels of cccDNA after combination therapy. Each dot represents one patient, and each solid line represents the changed level of cccDNA in each patient.

TABLE III. Association Between Intrahepatic cccDNA Content and Relapse Rates

	Serum HBV DNA levels <2.1 log copies/ml at the end of the treatment (n = 14)		*P* value
	Cases with non-relapse (n = 11)	Cases with relapse (n = 3)	
No. of cases with high cccDNA level	2/11 (18%)	3/3 (100%)	0.027

Cases with a decrease in serum DNA concentrations to less than 2.1 log copies/ml at the end of the treatment were subjected to analysis. Relapse was defined as serum DNA ≥ 4.0 log at the end of follow-up.
Relapse: serum HBV DNA at 4.0 log copies/ml or higher at the end of follow-up.
Non-relapse: serum HBV DNA less than 4.0 log copies/ml at the end of follow-up.
P value by Fisher's exact test.
High cccDNA level: intrahepatic cccDNA ≥ 4.5 log at the end of treatment.

with a complete response increased to 65% (11/14). For HBeAg-positive patients, the number with a complete response increased from 3 of 11 (27%) at the end of the treatment phase to 8 of 11 (73%) at the end of the follow-up phase. It has been reported that IFN treatment can induce HBeAg seroconversion via immunological mechanisms during and after treatment [Lau, 2009]. From this perspective, the high percentage of patients who achieved complete response after the conclusion of combination therapy can be attributed primarily to the compensatory, immune-modulator effects of IFN.

Previously, the effectiveness of PEG-IFN α-2a monotherapy or combination therapy with LVD was compared among patients with HBeAg-positive chronic hepatitis B [Lau et al., 2005]. However, PEG-IFN α-2a monotherapy failed to reach the level of superiority of the combination therapy with regard to HBeAg seroconversion and virological response; HBeAg seroconversion occurred in 29–32% of patients treated with PEG-IFN α-2a monotherapy and in 27–29% of patients who received combination therapy (Supplementary Table II). Therefore, the authors concluded that combination therapy with LVD does not yield additive effects, and this conclusion supported the use of PEG-IFN α-2a monotherapy as the first-line treatment [Janssen et al., 2005; Lau et al., 2005]. In contrast, by replacing LVD with ETV, HBeAg seroconversion was observed in 73% of study patients at the end of the follow-up phase (week 72) and virological response in 82% of HBeAg-positive patients, which is significantly higher than that in the previous report. Furthermore, our definition of a "positive" virological response (<4.0 log copies/ml) was more strict than the previously reported definition (<5.0 log copies/ml), and therefore, it is speculated that the combination of PEG-IFN and EVT is more effective than the combination of PEG-IFN and LVD. The superiority of the PEG-IFN and ETV combination was observed even in HBeAg-negative patients. The biological and virological response rates of PEG-IFN alone was 59% and 43%, respectively, and the rates of a PEG-IFN and LVD combination were 60% and 44%, respectively (Supplementary Table II). In contrast, the biological and virological response rates to combined PEG-IFN and ETV treatment were 67% and 50%, respectively. Based on the response levels of ALT and HBV DNA in our cohort, all HBeAg-positive patients who had achieved virological and biological response also exhibited HBeAg seroconversion at the end of the follow-up phase and vice versa. From this perspective, achievement of both virological and biological response at the end of follow-up phase was considered to be critical to HBeAg-positive and HBeAg-negative patients. Therefore, the definition of complete response was modified to include HBeAg-negative patients and the overall complete response ratio was calculated. In the present cohort, 65% achieved complete response (Fig. 4D), which was considerably high for the treatment of chronic hepatitis B. Considering these data, it is speculated that PEG-IFN and ETV may function as a more powerful and ideal combination treatment that yields additive effects.

Recent studies have shown that relapse of hepatitis sometimes occurs after antiviral therapy is discontinued, even in patients who exhibited decreased levels of serum HBV DNA, because of the protracted half-life of cccDNA in infected hepatocytes [Moraleda et al., 1997; Le Guerhier et al., 2000; Mommeja-Marin et al., 2003; Werle-Lapostolle et al., 2004; Sung et al., 2005; Laras et al., 2006]. Therefore, a critical component of any assessment of anti-HBV therapy is the reduction of intrahepatic cccDNA. In the present study, intrahepatic cccDNA dropped by a mean of 1.4 log copies/μg by the end of the treatment period.

This study investigated the relationship between intrahepatic cccDNA at the end of the treatment period and viral reactivation at the end of the follow-up phase in patients with undetectable levels of serum HBV DNA at the end of the treatment period. All relapsed patients displayed high levels of intrahepatic cccDNA ($\geq$4.5 log copies/μg) at the end of the treatment period, whereas, only 2 of the 11 non-relapse patients displayed high levels of intrahepatic cccDNA. The findings from both the present study and previous reports [Moraleda et al., 1997; Le Guerhier et al., 2000; Werle-Lapostolle et al., 2004; Laras et al., 2006] support the notion that cccDNA levels predict long-term suppression of HBV more accurately than serum HBV DNA. Subsequently, non-invasive serum markers that may reflect intrahepatic levels of cccDNA were evaluated. The mechanism of action of nucleotide analogues barely influences the transcription of cccDNA to mRNA or translation to various viral proteins. Therefore, treatment with nucleotide analogues should still allow HBsAg, HBcrAg [Rokuhara et al., 2003; Tanaka et al., 2008; Matsumoto et al., 2012], and HBV RNA levels to function as surrogate markers of intrahepatic cccDNA. Nevertheless, the levels of cccDNA did not correlate with any of these markers in this study. It is possible that maldistribution of cccDNA in liver tissue presents a critical issue to quantification. Therefore, the quantification of cccDNA from multiple liver biopsies from different sites would be ideal for determining the accurate level of cccDNA in each patient. However, because of ethical limitations, only one liver biopsy was performed on each patient and thus the distribution differences from single liver tissue samples could not be evaluated. Nevertheless, the amount of cccDNA observed before treatment initiation correlated significantly with serum HBV DNA ($r = 0.85$, $P < 0.001$) and serum HBsAg ($r = 0.70$, $P = 0.002$), and thus, the levels of cccDNA determined in this study are probably sound. Because of this study's limited number of patients, future studies may yield controversial results with regard to the levels of cccDNA, HBcrAg, and HBV RNA.

Further investigation should be conducted on a larger number of patients.

In summary, this study systematically analyzed the efficacy of a new combination therapy that consists of PEG-IFN α-2b and ETV administered over a 48-week period. In particular, the effectiveness of this combination approach was evident by decreases in intrahepatic cccDNA. Given the robustness of the data, these results are clinically significant with respect to the achievement of sustained viral response, which is the ultimate goal for chronic hepatitis B treatment. However, further investigation is needed on a larger number of patients and to compare combination therapy to PEG-IFN monotherapy. To address these important issues, and validate the results of the present study, a prospective multicenter trial of patients using combination therapy will be conducted.

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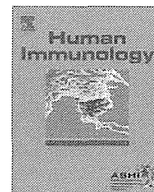

Rapid Communication

Association analysis of toll-like receptor 4 polymorphisms in Japanese primary biliary cirrhosis

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ABSTRACT

Primary biliary cirrhosis (PBC) is characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts that often result in liver failure. Toll-like receptor (TLR) 4 recognizes lipopolysaccharides of Gram-negative bacteria. Infectious agents have been suspected to play a crucial role in PBC pathogenesis since TLR4 expression was found in bile duct epithelial cells and periportal hepatocytes in liver tissues of PBC. To assess the potential contribution of *TLR4* SNPs to the development of this disease, we genotyped five SNPs in *TLR4* in 261 PBC patients and 359 controls using a TaqMan assay. No significant positive associations with either PBC susceptibility or progression were uncovered. These results indicate that *TLR4* polymorphisms do not play a prominent role in the development of PBC in Japanese patients.
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1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts that often result in cirrhosis and liver failure [1]. The cause of PBC remains poorly understood [2], although population and family studies suggest that genetic factors contribute to disease susceptibility and severity [3]. Significant associations of genetic factors, including HLA alleles [4–6], cytotoxic T-lymphocyte antigen 4 [7–10], and other loci [11] have been reported for PBC. Only HLA has consistently been associated with PBC among these susceptibility genes. Specifically, the *DRB1*08* family of alleles has been the most frequently described determinant for this disease [4–6].

Toll-like receptors (TLRs) are a class of evolutionarily conserved pathogen recognition receptors that play an important role in innate identification of foreign material [12]. Activation of TLRs induces both innate and adaptive immune reactions against invading pathogens. TLR4 is a receptor for bacterial lipopolysaccharide (LPS) which selectively binds the lipid A portion of LPS. It was also found to be expressed in bile duct epithelial cells and periportal hepatocytes in PBC patient liver tissues [13,14]. Since several bacterial products were detected in sera or liver tissues of PBC patients [15–17], infectious agents might play a crucial role in disease pathogenesis [18]. *TLR4* single nucleotide polymorphisms (SNPs) have been reported to be associated with genetic susceptibility to autoimmune diseases [19–22], but these genes have not been examined with respect to PBC. As such, we hypothesized that *TLR4* SNPs may be associated with PBC in the Japanese population and examined eight SNPs for associations with susceptibility and progression in Japanese patients.

2. Subjects and methods

2.1. Study subjects

Between January 2005 and December 2011, a total of 261 patients with PBC (234 women, median age: 58 years, range: 27–86 years) and 359 healthy subjects (319 women) participated in this study. All control subjects had indicated the absence of major illness on a standard questionnaire. Racial backgrounds were all

Abbreviations: PBC, primary biliary cirrhosis; TLR, toll-like receptor; LPS, lipopolysaccharide; SNPs, single nucleotide polymorphisms.

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Japanese. The diagnosis of PBC in all patients was based on criteria from the American Association for the Study of Liver Diseases [23]. Serum anti-mitochondrial antibody-M2 was determined by ELISA, where a >7.0 index was considered to be positive, as previously reported [24]. All patients were negative for hepatitis B surface antigen and antibodies to hepatitis B core antigen, hepatitis C virus, and human immunodeficiency virus. Patients were grouped into two stages of PBC based on their most recent follow-up: early stage patients were histologically classified as Scheuer stage I or II [25] or of unknown histological stage without liver cirrhosis, and late stage patients were histologically Scheuer stage III or IV or clinically diagnosed with liver cirrhosis or hepatic failure [10]. Liver cirrhosis was diagnosed by histological examination and/or characteristic clinical signs of advanced liver disease [26]. Patients with late stage disease or cirrhosis were 53 (20%) and 44 (17%), respectively. All subjects and controls provided written informed consent for testing of DNA samples. This study was approved by the institutional ethics committee.

2.2. Genotypoing of TLR4 SNPs

Genomic DNA was isolated from whole blood extracts for all patients and controls using QuickGene-800 (FUJIFILM, Tokyo, Japan) and adjusted to 10–15 ng/μl. TLR4 is composed of four exons and has four transcript isoforms. We evaluated five SNPs (rs10759930, rs2149356, rs11536889, rs7037117, and rs7045953) which were localized within the exons and introns of the *TLR4* gene. SNPs were selected from among previous reports [27,28] and had minor allele frequencies of >5%. SNP spans were approximately 1–5 kb and included 5 kb of the predicted 5′-untranslated region and 6 kb of the predicted 3′-untranslated region of the *TLR4* gene. Genotyping of all SNPs was performed with a TaqMan 5′ exonuclease assay using primers supplied by ABI (Applied Biosystems, Foster City, CA, USA). The probe fluorescence signals were detected with a TaqMan Assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems) according to the manufacturer's instructions.

HLA typing was carried out using a Luminex multi-analysis profiling system with a LAB type® SSO OneLambda typing kit One (Lamda, Ganoga Park, CA), which is based on polymerase chain reaction sequence-specific oligonucleotide probes. HLA genotypes were determined by sequence-based typing [6].

2.3. Statistical analysis

The Hardy–Weinberg equilibrium test was performed for each SNP between control and patient groups. Pairwise linkage disequilibrium pattern, haplotype block structure, and haplotype frequency analysis were assessed for all SNPs by the block definition by Gabriel et al. [29], and were based on a 95% confidence interval (CI) of D′ with Haploview version 4.2 software [30]. We plotted r^2 values. The significance of allele distribution between patients with PBC and healthy subjects was evaluated using the χ^2 test for 2×2 comparisons. A P value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 18.0J; SPSS, Chicago, IL).

3. Results

A total of five SNPs in the *TLR4* gene were genotyped in 261 patients with PBC and 359 healthy subjects. The observed genotype frequencies for patients and controls were all in Hardy-Weinberg equilibrium, and the minor allele frequencies of all SNPs were >5%. All five SNPs were located in one haplotype block, and the magnitude of linkage disequilibrium between each SNP was high (Fig. 1). Analysis of allelic frequencies revealed no significant differences between PBC and controls for *TLR4* SNPs (Table 1).

The haplotype frequency of the five SNPs was estimated with the expectation-maximization algorithm. Six unique SNP haplotypes were identified, and five of them had frequencies of >5% (Table 2). Association analysis using haplotypes calculated by expectation-maximization algorithms showed that none of them were associated with either susceptibility or resistance to PBC.

Since we previously reported that the HLA *DRB1*08:03-DQ*06:01* haplotype was associated with PBC in Japan, we further investigated the genetic association between this haplotype and the *TLR4* SNPs. Analysis of allelic frequencies revealed no significant differences between the presence and absence of the HLA *DRB1*08:03-DQ*06:01* haplotype and these SNPs (data not shown).

Next, we examined associations between the five *TLR4* SNPs and disease progression. There were neither significant allelic associations nor significant haplotype associations found in comparisons of early and late stage groups with regard to liver cirrhosis or non-cirrhosis (data not shown).

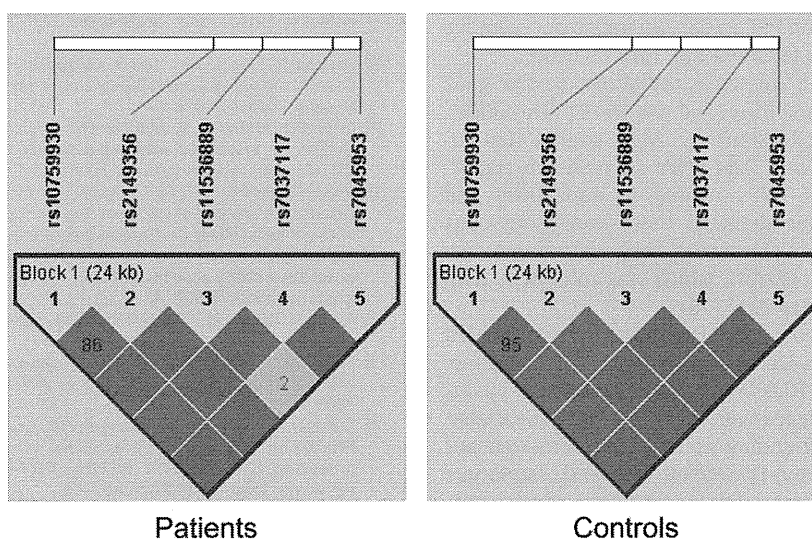

Fig. 1. Linkage disequilibrium plot of five SNPs of the *TLR4* gene in 261 patients with primary biliary cirrhosis and 359 healthy controls. Values of r^2 corresponding to each SNP pair are expressed as a percentage and shown within the respective squares. Higher D' values are indicated by a brighter red color. The five SNPs constitute a haplotype block spanning 24 kb of the *TLR4* gene.

Table 1
Allele frequencies of SNPs in the *TLR4* gene of PBC patients and healthy subjects.

SNP no.	dbSNP	Position (bp)	Minor allele	MAF in PBC	MAF in controls	*P* value	OR	95%CI
1	rs10759930	119,501,442	C	0.366	0.350	0.55	1.07	0.85-1.36
2	rs2149356	119,514,020	T	0.337	0.345	0.76	0.96	0.76-1.22
3	rs11536889	119,517,952	C	0.226	0.253	0.27	0.86	0.66-1.12
4	rs7037117	119,523,484	G	0.190	0.187	0.89	1.02	0.76-1.36
5	rs7045953	119,525,616	G	0.090	0.078	0.45	1.17	0.78-1.75

CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; PBC, primary biliary cirrhosis; TLR4; toll-like receptor 4; SNP, single nucleotide polymorphism. *P* values were calculated with a χ^2-test 2 × 2 contingency table ($df = 1$).

Table 2
TLR4 haplotypes in PBC patients and healthy subjects.

Haplotype	SNPs					Haplotype frequencies		*P* value
	1	2	3	4	5	PBC	Controls	
1	T	G	G	A	A	0.409	0.402	0.78
2	T	G	C	A	A	0.225	0.243	0.46
3	C	T	G	A	A	0.144	0.151	0.73
4	C	T	G	G	A	0.098	0.106	0.65
5	C	T	G	G	G	0.090	0.078	0.44

PBC, primary biliary cirrhosis; TLR4, toll-like receptor 4.

4. Discussion

In the present study, we investigated the possibility of an association between *TLR4* SNPs and PBC in Japan. We found no associations for any of the SNPs analyzed. Several infectious organisms have been proposed as potential causes of PBC [15–17], and TLR4, a specific receptor for LPS, was found in bile duct epithelial cells and periportal hepatocytes in liver tissues of PBC patients [13,14]. Ballot et al. [31] reported that 64% of PBC sera was positive for IgM antibodies against lipid A, an immunogenic and toxic component of LPS. This finding was specific for the disease and correlated with more florid histological lesions. Moreover, Mao et al. reported that PBC patients were hyper-responsive to LPS stimulation, and suggested that aberrant signaling through TLR4 may precipitate disease onset [32]. Therefore, it has been hypothesized that TLR4 and its ligands would be implicated in the development of PBC, but the results of our SNP analysis indicated otherwise. Until now, no reports have been published regarding an association between PBC and *TLR4* SNPs in other ethnicities. Furthermore, genome-wide association studies have shown no significant associations between *TLR4* SNPs and PBC in Caucasians, so our negative association of *TLR4* SNPs with Japanese PBC may be valid.

Of the two co-segregating missense mutations in the gene encoding *TLR4* rs4986790 (Asp299Gly) and rs4986791 (Thr399Ile), only rs4986790 interrupts TLR4 signaling. Most studies that reported disease associations with *TLR4* SNPs have shown significantly higher frequencies of SNPs related to Asp299Gly and Thr399Ile [33], but none have detected these nonsynonymous mutations in Asian populations. Moreover, they were monomorphic in our Japanese healthy controls, which was consistent with other reports, including HapMap data [28].

The *HLA DRB1*08:03-DQB1*06:01* haplotype has been associated with susceptibility to PBC in a Japanese population [6]. Therefore, we investigated whether the HLA *DRB1*08:03-DQB1*06:01* haplotype and *TLR4* SNPs or haplotypes were independently associated with PBC, but found no confounding associations. Although our prior study showed that the HLA *DRB1*09:01-DQ*03:03* haplotype was associated with disease progression [6], we observed no significant associations between *TLR4* SNPs or haplotypes with late stage PBC or cirrhosis in this study.

In conclusion, it appears that *TLR4* SNPs and haplotypes are not associated with susceptibility to PBC in Japan. Genetic variations associated with PBC vulnerability remain open for further investigation, indicating the need for a genome-wide association study of PBC in Japan.

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Original Article

Fibrosis score consisting of four serum markers successfully predicts pathological fibrotic stages of chronic hepatitis B

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Aim: In order to evaluate and judge a fibrotic stage of patients with chronic hepatitis B, multivariate regression analysis was performed using multiple fibrosis markers.

Method: A total of 227 patients from seven hepatology units and institutes were diagnosed by needle biopsy as having chronic liver disease caused by hepatitis B virus. Twenty-three variables and their natural logarithmic transformation were employed in the multivariate analysis. Multiple regression function was generated from data of 158 patients in one hospital, and validation was performed using the other data of 69 patients from six other hospitals.

Results: After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $- 9.15$. Median values of fibrosis scores of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively. Multiple regression coefficient and coefficient of determination were 0.646 and 0.418, respectively. Validation with patient data from other institutions demonstrated good reproducibility of fibrosis score for hepatitis B (FSB), showing 1.33 in F1 ($n = 27$), 2.20 in F2 ($n = 20$), 3.11 in F3 ($n = 20$) and 5.30 in F4 ($n = 2$), respectively.

Conclusion: A concise multiple regression function using four laboratory parameters successfully predicted pathological fibrosis stage of patients with hepatitis B virus infection.

Key words: chronic hepatitis, hepatitis B virus, liver cirrhosis, liver fibrosis, multiple regression analysis, stage

INTRODUCTION

WHEN HEPATITIS B virus (HBV)-related chronic liver disease is found by biochemical and virological examination, liver biopsy can establish the definitive diagnosis of chronic hepatitis and its fibrotic staging. Although these pathological procedures are reliable and informative both in diagnosis and treatment, they sometimes require medical invasion and financial costs, including the risk of bleeding from needle puncture, some pain experienced during the procedure and hospital stays of a few days. The pathological examination is, therefore, rarely performed repeatedly in a short period of time, unless disease activity is severe or progression of liver disease is highly suspected. Recently, many authors described the usefulness of ultrasonographic elastography and multiple resonance imaging technology in the estimation of staging of chronic hepatitis and cirrhosis.[1-5] These ways of estimation using the imaging apparatuses seem truly useful for current patients, but they cannot evaluate and compare with past fibrotic states of patients retrospectively. Moreover,

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the same apparatus for elastometry will not be available for repeated measurement for a follow-up examination, for example, several years later.

In spite of the accuracy of biopsy and convenience of elastography in chronic liver disease, clinical diagnosis based on biochemistry and hematology is still indispensable for the daily practice of many patients with HBV-related liver disease. Recently, several studies were published about estimation of hepatitis stages, using one or more serum biomarkers. Discriminant functions or multivariate analyses demonstrated that approximately 60–90% of patients with chronic hepatitis B were correctly classified as having mild hepatitis and severe hepatitis with advanced fibrosis.[2,6–13] Up to the present time, however, the usefulness of the discriminant functions are less valuable for a few reasons. First, these functions were made for the purpose of discrimination of severe hepatic fibrosis from mild fibrosis, and four histological classifications (F1–F4) were neglected in almost of the studies. Second, some studies analyzed both hepatitis B and hepatitis C virus infection, although the significance and actual values of each liver function test in the evaluation of the severity of liver disease were not similar among each viral hepatitis and alcoholic liver disease. Third, biochemical markers for liver fibrosis (e.g. hyaluronic acid, type IV collagen, procollagen III peptide)[14–16] were not always included in those previous studies.

We tried to generate a function estimating fibrotic stages of HBV-related chronic hepatitis, which were objectively diagnosed by liver biopsy. The purpose of this study is, therefore, to make a reliable multiple regression function and to obtain practical coefficients for significant variables also using fibrosis markers.

METHODS

Patients

A TOTAL OF 273 Japanese patients with chronic hepatitis B were recruited for the study from seven hospitals in Japan: Toranomon Hospital, Hiroshima University Hospital (K. Chayama, M.D.), Ehime University Hospital (M. Onji, M.D.), Musashino Red Cross Hospital (N. Izumi, MD), Shishu University Hospital (E. Tanaka, M.D.), Showa University Hospital (M. Imawari, M.D.) and Osaka University Hospital (T. Takehara, M.D.). Inclusion criteria for this study were: (i) positive hepatitis B surface antigen for more than 6 months; (ii) persistent or intermittent elevation in aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels; and (iii) liver biopsy showing chronic hepatitis (F1–F4). We excluded those patients with overt alcoholic liver disease or fatty liver, association of other types of liver disease (e.g. hepatitis C, primary biliary cirrhosis, autoimmune hepatitis), or those associated with hepatocellular carcinoma or other malignancy. Among the patients, 244 patients fulfilled the conditions for the study: complete demographic data, basic laboratory data of hematology and biochemistry, required liver biopsy specimens, and sufficient amount of frozen sera. Also, we excluded additional 17 patients with eventual histological diagnosis as F0 stage.

Finally, a total of 227 patients who were diagnosed as having chronic hepatitis or cirrhosis (F1–F4) were analyzed for the following hematological, biochemical and histopathological examination. There were 172 males and 55 females aged 16–70 years (median, 39 years).

All the patients presented written informed consent in individual hospitals and medical centers, and the study was approved in each ethical committee.

Hematological and biochemical examination

Hematological and standard biochemical evaluation had been performed in each medical institution: white blood cells, red blood cells, hemoglobin, platelets, total bilirubin, AST, ALT, AST/ALT ratio (AAR), γ-glutamyl transpeptidase (γ-GTP), total protein, albumin and γ-globulin.

Special biochemical examinations including "fibrosis markers" were carried out using stored frozen sera at −20°C or lower: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, procollagen III peptide and type IV collagen 7S.

Histological diagnosis of chronic hepatitis and cirrhosis

All the 227 cases fulfilled required standards of histological evaluation: sufficient length of specimen, hematoxylin–eosin staining, and at least one specimen with fiber staining. Four independent pathologists (Y. T., J. F., F. K. and T. F.), who were not informed of patients' background and laboratory features except for age and sex, evaluated the 227 specimens regarding the stages of fibrosis and activity. Pathological classification of chronic hepatitis staging was based on Desmet *et al.*[17]

Before judgment of histological staging of individual specimens, the pathologists discussed the objective and reproducible judgment of pathological diagnosis of

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hepatitis. They made a panel about obvious criteria using typical microscopic pictures for each stage, and it was always referred to during the procedure of pathological judgment. When inconsistent results were found in the diagnosis of hepatitis stage among the pathologists, the final judgment accepted majority rule among them.

Statistical analysis

Non-parametric procedures were employed for the analysis of background characteristics and laboratory data among patients in each stage, including Mann–Whitney *U*-test, Kruskal–Wallis test and χ^2-test.

The normality of the distribution of the data was evaluated by a Kolmogorov–Smirnov one-sample test. Because certain variables partly did not conform to a normal distribution, natural logarithmic transformation of bilirubin, AST, ALT, γ-GTP, α-2-macroglobulin, hyaluronic acid, type IV collagen 7S and TIMP-2 were also analyzed in the following calculation. The natural logarithmic transformation of the results yielded a normal distribution or symmetrical distribution for all the analyzed factors. After the procedures, the following multiple regression analysis became rationally robust against deviations from normal distribution. In order to avoid introducing into the model any variables that were mutually correlated, we checked the interaction between all pairs of the variables by calculating variance inflation factors. Of the highly correlated variables, less significant factors were removed from the viewpoint of multicollinearity.

Multivariate regression analysis was performed using 158 patient data from Toranomon Hospital (training dataset) to generate a training data of predicting function. We used a stepwise method for selection of informative subsets of explanatory variables in the model. Multiple regression coefficient and coefficient of determination were also taken into account in the selection of variables. Next, we validated the obtained predictive function using the remaining 69 patient data from the other six liver institutions (validation dataset).

A *P*-value of less than 0.05 with two-tailed test was considered to be significant. Data analysis was performed using the computer program SPSS ver. 19.[18]

For evaluation of the efficiency and usefulness of obtained function for fibrosis estimation, we compared various fibrosis scores for hepatitis B and C, including AAR,[19] AST-to-platelet ratio index (APRI),[20] FIB-4,[21] FibroTest[22] and discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

RESULTS

Pathological diagnosis

FOUR PATHOLOGISTS INDEPENDENTLY judged the fibrotic stages and inflammatory activity for 227 specimens of chronic hepatitis/cirrhosis caused by HBV. One hundred patients (44.1%) had a fibrosis stage of F1, 62 (27.3%) F2, 51 (22.5%) F3 and 14 (6.2%) F4. In the subgroup of the 158 patients in the training group, judgment as F1 was made in 73 cases, F2 in 42, F3 in 31 and F4 in 12. Of the 69 patients in the validation group, judgment as F1 was made in 27, F2 in 20, F3 in 20 and F4 in two.

According to hepatitis activity classification, A0 was found in five (2.2%), A1 in 100 (44.1%), A2 in 107 (47.1%) and A3 in 15 (6.6%).

Laboratory data of each hepatitis stage in the training group

There were 124 men and 34 women with a median age of 39 years ranged 16–70 years. Laboratory data of 158 patients in the training group are shown in Table 1. Although several individual items were well correlated with the severity of hepatic fibrosis, significant overlap values were noted among F1–F4 stages: platelet count, γ-globulin, α-2-macroglobulin, haptoglobin, hyaluronic acid, TIMP-2 and type IV collagen 7S.

Significant variables serving staging of hepatitis

Univariate analyses using trend analysis with the Cochran–Armitage method showed that the fibrotic stage of chronic hepatitis B (FSB) was significantly correlated with platelet count (Spearman: $r = -0.45$, $P < 0.001$), γ-GTP ($r = 0.19$, $P = 0.017$), γ-globulin ($r = 0.29$, $P < 0.001$), α-2-macroglobulin ($r = 0.32$, $P < 0.001$), hyaluronic acid ($r = 0.36$, $P < 0.001$), TIMP-2 ($r = 0.16$, $P = 0.043$), procollagen III peptide ($r = 0.30$, $P < 0.001$) and type IV collagen 7S ($r = 0.55$, $P < 0.001$).

Regression function generated from training patient group

After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (TIMP-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $-$ 9.15. Median values of the fibrosis score of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively

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