serum ALT levels to within the normal range; *virological response* was defined as a decrease in serum HBV DNA to $<10^4$ copies/mL; and a *serological response* was defined as loss of serum HBeAg. A sustained response was defined as fulfillment of the criteria for combined biochemical, virological, and serological responses 24 weeks after the end of therapy.

Assays

The following variables were determined for all enrolled patients: complete blood counts; serum ALT level; HBsAg, HBeAg, anti-HBe, HBcrAg, and HBV DNA levels; HBV genotypes; proportion of mutants in the precore and basal core promoter regions of HBV DNA; and drug-resistant mutations in the HBV polymerase gene.

Complete blood counts and serum ALT (upper limit of normal, 30 IU/L) were determined by standard procedures. HBsAg was measured with a chemiluminescent microparticle immunoassay (Architect HBsAg QT; Abbott Japan, Tokyo, Japan) as described elsewhere [25]. HBeAg and anti-HBe were detected with chemiluminescence enzyme immunoassays. HBcrAg was also detected with a chemiluminescence enzyme immunoassay (Fuji-Rebio, Tokyo, Japan) [23]. HBV DNA was measured with a real-time polymerase chain reaction (PCR) assay (COBAS TaqMan HBV Test v2.0; Roche Diagnostics, Tokyo, Japan) [26]. Genotypes of HBV were identified by enzyme-linked immunosorbent assay with monoclonal antibodies to type-specific epitopes in the preS2-region (Institute of Immunology, Tokyo, Japan) [27]. Mutations at nucleotide (*nt*) 1896 in the precore region and at *nt* 1762 and *nt* 1764 in the basal core promoter region of HBV DNA were found by means of an enzyme-linked minisequence assay (Genome Science Laboratory, Tokyo, Japan). Drug-resistant mutations (at codons 180, 181, 184, 202, 204, 236, and 250 of the HBV reverse transcriptase domain) were detected by PCR-Invader technology (BML, Tokyo, Japan) [28].

Histopathology

When informed consent had been obtained, a liver biopsy was performed before the patient started therapy. Histopathological findings were assessed by grading inflammatory activity and staging fibrosis according to the METAVIR scoring system [29]. An experienced pathologist blinded to the clinical data performed these evaluations.

Statistical analysis

Statistical analysis was performed with SAS, version 9.2 for Windows (SAS Institute, Cary, NC, USA). Distributions of continuous variables were analyzed with the non-parametric Mann–Whitney *U*-test. Differences in proportions were tested by Fisher's exact test. The significance of changes in values between two time points was evaluated by the Wilcoxon signed-rank test. A two-tailed *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Rate of response to therapy

Although common interferon-α-related side effects included pyrexia, fatigue, headache, and myalgia, the therapy was well tolerated, and all patients completed the treatment according to the protocol. The proportions of patients with biochemical, virological, and serological responses during and after sequential therapy with entecavir and interferon-α are shown in Fig. 1. Drug-resistant mutant variants did not emerge in any patient during entecavir treatment. At the start of interferon-α treatment (about 1 year after the start of the entecavir treatment), most patients had normal ALT levels and serum HBV DNA levels of $<10^4$ copies/mL

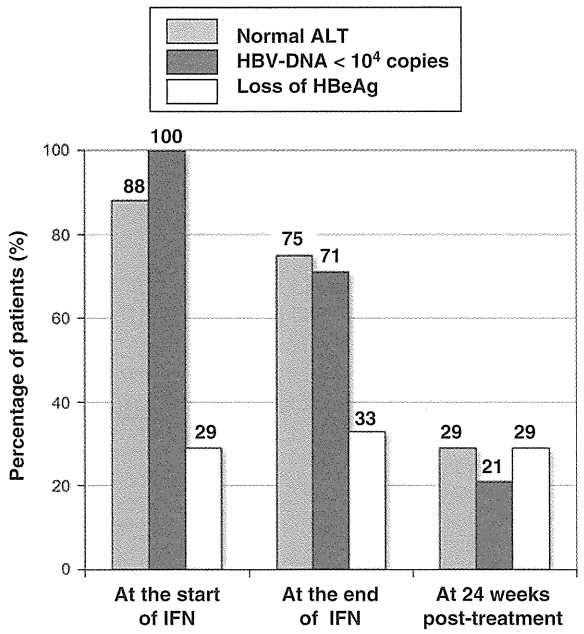

Fig. 1 Rate of biochemical, virological, and serological responses during and after sequential therapy with entecavir and interferon-α. Combined sustained biochemical, virological, and serological response was achieved in 5 (21 %) of the 24 enrolled patients 24 weeks after completion of the sequential therapy. *ALT* Alanine aminotransferase, *HBeAg* hepatitis B envelope antigen, *HBV* hepatitis B virus, *IFN* interferon

(88 and 100 %, respectively). However, loss of HBeAg was achieved in a minority of patients (29 %) during the entecavir treatment.

In most patients without HBeAg loss at the end of the entecavir treatment, serum ALT and HBV DNA levels increased even during the interferon-α treatment. Hepatitis flare (defined as a rise in ALT equivalent to 10 times higher than the upper limit of normal and more than twice the baseline value) occurred in 3 patients after the withdrawal of entecavir. Although peak ALT levels in these patients were 693, 721, and 876 IU/L, respectively, none had jaundice or hepatic decompensation. At the end of the interferon-α treatment, the percentages of patients with normal ALT, HBV DNA $<10^4$ copies/mL, and loss of HBeAg were 75, 71, and 33 %, respectively.

Lastly, 24 weeks after the completion of the sequential therapy, a sustained biochemical, virological, and serological response was achieved in 5 (21 %) of the 24 patients. No patient had loss of serum HBsAg in response to the sequential therapy.

Changes in HBsAg and HBcrAg during and after sequential therapy

Changes in serum HBsAg and HBcrAg levels during and after the sequential therapy with entecavir and interferon-α are shown in Fig. 2. The serum HBsAg level did not change significantly during or after the therapy (Fig. 2a). The serum HBcrAg levels were significantly decreased at the start ($P < 0.0001$) and at the end of interferon-α treatment ($P < 0.0001$), but returned to baseline levels after completion of the sequential treatment (Fig. 2b). The serum HBsAg level did not differ significantly between patients with a sustained response and those with no response (Fig. 2c). In contrast, the serum HBcrAg level was significantly lower in patients with a sustained response than in those with no response at the end of the interferon-α therapy ($P = 0.013$) and 24 weeks post-treatment ($P = 0.031$) (Fig. 2d).

Characteristics of patients at the start of entecavir treatment

The baseline demographic, biochemical, virological, and histological characteristics of patients at the start of entecavir treatment, classified according to the response to sequential therapy, are listed in Table 1. The mean age of patients with a sustained response was more than 10 years less than that of the patients with no response, but this difference did not reach statistical significance ($P = 0.102$). There were no significant differences between the two groups with respect to sex ratio, proportion of patients with a history of interferon treatment, ALT level, HBV DNA level, ratios of HBV genotypes, ratios of precore or basal core promoter mutants, or histopathological findings in the liver.

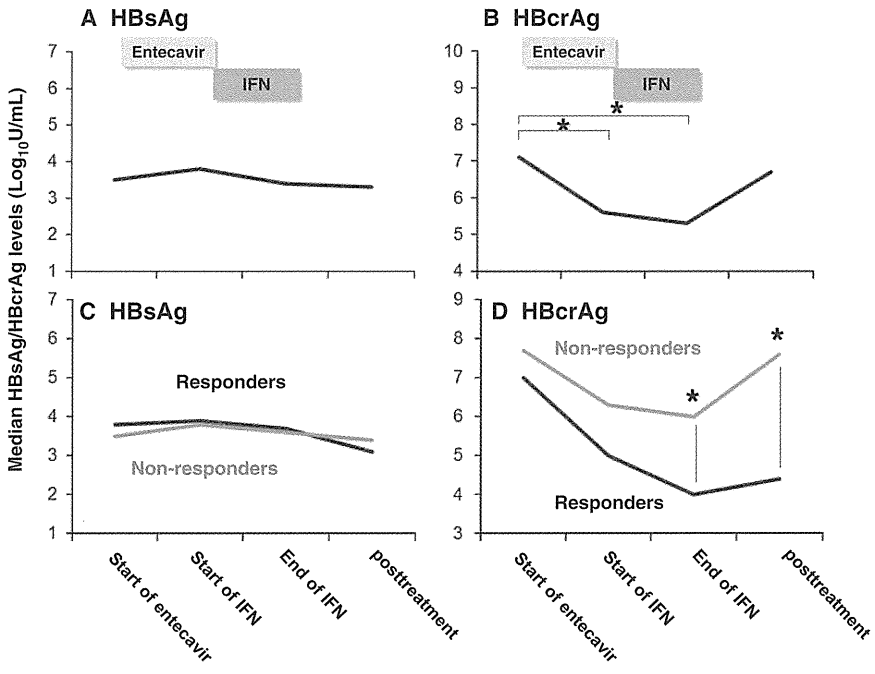

Fig. 2 Changes in serum levels of hepatitis B surface antigen (*HBsAg*) and hepatitis B core-related antigen (*HBcrAg*) during and after sequential therapy with entecavir and interferon-α. Serum HBsAg levels did not change during or after therapy (**a**). As compared with the baseline value, the serum HBcrAg level was significantly decreased at the start ($P < 0.0001$) and at the end of interferon-α treatment ($P < 0.0001$) (*asterisks*) (**b**). When sustained responders were compared with non-responders, there was no significant difference in the serum HBsAg level (**c**). In contrast, the serum HBcrAg level was significantly lower in sustained responders than in non-responders at the end of interferon-α therapy ($P = 0.013$) and 24 weeks post-treatment ($P = 0.031$) (*asterisks*) (**d**)

Table 1 Baseline characteristics of patients at the start of entecavir treatment

Characteristics	Sustained responders ($n = 5$)	Non-responders ($n = 19$)	P values
Age (years)	29 ± 6	41 ± 5	0.10
Male sex (%)	5 (100 %)	18 (95 %)	0.99
History of interferon treatment (%)	3 (60 %)	12 (63 %)	0.99
ALT (IU/L)	85 (65, 322)	210 (79, 531)	0.37
HBV DNA ($\log_{10}$ copies/mL)	7.7 ± 0.4	7.8 ± 0.8	0.31
Genotype (A/B/C/D)	0/0/5/0	1/0/18/0	0.99
Precore (wild/mixed/mutant)	0/4/1	9/9/1	0.12
Basal core promoter (wild/mixed/mutant)	1/0/4	5/8/6	0.070
Grade of inflammation (mild/moderate/severe)	2/3/0	9/7/2	0.60
Stage of fibrosis (mild/moderate/severe/cirrhosis)	2/2/0/1	10/5/3/0	0.19

Values are means ± SDs for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables

ALT alanine aminotransferase, *HBV* hepatitis B virus

Table 2 Characteristics of patients at the start of interferon-α treatment

Characteristics	Sustained responders ($n = 5$)	Non-responders ($n = 19$)	P values
ALT (IU/L)	24 (23, 35)	20 (15, 32)	0.27
ALT normal (%)	5 (100 %)	16 (84 %)	0.99
HBV DNA ($\log_{10}$ copies/mL)	2.1 ± 0.3	2.3 ± 0.4	0.18
HBV DNA negative (%)	3 (60 %)	6 (32 %)	0.33
HBeAg loss (%)	4 (80 %)	3 (16 %)	0.015

Values are means ± SDs for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables

HBeAg hepatitis B envelope antigen

Characteristics of patients at the start of interferon-α treatment

The characteristics of the patients at the start of interferon-α treatment, classified according to the response to sequential therapy, are shown in Table 2. The responders and non-responders did not differ significantly with respect to ALT level or HBV DNA level at the start of interferon-α treatment. The proportion of patients in whom HBeAg was lost during entecavir treatment was significantly higher among those with a sustained response than among those with no response ($P = 0.015$). In another comparison, a sustained response was achieved in 4 (57 %) of the 7 patients with loss of HBeAg during entecavir treatment, as compared with 1 (5.9 %) of the 17 patients without loss of HBeAg during treatment; this difference was also statistically significant ($P = 0.015$).

Case presentation

A 24-year-old man with no response to previous treatment with interferon-α was referred to us (Fig. 3). His ALT level

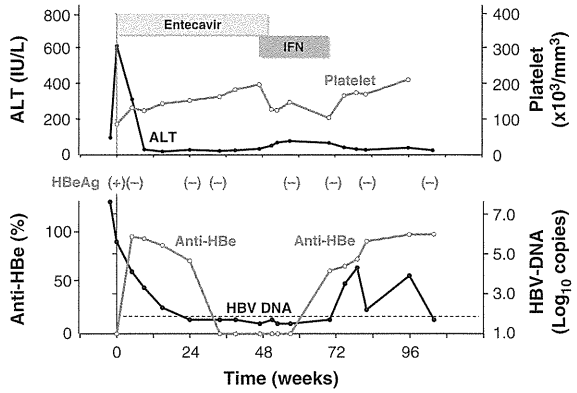

Fig. 3 Changes in platelet count, ALT, HBeAg, anti-HBe, and HBV DNA in a 24-year-old man with sustained response to sequential therapy with entecavir and interferon-α. In the *upper panel*, the changes in ALT levels (*filled circles*) and platelet counts (*open circles*) are shown. In the *lower panel*, the changes in HBV DNA (*filled circles*) and anti-HBe (*open circles*) titers are shown. During 1 year of entecavir treatment, the platelet count rose from 87,000 to 199,000/mm^3. After the patient was switched to interferon-α, his anti-HBe antibody titer increased. At the most recent hospital visit, the patient's ALT level was normal, HBeAg was negative, and HBV DNA was negative; the patient has remained drug-free since the completion of treatment

was 617 IU/L, HBV DNA level was 7.6 $\log_{10}$ copies/mL, and HBV genotype was C. A precore stop codon mutation at *nt* 1896 and basal core promoter mutations at *nt* 1762 and *nt* 1764 were detected. A liver biopsy showed moderate inflammation and cirrhosis. Although the patient was young, interferon-α was not indicated because of a low platelet count and concern about exacerbation of hepatitis. However, during 1 year of entecavir treatment, his ALT level became normal, and his platelet count rose from 87,000 to 199,000/mm^3. After switching to interferon-α, his HBV DNA rose transiently, but his anti-HBe antibody titer increased. At the most recent hospital visit (up to 35 weeks after the completion of treatment), his ALT level was normal and HBeAg and HBV DNA were negative; the

patient has remained drug-free since the completion of treatment.

Discussion

Several groups have evaluated protocols for sequential therapy with lamivudine and interferon-α, and their protocols were similar to that originally described by Serfaty et al. [10]. Manesis et al. [11], from Greece, where HBV genotype D is predominant, found that in HBeAg-*negative* patients, the rate of sustained biochemical and virological response was 22 %, which did not differ from that obtained in an age/sex-matched historical control group treated with interferon-α alone. In another report from Greece [12], sequential combination therapy significantly prevented the emergence of resistance to lamivudine, but the rate of sustained virological response was only 17 % among HBeAg-*negative* patients. A group from China, where genotype B or C is predominant, reported very similar results [13]. To date, only the study by Moucari et al. [17] has used adefovir dipivoxil instead of lamivudine. Sustained virological response was achieved in 50 % of their subjects, although only 20 HBeAg-*negative* patients were included.

In Japan and other countries in East Asia, genotype C is the most prevalent type of HBV [18, 19], and most patients with chronic hepatitis B acquire the virus perinatally or in early childhood [7]. The rates of response to interferon are thus lower than those reported in Europe and the United States. In our previous study [14], using a sequential therapy protocol similar to that described by Serfaty et al. [10], we found that the rate of sustained response was only 29 % among 24 HBeAg-*positive* patients. The patients with a sustained response were significantly younger and had a significantly lower HBV DNA level at the start of interferon than did those with no response. The rate of HBeAg loss during lamivudine treatment was slightly but not significantly higher among sustained responders than among non-responders. Minami and Okanoue [15] also found that patients who lost HBeAg during lamivudine treatment were more likely to have a sustained response to sequential therapy. Okuse et al. [16] reported that sequential therapy was effective for patients with acute exacerbations of chronic hepatitis B, particularly those in whom HBeAg had become negative during lamivudine treatment.

One objective of sequential therapy is to lower the viral load by the use of a nucleos(t)ide analogue, thereby restoring sensitivity to interferon treatment. In clinical studies, a low HBV DNA level is predictive of a favorable response to interferon-α [30, 31]. In basic studies, a high viral load is associated with T-cell hyporesponsiveness [32], and treatment with nucleos(t)ide analogues restores cellular immune response in chronic HBV infection [33]. Although lamivudine had been administered for about half a year before the start of interferon administration in previous studies (including ours) [10–16], we administered entecavir, a more potent antiviral agent, for about 1 year in the present study. Treatment with entecavir was given for a longer period because it has been reported in previous studies that patients in whom HBeAg and HBV DNA levels were lowered by lamivudine were more likely to have a sustained response and because few entecavir-resistant variants emerge within the first few years [34]. However, the use of entecavir for a longer duration did not raise the rate of off-treatment sustained response to sequential therapy in the present study, although the rate of on-treatment biochemical and virological responses was higher with entecavir than that obtained with lamivudine in our previous study [14].

Another objective of sequential therapy is to prevent the relapse of hepatitis after discontinuation of the nucleos(t)ide analogue through the use of interferon-α. Nucleos(t)ide analogues rapidly decrease serum HBV DNA levels by suppressing the reverse transcription of pregenomic HBV RNA, but viral relapse commonly occurs after the cessation of treatment. This high risk of viral relapse may be attributed to the persistence of HBV replicative intermediate covalently closed circular DNA (cccDNA) in the liver even during nucleos(t)ide treatment. The measurement of HBV antigens in serum is thus clinically important as a surrogate marker of intrahepatic cccDNA. In particular, a decline in serum levels of HBsAg is strongly associated with response to interferon-α [35]. The HBcrAg assay measures serum levels of all antigens transcribed from the precore/core gene, including hepatitis B core and e antigens, by using monoclonal antibodies that recognize common epitopes of the denatured antigens [23, 24]. Matsumoto et al. [36] recently proposed a model for predicting relapse of hepatitis after discontinuation of nucleos(t)ide analogue administration, in which cut-off values were set at 1.9–2.9 $\log_{10}$ IU/mL of HBsAg and 3.0–4.0 $\log_{10}$ U/mL of HBcrAg at the withdrawal of treatment. In our study, only one patient had a decrease in HBsAg to between 1.9 and 2.9 $\log_{10}$ IU/mL and another one had a decrease in HBcrAg to between 3.0 and 4.0 $\log_{10}$ U/mL at the withdrawal of entecavir (data not shown), probably because of an insufficient duration of entecavir treatment in our protocol. The finding that at least 21 % of our patients with insufficient HBsAg and HBcrAg decline during entecavir treatment achieved a sustained response to sequential therapy suggests that switching to interferon-α contributes to the safe termination of nucleos(t)ide analogue treatment in some patients.

The major advantages of interferon-α include a finite course of treatment, the opportunity to obtain an off-treatment durable response to therapy, and absence of drug resistance. The advantages of nucleos(t)ide analogues include good tolerance and potent antiviral activity associated with high rates of on-treatment response to therapy. Guidelines proposed by the Japanese Study Group of the Standardization of Treatment of Viral Hepatitis basically recommend interferon-α as the first-line treatment for patients with chronic hepatitis B who are younger than 35 years, to attain a 'drug-free state'; and entecavir for patients who are 35 years or older, to persistently suppress HBV DNA [37]. Consistent with the findings of previous studies [14–16], our results show that sequential therapy is best indicated for patients who have lost HBeAg during nucleoside analogue treatment, because such patients have a higher probability of a sustained response. As shown in Fig. 3, patients who are young but have exacerbation of hepatitis, cirrhosis, or both, were also good candidates for sequential therapy, because interferon-α is generally not recommended for such patients because of concern about hepatic decompensation, and the preceding use of a nucleos(t)ide analogue can reduce such risk.

Our study had several limitations. First, it was not a randomized controlled trial. The reported rate of HBeAg seroconversion obtained by 6-month interferon-α monotherapy among Japanese patients was about 20 % [38], which is similar to the rate obtained by the sequential therapy used in our study (21 %). As compared with our previous study of lamivudine [14], the rate of sustained response in our present study of entecavir did not differ significantly (21 % in the entecavir group vs. 29 % in the lamivudine group). Although the patients were not randomly assigned to treatment, the baseline characteristics of the subjects did not differ between those in our previous study of lamivudine and those in the present study of entecavir with respect to mean age, sex ratio, ALT level, HBV DNA level, ratios of HBV genotypes, ratios of precore or basal core promoter mutants, or histopathological findings (data not shown). Thus, we cannot conclude that sequential therapy with entecavir and interferon-α is more effective than interferon-α monotherapy or sequential therapy with lamivudine and interferon-α. Second, we gave patients non-pegylated interferon-α for 6 months, because pegylated interferon-α had not been approved for the treatment of chronic hepatitis B by the Japanese medical insurance system during the study period. Further studies are thus needed to evaluate the efficacy of sequential therapy with entecavir and pegylated interferon-α.

To our knowledge, this is the first study to report on the response to sequential therapy with entecavir and interferon-α in patients with chronic hepatitis B. In summary, an off-treatment sustained response to sequential therapy with entecavir and interferon-α was achieved in 21 % of HBeAg-*positive* patients with chronic hepatitis B in Japan, where genotype C is predominant. This rate of response was not higher than that in our previous study using lamivudine [14]. Patients who had loss of HBeAg during entecavir treatment were more likely to have a sustained response to sequential therapy.

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Conflict of interest Dr. Shuhei Nishiguchi has received research grants from Bristol-Myers K.K. and Otsuka Pharmaceutical Co., Ltd. Dr. Norifumi Kawada has received research grants from Bristol-Myers K.K. and Otsuka Pharmaceutical Co., Ltd.

Appendix

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Relevance of the Core 70 and IL-28B polymorphism and response-guided therapy of peginterferon alfa-2a ± ribavirin for chronic hepatitis C of Genotype 1b: a multicenter randomized trial, ReGIT-J study

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Abstract
Background We conducted a multicenter randomized clinical trial to determine the optimal treatment strategy against chronic hepatitis C virus (HCV) with genotype 1b and a high viral load (G1b/high).
Methods The study subjects included 153 patients with G1b/high. Patients were initially treated with PEG-IFNα-2a alone and then randomly assigned to receive different treatment regimens. Ribavirin (RBV) was administered to all patients with HCV RNA at week 4. Patients negative for HCV RNA at week 4 were randomly assigned to receive PEG-IFNα-2a (group A) or PEG-IFNα-2a/RBV (group B). Patients who showed HCV RNA at week 4 but were negative at week 12 were randomly assigned to receive weekly PEG-IFNα-2a (group C) or biweekly therapy (group D). Patients who showed HCV RNA at week 12 but were negative at week 24 were randomly assigned to receive PEG-IFNα-2a/RBV (group E) or PEG-IFNα-2a/RBV/fluvastatin (group F).
Results Overall, the rate of sustained virological response (SVR) was 46 % (70/153). The total SVR rate in the group (A, D, and F) of response-guided therapy was significantly higher than that in the group (B, C, and E) of conventional therapy [70 % (38/54) versus 52 % (32/61), $p = 0.049$]. Although IL28-B polymorphism and Core 70 mutation were significantly associated with efficacy, patients with rapid virological response (RVR) and complete early virological response (cEVR) achieved high SVR rates regardless of their status of IL-28B polymorphism and Core 70 mutation.
Conclusion In addition to knowing the IL-28B polymorphism and Core 70 mutation status, understanding the likelihood of virological response during treatment is critical in determining the appropriate treatment strategy.

Keywords Chronic hepatitis C · IL-28B · Peginterferon alfa-2a · Ribavirin · Response-guided therapy

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Introduction

The introduction of combined treatment with peginterferon (PEG-IFN) and ribavirin (RBV) has dramatically increased the rate of sustained virological response (SVR) in patients with genotype 1 high virus titer chronic hepatitis C (HCV RNA titer $\geq$ 5 Log IU/mL), a disease generally considered intractable, to approximately 50 % [1–4]. Currently, a protease inhibitor, telaprevir, can be used for the treatment of chronic hepatitis C, further increasing the SVR rate to approximately 70 % after initial treatment; however, adverse events such as severe anemia, dermatopathy, and renal dysfunction due to increased creatinine level have been reported [5, 6].

RBV is also associated with adverse events, such as anemia, dermatopathy and taste disturbance, and these events can be accentuated in elderly patients or patients with renal dysfunction or anemia. In Japan, there are many elderly patients with chronic hepatitis C and they often cannot tolerate a treatment combination involving RBV [7]. For such patients, PEG-IFN monotherapy could be a treatment option. It has been reported that patients with genotype 1 high virus titer chronic hepatitis C are more likely to achieve SVR if their HCV RNA becomes negative within 4 weeks after initiation of PEG-IFN monotherapy (Rapid Virological Response: RVR) [8].

Patients receiving the PEG-IFNα-2a/RBV combination therapy can also achieve an excellent SVR rate if their HCV RNA becomes negative within 12 weeks after initiation of treatment, whereas the rate is known to decrease with a delay in the timing of HCV RNA-negative conversion [3]. Based on these findings, we propose the use of "response-guided therapy", in which a treatment regimen is modified according to viral kinetics. For the treatment of genotype 1 chronic hepatitis C, proposed treatment strategies include shortening of treatment period in patients with RVR and extension of treatment period in patients with a delayed response to the initial treatment as judged at week 12 [9–17]. For the treatment of genotype 1 high virus titer chronic hepatitis C, shortening of the treatment period may not be recommended even if RVR is achieved because of a possible reduction in the SVR rate, whereas extension of the treatment period to 72 weeks has been reported to increase the SVR rate in patients showing a delayed response to the initial treatment [12, 14–18]. In addition, combined use of HMG-CoA reductase inhibitors and IFN has been shown to enhance the antiviral effects in a synergistic manner [19]. Addition of fluvastatin (FLV), an HMG-CoA reductase inhibitor reported to exhibit the highest antiproliferative activity against hepatitis C virus, to PEG-IFNα-2a/RBV combination therapy has improved the SVR rate [20–22].

Factors affecting the efficacy of PEG-IFN/RBV combination therapy can be divided into viral and host factors. The viral factors include virus titer, genotype, amino acid substitution at position 70 of the core protein (Core 70) and mutations in the interferon sensitivity-determining region (ISDR) in the HCV NS5A region [23–27]. The host factors include age, sex, the degree of liver fibrosis, and a single nucleotide polymorphism (SNP) close to the IL-28B gene [28–33].

We therefore conducted a randomized trial to explore the optimal treatment strategy for patients with genotype 1 high virus titer chronic hepatitis C by comparing several treatment regimens modified according to the concept of "response-guided therapy" in consideration of tolerability (PEG-IFNα-2a monotherapy, PEG-IFNα-2a weekly or biweekly/RBV combination, and PEG-IFNα-2a/RBV/FLV combination therapy). We also evaluated the relations of IL-28B polymorphism and Core 70 mutation to the rate of HCV-RNA-negative conversion and SVR.

Patients and methods

Patients

The study subjects included 153 patients with genotype 1b high virus titer chronic hepatitis C (HCV RNA $\geq$ 5 Log IU/mL) who visited 17 institutions from April 2007 to December 2010 and met the following inclusion criteria: laboratory data before study treatment of white blood cell count $\geq$ 3,000/mm^3, neutrophil count $\geq$ 1,500/mm^3, platelet count $\geq$ 90,000/mm^3, and hemoglobin $\geq$ 12 g/dL. Before the study treatments were carried out, all patients gave written informed consent after receiving a sufficient explanation of the therapy. All patients had genotype 1b chronic hepatitis C with a mean HCV RNA titer of 6.4 Log IU/mL. There were 63 male and 90 female patients with a mean age of 56.5 years. Sixty patients had received prior treatment with IFN, though it was ineffective in 30 of these patients (Table 1).

Treatment protocol

The study design is shown in Fig. 1. After a lead-in therapy with PEG-IFNα-2a 180 μg/week alone (for 4 weeks), RBV was added to the treatment for patients without HCV RNA-negative conversion (according to their weight; $\leq$ 60 kg, 600 mg/day; 60–80 kg, 800 mg/day; and >80 kg, 1,000 mg/day). Patients with negative HCV RNA (TaqMan < 1.2 Log IU/mL) at week 4 (rapid virological response, RVR) were randomly assigned to receive PEG-IFNα-2a alone (group A) or PEG-IFNα-2a/RBV combination (group B). Patients with negative HCV RNA

Table 1 Baseline characteristics of patients ($n = 153$)

Age (years)	56.5 ± 11.1
Gender (male/female)	63/90
HCV RNA (Log IU/mL)	6.4 ± 0.7
BMI (kg/m^2)	22.8 ± 3.3
ALT (IU/L)	60.5 ± 41.3
AST (IU/L)	51.7 ± 31.5
Previous IFN (no/yes)	93/60 (non-responder for 30)
Fibrosis (F0-2/F3-4)	72/32 (unknown for 49)
Activity (A0-1/A2-3)	49/56 (unknown for 48)
Core 70 (wild/mutant)	54/38 (unknown for 61)
IL-28B, rs8099917 (TT/non-TT)	43/26 (unknown for 84)

Values are mean ± standard deviation (SD)

BMI body mass index, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase

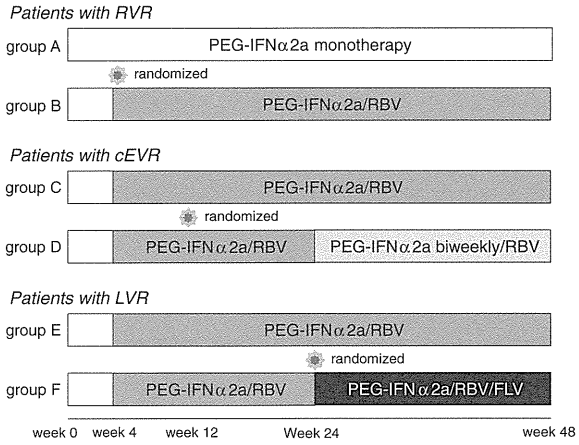

Fig. 1 Study design. After a lead-in therapy with PEG-IFNα-2a for 4 weeks, patients with negative HCV RNA at week 4 (RVR) were randomly assigned to receive PEG-IFNα-2a alone (group A) or PEG-IFNα-2a/RBV combination (group B). Patients with negative HCV RNA at week 12 (cEVR) were randomly assigned to receive weekly PEG-IFNα-2a/RBV combination (group C) or biweekly PEG-IFNα-2a/RBV combination (group D). Patients with negative HCV RNA at week 24 (LVR) were randomly assigned to receive PEG-IFNα-2a/RBV combination (group E) or PEG-IFNα-2a/RBV/fluvastatin (FLV) combination (group F)

at week 12 (complete early virological response, cEVR) were randomly assigned to receive weekly PEG-IFNα-2a/RBV combination (group C) or biweekly PEG-IFNα-2a/RBV combination (group D). Patients with negative HCV RNA at week 24 (late virological response, LVR) were randomly assigned to receive PEG-IFNα-2a/RBV combination (group E) or PEG-IFNα-2a/RBV/fluvastatin (FLV) combination (group F). For assignment, we used Microsoft Access to generate random numbers.

Cases with RVR: evaluation of necessity of RBV (PEG-IFNα-2a monotherapy versus PEG-IFNα-2a/RBV combination therapy)

Patients with negative HCV RNA at week 4 after the introduction of lead-in therapy with PEG-IFNα-2a alone (RVR) were randomly assigned to receive PEG-IFNα-2a alone (group A) or PEG-IFNα-2a/RBV combination (group B) to compare the efficacy and safety between the treatment groups and to evaluate the significance of addition of RBV in RVR cases.

Cases with cEVR: evaluation of dosage interval of PEG-IFNα-2a (weekly versus biweekly PEG-IFNα-2a in combination of RBV)

Patients positive for HCV RNA at week 4 but negative at week 12 (cEVR) were randomly assigned to receive weekly PEG-IFNα-2a/RBV combination (group C) or biweekly PEG-IFNα-2a/RBV combination (group D) after week 24, to compare the efficacy and safety between the treatment groups and to evaluate the dosage interval of PEG-IFNα-2a.

Cases with LVR: evaluation of clinical significance of addition of fluvastatin (PEG-IFNα-2a/RBV combination therapy versus PEG-IFNα-2a/RBV/FLV combination therapy)

Patients with positive HCV RNA at week 4 and 12 but negative HCV RNA at week 24 (LVR) were randomly assigned to a treatment group of PEG-IFNα-2a/RBV (group E) or PEG-IFNα-2a/RBV/FLV (group F) to compare the efficacy and safety between the treatment groups and to evaluate the significance of adding FLV. The dosage of FLV was set to 20 mg/day.

The primary efficacy endpoint was SVR. We also investigated correlations of IL-28B polymorphism (rs8099917) and Core 70 mutation with the rate of HCV RNA-negative conversion and SVR. The IL-28B polymorphism and Core 70 mutation were measured only in patients who wished to have this done. The genetic testing (IL-28B) was performed only in patients who gave written informed consent after obtaining the approval from the ethical committee. This study was a multicenter trial, and the numbers of patients with available HCV-RNA data were different for the week-4, -12, and -24 responses, because not all of the participating institutions completed all of these time points. Therefore, the numbers of patients with regard to IL28B and Core 70 mutation did not completely match at each time point.

If a decrease in the neutrophil count, platelet count, or Hb level reached a critical level or other adverse events

occurred, dose reduction or discontinuation of PEG-IFNα-2a or RBV was performed.

Statistical analysis

All statistical analyses were done using JMP version 9 (SAS). We used the *t* test, Chi-square test, and Fisher's exact test for univariate analysis. To identify factors affecting the SVR rate, we used the logistic regression test. A *p* value of less than 0.05 was considered statistically significant.

Results

Flowchart of the study

A flowchart of the study is shown in Fig. 2. PEG-IFNα-2a monotherapy was initiated in 153 patients, out of which 15 patients necessitated treatment discontinuation due to the patient's hope of recovery or adverse events. The timing of treatment discontinuation was within 4 weeks in three patients, between 5 and 12 weeks in nine patients, and between 13 and 24 weeks in three patients. RVR, cEVR, and LVR were achieved in 18, 70, and 27 patients, respectively, and these 115 patients were randomly assigned to treatment groups according to the response-guided therapy. However, 23 patients remained positive for HCV RNA (non-virological response, NVR) at week 24 and were finally judged as non-SVR.

Of 18 patients with RVR, 10 were assigned to group A (PEG-IFNα-2a monotherapy) and eight to group B (PEG-IFNα-2a/RBV combination); of 70 patients with cEVR, 39 were assigned to group C (weekly PEG-IFNα-2/RBV combination) and 31 to group D (biweekly PEG-IFNα-2/RBV combination); and of 27 patients with LVR, 14 were assigned to group E (PEG-IFNα-2a/RBV combination) and 13 to group F (PEG-IFNα-2a/RBV/FLV combination).

PEG-IFNα-2a monotherapy versus PEG-IFNα-2a/RBV combination therapy in cases with RVR (group A versus group B)

The SVR rate in 18 patients with negative HCV RNA at week 4 after initiation of PEG-IFNα-2a monotherapy (RVR) was 100 % (10/10) in group A (PEG-IFNα-2a monotherapy) and 87.5 % (7/8) in group B (PEG-IFNα-2a/RBV combination), showing no significant difference between the two groups ($p = 0.444$). The rate of treatment discontinuation was 0 % (0/10) in group A. However, treatment discontinuation was required in one patient (12.5 %) in group B due to hemolytic anemia caused by RBV, resulting in non-SVR. Although the rate of RVR by PEG-IFNα-2a monotherapy was only 12 % (18/153), once

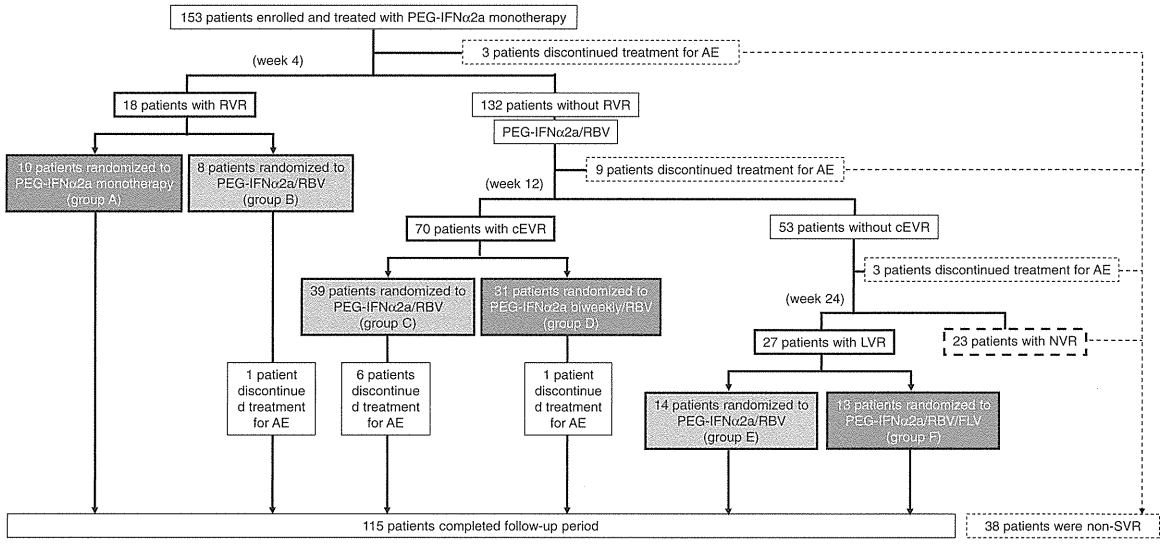

Fig. 2 Flowchart of the study. PEG-IFNα-2a monotherapy was initiated in 153 patients, of whom 15 patients necessitated treatment discontinuation. A total of 115 patients with RVR, cEVR, or LVR were randomly assigned to treatment groups, while 23 patients remained positive for HCV RNA (non-virological response, NVR) at week 24 and were finally judged as non-SVR. Of 18 patients with RVR, 10 were assigned to group A (PEG-IFNα-2a monotherapy) and eight to group B (PEG-IFNα-2a/RBV combination); of 70 patients with cEVR, 39 were assigned to group C (weekly PEG-IFNα-2/RBV combination) and 31 to group D (biweekly PEG-IFNα-2/RBV combination); and of 27 patients with LVR, 14 were assigned to group E (PEG-IFNα-2a/RBV combination) and 13 to group F (PEG-IFNα-2a/RBV/FLV combination)

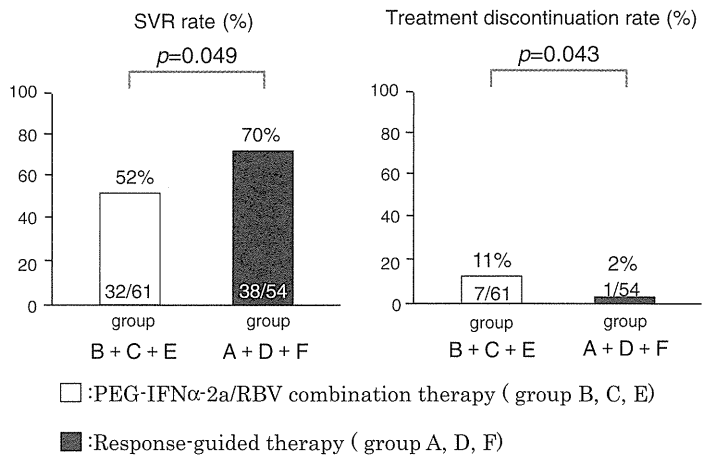

Fig. 3 The SVR and treatment discontinuation rate in the group (A + D + F) of treatment regimens modified according to response-guided therapy and in the group (B + C + E) of PEG-IFNα-2a/RBV combination therapy

RVR is achieved, PEG-IFNα-2a monotherapy without addition of RBV can induce SVR at a high rate with a high tolerability.

Weekly PEG-IFNα-2/RBV combination versus biweekly PEG-IFNα-2/RBV combination therapy in patients with cEVR (group C versus group D)

The SVR rate in 70 patients with cEVR was 54 % (21/39) in group C (weekly PEG-IFNα-2/RBV combination) and 65 % (20/31) in group D (biweekly PEG-IFNα-2/RBV combination). Adverse events leading to treatment discontinuation occurred in six patients (15 %) in group C (a decrease in Hb level, chest pain, fatigue, dizziness, a sense of feeling bad, and a suspicion of HCC) but in only one patient (3 %) in group D (depression), suggesting that the rate of treatment discontinuation tended to be higher in group C than in group D ($p = 0.123$). The difference in the SVR rates between groups C and D may reflect the difference in the rate of treatment discontinuation between the groups.

PEG-IFNα-2a/RBV combination versus PEG-IFNα-2a/RBV/FLV combination therapy in patients with LVR (group E versus group F)

The SVR rate in 27 patients with LVR was 29 % (4/14) in group E (PEG-IFNα-2a/RBV combination therapy) and 62 % (7/13) in group F (PEG-IFNα-2a/RBV/FLV combination therapy), suggesting that the rate tended to be higher in group F than in group E ($p = 0.085$). Thus, addition of an HMG-CoA inhibitor, FLV, increased the SVR rate even in patients with LVR showing delayed negative conversion of HCV RNA. There were no adverse events leading to treatment discontinuation in both groups, and FLV did not augment the adverse events in group F.

Group with PEG-IFNα-2a/RBV combination therapy versus group with response-guided therapy (groups B + C + E versus groups A + D + F)

We then divided all of these groups into two groups according to treatment regimens, a group (A + D + F) in which treatment regimen was modified according to response-guided therapy and a group (B + C + E) of PEG-IFNα-2a/RBV combination therapy. The SVR rate in the response-guided therapy group was significantly higher than in the PEG-IFNα-2a/RBV combination therapy group [70 % (38/54) versus 52 % (32/61), $p = 0.049$].

The rate of treatment discontinuation due to adverse events was significantly lower in the response-guided therapy group than in the PEG-IFNα-2a/RBV combination therapy group [11 % (7/61) versus 2 % (1/54), $p = 0.043$] (Fig. 3).

Factors influencing negative conversion of HCV RNA at week 4, 12, and 24

Factors influencing negative conversion of HCV RNA at week 4 were analyzed in 18 patients with negative HCV RNA and 132 patients with positive HCV RNA. Factors identified as significantly different between the negative and positive groups were age and HCV RNA titer before study treatment, but IL-28B polymorphism and Core 70 mutation were not associated with negative conversion at this time point. Comparison between 88 negative and 53 positive HCV RNA patients at week 12 and that between 115 negative and 23 positive HCV RNA patients at week 24 identified IL-28B polymorphism and Core 70 mutation as factors, showing differences with a statistical significance (Table 2).

Table 2 Characteristics of HCV RNA-negative or positive patients at week 4, 12, and 24

At week 4	Negative ($n = 18$)	Positive ($n = 132$)	p value
Age (years)	49.5 ± 14.6	57.6 ± 10.3	0.003
HCV RNA (Log IU/mL)	6.0 ± 0.7	6.4 ± 0.7	0.009
At week 12	Negative ($n = 88$)	Positive ($n = 53$)	p value
Core 70 substitution (wild/mutant)	39/14	13/22	<0.001
IL-28B, rs8099917 (TT/non-TT)	31/8	10/18	<0.001
At week 24	Negative ($n = 115$)	Positive ($n = 23$)	p value
Core 70 substitution (wild/mutant)	48/23	4/11	0.003
IL-28B, rs8099917 (TT/non-TT)	38/14	3/10	0.003

Value are mean ± standard deviation (SD)

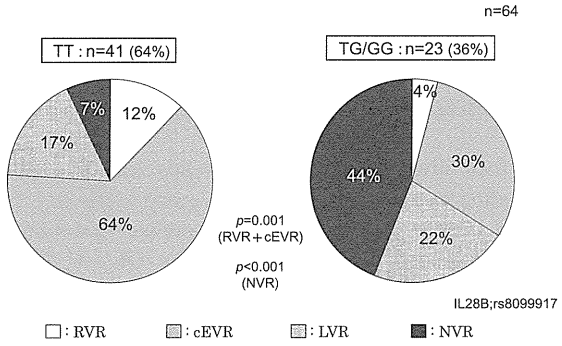

Fig. 4 Treatment response to PEG-IFNα-2a with or without RBV according to the IL-28B single nucleotide polymorphisms (TT versus TG/GG genotype)

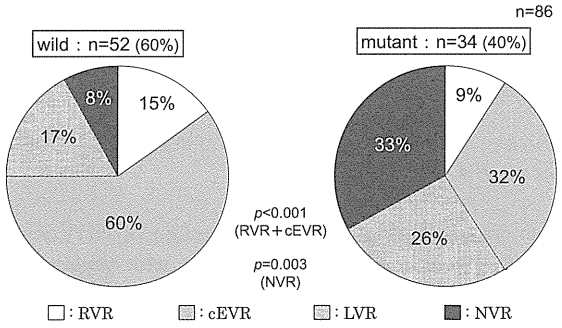

Fig. 5 Treatment response to PEG-IFNα-2a with or without RBV according to the Core 70 mutation (wild-type versus mutant Core 70)

We also investigated the correlation between IL-28B polymorphism and HCV RNA-negative conversion within 12 weeks (RVR + cEVR) in 64 patients in whom IL-28B polymorphism was examined. Negative HCV RNA was achieved within 12 weeks in 76 % of 41 patients with IL-28B TT genotype (major) and in 34 % of 23 patients with IL-28B TG or GG genotype (minor), showing a significant difference between them ($p = 0.001$). Especially in cases with NVR, negative HCV RNA was achieved in 7 % of patients with IL-28B major genotype and in 44 % of patients with IL-28B minor genotype ($p < 0.001$), suggesting that IL-28B polymorphism is strongly associated with treatment response (Fig. 4). Similarly, in 86 patients with determined Core 70 mutation status, negative HCV RNA was achieved within 12 weeks in 75 % of 52 patients with wild-type Core 70 and 41 % of 34 patients with mutant Core 70, showing a significant difference between them ($p < 0.001$). In patients with NVR, the rate of becoming HCV RNA-negative within 12 weeks was 8 % in patients with wild-type Core 70 and 33 % in those with mutant Core 70 ($p = 0.003$) (Fig. 5).

The SVR rates at different time points of HCV RNA-negative conversion by IL-28B polymorphism and Core 70 mutation

The SVR rates were investigated in patients with different time points of HCV RNA-negative conversion (RVR in six patients, cEVR in 33, LVR in 13, and NVR in 13) according to the IL-28B genotypes. The SVR rate was 100 % (5/5) in patients with RVR, 65 % (17/26) in patients with cEVR, 57 % (4/7) in patients with LVR, and 0 % (0/3) in patients with NVR with IL-28B major genotype; whereas the rate was 100 % (1/1) in patients with RVR, 43 % (3/7) in patients with cEVR, 83 % (5/6) in patients with LVR, and 0 % (0/10) in patients with NVR with IL-28B minor genotype. Similarly, the SVR rates were investigated in patients with different time points of HCV RNA-negative conversion (RVR in 11 patients, cEVR in 42, LVR in 18, and NVR in 15) according to the Core 70

Table 3 Characteristics of sustained virological response (SVR) and non-SVR patients

	SVR ($n = 70$)	Non-SVR ($n = 83$)	p value
Age (years)	53.1 ± 12.7	59.4 ± 8.7	<0.001
Gender (male/female)	29/41	34/49	0.954
HCV RNA (Log IU/mL)	6.4 ± 0.7	6.4 ± 0.7	0.782
BMI (kg/m^2)	22.7 ± 3.9	22.9 ± 2.8	0.815
Previous IFN (no/yes)	49/21	44/39	0.032
Fibrosis (F0-2/F3-4)	41/9	31/23	0.007
Activity (A0-1/A2-3)	24/27	25/29	0.938
NS5A mutation, n (0-1/2-)	31/10	47/3	0.013
Core 70 substitution (wild/mutant)	30/11	24/27	0.012
IL-28B, rs8099917 (TT/non-TT)	26/9	17/18	0.027
HCV RNA-negative at week 12 (yes/no)	58/12	30/41	<0.001
Treatment group (B,C,E/A,D,F)	32/38	29/16	0.049

Values are mean ± standard deviation (SD)

BMI body mass index

Table 4 Associated factors with sustained virological response (SVR) by multivariate logistic regression analysis

Factor	Odds ratio	95 % CI	p value
Age (per 1 year)	0.94	0.89–0.98	0.005
Previous IFN (no/yes)	1.62	0.62–4.27	0.323
Fibrosis (F0-2/F3-4)	3.38	1.15–10.8	0.026
NS5A mutation, n (2-/0-1)	7.18	1.32–61.0	0.021
Core 70 substitution (wild/mutant)	2.49	1.51–8.28	0.044
IL-28B, rs8099917 (TT/non-TT)	1.85	0.85-8.61	0.563
HCV RNA-negative at week 12 (yes/no)	7.89	2.92–24.0	<0.001

mutation status. The SVR rate was 100 % (RVR), 58 % (cEVR), 44 % (LVR), and 0 % (NVR) in patients with wild-type Core 70; whereas the rate was 67 % (RVR), 55 % (cEVR), 33 % (LVR), and 0 % (NVR) in patients with mutant Core 70. Thus, when the SVR rates were investigated according to the different time points of HCV RNA-negative conversion, there was no association of IL-28B polymorphism or Core 70 mutation with the SVR rates.

Factors affecting the SVR rate

An univariate analysis in 70 SVR patients and 83 non-SVR patients identified age, previous IFN treatment, fibrosis, NS5A mutation, Core 70 mutation, EVR, IL-28B, and treatment group as factors affecting the SVR rate (Table 3). In this analysis, we examined 83 non-SVR patients: 45 non-SVR patients are presented in Fig. 3, and 38 non-SVR patients (23 patients with NVR and 15 patients who discontinued the Peg-IFN-RBV treatment prior to the enrollment of the randomized trial) are presented in Fig. 2. Multivariate analysis using a logistic regression analysis revealed age (younger), fibrosis (mild), NS5A mutation (two or more mutations), Core 70 status (wild-type), and EVR (RVR + cEVR), to be independent factors affecting the SVR rate, and among them EVR was the most significant factor (odds ratio, 7.89; $p < 0.001$) (Table 4). Therefore, even in patients considered intractable based on the IL-28B genotype or Core 70 mutation status, SVR is expected to be achieved once RVR or cEVR is reached during treatment.

Discussion

The introduction of combined treatment with PEG-IFN and RBV has increased the SVR rate to approximately 40–50 % even in intractable cases with genotype 1b high virus titer chronic hepatitis C after a standard treatment course of 48 weeks [1–4]. In an attempt to further improve the SVR rate, we propose a concept of "response-guided therapy", in which the treatment regimen (such as an extension of a treatment period) is determined according to the viral response to the initial treatment [7–15]. In cases with positive HCV RNA at week 4 or 12, extension of the treatment period from 48 to 72 weeks has been reported to prevent the recurrence and improve the SVR rate [12–14]. Recently, Miyase et al. [34] showed that PEG-IFNα-2a/ribavirin combination therapy resulted in better SVR rates than PEG-IFNα-2b/ribavirin combination therapy in female, older or low-weight patients. In addition, Minami et al. [35] reported that the rate of severe adverse events was not negligible in PEG-IFN/ribavirin combination therapy, and the rate was affected by treatment regimens. Therefore, it is important to establish a treatment regimen of PEG-IFN/RBV combination therapy that has a high efficacy with minimal adverse events. We herein investigated the treatment regimens based on the concept of response-guided therapy to minimize the rate of treatment discontinuation, without changing the treatment period, in consideration of aged patients in Japan.

Factors influencing SVR have been evaluated in many studies that reported IL-28B (a host factor) and Core 70 mutation (a viral factor) as factors predicting the treatment outcome [23, 24, 36–38]. Our present study also demonstrate that the SVR rate was lower in patients with IL-28B minor genotype and those with mutant Core 70, suggesting that IL-28B polymorphism and Core 70 mutation represent factors largely influencing the negative conversion of HCV RNA. Regarding the correlation between treatment response and SVR, Thompson et al. [38] reported that RVR and cEVR rates were lower in patients with the IL-28B minor genotype than in those with the major genotype but the SVR rate was not affected by the IL-28B genotype in patients with RVR or cEVR. In recent studies published after recognition of IL-28B polymorphism, virological response at week 4 and 12 was highly associated with SVR [39, 40]. In our present results, if RVR or EVR is achieved, a high SVR rate can be obtained regardless of the IL-28B polymorphism or Core 70 mutation status.

If RVR is achieved, PEG-IFNα-2a monotherapy exhibits a treatment effect equivalent to that of PEG-IFNα-2a/RBV combination therapy. Conversely, one patient receiving PEG-IFNα-2a/RBV combination therapy developed anemia caused by RBV, resulting in treatment discontinuation and non-SVR. In a phase III clinical trial in Japanese patients, the SVR rate in patients with RVR was 100 % (14/14) in control patients receiving PEG-IFNα-2a monotherapy but was 78 % (18/23) in those receiving PEG-IFNα-2a/RBV combination therapy [41]. Therefore, in terms of preventing treatment discontinuation due to adverse events of RBV, PEG-IFNα-2a monotherapy is recommended in cases with RVR.

In cases with cEVR, the SVR rate in patients who received biweekly PEG-IFNα-2/RBV combination therapy was comparable or even higher as compared to those who received weekly PEG-IFNα-2/RBV combination therapy. This means that biweekly PEG-IFNα-2a in a later treatment period did not reduce the antiviral effects in a subset of cases achieving a good antiviral effect (cEVR). This is partly because the half-life of PEG-IFNα2a is longer than that of PEG-IFNα2b [42–44], thus enabling the maintenance of antiviral effects. Therefore, this biweekly regimen appears possible only with PEG-IFNα2a. Regarding treatment discontinuation, the rate of treatment discontinuation was 3 % (1/31) in patients receiving biweekly PEG-IFNα-2 and 15 % (6/39) in those receiving weekly PEG-IFNα-2, suggesting that the reduced rate of adverse events and subsequent treatment discontinuation by biweekly administration may lead to the increased SVR rate.

Ikeda et al. [19] reported that one of the HMG-CoA reductase inhibitors, FLV, exhibits inhibitory effects on HCV RNA replication in a system of HCV RNA replication clone. In the clinical setting, Sezaki et al. and Rao and Pandya [20–22] reported that combined use of FLV from the treatment initiation period improved the SVR rate [21]. The HCV RNA is replicated using the lipid droplet in hepatocytes [45, 46], and HMG-CoA reductase inhibitors are reported to inhibit the proliferation of HCV RNA by suppressing the synthesis of mevalonic acid through geranylgeranylation [47].

We investigated whether the SVR rate is improved by the addition of FLV only in cases with LVR, because a high SVR rate is expected in patients showing rapid negative conversion of HCV RNA (such as RVR and cEVR cases) without the combined use of FLV. Our results showed that combined use of FLV yielded a higher SVR rate (62 %) as compared to the rate (29 %) obtained without the use of FLV, suggesting that the difference in the recurrence rate may reflect the difference in the SVR rate in patients negative for HCV RNA. Thus, because we used FLV in patients with LVR at high risk of recurrence, but not in those with RVR or cEVR at low risk of recurrence, the difference in anti-HCV activities by FLV was more pronounced. It has been reported that treatment with HMG-CoA reductase inhibitors does not increase the risk of severe hepatotoxicity in patients with chronic hepatitis C [48], which is consistent with our present results showing no adverse events associated with the addition of FLV.

In summary, the SVR rate was 52 % (32/61) in the group receiving PEG-IFNα-2a/RBV combination therapy and 70 % (38/54) in the group receiving modified treatment regimens according to response-guided therapy, showing a significant increase in the latter group. This result may be attributed to the difference in the rate of treatment discontinuation, which was significantly lower in the response-guided therapy group [2 % (1/54)] than in the PEG-IFNα-2a/RBV combination group [11 % (7/61)]. In addition, anti-HCV effects of FLV in patients with LVR at high risk of recurrence may contribute to the improved SVR in the response-guided therapy group. Our results demonstrated the safety and efficacy of PEG-IFNα-2a monotherapy in patients with RVR, biweekly PEG-IFNα-2a/RBV combination therapy in those with cEVR, and PEG-IFNα-2a/RBV/FLV combination therapy in those with LVR.

In conclusion, for the treatment of genotype 1b high virus titer chronic hepatitis C, the selection of an optimal response-guided therapy option, taking into consideration the viral response to initial treatment, the IL-28B polymorphism and Core 70 mutation status, and the safety of individual patients, can improve the SVR rate.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

New malignant grading system for hepatocellular carcinoma using the Sonazoid contrast agent for ultrasonography

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Abstract
Background The ultrasonography contrast agent Sonazoid provides parenchyma-specific contrast imaging (Kupffer imaging) based on its accumulation in Kupffer cells. This agent also facilitates imaging of the fine vascular architecture in tumors through maximum intensity projection (MIP). We examined the clinical utility of the malignancy grading system for hepatocellular carcinoma (HCC) using a combination of 2 different contrast-enhanced ultrasonography images.
Methods We studied 121 histologically confirmed cases of HCC (well-differentiated, 45; moderately differentiated, 70; poorly differentiated, 6). The results of Kupffer imaging were classified as (1) iso-echoic pattern or (2) hypo-echoic pattern. The MIP patterns produced were classified into one of the following categories: fine, tumor vessels were not clearly visualized and only fine vessels were visualized; vascular, tumor vessels were visualized clearly; irregular, tumor vessels were thick and irregular. Based on the combined assessment of Kupffer imaging and the MIP pattern, the samples were classified into 4 grades: Grade 1 (iso-fine/vascular), Grade 2 (hypo-fine), Grade 3 (hypo-vascular), and Grade 4 (hypo-irregular).
Results The distribution of moderately and poorly differentiated HCCs was as follows: Grade 1, 4 % (1/24); Grade 2, 52 % (15/29); Grade 3, 85 % (44/52); and Grade 4, 100 % (16/16). The grading system also predicted portal vein invasion in 72 resected HCCs: Grade 1, 0 % (0/4); Grade 2, 13 % (1/8); Grade 3, 23 % (11/48); and Grade 4, 67 % (8/12).
Conclusions This new malignant grading system is useful for estimation of histological differentiation and portal vein invasion of HCC.

Keywords Hepatocellular carcinoma · Contrast enhanced ultrasonography · Sonazoid · Malignant grade

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Introduction

Hepatocellular carcinoma (HCC) represents the most common liver cancer and the third most common cause of cancer-related deaths [1, 2]. Knowledge of the histological grade of differentiation of HCC is useful in establishing a therapeutic strategy and in predicting therapeutic outcome, prognosis [3], and recurrence (especially in the case of internal metastases) [4]. However, tumor biopsy is the only strategy available for obtaining tumor tissue prior to therapy. Performing a biopsy of HCC has traditionally been avoided, because several cases of tumor seeding after biopsy have been reported [5, 6]. The risk of seeding is in

addition to the risk of complications, such as bleeding. Therefore, in order to determine the ideal therapeutic strategy, alternatives to biopsy are required.

The principal methods of diagnosis for HCC are imaging studies such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI). CT and MRI are superior to US in terms of objectivity. However, prediction of histological differentiation of HCC only by contrast-enhanced CT and MRI has limited effectiveness, because most classical HCCs are hyper-vascular in the arterial phase and hypo-vascular in the portal phase. Thus, it is difficult to distinguish histological differentiation by factors such as the vascular structure of the tumor.

Currently, there are only 2 US contrast agents available, Sonazoid and Levovist, which can be used for Kupffer imaging in the post-vascular phase (i.e., 10 min after injecting these agents). Bubbles made from Levovist, the first-generation US contrast agent, are very fragile and are easily collapsed by US emissions. Therefore, Kupffer imaging in the post-vascular phase using Levovist should be performed using a single sweep scan of the liver, which is insufficient for surveillance. However, Sonazoid, a second-generation US contrast agent, is a lipid-stabilized suspension of perfluorobutane and is composed of a hard shell containing bubbles. Due to this structure, microbubbles made from Sonazoid are chemically stable in blood vessels [7–9] and produce stable, non-linear oscillations in the low-power acoustic field. This feature allows Sonazoid to provide detailed perfusion features during imaging in the vascular phase and Kupffer imaging in the post-vascular phase, without bubble collapse. Specifically, Sonazoid is stable for at least 3 h after injection and, since the Sonazoid microbubbles are phagocytosed by liver Kupffer cells, this agent allows for multiple real-time scans. Malignant hepatic tumors, including HCC, contain few or no Kupffer cells, leading to an area clear of contrast material or a perfusion defect in Kupffer imaging. Therefore, perfusion defects seen on Kupffer imaging and the degree of histological malignancy of HCC are correlated [10–12]. Moderately or poorly differentiated HCC requires prompt therapy and Kupffer imaging has been a key imaging modality for the estimation of these histologic grades.

Moreover, using maximum intensity projection (MIP) [13], this type of contrast-enhanced ultrasonography (CEUS) could enable visualization of the fine vascular architecture in tumors, which also has been correlated with the histological differentiation of HCC [14]. The MIP pattern is an image that takes advantage of Sonazoid CEUS characteristics, such as high time and spatial resolution.

Hence, in comparison with other imaging modalities, these 2 imaging techniques, Kupffer imaging and MIP, could provide more relevant information for estimating the malignant grade of HCC. In this study, we examined the clinical utility of the malignant grading system for HCC using a combination of 2 different CEUS images, namely, Kupffer imaging and the MIP pattern.

Methods

Patients

We studied 116 patients with histologically confirmed HCC who were admitted to our institution between January 2008 and October 2010. Eighty patients were male and 36 were female, with a mean age of 69.9 years (range 38–92 years). Most of the 116 patients had a history of chronic liver disease, including hepatitis C virus (HCV) infection in 79 (68.1 %) patients and hepatitis B virus (HBV) infection in 14 (12.1 %) patients. Of the 116 patients included, 23 (19.8 %) were negative for both HCV and HBV. For the 23 patients that were negative for both viral markers, 13 (11 %) patients had alcoholic liver disease, 2 (2 %) patients had autoimmune hepatitis (AIH), 1 (1 %) patient had non-alcoholic steatohepatitis (NASH), and 7 (6 %) patients had cryptogenic hepatitis.

Liver specimens of 72 tumors (obtained from 71 patients who underwent partial hepatectomy) were analyzed in this study. The liver specimens obtained from 71 patients showed 34 patients with cirrhosis and the remaining 37 patients with chronic hepatitis. Presence of portal vein tumor invasion in these resected tumors was diagnosed by histological examination. An additional 49 tumors, obtained by 21-gauge needle core biopsy (Majima needle, Top Surgical Manufacturing, Tokyo, Japan) from 45 patients, were also evaluated for validation of the system. HCCs with regions of varying histological grades were classified as belonging to the predominating histological characteristic. The degree of differentiation was determined according to the International Working Party classification [15]. The final histological diagnoses of the 121 HCCs were as follows: 45 (37 %) well-differentiated, 70 (58 %) moderately differentiated, and 6 (5 %) poorly differentiated. This study was approved by the institutional ethics review board of Hyogo College of Medicine, Hyogo, Japan and all patients provided informed consent.

Sonologists

Two sonologists from our institution, with 20 (HT) and 30 (HI) years of experience in liver US imaging, were involved in this retrospective study. Each sonologist had at least 10 years of experience in microbubble contrast-enhanced US of the liver. They were aware of the patients' clinical histories and were blinded to the biopsy results.

Contrast-enhanced US study

The intravenously injected sonographic contrast agent, Sonazoid (Daiichi Sankyo, Tokyo, Japan; GE Healthcare, Little Chalfont, UK), was used in all studies. The suspension was prepared by vigorously shaking the powder with 2 mL of sterile water for 5–10 s. After the suspension was allowed to stand for 2 min to achieve equilibrium and the dissolution of large bubbles, the suspension was injected into an antecubital vein through a 21-gauge cannula at a speed of 1 mL/s and immediately flushed with 5–10 mL of normal saline.

US equipment included SSA-770A, SSA-790A, and TUS-A500 (Aplio; Toshiba Medical Systems, Tokyo, Japan) with a 3.75-MHz convex transducer (PSK-375BT). The imaging mode was wideband harmonic imaging (commercially called pulse subtraction) with transmission and reception frequencies of 3.75 and 7.5 MHz, respectively. When a suspected lesion was identified, CEUS was performed with the focus depth beyond the lesion of interest using the following settings: frame rate, 15 fps and dynamic range, 35 dB. A low mechanical index (MI) (0.16–0.30) was selected to avoid the disruption of microbubbles.

The region of interest was observed continuously for approximately 3 min from the time of injection. The arterial phase was timed for 45 s after completion of the flash. Approximately 20 min after the injection via the peripheral venous line, the liver was scanned again to observe Kupffer imaging (Fig. 1). Arterial-phase findings and Kupffer imaging were classified as follows: (1) hyper-echoic pattern, (2) iso-echoic pattern, and (3) hypo-echoic pattern.

After the Kupffer imaging was acquired, an MIP pattern was evaluated by reinjection of Sonazoid using micro-flow imaging (MFI), as was introduced by Sugimoto et al. [14]. Briefly, the maximum-hold processing started just after the burst scan. The burst scan consisted of high-MI (1.3–1.6) scanning of 5 frames. Low-MI (0.16–0.30) scanning was started again, just after the MI burst scanning, to visualize fresh microbubble contrast agent flowing into the scanning volume. The maximum intensity holding sequence was started simultaneously with flash replenishment low-MI imaging, which maintained maximum brightness on each pixel and was displayed as a persistent vision. The accumulation time for each MFI sequence was 10–15 s, depending on the perfusion of the target tissue.

MIP classification

The MIP pattern was classified into 1 of the following 3 patterns: (1) fine pattern: where tumor vessels were not clearly visualized and only fine vessels were visualized; (2) vascular pattern: where tumor vessels were visualized clearly; and (3) irregular pattern: where tumor vessels were thick and irregular (Fig. 1). The "tumor vessels" were defined as the vascular pattern in which vessels were obvious to the surrounding fifth or sixth branches. In cases where the vascular pattern was similar to that of the surrounding vessels, the tumor was classified as fine pattern. For accurate diagnose of the MIP pattern, the entire tumor was observed in vascular phase and the most suitable cross-sectional direction was chosen to enable identification of important signs of vascular and irregular patterns.

Malignant grading system

The combination of Kupffer imaging and MIP patterns classified HCCs into 4 grades: Grade 1 (iso-fine/vascular),

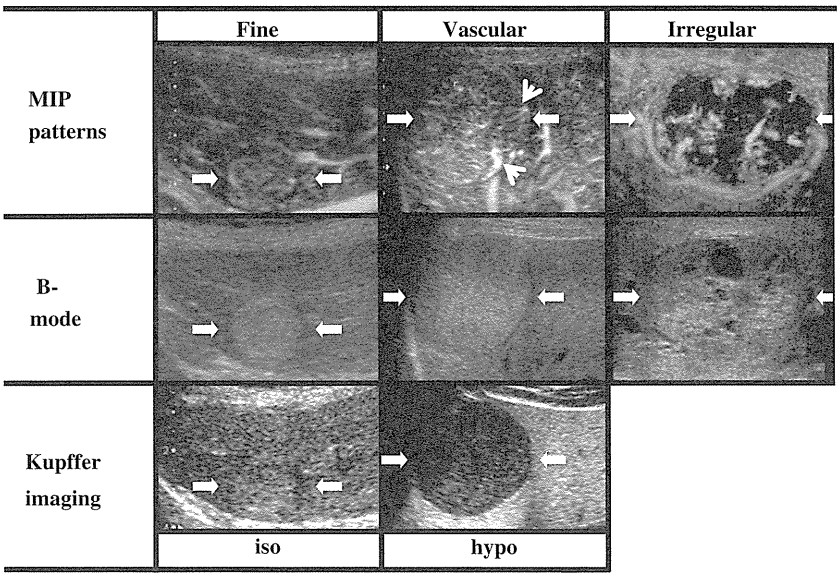

Fig. 1 Classification of MIP and Kupffer imaging. The MIP pattern is classified as 1 of the following 3 patterns: (1) fine pattern: where tumor vessels were not clearly visualized and only fine vessels were visualized; (2) vascular pattern: where tumor vessels were visualized clearly; and (3) irregular pattern: where tumor vessels were thick and irregular. *Small arrows* in "vascular" category of MIP patterns show tumor vessels of vascular pattern. Kupffer imaging is classified as 1 of following 2 patterns: (1) iso-echoic pattern, (2) hypo-echoic pattern

Grade 2 (hypo-fine), Grade 3 (hypo-vascular), and Grade 4 (hypo-irregular).

Statistical analysis

In the case of categorical variables, statistical analysis was performed using the Fisher's exact test. The Kruskal–Wallis H test was used for continuous variables. The Tukey–Kramer honestly significant difference was used for multiple comparisons. Relationships among the clinical parameters, such as malignant grade, tumor size, portal vein invasion, and histological differentiation were analyzed using Spearman's rank correlation coefficient. Unless otherwise noted, all data are presented as mean ± SD. $P < 0.05$ was considered statistically significant. The statistical analysis was performed with the JMP 8 (SAS Institute Inc., Cary, NC, USA).

Results

Arterial phase of HCCs and Kupffer imaging of HCCs

When compared with the adjacent liver tissue, 68 (94 %) lesions showed a hyper-echoic pattern and 4 (6 %) lesions showed an iso-echoic pattern during the arterial phase. As the degree of histological differentiation of HCC decreased, tumor hyper-vascularity increased: well-differentiated, 80 % (8/10); moderately differentiated, 96 % (54/56); and poorly differentiated, 100 % (6/6) (Table 1). Kupffer imaging of HCC according to histological differentiation showed the opposite tendency in that tumor hypo-intensity increased as the degree of histological differentiation of HCC decreased [well-differentiated, 60 % (6/10); moderately differentiated, 98 % (55/56); and poorly differentiated, 100 % (6/6)]. In Kupffer imaging, a hypo-echoic pattern was significantly larger in moderately or poorly differentiated HCCs, compared to well-differentiated HCCs ($P < 0.001$).

Table 1 Correlations between histological differentiated and patterns of contrast enhanced ultrasonography

	n	Arterial phase			Kupffer phase		
		Iso	Hyper	P^a	Iso	Hypo	P^b
Well	10	2	8	0.090	4	6	<0.001
Mod	56	2	54		1	55	
Poor	6	0	6		0	6	
Total	72	4	68		5	67	

Well Well differentiated HCC, *mod* moderately differentiated HCC, *poor* poorly differentiated HCC

[a] Proportion of hyper in arterial phase (well vs. mod/poor)

[b] Proportion of hyper in arterial phase (well vs. mod/poor)

Relationship between MIP patterns and histological differentiation or CEUS findings

In this study, intratumoral vessels of 72 tumors were clearly delineated using MIP. The vascular architecture of the tumors was as follows: fine, 12 (17 %); vascular, 48 (67 %); and irregular, 12 (17 %). Correlations between the MIP patterns and CEUS findings are presented in Fig. 2a, b. Most cases were hyper-echoic pattern in arterial phase (94 %) and hypo-echoic pattern in Kupffer imaging (93 %) and it was difficult to classify the MIP patterns using the CEUS patterns. Next, the correlations between the MIP patterns and histological differentiation were examined (Fig. 2c). We observed that 50 % (5/10) of well-differentiated HCCs showed a Fine pattern and 77 % (43/56) of moderately differentiated HCCs showed a vascular pattern, while the all poorly differentiated HCCs showed an irregular pattern. Furthermore, the irregular pattern was found only in moderately and poorly differentiated HCCs (Fig. 2c).

Histological differentiation and CEUS malignant grading system

As shown in Fig. 3a, Grade 1 included no moderately differentiated HCC tumors. In contrast, all poorly differentiated HCCs were Grade 4. This tendency became more clear when we considered the relationship among all 121 nodules, including biopsy-confirmed HCCs. There was a close relationship between malignant grade and histologic differentiation ($r = 0.712$, $P < 0.0001$). Thus, this grading system could predict moderately and poorly differentiated HCCs: Grade 1, 4 % (1/24); Grade 2, 52 % (15/29); Grade 3, 85 % (44/52); and Grade 4, 100 % (16/16).

Tumor size and malignant grading system

When evaluating tumor size according to the grading system, mean tumor size increased: Grade 1, 18.2 ± 4.7 mm; Grade 2, 16.6 ± 4.2 mm; Grade 3, 30.6 ± 14.7 mm; and Grade 4, 53.2 ± 21.3 mm ($r = 0.590$, $P < 0.001$). Tumor sizes were similar between Grades 1 and 2, but there was a greater increase in size between Grades 3 and 4 (Fig. 4a).

Portal vein invasion and clinical parameters

When portal vein invasion was compared with 72 resected HCCs, all HCCs with portal vein invasion were high-echoic pattern in arterial phase and hypo-echoic pattern in Kupffer imaging. There were only 4 (8 %) HCCs without portal vein invasion that showed an iso-echoic pattern