however, may have actively eaten more because they were receiving a "specific" nutritional treatment. Therefore, we cannot exclude the possibility that improved nutrition and serum albumin may stem also from a larger energy and protein intake, which might be an indirect effect of BCAAs.

We found no significant improvement in the SF-36 scores. The lack of such an effect might be due to the 3-mo duration of the study, because a significant improvement in QOL required 1–2 y in granulated-BCAA studies [11,13]. Moreover, the study's sample was too small to show significant differences. In this study we measured RQ in the hospital, which made it difficult to recruit patients. In addition, the SF-36 is a scale that evaluates health-related QOL comprehensively, but does not specifically reflect symptoms of a certain condition. Therefore, improved weakness in both LES groups and easy fatigability in the BCAA-LES group might show significant usefulness of LESs and BCAAs regarding QOL.

There are some limitations to this study: 1) we could not avoid observer bias in treatment of BCAAs because neither the patients nor investigators were blinded to the treatment allocation; 2) although we found significant improvement in serum albumin, its change was small, and a longer study period is required to confirm this change; 3) the sample was not large enough to show significant differences in some indices; and 4) the sex distribution between the two groups was unbalanced, with more men in the BCAA group, although this was not statistically significant. The sex of the patient may play a role in the effects on nitrogen balance. Alberino et al. [31] found that women with cirrhosis are better able to retain body muscle stores than are men, but men are better able to retain fat stores, suggesting that men might respond more to provision of an evening meal.

No serious adverse effects were found. Non-compliance or withdrawals of consent in previous BCAA studies were mainly due to the bad taste of the supplements [11,32]. Some patients complained of poor palatability of the formula in the present study, and one withdrew. Recently, a new, more palatable BCAA formula has been developed [33]. Thus, the problem concerning palatability of the BCAA mixture can be overcome at least to some extent.

Conclusions

A BCAA mixture as a LES is a favorable nutritional intervention for liver cirrhosis to repair hypercatabolism and improve nutritional states, such as nitrogen balance and serum albumin. Symptoms and QOL could have recovered, but this effect requires confirmation by future studies.

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Pharmacokinetics and enhanced PKR response in patients with chronic hepatitis C treated with pegylated interferon alpha-2b and ribavirin

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SUMMARY. This study investigated the molecular and pharmacokinetic mechanisms of the enhanced antiviral efficacy associated with pegylated interferon (PEG-IFN) alpha-2b and ribavirin. The study involved comparing the expression of serial double-stranded RNA-activated protein kinase (PKR) before and during treatment in 26 PEG-IFN alpha-2b and 26 conventional IFN alpha-2b recipients matched for age, body weight and dose of ribavirin. The pharmacokinetics of PEG-IFN alpha-2b and ribavirin was analysed in 15 of the 26 PEG-IFN recipients. There was a rapid increase in PKR expression in both treatment groups, although expression from day 2 onwards was maintained at a significantly higher level in the PEG-IFN recipients ($P < 0.05$). C_{max} of PEG-IFN occurred 12–48 h after the initial administration, with $t_{1/2}$ and C_{min} being 49 h and 190 pg/mL, respectively. In contrast to ribavirin, accumulation of PEG-IFN was minimal. There was no association between serum PEG-IFN and ribavirin levels and virological response. Although baseline expression of PKR before treatment was marginally higher in nonresponders (NRs), from day 2 onwards, sequential PKR expression in response to PEG-IFN was higher in sustained viral responders compared with the NRs ($P < 0.05$). Significant correlations were found between kinetics of PKR expression and viral decline rates in each phase of hepatitis C virus dynamics (first phase, $r = 0.67$, $P = 0.0006$; second phase, $r = 0.67$, $P = 0.001$). In conclusion, improvement in pharmacokinetics following pegylation led to higher intracellular PKR expression, which was associated with enhanced virological efficacy of PEG-IFN-based combination therapy. The concentrations of both ribavirin and PEG-IFN alpha-2b were not associated with viral response and PKR expression.

Keywords: hepatitis C virus, hepatitis C virus dynamics, interferon-stimulated gene, treatment.

INTRODUCTION

Combination therapy with pegylated interferon (PEG-IFN) alpha and ribavirin results in a higher sustained virological response (SVR) rate than conventional IFN alpha and ribavirin therapy [1,2] and is now established as the standard treatment for chronic hepatitis C virus (HCV) infection. However, the mechanism responsible for this improved response rate remains to be elucidated.

Interferon induces transcription of IFN-stimulated genes (ISG), including double-stranded RNA-activated protein kinase (PKR) [3]. PKR has many cellular roles, including inhibition of translational responses to viral infection, growth control, differentiation activity and proapoptotic functionality [4,5]. However, the clinical significance of PKR expression during PEG-IFN therapy is not fully understood. Moreover, the pharmacokinetic effects of PEG-IFN on PKR expression and the relationship between the expression of PKR and viral response remain unknown.

In addition, although the serum concentration of ribavirin has been reported to affect the outcome of conventional IFN alpha and ribavirin combination therapy [6], the relationship between serum ribavirin, PEG-IFN concentrations and viral response has not been studied.

Abbreviations: PEG-IFN, pegylated interferon; SVR, sustained virological response; HCV, hepatitis C virus; ISG, IFN-stimulated genes; PKR, double-stranded RNA-activated protein kinase; ALT, alanine aminotransferase; NR, nonresponder; PBMC, peripheral blood mononuclear cells; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

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In the present study, we sought to elucidate the underlying mechanism of the enhanced antiviral efficacy seen with PEG-IFN alpha-2b and ribavirin combination therapy by analysing PKR gene expression and pharmacokinetics of PEG-IFN and ribavirin in patients with chronic HCV genotype 1b infections. The relationships between the viral response and PKR expression and pharmacokinetics of PEG-IFN and ribavirin were also studied.

MATERIALS AND METHODS

Patients

Fifty-two patients infected with chronic hepatitis C of genotype 1b and high viral load, admitted between November 2001 and June 2002, were included in the study. Twenty-six patients were treated with PEG-IFN alpha-2b and ribavirin combination therapy, with the remaining 26 patients matched for age, body weight and dose of ribavirin being treated with conventional IFN alpha-2b and ribavirin. The inclusion criteria for the study were as follows: Persistent elevation of serum alanine aminotransferase (ALT) levels above the upper limit of the normal for ≥6 months prior to therapy; the presence of HCV genotype 1b in the serum; the presence of serum HCV-RNA of >100 000 IU/mL detected by the Amplicor-HCV monitor assay (Roche Molecular Diagnostic Co., Tokyo, Japan); no evidence of hepatocellular carcinoma in an ultrasound examination; a haemoglobin level ≥14 g/dL, neutrophil count ≥1500/mm^3, platelet count ≥100 × 10^3/mm^3, creatinine clearance ≥51 mL/min and fasting blood sugar <110 mg/dL. Exclusion criteria included the presence of hepatitis B surface antigen or human immunodeficiency viral antibodies and a history of excess alcohol consumption. Eleven of the 26 PEG-IFN alpha-2b recipients and all 26 conventional IFN alpha-2b recipients had been enrolled previously in a viral dynamics study [7].

Written informed consent was obtained from all the patients and the study protocol was approved by the institutional ethical committee in accordance with the revised version of the Helsinki Declaration of 1983.

Treatment

Twenty-six patients were treated for 48 weeks with subcutaneous injections of PEG-IFN alpha-2b (PegIntron®; Schering-Plough Corporation, Kenilworth, NJ, USA) at a dose of 1.5 μg/kg/week. Ribavirin (Rebetol®, Schering-Plough Corporation) was administered concomitantly over the 48-week period, provided orally twice daily at a total daily dose of 800 mg. At the start of the study, 400 mg of ribavirin was administered, with serum concentrations being measured after 48 h. As the body weight of the patients in the study ranged between 60 and 80 kg, the dose of ribavirin for the remainder of the study period was fixed at 800 mg/day. The dose of PEG-IFN alpha-2b was reduced to 0.75 μg/kg/week when either the neutrophil count was <750/mm^3 or the platelet count was <80 × 10^3/mm^3. The dose of ribavirin was reduced to 600 mg/day when the haemoglobin concentration decreased to <10 g/dL.

The remaining 26 patients were treated for 48 weeks with intramuscular IFN alpha-2b (Intron-A®; Schering-Plough Corporation) in combination with daily oral ribavirin at a dose of 800 mg. For the first 2 weeks of therapy, 6 MU of IFN alpha-2b was administered daily, followed for the next 46 weeks by 6 MU given three times a week.

Measurement of PKR mRNA before and during therapy

Serial measurements of PKR expression before and during treatment were determined in both treatment groups. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood samples collected before, and at 4, 8, 24 h and 2, 4, 7, 14, 21, 28, 56, 84, 112, 140, 168 and 336 days after the initiation of either PEG- or conventional IFN alpha-2b and ribavirin combination therapy. After extraction of total RNA from the PBMCs, the expression of PKR mRNA was quantified at each specified time point using real-time quantitative polymerase chain reaction (PCR) as described previously [8]. The assays were performed in triplicate, and as an internal control, the expression levels of PKR transcript were normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression quantified by real-time quantitative PCR. The level of PKR gene expression at each time point during IFN treatment was calculated relative to baseline expression levels measured prior to IFN treatment.

Pharmacokinetics of pegylated interferon alpha-2b and ribavirin

The pharmacokinetics of PEG-IFN and ribavirin was analysed in 15 PEG-IFN alpha-2b recipients who consented to be enrolled in the additional pharmacokinetic study. Of these 15 patients, two were naïve , nine had relapsed and four had not responded to previous conventional IFN monotherapy. Blood samples were collected immediately before, and at 2, 4, 6, 8, 10, 12, 14, 16, 24, 36, 48, 72, 96, 120, 144 and 168 h after the first dose of PEG-IFN alpha-2b and ribavirin. Blood samples were also collected immediately before each administration at weeks 5, 9, 13, 25 and 37 and the trough values measured. At week 48 (final dose), blood was drawn immediately before, and at 2, 4, 6, 8, 10, 12, 14, 16, 36, 48, 72, 96, 120, 144, 168, 366, 504 and 672 h after administration. The sera were harvested immediately after blood collection and stored frozen at −20°C.

Serum PEG-IFN alpha-2b levels were determined using an electrochemiluminescent immunoassay (IGEN International, Inc., Gaithersburg, MD, USA), with the lower limit of detection for this assay being 27 pg/mL. Serum ribavirin levels were measured by high-performance liquid chromatography

in conjunction with tandem mass spectroscopy (MDS Pharma Services Inc., Montreal, QC, Canada) according to a method reported previously [9]. The maximum serum concentration (C_{max}), time to maximum serum concentration (t_{max}) and C_{168h} (trough value of ribavirin) were then determined. Confirmation of the steady state using circadian changes of the trough value, estimation of the time to reach the steady state, the cumulative coefficient (Rods) based on the area under the curve (AUC), the clearance half-life in the terminal excretion phase ($t_{1/2\lambda_z}$) and comparison of AUC_{0-168h} (PEG-IFN alpha-2b) or AUC_{0-12h} for the first and final administrations were also determined. One patient whose IFN concentration exceeded the upper limit of the therapeutic range was excluded from this analysis.

Final virological response and hepatitis C virus dynamics in serum

Patients who were HCV-RNA negative at week 24 following completion of treatment were defined as having achieved an SVR. Patients who did not achieve an SVR were classified as nonresponders (NRs).

To analyse the effect of treatment on HCV dynamics, the amount of HCV-RNA was quantified at the following time points: immediately before initiation of the therapy and 4, 8, 24 h and 2, 4, 7, 14, 21, 28, 56, 84, 112, 140, 168 and 336 days after initiating therapy. The total RNA was extracted from the serum, and the amount of HCV-RNA at each time point was quantified by real-time detection PCR as reported previously [7,10]. The detection sensitivity of this assay was approximately 10 copies/mL, and the dynamic range for the method was from 10 to 1×10^8 copies/mL [11]. The viral decline curve was plotted on a semilogarithmic graph, and the slope of the exponential viral decline was calculated individually by a straight-line fit to the data for each viral decline phase.

Statistical analysis

Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables in the two treatment groups were analysed by Student's *t*-test. All tests of the confidence interval were two tailed, with the level of confidence level being set at 95%. *P*-values of <0.05 were considered statistically significant.

In order to analyse the pharmacokinetics of PEG-IFN alpha-2b and ribavirin, descriptive statistics were calculated at each blood collection, and the relationship between the time point of blood collection and the measured levels of the two drugs displayed graphically for each subject. These graphs included the mean value, standard error and the measured concentrations of the drugs at the first and after the final administration. In addition, these analyses were used to confirm the circadian trough values and to estimate the time to reach the steady state, based on AUC (Rods) and clearance half-life ($t_{1/2\lambda_z}$).

RESULTS

The demographics of the patients are shown in Table 1. No significant differences were found in mean age, gender proportionality, activity and stage of liver histology, serum ALT level and initial viral load between the PEG-IFN alpha-2b and non-PEG-IFN alpha-2b treatment groups. SVR rates in the PEG-IFN alpha-2b and non-PEG-IFN alpha-2b treatment groups were 69% (18/26) and 31% (8/26), respectively.

Differences in PKR mRNA expression in response to the different interferon treatment regimens

Sequential transcript analysis demonstrated an approximately 15-fold increase in PKR mRNA expression within 4 h following administration of conventional IFN alpha-2b. At

Table 1 Clinical characteristics of the patients in the two treatment groups of the study

	PEG-IFN alpha-2b plus ribavirin	IFN alpha-2b plus ribavirin	*P*-value (95% CI)
No. of patients	26	26	
Age (years), median (range)	53 (29–67)	53 (29–70)	0.66 (−4.18–6.57)*
Gender (male/female)	14/12	13/13	0.78†
Histology of the liver			
A1/A2/A3	12/11/3	14/11/1	0.56†
F1/F2/F3	14/10/2	13/7/6	0.28†
ALT (IU/L)	93 (72–113)	84 (63–105)	0.54 (−38.2–20.2)*
Haemoglobin (g/dL)	14.6 (14.2–15.0)	14.2 (13.6–14.9)	0.26 (−1.11–0.31)*
Platelet count ($\times 10^3$/mL)	179 (164–195)	171 (151–190)	0.47 (−3.32–1.56)*
Viral load ($\times 10^6$ copies/mL)	14.6 (9.00–20.2)	8.35 (3.77–12.9)	0.11 (−14.1–1.55)*
Ribavirin concentration at 4 W (ng/mL)	2413 (1451–3376)	2266 (1568–2963)	0.79 (−1281–985)*

Values are expressed as mean (95% CI).
*Unpaired *t*-test. †Chi-square test.

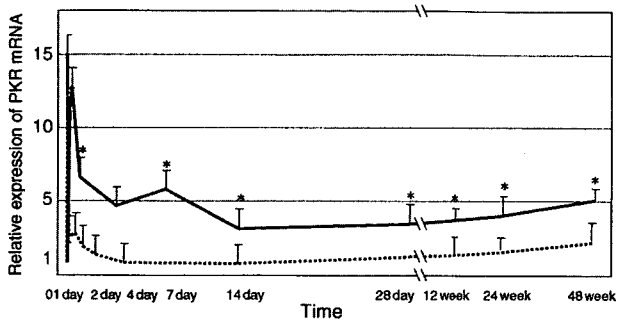

Fig. 1 Sequential expression of PKR mRNA in PBMCs during PEG- (solid line) and conventional (dotted line) IFN alpha-2b and ribavirin combination therapy. Expression of mRNA is shown as the expression level relative to baseline expression. The error bars indicate the standard error. An asterisk indicates a statistically significant difference in relative expression values between the two different IFN regimens ($P < 0.05$).

8 h, the level of PKR mRNA had fallen to a level that was twofold greater than the pre-treatment level (Fig. 1). With PEG-IFN alpha-2b administration, PKR mRNA expression reached a peak at 8 h at a level 12-fold greater than the pre-treatment level. At 24 h post-administration, the level of PKR mRNA had fallen but was still sixfold greater than the pre-treatment level (Fig. 1). This level was maintained until the next dose. No significant difference was observed in peak PKR mRNA expression between conventional IFN alpha-2b and PEG-IFN alpha-2b. However, from the second day of administration onwards, the expression was maintained at a significantly higher level in the PEG-IFN alpha-2b group compared with the conventional IFN alpha-2b group ($P < 0.05$) (Fig. 1).

Pharmacokinetics of serum pegylated interferon alpha-2b

The pharmacokinetic parameters for PEG-IFN alpha-2b at weeks 1 (first administration) and 48 (final administration) are shown in Table 2. Although the trough value of serum PEG-IFN alpha-2b varied between individuals, it almost reached a plateau at week 8. Accumulation of IFN was minimal in the PEG-IFN alpha-2b treatment regimen.

The level of serum PEG-IFN alpha-2b at week 1 increased gradually up to 12–24 h with a $t_{1/2\lambda_z}$ of 40.2 h. These levels were measurable up to 168 h after administration or immediately before the next administration. The trough value following administration showed no significant increase during the 48-week treatment phase (Fig. 2). The blood level after the final administration increased gradually for 12–24 h, remained high for approximately 48 h, and then decreased slowly with a $t_{1/2\lambda_z}$ of 55.3 h. The drug remained measurable up to 2 weeks post-administration. The cumulative coefficients (Rods) of repeated administrations calculated on the basis of C_{max}, C_{168h} and AUC_{0-168h} were 0.917, 2.11 and 1.12, respectively. When a comparison was made between the first and final administrations (weeks 1 and 48), $t_{1/2}$ of serum PEG-IFN alpha-2b levels was slightly prolonged after the final administration, although no changes were observed in C_{max}, AUC and plasma clearance (CL/F) (Table 2; Fig. 3).

Pharmacokinetics of serum ribavirin

The pharmacokinetic parameters for ribavirin at weeks 1 (first administration) and 48 (final administration) are summarized in Table 3. The trough value of serum ribavirin almost reached a plateau 8 weeks after the initial administration. In contrast to PEG-IFN alpha-2b, ribavirin was accumulated significantly during the first 4–8 weeks.

Serum ribavirin levels after the first administration (first day) reached t_{max} by 3.33 h and then decreased rapidly with a $t_{1/2\lambda_z}$ of 27.1 h. In contrast, serum ribavirin levels reached t_{max} by 2.73 h after the final administration and then decreased slowly with a $t_{1/2\lambda_z}$ of 296 h. A comparison of the cumulative coefficient (Rods) in the steady state was made between the first and final administrations and was calculated on the basis of C_{max}, C_{12h} and AUC_{0-12h}. This showed that by the final administration, there was a marked increase in C_{max} and AUC in serum ribavirin levels, an approximately 10-fold prolongation of $t_{1/2\lambda_z}$, a decrease in CL/F of about 1/3, and an approximately threefold increase in Vz/F. There was no change evident in t_{max} (Table 3; Fig. 4).

Clinical and virological response and serum pegylated interferon alpha-2b and ribavirin levels

The dose of PEG-IFN alpha-2b was reduced in two patients after 4 and 25 weeks of treatment because of neutropoenia. Similarly, the dose of ribavirin was reduced in three patients

Table 2 Pharmacokinetic parameters of the patients who received PEG-IFN alpha-2b at weeks 1 (first administration) and 48 (final administration)

	t_{max} (h)	C_{max} (pg/mL)	C_{168h} (pg/mL)	$t_{1/2\lambda_z}$ (h)	AUC (pg h/mL) 0–168 h	CL/F (mL/h/kg)	Vz/F (L/kg)
First	23.1	874	99	40.2	68 926	21.4	1.18
Final	22.2	774	185	55.3	77 039	–	–
Rods	–	0.917	2.11	–	1.12	–	–

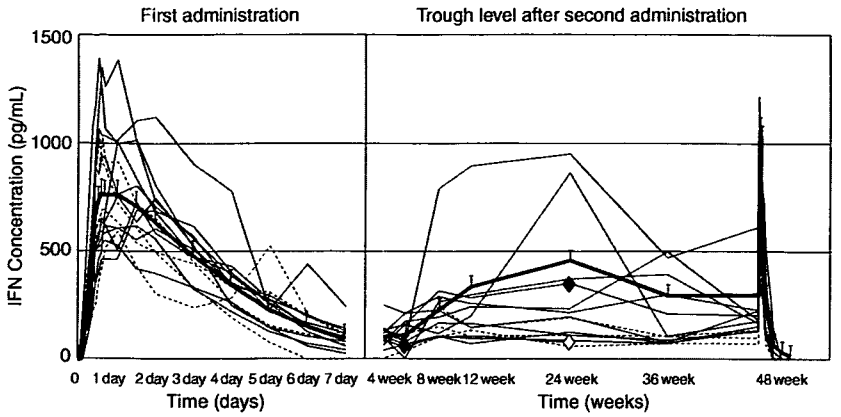

Fig. 2 Changes in serum IFN levels during PEG-IFN alpha-2b and ribavirin combination therapy. No significant increase in the trough value of serum IFN level was found during the 48-week treatment period. The bold lines indicate mean values, while the error bars indicate the standard error. Fine solid lines indicate a sustained virological responder and broken lines a nonresponder. The diamond-shaped symbol indicates a time point and IFN concentration at which either dose reduction (closed diamonds) or discontinuation (open diamonds) occurred.

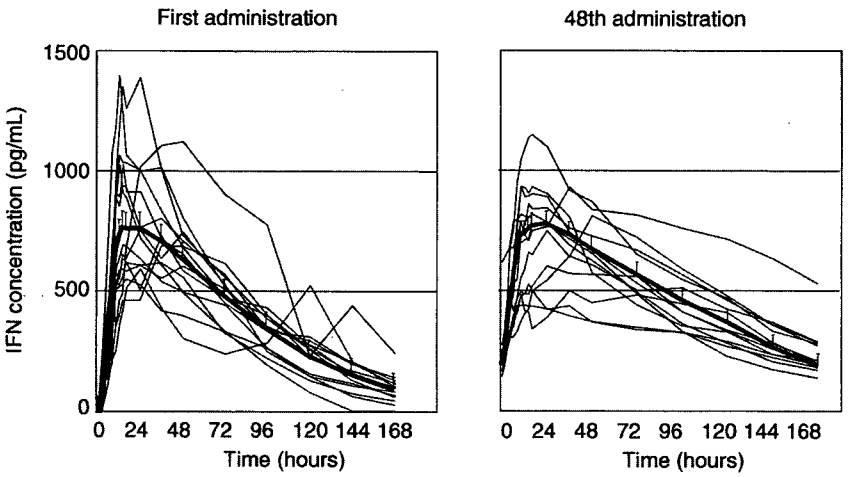

Fig. 3 A comparison of serum IFN levels between the first and 48th doses. Both show very similar values and no accumulation of IFN. It should be noted that PEG-IFN alpha-2b was detectable in all but one patient at 168 h after initial administration. Bold lines indicate mean values and the error bars indicate the standard error.

Table 3 Pharmacokinetic parameters of the patients who received ribavirin at weeks 1 (first administration) and 48 (final administration)

	t_{max} (h)	C_{max} (pg/mL)	C_{168h} (pg/mL)	$t_{1/2\lambda_z}$ (h)	AUC(pg h/mL) 0–168 h	CL/F (mL/h/kg)	Vz/F (L/kg)
First	3.33	604	221	27.1	4019	37.8	1472
Final	2.73	3449	2422	296	33 060	12.7	5374
Rods	–	6.53	12.2	–	9.42	–	–

after 12 and 16 weeks of treatment because of anaemia. In Figs 2 & 4, the individual time points and drug concentration following dose reduction are indicated by closed diamonds. No association could be found between dose reduction and serum concentration for both agents. Treatment was discontinued in 1 of the 15 patients because of depression, as indicated by open diamonds in Figs 2 & 4. Eleven patients including this patient achieved an SVR, with the remaining four patients being classified as NRs.

In order to demonstrate the association between virological response and pharmacokinetics, the final virological response for each individual is indicated in Figs 2 & 4. Serum IFN levels at 2 weeks post-dose tended to be slightly higher in NRs when compared with patients who achieved an SVR. This difference was not statistically significant. There was also no significant difference in serum ribavirin levels between these two groups from the time of the first administration until the completion of the 48-week treatment period.

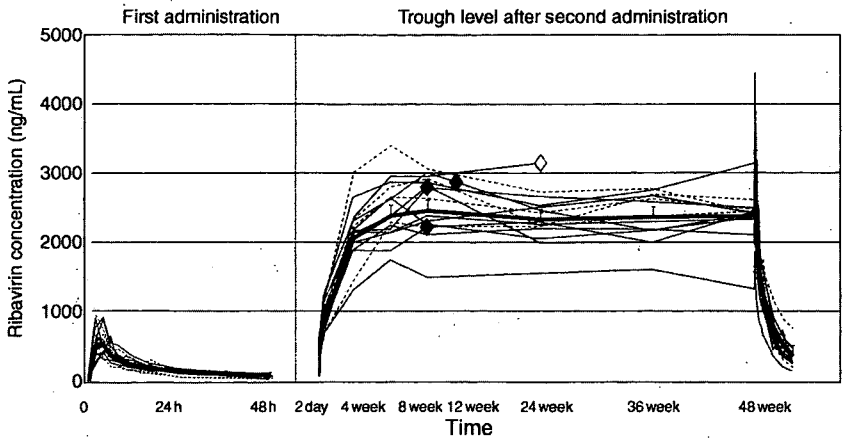

Fig. 4 Changes in serum ribavirin levels during PEG-IFN alpha-2b and ribavirin combination therapy. Serum ribavirin levels reached a peak by the eighth week and then plateaued. Bold lines indicate mean values and the error bars indicate the standard error. Fine solid lines indicate a sustained virological responder and broken lines a nonresponder. The diamond-shaped symbols indicate a time point and ribavirin concentration at which either dose reduction (closed diamonds) or discontinuation (open diamonds) occurred.

Association between PKR mRNA expression and virological response

The absolute expression levels of PKR mRNA at baseline prior to treatment were slightly higher in NRs than in SVR patients (1.8×10^{-2} *vs* 1.3×10^{-2} copies/one copy of G3PDH), although this difference was not statistically significant. Interestingly, in the PEG-IFN alpha-2b group, sequential PKR mRNA expression in response to PEG-IFN administration was significantly higher in patients who achieved an SVR compared with patients classified as NRs ($P < 0.05$) (Fig. 5).

The serum HCV dynamics during PEG-IFN alpha-2b and ribavirin combination therapy showed a biphasic pattern consisting of a rapid decrease within 24 h of initiation of the treatment (first phase), followed by a subsequent slow decrease. The mean viral decay during the first phase was 3.0 $\log_{10}$/day (95% CI: 2.4–3.5) and that calculated from day 2 onwards (the second phase of the response) was 0.075 (95% CI: 0.028–0.12) $\log_{10}$/day. Significant correlation was found between PKR expression at day 1 and viral decline rate calculated from the first phase of HCV dynamics ($r = 0.67$, $P = 0.0006$) (Fig. 6a). Moreover, significant correlation was also found between PKR expression at day 84 and second phase viral decline rate ($r = 0.67$, $P = 0.001$) (Fig. 6b). No significant associations were found between PEG-IFN or ribavirin concentration and kinetics of PKR expression.

DISCUSSION

The data of this study suggests that the higher expression levels of PKR transcripts seen with PEG-IFN alpha-2b from the second day of administration onwards were related, at least in part, to the improved efficacy of PEG-IFN alpha-2b compared with conventional IFN alpha-2b. Our pharmacokinetic study suggests that pegylation may be responsible for the dramatic effect on induction of PKR associated with the PEG-IFN regimen, possibly as a consequence of maintaining blood levels of IFN within the therapeutic range. This concept is supported by our previous work [8], in which we demonstrated that intracellular expression of PKR during the second phase was maintained at a significantly higher level when IFN-beta was administered twice daily.

The expression of PKR transcripts was induced very rapidly following the first administration, and PKR expression at day 1 was significantly correlated with the first phase viral decline rate of HCV dynamics. It is likely this increase in PKR transcripts was associated with the rapid decline of HCV seen in the first phase of serum HCV dynamics, and this change is believed to be a result of the direct effect of IFN on virion production and release from infected target cells [12]. Although we found that there was no significant difference in peak PKR mRNA expression between the PEG-IFN alpha-2b and IFN alpha-2b groups, the expression of PKR transcripts from 24 h onwards was significantly higher with PEG-IFN alpha-2b than conventional IFN alpha-2b administration. The decline in viral numbers and activity seen after the second day (second phase viral decline of HCV dynamics) is believed to reflect the presumed elimination of viral-infected cells in addition to the direct antiviral properties of IFN [12]. It has been suggested recently that apoptosis of HCV-infected cells induced by IFN-stimulated PKR may be an important mechanism for the elimination of viruses [13]. In the present study, expression of PKR transcripts in response to PEG-IFN administration was higher in patients who achieved SVR compared with NR patients, and expression of PKR at day 84 was significantly associated with the viral decline rate calculated from the second phase of HCV dynamics. Therefore, the increased expression of PKR transcripts we observed

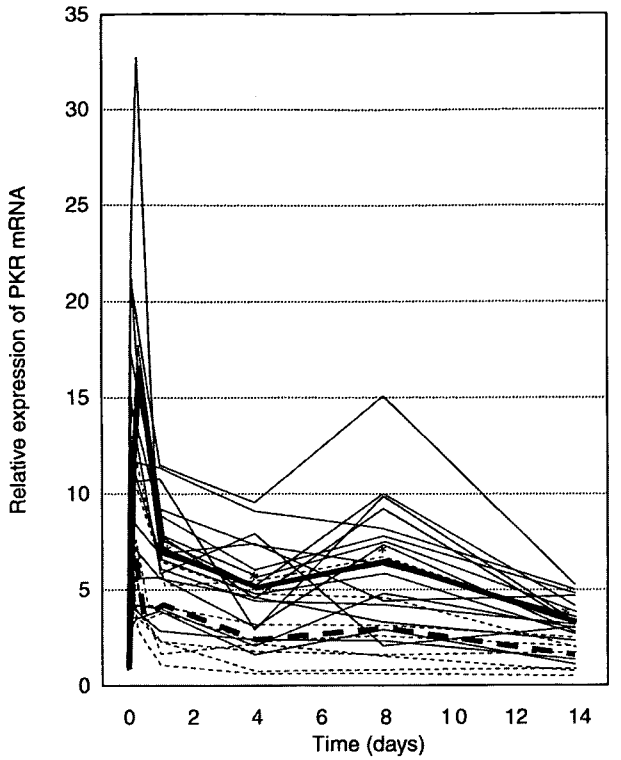

Fig. 5 Sequential expression of PKR mRNA in PBMCs in sustained viral responders (solid line, $n = 18$) and nonresponders (dotted line, $n = 8$). The bold line indicates the mean value for each group. Expression of mRNA is shown as the expression level relative to baseline expression. An asterisk indicates a statistically significant difference in relative expression value between the two different virological responses ($P < 0.05$).

after the second day may be associated with the enhanced efficacy of PEG-IFN alpha-2b. Again, this increased expression may have been due to an improvement in the pharmacokinetics of IFN following pegylation that results in prolonged clearance of IFN from serum.

Gerotto et al. [14] reported previously that higher baseline PKR expression was observed in NR patients compared with patients who achieved an SVR, although no significant difference was found in 'absolute' expression of PKR during treatment between these patients. We observed a similar trend in baseline expression in our study, although the relatively small number of cases meant that this difference did not achieve statistical significance. However, in our study, increased expression of PKR in response to PEG-IFN treatment was found in patients with an SVR. We analysed the changes in PKR expression during treatment relative to baseline expression levels. Because the absolute expression of PKR in response to IFN varies between patients (data not shown), we believe that calculating the level of expression during IFN treatment relative to the level of baseline expression is suitable in comparing PKR responses between patients. While this issue still remains controversial, our results imply that no or low responsiveness of PKR (i.e. less than a twofold increase from baseline) is associated negatively with an SVR, although high responsiveness of PKR during PEG-IFN administration does not always assure an SVR.

Although PBMCs were used as a model to quantify the serial gene expression of PKR, expression of PKR should be studied with hepatocytes, the target cell of HCV. Using liver tissue for sequential analysis is more ideal but ethically impossible. To address this point, we previously demonstrated a significant correlation between basal expression of PKR in liver tissue and the corresponding PBMC [8].

One of the limitation of the present study is that our results specifically concern PKR. Therefore, our present findings cannot be extrapolated to other ISGs such as MxA and 2′,5′-oligoadenylate synthetase. Although expression and response of ISGs to therapy may differ among different ISGs, we previously found significant correlation between sequential expression levels for PKR and MxA during IFN treatment [8].

In the present study, PEG-IFN alpha-2b was detectable in all but one patient at 168 h after initial administration in

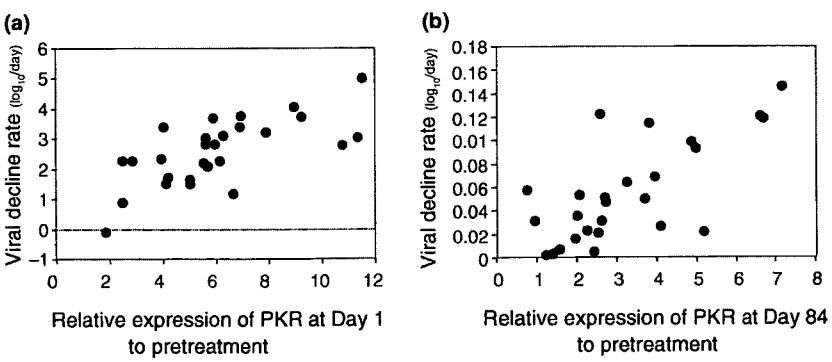

Fig. 6 (a) Significant correlation between expression of PKR mRNA at day 1 and viral decline rate calculated from the first phase of HCV dynamics ($r = 0.67$, $P = 0.0006$). (b) Significant correlation between expression of PKR mRNA at day 84 and viral decline rate calculated from the second phase of HCV dynamics ($r = 0.67$, $P = 0.001$).

contrast to a study reported by Bruno et al. [15]. However, as in that study, no significant accumulation of PEG-IFN alpha-2b was found during therapy, which is marked contrast to the data from PEG-IFN alpha-2a (40 kD) plus ribavirin therapy [15]. In our study, the viral response was not associated with serum PEG-IFN concentration, but it was associated with cellular responses to IFN such as PKR expression. Although both PEG-IFNs appear to show different profiles in absorption, distribution and clearance, it remains unknown how these differences relate to differences in cellular responses *in vivo* such as PKR and the primary clinical endpoint, SVR.

The serum level of ribavirin has been reported previously to be associated with the observed clinical effects [6]. With ribavirin combination therapy, the antiviral effect was more potent after 3 weeks, at which time serum ribavirin levels were shown to have increased [7]. Therefore, accumulation of ribavirin from the third week of administration onwards, during which viral suppression is important for SVR, may be associated with the viral response seen with combination therapy. However, in our study, we found no significant difference in serum ribavirin levels between patients who achieved an SVR and NR patients. There was also no significant difference in serum IFN levels between the SVR and NR patients. As there are only a small number of studies that have reported serum ribavirin levels and associated virological effects in detail, further more comprehensive investigations are therefore required.

In conclusion, the pharmacokinetic improvement provided by pegylation of IFN leads to dramatic changes in PKR transcript expression patterns. In contrast, serum ribavirin concentrations appear not to be associated with the viral response and PKR expression. Our data suggest that the higher intracellular expression of PKR transcripts from the second day onwards is associated with the enhanced virological efficacy of PEG-IFN alpha-2b and ribavirin combination therapy.

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The presence of steatosis and elevation of alanine aminotransferase level are associated with fibrosis progression in chronic hepatitis C with non-response to interferon therapy.

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Title: Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response

Short title: Innate immunity and therapeutic response

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Technology; and the Japanese Ministry of Welfare, Health and Labor.

Abbreviations: HCV, hepatitis C virus; PEG-IFN, pegylated interferon; NVR, non-virological responders; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation associated gene 5; CARD, Caspase-recruiting domain; Cardif, caspase-recruiting domain adaptor inducing IFN-beta; IPS-1, IFN-beta promoter stimulator I; MAVS, mitochondrial antiviral signaling protein; VISA, virus-induced signaling adaptor; RNF125, ring-finger protein 125; ISG15, IFN-stimulated gene 15; USP18, ubiquitin-specific protease 18; UBP43, ubiquitin-specific protease 43; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethylbilane synthase; PBMC, peripheral blood mononuclear cell; SVR, sustained viral responder; TR, transient responder; ROC, receiver operator characteristic; JAK, Janus kinase.

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Reviewer #1: None

Reviewer #2: No further comments.

External Validation of FIB-4: Diagnostic Accuracy Is Limited in Elderly Populations

To the Editor:

We read with interest the articles by Stering et al.[1] and Vallet-Pichard et al.[2] The former authors developed the FIB-4 index, a noninvasive method for assessing liver fibrosis in patients with HIV/HCV coinfection. The variables used are age, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet (PLT) count, and the formula is as follows: (age [yr] × AST [U/L])/ ((PLT[10^9/L]) × (ALT[U/L])$^{1/2}$). They showed that over 70% of patients could be classified into either absence or presence of advanced fibrosis by cutoff of <1.45 or >3.25 respectively, with diagnostic accuracy of 87%. The latter authors expanded the applicability of the FIB-4 index to HCV-monoinfected patients and showed that 73% of patients were classified with diagnostic accuracy of 93%, an excellent performance in both classification and accuracy of diagnosis.

Because the mean age of patients was young in these studies (40 years[1] and 44 years[2]), we wondered whether this index could also fit to Japanese patients who are rather older than the Western patients. We validated the FIB-4 index in a retrospective cohort of 1,405 patients who underwent liver biopsy at our hospital. The mean age was 55 ± 12 years. The distribution of METAVIR fibrosis scores was as follows: 1.6% showed no fibrosis (F0), 44.8% showed mild fibrosis (F1), 29.5% showed moderate fibrosis (F2), 20.2% showed severe fibrosis (F3), and 3.9% showed cirrhosis (F4). The proportion of advanced fibrosis (F3 or F4) was slightly higher in our population compared to the former studies (24.1% vs. 20.7% [1] and 17.2% [2]). As shown in Table 1, only 53% of patients were classified to either <1.45 or >3.25, a much lower rate than previous reports. The diagnostic accuracy was excellent in patients with a FIB-4 index <1.45 (94%), however, it was relatively poor in patients with a FIB-4 index >3.25 (50%) making the overall accuracy as low as 67%.

We supposed this discordance with previous reports may be derived from the older age of our populations and thus we categorized patients into three groups according to age and analyzed separately. In patients with age ≤50 years, 64% of patients were classified, and the diagnostic accuracy was 94% for a FIB-4 index <1.45 and 68% for a FIB-4 index >3.25 making the overall accuracy of 90%, a result comparable to previous reports. In older patients, however, diagnostic accuracy was significantly low compared to those with age ≤50 years (56% for age 51-60 years, $P < 0.0001$ and 51% for age ≥60 years, $P < 0.0001$). Because patients with a FIB-4 index >3.25 increased according to age (6%, 34%, and 53% for ages ≤50, 51-60 and >60 years), and the diagnostic accuracy was low in these patients (48% to 50%), these results suggest that, in elderly patients, a variable "age" generates excessively high FIB-4 index leading to misclassification of no-moderate fibrosis (F0-F2) into a FIB-4 index >3.25.

In conclusion, the FIB-4 index could accurately differentiate advanced fibrosis in young Japanese patients with chronic hepatitis C but the diagnostic accuracy is limited in the elderly. Thus, in elderly patients, some sort of adjustment for the effect of age on FIB-4 index may be necessary for more precise classification.

Table 1. Comparison of FIB-4 Index and Liver Biopsy Results in Terms of Age

		METAVIR Fibrosis Score			
	FIB-4	F0-2	F3-4	Total	Diagnostic Accuracy
All patients					
	<1.45	283 (20%)	18 (1%)	301 (21%)	94%
	>3.25	228 (16%)	226 (16%)	454 (32%)	50%
	1.45-3.25	556 (40%)	94 (7%)	650 (47%)	
	Total	1067 (76%)	338 (24%)	1405 (100%)	67%
Age ≤50	<1.45	240 (54%)	16 (4%)	256 (58%)	94%
(Mean 40 yrs)	>3.25	9 (2%)	19 (4%)	28 (6%)	68%
	1.45-3.25	126 (28%)	38 (8%)	164 (36%)	
	Total	375 (84%)	73 (16%)	448 (100%)	90%
Age 51-60	<1.45	30 (7%)	2 (1%)	32 (8%)	94%
(Mean 56 yrs)	>3.25	76 (18%)	69 (16%)	145 (34%)	48%
	1.45-3.25	215 (50%)	36 (8%)	251 (58%)	
	Total	321 (75%)	107 (25%)	428 (100%)	56%
Age >60	<1.45	13 (2%)	0 (0%)	13 (2%)	100%
(Mean 66 yrs)	>3.25	143 (27%)	138 (26%)	281 (53%)	49%
	1.45-3.25	215 (41%)	20 (4%)	235 (45%)	
	Total	371 (70%)	158 (30%)	529 (100%)	51%

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Pretreatment predictor of response, time to progression, and survival to intraarterial 5-fluorouracil/interferon combination therapy in patients with advanced hepatocellular carcinoma

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Background. Several studies have reported survival benefits of combination therapy with intraarterial 5-fluorouracil (5-FU) and subcutaneous interferon (IFN) α for advanced hepatocellular carcinoma (HCC) with portal vein tumor thrombosis (PVTT). We investigated the pretreatment predictive factors of early response, time to progression (TTP), and survival in response to intraarterial 5-FU/IFN combination therapy. ***Methods.*** Patients with nonresectable HCC and variable PVTT grades (without PVTT to PVTT in the trunk) received intraarterial 5-FU/IFN combination therapy (n = 55). ***Results.*** After two courses of the combination therapy, 1 (2%), 15 (27%), 16 (29%), 12 (22%), and 11 (20%) of 55 patients showed complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), or had dropped out (DO), respectively, when their early response to treatment was assessed. Univariate analysis identified only hepatitis C virus (HCV) antibody positivity as having significantly influenced the early response (P = 0.028) and TTP (P = 0.021). Multivariate analysis identified performance status (P = 0.003) and HCV antibody positivity (P = 0.007) as significant and independent determinants of survival. PVTT grade did not influence early response, TTP, or survival. The survival rate was significantly higher in patients who achieved CR or PR than in those that assessed as SD or PD, or DO (P < 0.0001, each). ***Conclusions.*** HCV antibody positivity may be a significant pretreatment predictor of early response, TTP, and survival of patients with advanced HCC treated with 5-FU/IFN. CR or PR as the early response to the combination therapy might indicate a more favorable prognosis in patients with advanced HCC. PVTT grade did not seem to influence the efficacy of combination therapy.

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Introduction

Hepatocellular carcinoma (HCC) is a life-threatening neoplasm and one of the most common neoplasms in Africa and Asia, including Japan. Deaths due to HCC are increasing worldwide.[1–3] Advances in biotechnology have resulted in new diagnostic techniques, such as ultrasonography, computed tomography (CT), magnetic resonance imaging, and angiography. Similarly, new treatment options have become available, such as surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), and transcatheter arterial chemoembolization (TACE). As a result, the prognosis of HCC patients has gradually improved. Nevertheless, the survival rates of patients with advanced HCC and complications such as portal vein tumor thrombosis (PVTT) or distant metastasis remains extremely poor.[4–8]

Advances in implantable drug delivery systems have allowed repeated arterial infusions of anticancer agents. First, monotherapy with intraarterial 5-fluorouracil (5-FU) for unresectable HCC was reported.[9,10] However, such treatment resulted in a low response rate (13.0% and 22.0%). Next, several authors reported favorable results with low-dose cisplatin and 5-FU for advanced HCC with PVTT, with a response rate ranging from 33.0% to 48.0%.[11–13] Recently, several studies have reported survival benefits of combination therapy with intraarterial 5-FU and subcutaneous interferon (IFN) α for advanced HCC with PVTT, with a response rate ranging from 43.6% to 72.7%.[14–17] In these studies, only HCC patients with PVTT (in the main trunk or first branch) without distant metastases were treated. The

pretreatment predictive factors of response, time to progression (TTP), and survival of HCC patients treated with the combination therapy remain unclear. At present, some patients with nonresectable HCC are treated with TACE. However, some patients are not suitable candidates for TACE because of PVTT or poor response to TACE. Because of the poor prognosis of patients with nonresectable HCC who are not treatable by TACE, effective treatment is needed. There is little information about assessment of patients with advanced HCC (e.g., nonresectable HCC with PVTT in the second branch or nonresectable HCC without PVTT but with poor response to TACE) treated with combination therapy of intraarterial 5-FU and IFN. In the present retrospective cohort study, we assessed the efficacy of intraarterial 5-FU with IFN for various types of nonresectable advanced HCC and investigated the pretreatment predictive factors of early response, TTP, and survival in response to the combination therapy.

Materials and methods

Patients

From June 2003 to December 2006, 265 consecutive patients with unresectable HCC were admitted to our hospital. Of the 265 patients with advanced HCC, 94 were treated with TACE, 34 patients received systemic chemotherapy, and 13 patients received best supportive care. The remaining 124 patients were selected as suitable candidates for intraarterial 5-FU and IFN combination therapy. Forty-one patients refused the therapy. Thus, 83 patients with advanced HCC were treated with intraarterial 5-FU and IFN. Of these 83 patients, 24 with distant metastases and four with hepatic venous invasion were excluded from this study, so we assessed 55 patients without distant metastases or hepatic venous invasion in this retrospective cohort study. Of the 55 patients, 30 had been treated with TACE before enrollment. Table 1 lists the baseline characteristics of the 55 patients. PVTT grade, based on the location of the tumor thrombus, was determined according to the criteria of the Liver Cancer Study Group of Japan (LCSGJ).[18] PVTT grading was as follows: Vp 0, no PVTT; Vp 1, tumor thrombus in a third or more of the peripheral branches of the portal vein; Vp 2, tumor thrombus in a second branch of the portal vein; Vp 3, tumor thrombus in the first branch of the portal vein; and Vp 4, tumor thrombus in the trunk of the portal vein. Tumor staging was defined based on the TNM staging system of the LCSGJ:[18] stage I (fulfilling three intrahepatic conditions: solitary, <2 cm, no vessel invasion), stage II (fulfilling two of the three intrahepatic conditions), stage III (fulfilling one of the three intrahepatic conditions), stage IVA (fulfilling none of the three intrahepatic conditions with no distant metastases or any intrahepatic conditions with lymph node metastases), and stage IVB (any intrahepatic conditions with distant metastases).

Eligibility

This was a retrospective cohort study to investigate pretreatment predictive factors of TTP, survival, and

Table 1. Clinical profile of the 55 HCC patients

Age (years)[a]	67 (38–79)
Sex (M/F)	44/11
Etiology: HBV/HCV/other	15/36/4
Total bilirubin (mg/dl)	1.1 (0.4–6.4)
Platelet count ($\times 10^4$ mg/dl)	13.0 (5.1–54.5)
Albumin (mg/dl)	3.5 (2.4–4.8)
Child Pugh stage (A/B/C)	43/10/2
PS (0/1)	45/10
Intrahepatic tumor volume (≤50%/>50%)	38/17
Tumor stage (III/IVA)	20/35
Vp[a] (0/2/3/4)	20/6/15/14
AFP (ng/ml)	934 (14.3–525 900)
AFP-L3 (%)	47.3 (<0.5–87.6)
DCP (mAU/ml)	3729 (10–722 140)
Previous treatment (performed/not performed)	30/25

Data are expressed as median with range values in parentheses, or number of patients HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; PS, Eastern Cooperative Oncology Group performance status; AFP, α-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of α-fetoprotein; DCP, des-γ-carboxy prothrombin; PVTT, portal vein tumor thrombosis

[a] PVTT grade: Vp 0, no PVTT; Vp 1, tumor thrombus in a third or more of the peripheral branches of the portal vein; Vp 2, tumor thrombus in a second branch of the portal vein; Vp 3, tumor thrombus in the first branch of the portal vein; Vp 4, tumor thrombus in the trunk of the portal vein