response to intraarterial 5-FU/IFN combination therapy. Eligibility criteria were as follows: age, 18–80 years; leukocyte count, >2000/μl; neutrophil count, >1200/μl; hemoglobin, >8 g/dl; platelet count, >50 000/μl; unresectable or not suitable for local ablation therapy, including RFA or PEI; with PVTT or TACE was ineffective; without hepatic venous invasion; without distant metastases; and Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–1.[19] There was no eligibility criterion regarding hepatic reserve function, including serum total bilirubin levels. All patients gave written informed consent to this study, which was approved by the Institutional Review Board of Hiroshima University.

Treatment protocol

Patients received repeated arterial infusions of anticancer agents via the injection port. One course of chemotherapy lasted 4 weeks. 5-FU (500 mg body weight/day, Kyowa Hakko, Tokyo, Japan) was administered over 5 h with a mechanical infusion pump on days 1 to 5 of the first and second weeks (5 g in one course). Recombinant IFN α-2b (Intron A, Schering-Plough Pharmaceuticals, Osaka, Japan); 3×10^6 U (3 MU), or natural IFN α (OIF, Otsuka Pharmaceuticals, Tokyo, Japan); 5×10^6 U (5 MU) was administered intramuscularly on days 1, 3, and 5 of each week (total dose, 36 and 60 MU, respectively). In principle, treatment was repeated several times unless PS changed to 3 or 4 during the treatment. A 2- to 4-week rest period of no treatment was allowed after each treatment course. As for the two types of IFN, we previously reported similar effects of recombinant IFN α-2b and natural IFN α when combined with intraarterial 5-FU for the treatment of advanced HCC.[20]

Implantation of the arterial catheter

A catheter was inserted through the right femoral artery by the Seldinger method. After localization of the HCC, a 3-French heparin-coated catheter was inserted and its tip advanced to the common hepatic artery or proper hepatic artery. The other end of the catheter was connected to the injection port, which was implanted in a subcutaneous pocket created in the right lower abdominal quadrant. The gastroduodenal artery and right gastric artery were occluded with steel coils to prevent gastroduodenal injury by the chemotherapeutic agents.

Evaluation

The early response to the combination therapy was assessed with contrast-enhanced CT after two courses of the combination therapy. The response was defined according to the criteria of the Response Evaluation Criteria in Solid Tumors (RECIST).[21] A complete response (CR) was defined as the complete disappearance of all target lesions. A partial response (PR) was defined as a decrease of at least 30% in the sum of the longest diameter of the target lesions with the baseline sum of the longest diameter of the target lesions as the reference. Progressive disease (PD) was defined as an increase of at least 20% in the sum of the longest diameter of target lesions. Stable disease (SD) was defined as meeting neither the PR nor the PD criteria. The duration of the response was measured from the date of the start of treatment to the date of documented progression. Adverse reactions were assessed with the National Cancer Institute Common Toxicity Criteria (NCI-CTC; version 3.0)[22] every week during the treatment.

Additional therapy

After two courses of the combination therapy, we assessed the response to therapy in all patients. According to the response, we provided various additional therapies such as RFA, TACE, or radiotherapy (RT) to patients treated with the combination therapy. These additional therapies were considered for patients with PS of 0–1 and a Child-Pugh stage of A or B. Patients assessed with PR continued to receive the combination therapy repeatedly. Then, when downstaging of advanced HCC was achieved (single tumor ≤50 mm in diameter or 1–3 tumors ≤30 mm in diameter) by the repeated combination therapy, RFA was considered. For patients assessed with SD or PD, in addition to the combination therapy, TACE with cisplatin–lipiodol suspension was performed. The catheter tip was advanced superselectively into the feeding artery so that sufficient anticancer agent was delivered. Among the patients assessed with SD or PD, RT was performed for PVTT if present. For patients assessed with CR, the clinical course was observed without adjuvant chemotherapy or additional therapy.

Statistical analysis

Statistical analysis was performed on 1 April 2007. Differences between groups were examined for statistical significance using the Mann-Whitney *U* test, logistic regression test, or χ-squared test as appropriate. Cumulative survival rate and TTP were calculated from the initial date of the combination therapy and assessed by the Kaplan-Meier life-table method, and differences were evaluated by the log rank test. Univariate and multivariate analyses of predictors for early response to the combination therapy were assessed by logistic

regression test. Univariate analysis of predictors of TTP and survival of patients with HCC who received the combination therapy was assessed by the Kaplan-Meier life-table method, and differences were evaluated by the log rank test. Multivariate analysis of predictors of TTP and survival was assessed by Cox proportional hazard model. Statistical significance was defined as a P value of less than 0.05. All analyses described above were performed with SPSS software (version 11, SPSS, Chicago, IL, USA). In this study, we investigated pretreatment predictive factors of early response, TTP, and survival in response to the combination therapy.

Results

Response to the combination therapy

The early response of the 55 patients was assessed after two courses of 5-FU/IFN combination therapy. As a result, 1 (2%), 15 (27%), 16 (29%), 12 (22%), and 11 (20%) patients showed CR, PR, SD, PD, or dropped out (DO), respectively. The reasons for DO were confusion (one patient), refusal after initiation of therapy (one patient), exanthema (one patient), infection around the catheter (four patients), and stenosis of the hepatic artery (four patients). We investigated the pretreatment determinants of the early response to the combination therapy. Univariate analysis identified positivity to HCV antibody as the only factor with significant influence on the early response (P = 0.028, Table 2, Fig. 1). Of the HCV antibody-positive patients, 38.9% (14/36) showed an early response of CR or PR, but only 10.5% (2/19) of other patients. When we compared the early response between patients with Vp 0–2 and those with Vp 3/4, 30.8% (8/26) of patients with Vp 0–2 and 27.6% (8/29) of those with Vp 3/4 achieved CR or PR, but the difference was not significant.

Time to progression

The median TTP in all 55 patients was 7.5 months [95% confidence interval (CI), 5.1–9.9 months], and the cumulative TTP rates at 6, 12, 18, and 24 months were 60%, 41%, 30%, and 24%, respectively. We investigated the pretreatment determinants of TTP after initiation of the combination therapy. Univariate analysis identified positivity for HCV antibody as the only factor with significant influence on TTP (P = 0.021, Table 3, Fig. 2). The median TTP in patients with Vp 0–2 and those with Vp 3/4 was 5.2 and 7.5 months, respectively. There was no significant difference in TTP between these two groups.

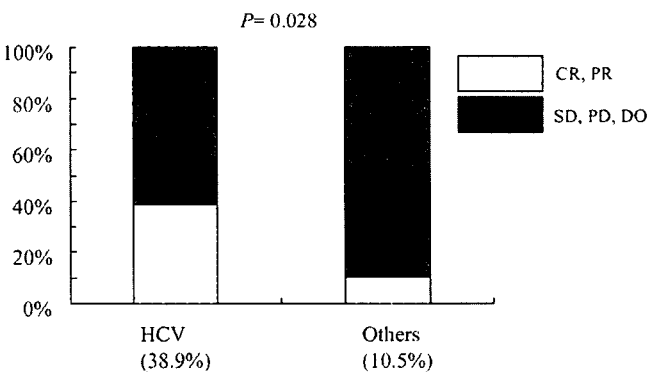

Fig. 1. Comparison of the early response rate between the hepatitis C virus (HCV)-positive group and others. The rate was significantly higher in the HCV-positive group (logistic regression test: P = 0.028). *CR*, complete response; *PR*, partial response; *SD*, stable disease; *PD*, progressive disease; *DO*, dropped out

Table 2. Univariate analysis of predictors for early response to 5-FU/IFN combination therapy

Variable	Odds Ratio	95% CI	P value
Age (≤65 vs. >65 years)	0.463	0.136–1.572	0.217
Sex (M vs. F)	2.327	0.445–12.168	0.317
HCV antibody (positive vs. negative)	6.071	1.216–30.314	0.028
Total bilirubin (≤1.5 vs. >1.5 mg/dl)	0.931	0.240–3.614	0.918
Platelet count (≤150000 vs. >150000 mg/dl)	0.978	0.278–3.437	0.972
Albumin (≤3.5 vs. >3.5 mg/dl)	1.390	0.441–4.376	0.574
Child Pugh stage (A vs. B, C)	2.172	0.413–11.420	0.360
PS (0 vs. 1)	4.965	0.576–42.810	0.145
Intrahepatic tumor volume (≤50% vs. >50%)	1.690	0.458–6.237	0.431
Tumor stage (III vs. IVA)	1.709	0.533–5.478	0.367
Vp (0–2 vs. 3, 4)	0.988	0.314–3.106	0.983
AFP (≤10000 vs. >10000 ng/ml)	0.978	0.278–3.437	0.972
AFP-L3 (≤50 vs. >50%)	0.776	0.229–2.625	0.683
DCP (≤10000 vs. >10000 mAU/ml)	0.606	0.186–1.974	0.406
Treatment (performed vs. not performed)	1.833	0.563–5.970	0.314

5-FU, 5-fluorouracil; IFN, interferon; CI, confidence interval

Table 3. Univariate analysis of predictors of time to progression

Variable	Hazard Ratio	95% CI	*P* value
Age (>65 vs. ≤65 years)	1.348	0.177–10.263	0.773
Sex (M vs. F)	1.788	0.403–7.935	0.445
HCV antibody (positive vs. negative)	2.775	1.169–6.590	0.021
Total bilirubin (≤1.5 vs. >1.5 mg/dl)	0.618	0.216–1.768	0.370
Platelet count (≤150000 vs. >150000 mg/dl)	0.739	0.307–1.777	0.500
Albumin (≤3.5 vs. >3.5 mg/dl)	0.705	0.300–1.655	0.421
Child Pugh stage (A vs. B, C)	2.381	0.314–18.045	0.401
PS (0 vs. 1)	1.348	0.177–10.263	0.773
Intrahepatic tumor volume (≤50% vs. >50%)	0.710	0.298–1.691	0.440
Tumor stage (III vs. IVA)	1.107	0.469–2.616	0.816
Vp (0–2 vs. 3, 4)	1.195	0.512–2.790	0.680
AFP (≤10000 vs. >10000 ng/ml)	1.325	0.484–3.626	0.584
AFP-L3 (≤50% vs. >50%)	2.371	0.696–8.076	0.167
DCP (≤10000 vs. >10000 mAU/ml)	1.145	0.486–2.701	0.756
Treatment (performed vs. not performed)	0.671	0.282–1.595	0.367

Table 4. Univariate analysis of predictors of survival of patients with HCC who received 5-FU/IFN combination therapy

Variable	Hazard Ratio	95% CI	*P* value
Age (≤65 vs. >65 years)	0.763	0.402–1.449	0.408
Sex (M vs. F)	1.208	0.527–2.769	0.655
HCV antibody (positive vs. negative)	2.283	1.165–4.474	0.016
Total bilirubin (≤1.5 vs. >1.5 mg/dl)	0.628	0.308–1.278	0.199
Platelet count (≤150000 vs. >150000 mg/dl)	0.690	0.355–1.340	0.273
Albumin (≤3.5 vs. >3.5 mg/dl)	0.760	0.398–1.451	0.406
Child Pugh stage (A vs. B, C)	0.527	0.228–1.216	0.133
PS (0 vs. 1)	3.413	1.391–8.375	0.007
Intrahepatic tumor volume (≤50% vs. >50%)	0.753	0.383–1.481	0.411
Tumor stage (III vs. IVA)	0.670	0.342–1.313	0.243
Vp (0–2 vs. 3, 4)	0.745	0.389–1.427	0.374
AFP (≤10000 vs. >10000 ng/ml)	0.947	0.445–2.017	0.888
AFP-L3 (≤50% vs. >50%)	0.898	0.430–1.871	0.773
DCP (≤10000 vs. >10000 mAU/ml)	0.753	0.394–1.438	0.390
Treatment (performed vs. not performed)	0.627	0.319–1.230	0.175
Additional therapy (performed vs. not performed)	1.129	0.583–2.188	0.719

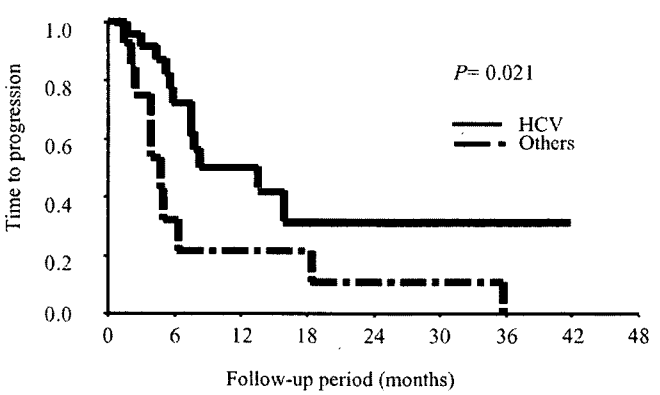

Fig. 2. Comparison of the time to progression between the HCV antibody-positive group and others. The rate was significantly higher in the HCV-positive group (log-rank test: $P = 0.021$)

Survival

The median survival in the whole group was 9.0 months (95% CI, 7.0–11.0 months), and the cumulative survival rates at 6, 12, 18, and 24 months were 67%, 39%, 22%, and 17%, respectively. We investigated the pretreatment determinants of survival after initiation of the 5-FU/IFN combination therapy. Univariate analysis identified PS = 0 ($P = 0.007$) and positivity for HCV antibody ($P = 0.016$) (Table 4, Fig. 3) as factors that significantly influenced survival. Since it was possible that the variables were mutually correlated, we performed a multivariate analysis and identified PS = 0 ($P = 0.003$) and positivity for HCV antibody ($P = 0.007$) as significant and independent determinants of survival (Table 5). The median survival time of patients with Vp 0–2 and of those with Vp 3/4 was 13.0 and 8.0 months, respectively. There was no significant difference in survival between these two groups.

Table 5. Multivariate analysis of predictors of survival of patients with HCC who received 5-FU/IFN combination therapy

Variable	Hazard Ratio	95% CI	*P* value
PS (0 vs. 1)	4.056	1.601–10.276	0.003
HCV antibody (positive vs. negative)	2.555	1.286–5.079	0.007

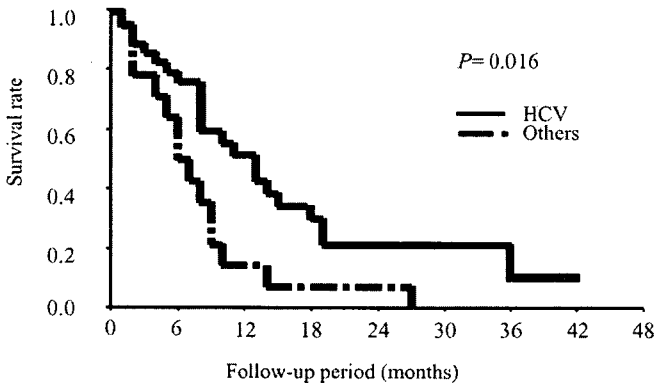

Fig. 3. Comparison of the cumulative survival rates between the HCV antibody-positive group and others. The rate was significantly higher in the HCV-positive group (log-rank test: $P = 0.016$)

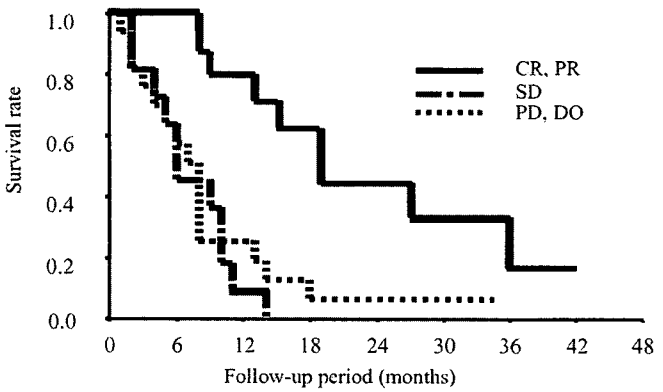

Fig. 4. Comparison of the cumulative survival rates among patients with CR/PR, SD, or PD/DO. The rate was significantly higher in patients who achieved CR/PR than those who showed SD (log-rank test: $P < 0.0001$) or PD/DO (log-rank test: $P < 0.0001$)

The cumulative survival rates of patients who achieved CR/PR at 6 and 12 months were 100% and 80%, respectively. On the other hand, the cumulative survival rates of patients who showed SD or PD/DO at 6 and 12 months were 64% and 9%, and 57% and 25%, respectively. The survival rate was significantly higher in patients who achieved CR/PR than in the other patients ($P < 0.0001$, Fig. 4).

Adverse reactions and complications

The most common adverse reactions were fever, nausea, and loss of appetite, but these were mostly NCI-CTC grade 1 or 2. Among patients with various NCI-CTC grade 3 adverse reactions, leukopenia was observed in seven (12.7%) patients, and thrombocytopenia in five (9.1%). None required administration of granulocyte colony-stimulating factor or blood transfusion. Five (9.1%) patients showed infection associated with the indwelling catheter. In this study, the number of patients with serum total bilirubin levels >3 mg/dl was three (3.7 mg/dl, 4.7 mg/dl, and 6.4 mg/dl). Other hepatic reserve functions and PS of the three patients was good (albumin, 4.1, 3.3, and 3.9 g/dl; prothrombin time, 60, 91, and 83%; PS, 0 in all cases). These three patients did not show any severe adverse reaction.

Additional therapy

Among the 55 patients, one (2%), ten (20%), and four (8%) patients were treated with RFA, TACE, and RT, respectively, as additional therapies for PVTT. The median survival time in patients receiving and in those not receiving additional therapies was the same at 9.0 months. There was no significant difference in survival between the two groups (Table 4).

Causes of death

Seventeen patients were still alive at the end of the observation period, and 38 patients had died. All 38 patients died of intrahepatic HCC-related disease.

Discussion

The median survival time of HCC patients with PVTT in the portal trunk is reported to be about 90 days with supportive care.[23] Recent studies have reported the efficacy and survival benefits of combination therapy with intraarterial 5-FU and IFN in a large number of patients with advanced HCC.[16,17] In particular, Ota et al.[16] assessed 55 patients with advanced HCC, multiple lesions, and Vp 3 or 4, and Obi et al.[17] assessed 116 patients with advanced HCC with Vp 3 or 4. These two studies assessed only patients with advanced HCC/

Vp 3 or 4. Thus, the favorable survival results they reported suggest that combination therapy with intraarterial 5-FU and IFN is potentially useful also for HCC with Vp 0–2. Although TACE is the standard treatment option for nonresectable HCC, many patients with nonresectable HCC either show a poor response to TACE or are not suitable candidates for TACE. The prognosis of patients with nonresectable HCC who are not treated with TACE is poor, so an effective treatment for such patients is needed. In this study, we treated a heterogeneous group of patients with advanced HCC (i.e., patients with nonresectable HCC and Vp 3 or 4, those with nonresectable HCC and Vp 2 who were not suitable candidates for TACE, and those with nonresectable HCC without PVTT who showed a poor response to TACE). There was no significant difference in early response, TTP, or survival between HCC patients with Vp 0–2 and those with Vp 3/4. Hence, with regard to the response to 5-FU/IFN combination therapy, PVTT grade does not seem to be an important factor.

The objective response rates (CR and PR patients/all patients) reported in the above two studies[16,17] were 43.6% (24/55 patients) and 52.6% (61/116 patients). In our study, the objective response rate, based on the early response, was 29% (16/55 patients). One reason for the discrepancy may be that the response was evaluated differently in the three studies. Ota et al.[16] and Obi et al.[17] used ECOG criteria, but we used RECIST criteria. Second, the inclusion criteria were different. Ota et al.[16] included patients with aspartate aminotransferase (AST) or alanine aminotransferase (ALT) of less than 100 IU/l and patients with total bilirubin of less than 1.4 mg/dl, whereas Obi et al.[17] mentioned no inclusion criteria related to AST or ALT, though they used a total bilirubin level of 3.0 mg/dl as a cutoff. In our study, we used no inclusion criteria related to AST or ALT. Third, the assessment day in our protocol may be earlier than that in the other two studies. In our study, the early response cumulative survival rate was significantly higher in patients who achieved CR/PR than in those with SD or PD/DO ($P < 0.0001$, each). The early response is an important posttreatment predictor of survival of patients with advanced HCC on 5-FU/IFN combination therapy. An early response of CR or PR promises a good prognosis.

The cumulative survival rates reported by Ota et al.[16] and Obi et al.[17] at 12 and 24 months were 48.9% and 28.8%, and 34% and 18%, respectively. In our study, the cumulative survival rates at 12 and 24 months were 39% and 17%, respectively. We obtained survival rates almost identical to those reported by Obi et al.,[17] but Ota et al.[16] obtained better survival rates. This discrepancy may be due to the differences in the inclusion criteria, as described above.

Our results indicated that HCV antibody positivity was a significant pretreatment predictor of early response, TTP, and survival of patients with advanced HCC treated with 5-FU/IFN. On the other hand, PVTT grade and total bilirubin levels were not significant predictors. Though we established no eligibility criterion regarding serum total bilirubin levels, the median total bilirubin level was 1.1 mg/dl (range, 0.4–6.4). Therefore, total bilirubin levels may not be statistically significant predictors in this study. In this study, three patients had serum total bilirubin levels >3 mg/dl. These patient achieved PR, SD, and PD. Though the three patients with high bilirubinemia (≥3 mg/dl) were safely treated in this study, we think that 5-FU/IFN combination therapy should be used with caution in patients with advanced HCC with high bilirubinemia. In general, the prognosis of HCC patients with Vp 3 or 4 is poorer than those with Vp 0–2. In this study, we treated a heterogeneous group of patients with advanced HCC as described above. Therefore, HCC with Vp 0–2 cases were advanced HCC cases in this study. All 55 patients were thought to have a poor prognosis at the time of enrollment in this study. Achievement of a good early response is important for good survival. A study with larger sample size may show the importance of PVTT grade and total bilirubin level.

Obi et al.[17] also reported that positivity to HCV antibody might be a predictor of CR in patients with advanced HCC treated with 5-FU/IFN. Why is HCV antibody positivity a predictor of the efficacy of combination therapy? One reason may be the underlying mechanisms associated with hepatocarcinogenesis. In our study, 36 patients were infected with HCV, 15 with hepatitis B virus (HBV), and four with non-B non-C hepatitis. Although the probability of hepatocarcinogenesis is high for both HBV and HCV infections, some differences have been noted with regard to their relationship with HCC.[24,25] HCV is an RNA virus, and viral genes are not integrated into the host genome. On the other hand, HBV is a DNA virus with reverse-transcriptase activity. HBV-mediated hepatocarcinogenesis is reported to be associated with the integration of viral DNA into the host genome.[26–28] The integration of the HBV genome into the host genome may diminish the effect of intraarterial 5-FU/IFN combination therapy. A second reason may be the differentiation of the cytokine pattern in HBV and HCV hepatitis.[29] Falasca et al.[29] reported the presence of high levels of Th1 cytokines, particularly during the course of chronic hepatitis B. They also reported that interleukin (IL)-18 and IL-6 levels might play important roles in both inflammation and hepatic injury, particularly during the course of hepatitis C infection. IFN may play a different role in patients with advanced HCC associated with HBV or HCV. In this study, the efficacy of the

combination therapy for advanced HCC patients with non-B non-C hepatitis was not clear because of the small number ($n = 4$) of those patients.

The DO proportion was high in this study (20%). Two major reasons for DO were infection around the catheter and stenosis of the hepatic artery. In this study, we established no eligibility criterion regarding the hepatic reserve function, including serum total bilirubin. Poor hepatic reserve function and high bilirubinemia might affect infection around the catheter. On the other hand, previous treatment with TACE might injury the hepatic artery and affect hepatic artery stenosis.

In conclusion, HCV antibody positivity might be a pretreatment predictive factor for early response, TTP, and survival of patients with advanced HCC treated with intraarterial 5-FU/IFN combination therapy. Early response to the combination therapy might be a significant posttreatment predictor of survival. Thus, patients who do not achieve CR or PR during the early phase of combination therapy should be switched to another treatment modality. Our results also showed that PVTT grade does not seem to be an important factor in the prognosis of patients with advanced HCC treated with 5-FU/IFN combination therapy. Further studies with long-term follow-up and a larger sample size are needed.

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Successful Treatment of an Entecavir-Resistant Hepatitis B Virus Variant

Hiromi Yatsuji,[1,2,3] Nobuhiko Hiraga,[1,2] Nami Mori,[1,2] Tsuyoshi Hatakeyama,[1,2] Masataka Tsuge,[1,2] Michio Imamura,[1,2] Shoichi Takahashi,[1,2] Yoshifumi Fujimoto,[2] Hidenori Ochi,[2,4] Hiromi Abe,[1,4] Toshiro Maekawa,[4] Fumitaka Suzuki,[3] Hiromitsu Kumada,[3] and Kazuaki Chayama[1,2,4]*

[1]*Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*
[2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[3]*Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan*
[4]*Laboratory for Liver Disease, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan*

Emergence of a lamivudine (LAM)-resistant hepatitis B virus (HBV) with amino acid substitutions in the YMDD motif is a well-documented problem during long-term LAM therapy. Entecavir (ETV) is a new drug approved for treatment of HBV infection with or without LAM-resistant mutants. This report describes an ETV-resistant strain of HBV, which emerged after prolonged ETV therapy in a patient who did not respond to LAM therapy. Direct sequence analysis of the ETV-resistant strain showed appearance of amino acid substitution rtS202G in the reverse transcriptase (RT) domain, together with rtL180M + M204V substitution that had developed at the emergence of LAM-resistant mutant. In vitro analysis demonstrated that the rtL180M + M204V + S202G mutant strain displayed a 200-fold and a 5-fold reduction in susceptibility to ETV compared with the wild-type and the rtL180M + M204V mutant strain, respectively. Adefovir was effective against the ETV-resistant strain both in vitro and during the clinical course. In conclusion, this study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV and confirmed that the addition of rtS202G substitution to the rtL180M + M204V mutant strain is responsible for ETV resistance and we could treat the resistant mutant successfully. ***J. Med. Virol. 79:1811–1817, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: HBV; rtS202G; lamivudine; adefovir; in vitro

INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma [Bruix and Llovet, 2003; Ganem and Prince, 2004]. To date, interferon and three nucleoside and nucleotide analogs (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and improve transaminase levels and liver histology [Nevens et al., 1997; Lai et al., 1998; Suzuki et al., 1999]. However, prolonged therapy results in the emergence of drug-resistant mutants.

LAM is associated with a higher rate of emergence of drug-resistant mutants than ADV or ETV, which is 24% and 70% after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels [Lai et al., 2003]. Most LAM-resistant

Abbreviations used: HBeAg,hepatitis B e antigenHBsAg,hepatitis B surface antigenHBV,hepatitis B virusORF,open reading framePCR,polymerase chain reactionRT,reverse transcriptase

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*Correspondence to: Kazuaki Chayama, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima-shi 734-8551, Japan.
E-mail: chayama@hiroshima-u.ac.jp

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strains show amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [Allen et al., 1998; Delaney et al., 2003].

Both in vitro and clinical studies have shown recently that ADV and ETV could suppress both wild-type and LAM-resistant strains and were confirmed as salvage therapy for LAM-refractory patients [Levine et al., 2003; Sherman et al., 2006; Rapti et al., 2007]. However, a few studies have already reported the emergence of resistant mutants to these drugs.

ADV-resistant mutations are infrequent and their appearance is delayed in treatment-naïve patients; mutation occurs at 0% after 1 year and 28% after 5 years and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [Maecellin and Asselah, 2005]. On the other hand, the emergence rate of ADV-resistant mutations in LAM-resistant patients was 18% after 48 weeks of ADV monotherapy [Lee et al., 2006]. A recent study reported patients treated with combination therapy of ADV with LAM did not develop resistance to ADV for 3 years [Rapti et al., 2007].

ETV is the most novel nucleotide analogue of the three drugs and displays greater in vitro potency than LAM or ADV against wild-type HBV. ETV-resistance is reported to be rare in treatment-naïve patients [Colonno et al., 2006]. However, ETV-resistant mutants appeared at 6–9% per year in LAM-refractory patients [Tenney et al., 2004, 2007; Sherman et al., 2006].

In the present study, an ETV-resistant strain of HBV was identified after prolonged ETV therapy in a patient who did not respond to LAM therapy. To our knowledge, this is the first report that breakthrough hepatitis was induced by emergence of an ETV-resistant strain and was successfully treated with ADV. This study checked the importance of amino acid substitutions in the HBV polymerase for resistance to ETV in vitro. Furthermore, the susceptibility of the mutant strain to ADV was analyzed.

MATERIALS AND METHODS

Antiviral Compounds

LAM [(−)-β-L-2′, 3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, UK). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and ETV {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingfold, CT).

Analysis of Virological Markers

Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) were determined by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using the Light Cycler (Roche, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAA-CAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Cloning of HBV-DNA and Plasmid Construction

HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. The full-length HBV-DNA was amplified using the above HBV-DNA samples by the method of Gunther et al. [1998]. Nucleotide sequence positions were numbered from the unique *Eco*RI site. The 1.4 genome lengths HBV-DNA amplified from the serum of a patient who showed ETV resistance was cloned into a plasmid vector pcDNA3 (Invitrogen, San Diego, CA). In brief, the PCR product amplified using serum from the patient was cleaved with *Bam*HI and *Apa*I (HBV positions 1,400–2,600) and cloned into pcDNA3, which was named pcDNA3-1. Similarly, the PCR product was cleaved with *Apa*I and *Bam*HI (HBV positions 2,600–3,215, 1–1,400) and cloned into pBluerscript SK+ (Stratagene, La Jolla, CA), which was named pB-1. The *Kpn*I-*Bam*HI fragment from pB-1 and *Kpn*I-*Apa*I fragment from pcDNA3-1 were cloned into pcDNA3-1. To introduce the nucleotide substitutions into the rtL180M, M204V, and S202G, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Four plasmids with/without amino acid substitutions were created and are listed in Table IV.

Cell Culture, Transfection, and Determination of IC_{50}

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C under 5% CO_2. Cells were seeded to semi-confluence in 6-well tissue culture plates. Transient transfection of the plasmids into HepG2 cell lines was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each anti-viral drug, various concentrations of LAM, ADV, and ETV were added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The medium containing the drugs was changed at days 1, 3, and 4. All experiments were performed in triplicate. GraphPad prism (GraphPad Prism Software, Inc., San Diego, CA) was used to determine the best-fit values for individual dose–response equations.

Analysis of Replicative Intermediate of HBV by Quantitation

The cells were harvested at 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH

7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000*g*. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec, and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Statistical Analysis

Data are expressed as mean ± SD. Group comparisons were performed using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Patient's Profile

An ETV-resistant strain of HBV was isolated from a 44-year-old Japanese woman with hepatitis B e antigen-positive chronic HBV infection (Fig. 1A). In this patient, LAM successfully reduced the HBV at the initial stage of treatment. However, viral breakthrough was observed at 11 months after the beginning of LAM therapy and the HBV viral load reached up to 7.5 log copies/ml. After 17 months of LAM, interferon was added to LAM therapy for 6 months. However, after withdrawal of IFN, the viral load and ALT rebounded. Thus, the patient was switched to 0.5 mg of ETV. This resulted in reduction of HBV-DNA and normalization of ALT. After 12 months of ETV therapy, the viral load rebounded, and following 12 more months of ETV, breakthrough hepatitis was observed. After stopping ETV, because of the inadequate effect of IFN monotherapy for one month, the patient was switched to 10 mg of ADV. This treatment reduced both the viral load and ALT level to acceptable levels (Fig. 1).

treatment		month	ALT (IU/L)	HBV-DNA (log copies/ml)
		-3	246	7.2
LAM		0	46	5.2
		5	28	3.7
		11	33	4.1
	IFN	17	72	7.5
		18	1184	5.6
		20	39	3.9
		23	34	3.4
		27	117	7.1
ETV		31	112	7.2
		39	40	2.9
		43	28	4.2
	IFN	56	140	6.8
ADV		57	313	6.8
		60	38	4
	LAM	71	24	3.3
		75	19	3.1

Fig. 1. Clinical course of a patient who developed entecavir resistant mutant.

Isolation of a Multiple Drug-Resistant Hepatitis Strain

Isolates from this patient were analyzed for substitutions in HBV reverse transcriptase (RT). Comparison of the nucleotide sequences by the direct sequence method obtained throughout the clinical course showed three amino acid substitutions in the RT domain of the polymerase (Table I). At the baseline of LAM, all three substitutions were of the wild-type by direct sequence analysis and clonal analysis (Table II). After breakthrough hepatitis induced by LAM, direct sequence analysis showed mixed type (YIDD and YVDD) mutant strain. The rtM204V mutant was detected in 65% of HBV clones and the rest were all the YIDD type. Importantly, at this point, there was no amino acid substitution at rt202. After 12 months of ETV therapy when the viral load was slightly increased, the rtL180M + M204V + S202G mutant was detected in 45% of the HBV clones, followed by decrease of the YIDD and YVDD mutants without substitution at rtS202G. Finally, after 24 months of ETV therapy, when the breakthrough hepatitis occurred, the rtL180M + M204V + S202G mutant was detected in 92% of the HBV clones and the rest were rtL180M + M204V mutants without substitution at rtS202G. Interestingly, the rtM204I + S202G strain never appeared during nucleotide therapy.

Susceptibility of Mutants to Entecavir In Vitro

To analyze the role of the rtL180M, rtG202S, and rtM204V substitutions in ETV resistance, four patient-specific strains were transfected into HepG2 cells (Table III). ETV was added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The core-associated HBV genome was extracted from cells and quantified by real-time PCR. The double amino acid substitutions rtL180M + M204V, which is related to LAM resistance, displayed a 38-fold decrease in susceptibility to ETV compared with the wild-type. Moreover, triple amino acid substitutions rtL180M + M204V + S202G, isolated from the patient

TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

	rt L180	rt S202	rt M204
(1) At the beginning of LMV	—	—	—
(2) At the beginning of ETV	L/M	—	I/V
(3) One year after ETV	M	G/S	V
(4) Two years after ETV	M	G	V

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC_{50} values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

	Relative rate (%) of clones (no. of clones/total)			
	Wild	M204I	L180M + M204V	L180M + M204V + S202G
(1) At the beginning of LMV	100 (6/6)	0	0	0
(2) At the beginning of ETV	0	35 (7/20)	65 (13/20)	0
(3) 12 months after ETV	0	14 (3/22)	41 (9/22)	45 (10/22)
(4) 24 months after ETV	0	0	8 (1/13)	92 (12/13)

LMV, lamivudine.

TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

				ETV	
	rt L180	rt M204	rt S202	IC_{50} (μM)	Resistance (fold)
Wild	—	—	—	0.00081	1
S202G	—	—	G	0.00054	0.67[a]
L180M + M204V	M	V	—	0.031	38**
L180M + M204V + S202G	M	V	G	0.16	198**

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo. Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

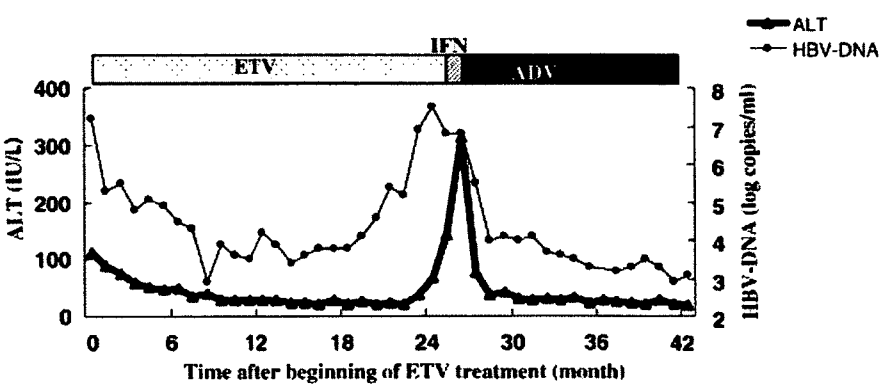

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

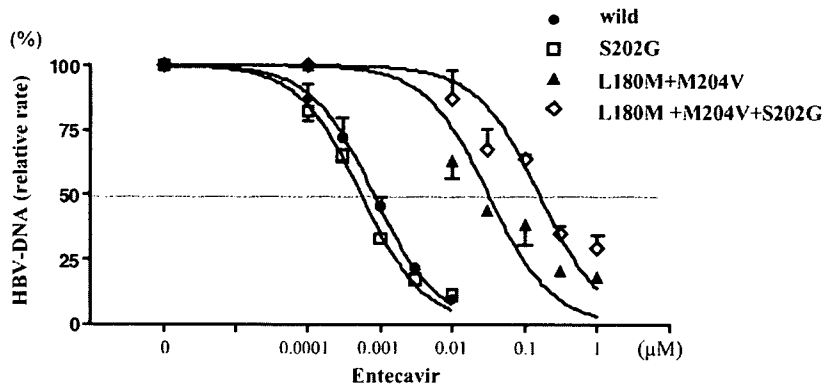

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose–response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC_{50} values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

	LAM		ADV	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
Wild	0.1	1	0.39	1
L180M + M204V	>100	>1,000**	—	—
L180M + M204V + S202G	>100	>1,000**	0.32	0.82[a]

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

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CLINICAL STUDIES

Similar effects of recombinant interferon-α-2b and natural interferon-α when combined with intra-arterial 5-fluorouracil for the treatment of advanced hepatocellular carcinoma

Kiminori Uka[1], Hiroshi Aikata[1], Shintaro Takaki[1], Daiki Miki[1], Soo Cheol Jeong[1], Akira Hiramatsu[1], Hideaki Kodama[1], Hiroo Shirakawa[1], Yoshiiku Kawakami[1], Shoichi Takahashi[1], Naoyuki Toyota[2], Katsuhide Ito[2] and Kazuaki Chayama[1]

1 Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
2 Department of Radiology, Hiroshima University Hospital, Hiroshima, Japan

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Correspondence
Hiroshi Aikata, MD, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
Tel: +81 82 257 5192
Fax: +81 82 257 5194
e-mail: aikata@hiroshima-u.ac.jp

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Abstract

Aim: Intra-arterial 5-fluorouracil (5-FU) plus interferon (IFN) combination therapy is effective against advanced hepatocellular carcinoma (HCC) with portal vein tumour thrombosis. In this study, we compared the efficiency and safety of recombinant IFN-α-2b with natural IFN-α as components of the combination therapy. *Methods:* Consecutive HCC patients ($n = 31$) with portal vein tumour thrombosis were enrolled in this prospective study. They received combination therapy of 5-FU and either recombinant IFN-α-2b (R group, $n = 15$) or natural IFN-α (N group, $n = 16$). We compared the two groups for the early response rate, adverse reactions, time to progression (TTP) and survival rates. In addition, we assessed the cost-effectiveness of each protocol. *Results:* The early response rate (R: 26.7%, N: 31.2%), median TTP (R: 5.8 months, N: 5.6 months) and median survival time (R: 7.5 months, N: 6.5 months) were not significantly different between the R and N groups. There were no differences in adverse reactions between the two groups. The estimated cost-effectiveness ratio of recombinant IFN-α-2b was better than natural IFN-α. *Conclusions:* In our protocol of combination therapy, there were no significant differences between recombinant IFN-α-2b and natural IFN-α with regard to early response to therapy, adverse effects, TTP and survival rates. 5-FU could be combined with either recombinant IFN-α-2b or natural IFN-α, although the cost-effectiveness of the former warrants its use clinically.

Hepatocellular carcinoma (HCC) is one of the most common neoplasms in Africa and Asia including Japan, and HCC-related deaths are increasing worldwide including Japan (1–3). Despite the progress in diagnostic techniques and therapeutic procedures, such as ultrasonography, computed tomography, magnetic resonance imaging, angiography, surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI) and transcatheter arterial chemoembolization (TACE), the prognosis of patients with HCC remains unsatisfactory. Furthermore, the survival rates of patients with advanced HCC and complications such as portal vein tumour thrombosis (PVTT) or distant metastasis remain extremely poor (4–8). PVTT frequently develops in HCC patients. In HCC patients with PVTT, tumour cells may spread out through the portal tract, resulting in intra-/extrahepatic metastases. Furthermore, portal vein occlusion may cause liver failure, ascites or variceal rupture. Thus, the performance status (PS) of HCC patients with PVTT gradually worsens, rendering them unsuitable for any treatment of HCC.

Advances in implantable drug-delivery systems have made it possible to administer repeated arterial infusions of anticancer agents. Recent studies reported the effectiveness of combination therapy of intra-arterial 5-fluorouracil (5-FU) and subcutaneous interferon (IFN)-α therapy for advanced HCC (9–11), with a response rate ranging from 47 to 73%. The majority of these studies used natural IFN-α. To our knowledge, there are no reports that have compared the effects of combination therapy of intra-arterial 5-FU and IFN

Table 1. Clinical profiles of the 31 patients with hepatocellular carcinoma

	Recombinant IFN-α-2b	Natural IFN-α	*P* value
N	15	16	
Age (years)	59 (26–79)	65(52–76)	NS
Sex (male/female)	12/3	14/2	NS
Grade of portal vein invasion (Vp 2/3/4)	2/3/10	1/8/7	NS
Main tumour size (mm)	52 (15–160)	57(25–140)	NS
Tumour volume (< 50%/≥ 50%)	9/6	10/6	NS
Child–Pugh grade (A/B/C)	10/5/0	13/3/0	NS
AFP (ng/mL)	3922.7 (51.2–708 100)	1957 (14.3–377 700)	NS
DCP (mAU/mL)	17 874 (< 10 – 233 450)	11 476 (46–722 140)	NS
Aetiology (HBV/HCV/others)	5/7/3	4/11/1	NS
Leucocyte count (/μL)	5790 (2210–8940)	6255 (2890–8910)	NS
Neutrophil count (/μL)	3395 (1260–6884)	4536 (1531–7008)	NS
Haemoglobin (g/dL)	12.5 (8.4–16.4)	12.9 (8.6–16.2)	NS
Platelet count (/μL) × 10^4	14.2 (5.1–54.4)	12.1 (5.9–34.2)	NS
Total bilirubin (mg/dL)	1.3 (0.6–2.7)	1.3 (0.7–2.8)	NS

Data are expressed as median values with ranges in parentheses, or number of patients. Portal invasion (Vp1, tumour thrombus in a third or more of the peripheral branches; Vp2, in the second branch; Vp3, in the first branch; Vp4, in the trunk).
AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; NS, not significant.

with those of recombinant IFN-α-2b and natural IFN-α. Such studies are important because the activity of recombinant IFN-α-2b differs from that of natural IFN (12, 13), and the former is less expensive than natural IFN-α. Thus, if the safety and efficacy of recombinant IFN-α-2b is equal to or better than that of natural IFN-α, recombinant IFN-α-2b may be recommended with regard to cost-effectiveness. In the present prospective study, we investigated the safety of intra-arterial 5-FU and IFN and compared recombinant IFN-α-2b with natural IFN-α. In addition, we assessed the cost-effectiveness of each treatment regimen.

Materials and methods

Study design and eligibility

This was a prospective study conducted at our hospital to compare the outcome of recombinant IFN-α-2b and natural IFN-α in combination with intra-arterial 5-FU. The eligibility criteria were as follows: age (18–80 years), Child–Pugh status A or B, leucocyte count > 2000/μL, neutrophil count > 1200/μL, haemoglobin > 8 g/dL, platelet count > 50 000/μL, total bilirubin < 3.0 mg/dL, serum creatinine < 1.5 mg/dL, unresectable or not suitable for local ablation therapy, main tumour size > 20 mm, tumour number > 2, presence of PVTT (in the second branch first branch or trunk), an Eastern Cooperative Oncology Group PS of 0–1 (14) and without extrahepatic metastases. All patients were asked to give their written informed consent to this study, which was approved by the Institutional Review Board of Hiroshima University.

From June 2003 to December 2006, 265 consecutive patients with unresectable HCC were admitted to our hospital. As a result of the progression of HCC (e.g. PVTT, extrahepatic metastases), these patients were not suitable candidates for either surgical resection or local ablation therapy, including RFA and PEI. Of the 265 patients with advanced HCC, 39 were considered to be suitable candidates for the intra-arterial 5-FU and IFN combination therapy. Eight patients could not be included because of refusal of enrolment. Thus, 31 patients with advanced HCC without extrahepatic metastases were enrolled in this prospective study. We treated the first 15 consecutive patients with recombinant IFN-α-2b (R group) and the second 16 consecutive patients with natural IFN-α (N group), combined with 5-FU.

Table 1 lists the baseline characteristics of the patients of the two groups (R group vs. N group). There were no differences between the two groups with respect to the sex ratio, age, proportion of patients with hepatitis B virus and hepatitis C virus infection, PVTT in the second branch (Vp2), major branch (Vp 3) and main trunk (Vp 4), median level of α-fetoprotein and des-γ-carboxy prothrombin, median leucocyte count, neutrophil count, haemoglobin, platelet count, total bilirubin and duration of the observation period [R: 10.5 (0.7–39.4 months), N: 6.3 (1.1–16.7 months)].

Treatment protocol

Patients received repeated arterial infusions of anticancer agents via an injection port. One course of

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chemotherapy represented 4 weeks. 5-FU (500 mg/body weight/day; Kyowa Hakko, Tokyo, Japan) was administered within 5 h using a mechanical infusion pump on days 1–5 of the first and second weeks (5 g in one course). Recombinant IFN-α-2b (Intron A®; Schering-Plough Pharmaceuticals Co., Osaka, Japan) at 3×10^6 U (3 MU), or natural IFN-α (OIF®; Otsuka Pharmaceuticals Co., Tokyo, Japan) at 5×10^6 U (5 MU) was administered intramuscularly on days 1, 3 and 5 of each week (total dose of 36 and 60 MU respectively). In our hospitals, the minimum dose of recombinant IFN-α-2b for the treatment of chronic hepatitis C is 3×10^6 U and that of natural IFN-α is 5×10^6 U. Previous reports used 5×10^6 U as the minimum dose of natural IFN-α. With regard to recombinant IFN-α-2, we selected the above dose as the minimum dose in order to avoid potential adverse effects. In principle, treatment was repeated several courses unless PS changed to 3 or 4 during the treatment. A 2–4-week rest period of no treatment was allowed after each treatment course.

Implantation of arterial catheter

The catheter was inserted through the right femoral artery using the Seldinger method. After the detection of HCC, a 3-French heparin-coated catheter (Clinical Supply, Gifu, Japan) was inserted and its tip was advanced to the common hepatic artery or proper hepatic artery. The other end of the catheter was connected to the injection port, which was implanted in a subcutaneous pocket created in the right lower abdominal quadrant. The gastroduodenal and right gastric arteries were occluded using steel coils to prevent potential gastroduodenal injury from the anticancer agents.

Evaluation

The response to treatment was assessed in all patients enrolled in this study. The response was defined according to the criteria of the Response Evaluation Criteria in Solid Tumors (RECIST) (15). A complete response (CR) was defined as the complete disappearance of all target lesions. A partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameter (LD) of target lesions with the baseline sum of the LD of target lesions as the reference. Progressive disease (PD) was defined as at least a 20% increase in the sum of the LD of target lesions. Stable disease (SD) was defined as neither PR nor PD criteria fulfilled. The duration of response was measured from the date of the start of treatment to the date of documented progression.

Adverse reactions were assessed every week during the treatment using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) (version 3.0) (16).

Cost-effectiveness

The cost of each IFN with two courses of treatment was calculated. The effectiveness of treatment was reflected by the percentages of patients who achieved CR or PR. The cost-effectiveness ratio was calculated using the formula: cost/effectiveness.

Additional therapy

After two courses of the combination therapy, we assessed the response to therapy in all patients. According to the response, we provided various additional therapies such as RFA, TACE and radiotherapy (RT) for patients treated with the combination therapy. Patients assessed as PR continued to receive the combination therapy, in addition to local ablation therapy when further decrease of HCC was not expected. All patients assessed as SD or PD received RT for PVTT. Furthermore, one session of TACE using a cisplatin–lipiodol suspension was repeated before the initiation of each course of intra-arterial 5-FU and IFN combination therapy unless the Child–Pugh status changed to C.

Statistical analysis

Statistical analysis was performed on 31 December 2006. Differences between groups were examined for statistical significance using the Mann–Whitney test (*U*-test) and χ^2 test where appropriate. Cumulative survival rate and time to progression (TTP) were assessed by the Kaplan–Meier life-table method, and differences were evaluated by the log-rank test. All analyses described above were performed using the SPSS program (version 11; SPSS Inc., Chicago, IL, USA).

Results

Early response rate

We assessed all patients after two courses of treatment. Of all 31 patients, one (3.2%), eight (25.8%), eight (25.8%), 10 (32.3%) and four (12.9%) patients showed CR, PR, SD, PD and drop-out (DO) respectively (Table 2). For the R group, zero (0%), four (26.7%), three (20%), five (33.3%) and three (20%) patients showed CR, PR, SD, PD and DO respectively. The reasons for DO were confusion (one patient), refusal after initiation of therapy (one patient) and exanthema (one patient). The overall response rate

Table 2. Response to treatment after two courses

	CR	PR	SD	PD	DO	Response rate*
Recombinant IFN-α-2b ($n = 15$)	0	4	3	5	3	26.7%
Natural IFN-α ($n = 16$)	1	4	5	5	1	31.2%

*Response rate = CR+PR/CR+PR+SD+PD.

CR, complete response; DO, drop out; IFN, interferon; PD, progressive disease; PR, partial response; SD, stable disease.

was 26.7% for the R group. For the N group, one (6.3%), four (25%), five (31.2%), five (31.2%) and one (6.3%) patients showed CR, PR, SD, PD and DO. The reason for DO was infection around the catheter (one patient). The overall response rate for the N group was 31.2%. There was no statistically significant difference in the early response between the two groups.

Adverse reactions and complications

Table 3 summarizes the adverse reactions and complications encountered during and after the treatment. The most common adverse reactions were fever, nausea and loss of appetite, but these were mostly NCI-CTC Grade 1 or 2. The percentages of patients with various NCI-CTC Grade 3 adverse reactions of the two treatment groups were not significantly different. With regard to the two patients of the R group with leucopenia, the initial leucocyte counts were 2210 and 3980/μL and the lowest counts were 1214 and 1524/μL respectively. As for the four patients with thrombocytopenia, the initial platelet counts were 61 000, 114 000, 185 000 and 227 000/μL, and the lowest counts were 31 000, 48 000, 45 000 and 37 000/μL respectively. None required administration of granulocyte-colony-stimulating factor (G-CSF) or blood transfusion, and none developed depression. Complications associated with the indwelling catheter were infection (one patient each).

Additional therapy after two courses of combination treatment

For the R group, four patients received TACE, one patient received RT and two patients received both TACE and RT. In the four patients who achieved PR, three continued to receive the combination therapy while the fourth achieved CR after completing four courses of the combination therapy, and undergoing RFA for the remaining HCC. With regard to the N group, four patients received TACE, two patients underwent RT and one patient received both TACE and RT. With regard to the four patients who achieved PR, three continued the combination therapy, while the fourth patient achieved CR after receiving three courses of the combination therapy, followed by RT for PVTT.

Table 3. Adverse reactions (National Cancer Institute Common Toxicity Criteria Grade 3) and complications during and after the combination treatment

	Recombinant IFN-α-2b	Natural IFN-α	*P* value
Leucopenia	2 (13.3%)	0	NS
Thrombocytopenia	1 (6.7%)	3 (18.8%)	NS
Nausea	1 (6.7%)	0	NS
Exanthema	1 (6.7%)	0	NS
Confusion	1 (6.7%)	0	NS
Infection around the catheter	1 (6.7%)	1 (6.3%)	NS
Pseudoaneurysm of the femoral artery	1 (6.7%)	1 (6.3%)	NS

IFN, interferon; NS, not significant.

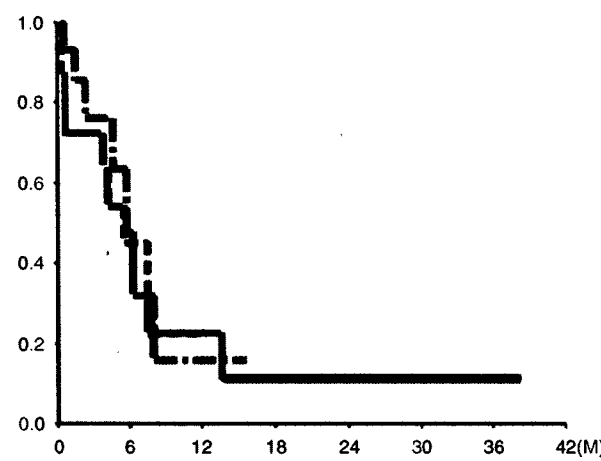

Fig. 1. Comparison of the time to progression of patients with hepatocellular carcinoma complicated with portal vein tumour thrombosis and treated with 5-fluorouracil (5-FU) and recombinant interferon (IFN)-α-2b (solid line) or with 5-FU combined with natural IFN-α (log-rank test: not significant).

Time to progression

The median TTP for all 31 patients was 5.8 months [95% confidence interval (CI), 3.9–7.7 months]. For the R and N groups, the median TTP was 5.6 months (95% CI, 3.0–8.2 months) and 5.8 months (95% CI,

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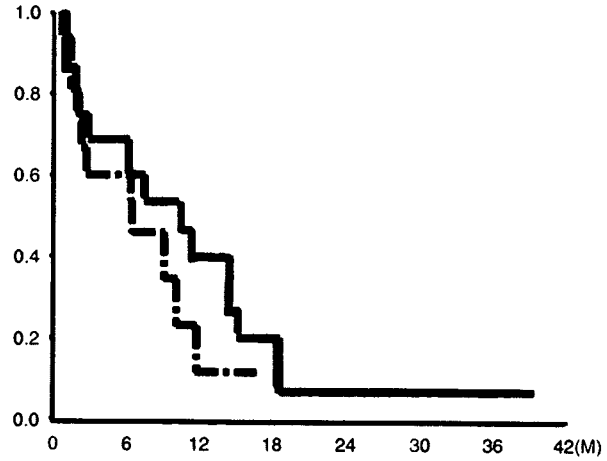

Fig. 2. Comparison of the survival rate of patients with hepatocellular carcinoma complicated with portal vein tumour thrombosis and treated with 5-fluorouracil (5-FU) and recombinant interferon (IFN)-α-2b (solid line) or with 5-FU combined with natural IFN-α (log-rank test: not significant).

4.0–7.6 months) respectively. There was no significant difference in TTP between the two groups (Fig. 1).

Survival rates

Of all 31 patients, the median survival time was 7.5 months (95% CI, 3.3–11.7 months) and the cumulative survival rates at 6, 12, 18 and 24 months were 64.5, 29.0, 16.5 and 5.6% respectively. The median survival time of the R group [10.5 months (95% CI, 0.0–21.4 months)] was not significantly different from that of the N group [6.5 months (95% CI, 3.7–9.3 months)] (Fig. 2).

Cost-effectiveness

The cost of IFN with two course of treatment was $US1052 for the R group and $US3060 for the N group. Four (26.7%) patients of the R group showed PR, while one (6.3%) and four (25%) patients of the N group showed CR and PR respectively. Thus, the estimated effectiveness was 26.7 for the R group and 31.3 for the N group. The cost-effectiveness ratios were 39.4 for the R group and 97.8 for the N groups, indicating that the R combination therapy was about three times more cost-efficient than the N combination therapy (Table 4).

Causes of death

Seven patients were still alive at the end of the observation period while 24 patients had died. All the 24 patients died of cancer-related disease.

Table 4. Cost-effectiveness of each interferon with two courses of the treatment

	Cost ($)	Effectiveness* (%)	Cost-effectiveness ratio†
Recombinant IFN-α-2b	1052	26.7	39.4
Natural IFN-α	3060	31.3	97.8

*Effectiveness; percentage of patients who showed CR or PR.
†Cost-effectiveness ratio = cost/effectiveness.
CR, complete response; IFN, interferon; PR, partial response.

Discussion

The prognosis of patients with advanced HCC complicated with PVTT remains poor, particularly in those patients with PVTT in the first branches or the portal trunk. The median survival time of HCC patients with PVTT in the portal trunk is reported to be about 90 days with supportive care (17). In this regard, Patt *et al.* (18) reported the efficiency of intravenous 5-FU combined with recombinant IFN-α-2b. Recently, several studies assessed the efficacy of combination therapy of intra-arterial 5-FU and IFN (9–11). However, most of the reports that analysed the effects of combination therapy of intra-arterial 5-FU and IFN used natural IFN-α. The use of recombinant IFN-α-2b has been reported in only one study (9). The response rates (CR and PR patients/all patients) reported in the two studies by Sakon *et al.* (10) and Obi *et al.* (11) were 73% (8/11 patients) and 52.6% (61/116 patients) respectively. In this study, for patients of the two groups, the objective response rate according to the early response was 29% (9/31 patients). The discrepancy between the studies may be because of the following reasons. First, the early response to our protocol was assessed after two courses of the treatment, while others evaluated the maximum response. Second, the method of evaluation of the response was different. The above two studies used the Eastern Cooperative Oncology Group criteria but we used the RECIST criteria. Third, the sample size was very small in the report by Sakon *et al.* (10). In our study, the survival rates of the patients of the two groups were almost identical to those reported by Obi *et al.* (11) (the survival rates at 6 and 12 months were 53 and 34% respectively). Thus, the protocol used in our study was considered to be suitable for patients with advanced HCC.

Both IFN-α and IFN-β induce the transcription of the p53 gene and contribute in boosting the responses to p53 activation, which suppresses cancer (19). IFN-α is also known to inhibit cancer cells directly as well as indirectly (20–26) and to have anti-angiogenic and

antiviral activities. The direct antineoplastic effects include cell damage (27), induction of cyclin-dependent kinase inhibitors involved in G1/G0 arrest (22) and delayed cell cycle (28). The indirect antineoplastic effects include activation of natural killer cells, T cells and macrophages (29–31). In various cultures of malignant cells, IFN-α exhibited a biomodulatory effect that enhanced the antineoplastic activity of 5-FU partly because of the arrangement of metabolism of 5-FU to fluoro-deoxy-uridylate (32–36). Furthermore, 5-FU and IFN-α synergize the antineoplastic effects of each other. The antineoplastic effects of the combination therapy of intra-arterial 5-FU and IFN are also considered to be mediated by modulating tumour necrosis factor-related apoptosis-inducing ligand receptor-induced cytotoxic pathway (37).

Several subtypes of natural IFN-α have been described (38), while only one subtype is available for recombinant IFN-α. Patients treated with natural IFN-α barely have antibodies to IFN, whereas circulating antibodies to IFN are sometimes detected in patients treated with recombinant IFN-α (12, 13). Antibodies to IFN weaken the therapeutic effects of IFN. Therefore, antibodies to IFN may dampen the effects of the combination therapy of intra-arterial 5-FU and IFN. This hypothesis favours the combination therapy of intra-arterial 5-FU and natural IFN-α relative to 5-FU and recombinant IFN-α.

Interferon-α subtypes exhibit several variations in biological activity. With regard to the antiviral activity, IFN-α8 is reported to be the most potent while IFN-α1 the least potent (39). IFN-α8 was the most potent in the induction of antineoplastic effect on renal cell carcinoma (40). OIF®, but not Intron A®, contains IFN-α8. Considered with the deficiency of the subtypes *in vitro*, the effect of combination therapy with IFN-α may be different based on the IFN.

Our results, however, showed no significant differences between the two groups with respect to the early response, adverse reactions, TTP and survival rate. What are the reasons for the lack of differences *in vivo*? One reason may relate to the dose and antineoplastic activity of IFN (19–31, 39, 40). Several groups have studied the impact of IFN treatment on HCC. Two controlled trials reported by Lai *et al.* (41, 42) using very high doses of IFN (50×10^6 IU/m^2) showed a 30% response rate and improvement in survival compared with no treatment. In comparison, another study using a low dose of IFN (3×10^6 IU/m^2) did not show any survival advantage (43). Considered together, it appears that for IFN alone to be effective against HCC, its dose must be higher than that used for the treatment of chronic hepatitis B and C. Administration of high-dose IFN may improve the effect of the combination therapy. However, under such circumstances, many patients could potentially DO because of the adverse reactions. Thus, our protocol is safe regardless of the type of IFN used. The second reason relates to the relationship between IFN subtype and the mechanism of action of the combination therapy (32–40). Although the mechanism is not yet clear, the direct effect of inhibition of cancer cells and the anti-angiogenic effect of IFN might play minor roles in our protocol *in vivo*. The most important mechanism of action of the combination therapy of our protocol may be enhancement of the antineoplastic effect of 5-FU by IFN. Thus, the IFN subtype does not seem to strongly influence the effect of the combination therapy. The third reason may relate to the several limitations in our study (e.g. small sample size, not randomized-controlled trial).

Most of the adverse reactions were controllable in the present cohort. The adverse reactions of anaemia, leucopenia and thrombocytopenia were controllable without G-CSF or blood transfusion. Depression owing to IFN was not observed in our patients. Thus, the lack of severe pancytopenia in patients with advanced HCC treated by the current protocol reflects the safety of intra-arterial 5-FU and IFN. It is recommended, however, that careful treatment should be provided to patients who develop pancytopenia.

Our study fell somewhat short of conclusiveness owing to the small number of patients. Thus, our study should be extended to include a long-term follow-up and a large sample size. In our protocol of the combination therapy, there were no significant differences between recombinant IFN-α-2b and natural IFN-α with regard to early response to therapy, adverse effects, TTP and survival rates. Recombinant IFN-α-2b is inexpensive compared with natural IFN-α. Our analysis showed a better cost-effectiveness ratio for recombinant IFN-α-2b than natural IFN-α. Thus, assuming no difference in outcomes between the two regimens, we recommend the use of recombinant IFN-α-2b based on the cost-effectiveness.

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