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Long-Term Outcome of Combined Interferon-α and 5-Fluorouracil Treatment for Advanced Hepatocellular Carcinoma with Major Portal Vein Thrombosis

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Key Words

Hepatocellular carcinoma · Interferon · Portal vein tumor thrombosis · Arterial infusion chemotherapy

Abstract

Background/Aim: We previously reported the beneficial effects of a combination therapy of interferon (IFN)-α/5-fluorouracil (FU) for advanced hepatocellular carcinoma (HCC) with tumor thrombi in the major portal branches. This report describes the results of longer follow-up and includes more than twice the number of patients relative to the previous report; it also evaluates the clinical predictor on the response to the combination therapy and long-term survival. ***Methods:*** The study subjects were 102 patients with advanced HCC and tumor thrombi in the major branches of the portal vein (Vp3 or 4). They were treated with at least 2 courses of IFN-α/5-FU. ***Results:*** No major treatment-related complications were noted. In the 102 patients, 40 (39.2%) showed objective response [11 (10.8%) showed complete response, 29 (28.4%) partial response], 8 (7.9%) showed no response and 54 (52.9%) showed progressive disease. ***Conclusion:*** IFN-α/5-FU combination therapy is a promising modality for advanced HCC with tumor thrombi in the major portal branches.

Introduction

The prognosis of patients with advanced hepatocellular carcinoma (HCC) remains poor, particularly in patients with tumor thrombi in the major trunk of the portal vein (Vp4) [1–3]. The mortality rate is very high in patients with unresectable tumors and the quality of life is poor due to intractable ascites or esophageal bleeding. Even in patients with resectable HCC, the prognosis is extremely poor despite aggressive surgery [4, 5]. In such a situation, conventional therapies generally have no clinical effect on HCC associated with portal tumor thrombi due to poor efficacy and possible complications [6, 7]. Arterial infusion chemotherapy has also been attempted, but its effectiveness is still unsatisfactory for portal venous tumor thrombus (PVTT) [8, 9]. Therefore, a new strategy is required for patients with intractable HCC and tumor thrombi in the major branch of the portal vein.

Several recent studies have indicated the beneficial effects of interferon (IFN)-α-based combination chemotherapies for HCC [10–15], in spite of the lack of satisfactory results from IFN-α monotherapy [16]. We also reported the clinical efficiency of IFN-α and 5-fluorouracil (5-FU) combination therapy for advanced HCC with PVTT and intrahepatic metastasis [17–22].

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Table 1. Patient characteristics

	Patients (n = 102)
Age, years	59.6 ± 9.4
Gender, male/female	94/12
Hepatitis virus	
HBV (+), HCV (–)	27
HBV (–), HCV (+)	54
HBV (+), HCV (+)	14
HBV (–), HCV (–)	9
Unknown	2
Granulocytes, /ml	4,420 ± 1,648
Platelets, $\times 10^4$/ml	13.3 ± 6.7
Serum albumin, g/dl	3.24 ± 0.44
Serum bilirubin, mg/dl	0.98 ± 0.36
Prothrombin time, s	17.9 ± 2.0
Child–Pugh classification	
A	38
B	63
C	4
Unknown	1
AFP, ng/ml	
<5	4
≥5	101
Unknown	1
PIVKA-II, mAU/ml	
<40	3
≥40	102
Unknown	1

The present study is the long-term outcome of the clinical effects of the combination therapy of subcutaneous IFN-α and arterial infusion of 5-FU in 102 patients with HCC associated with Vp4 and multiple intrahepatic metastases (IM3) [1], as an extension to our previous work [18, 19].

Patients and Methods

Patients and Selection Criteria

This was a single-arm open-label study, based on our pervious reports [18, 19]. Between December 1997 and December 2008, 102 patients with advanced HCC were enrolled. All patients were confirmed radiologically to have tumor thrombi in the main trunk of the portal vein (Vp4) and IM3. The diagnosis was based on liver function tests, serum α-fetoprotein (AFP), serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) and imaging techniques including computed tomography (CT) scan, magnetic resonance imaging (MRI), hepatic angiography and arterial portography.

The following were the eligibility criteria for selection for intra-arterial combination therapy: (1) age of more than 20 years and less than 75 years; (2) tumor thrombi invading at least one of the main branches of the portal vein; (3) presence of multiple intrahepatic metastases in more than three segments (IM3); (4) absence of extrahepatic metastases; (5) a granulocyte count of more than 2,500/μl and less than 12,000/μl; (6) a red blood cell count of more than 8.0 g/dl; (7) a platelet count exceeding 8×10^4/μl; (8) GOT and GPT of less than 100 IU/l; (9) total bilirubin less than 1.4 g/dl; (10) serum BUN less than 30 mg/dl; (11) serum creatinine less than 1.5 mg/dl; (12) successful implantation of intra-arterial catheter and drug delivery system; (13) a performance status of level 0–2 (Eastern Cooperative Oncology Group, ECOG) [23]. These eligibility criteria were based on our previous report [18, 19]. All patients signed informed consent documents approved by the institutional review board attesting to the fact that they were aware of the investigational nature of the study and were willing to try the combination therapy.

The baseline characteristics of the enrolled 102 patients who received IFN/5-FU combined treatment are shown in table 1 (age, gender, hepatitis virus, granulocytes, platelet, albumin, bilirubin, prothrombin time, Child-Pugh classification, AFP and PIVKA-II).

Treatment Protocol of IFN/5-FU Combination Therapy

In each of the 102 patients, an intra-arterial catheter was inserted through the subclavian or femoral artery, with a subcutaneously implanted drug delivery system [24]. Each patient was treated with subcutaneous IFN-α (OIF; Otsuka Pharmaceutical Co., Tokushima, Japan) and intra-arterial infusion of 5-FU (Kyowa Hakko Co., Tokyo, Japan). One cycle of the treatment consisted of 4 weeks. IFN-α (5×10^6 U, 5 MU) was administered subcutaneously on days 1, 3 and 5 of each week, resulting in a total dose of 60 MU in a cycle. Continuous infusion chemotherapy (5-FU, 300 mg/m^2/day) through the proper hepatic artery was performed on the 1st and 2nd weeks via a catheter connected to a subcutaneously implanted drug delivery system. Two- or three-week rest period (cessation of drug therapy) separated the treatment cycles. All anticancer therapies were discontinued when adverse effects reached level 2 of the ECOG classification [23] (with the exception of platelet and leukocyte counts of less than 40,000 and 2,000/mm^3, respectively, since these parameters were often low prior to treatment due to the associated liver cirrhosis) [18, 19].

Evaluation of Response to IFN/5-FU Combination Therapy

A pretreatment evaluation was conducted at the commencement of IFN-α/5-FU protocol and posttreatment evaluation after completion of the 2-cycle treatment, almost 3 months later. The evaluation was performed using CT or MRI, and changes in serum tumor markers, such as AFP and PIVKA-II. All cases were compared at these two time points for the evaluation of antitumor effect. The objective response was classified according to the ECOG criteria [23]. Complete response (CR) was defined as normalization of tumor markers and disappearance of all tumors and portal vein thrombosis on CT and/or MRI. Partial response (PR) represented a decrease in tumor markers and 50–99% regression on the two-dimensional measurement. No change (NC) represented less than 50% regression or less than 25% progression. Progressive disease (PD) represented more than 25% progression. In addition, we also evaluated progression-free and overall survival rates. The follow-up period was 12–120 months.

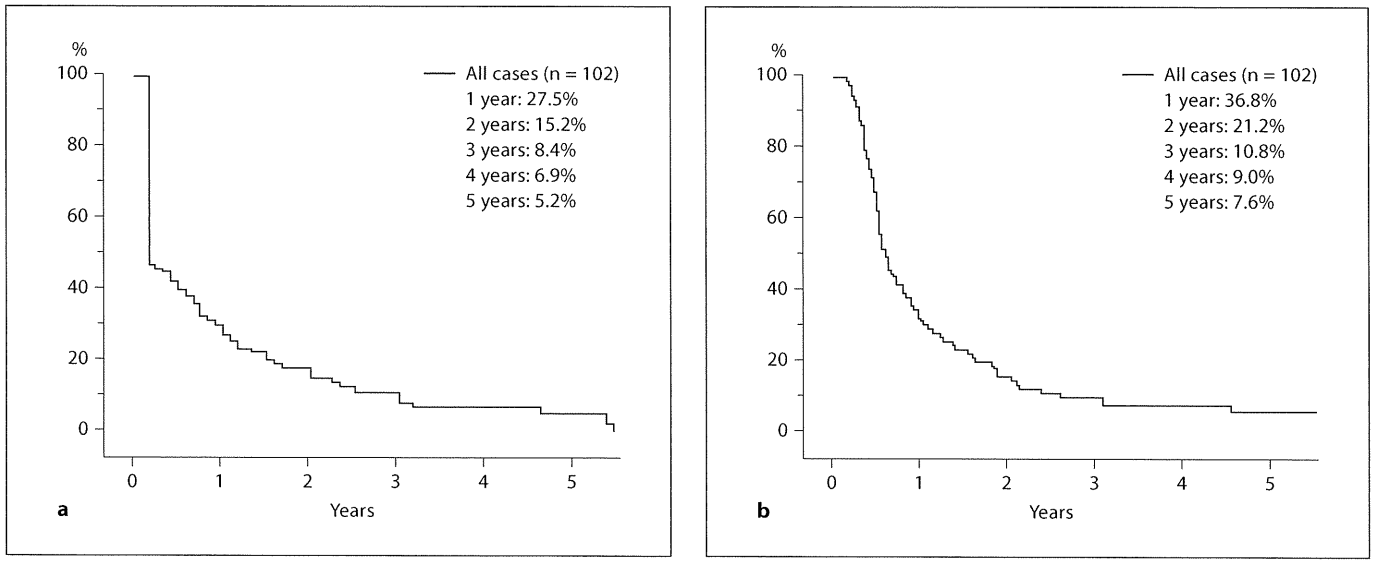

Fig. 1. Kaplan-Meyer analysis for efficiency of IFN/5-FU combination therapy. **a** Progression-free survival curve in all cases. The median progression-free survival period was 2.0 months, and the 1-, 3- and 5-year progression-free survival rates were 27.5, 8.4 and 5.2%, respectively. **b** Overall survival curve in all cases. The median overall survival period was 9 months, and the 1-, 3- and 5-year survival rates were 36.8, 10.8 and 7.6%, respectively.

Statistical Analysis

The Breslow-Gehan-Wilcoxon univariate test was used to examine the possible relationship between the effect of therapy (CR, PR vs. NC, PD), Child-Pugh score, serum AFP, serum PIVKA-II, Okuda score and CLIP score [3]. Survival curves were constructed using the Kaplan-Meier method. Differences in distribution between groups were compared by the χ^2 test and differences in mean values by Student's t test. All data were expressed as means ± SD. A p value less than 0.05 denoted the presence of a statistically significant difference.

Results

Clinical Response to Combination Therapy

All patients completed at least two cycles of the IFN/5-FU combination therapy. For patients who showed clinical response, we continued this combination therapy, while in those who showed no effect, we stopped the treatment after the completion of the second cycle, because of the extensive progression of HCC.

With regard to the clinical effect, 40 (39.2%) showed objective response, 11 (10.8%) showed CR, 29 (28.4%) showed PR, 8 (7.9%) showed NC and 54 (52.9%) showed PD. With respect to time to progression, the median progression-free survival period was 2.0 months, and the 1-, 3- and 5-year progression-free survival rates were 27.5, 8.4 and 5.2%, respectively. Furthermore, the median overall survival period was 9 months, and the 1-, 3- and 5-year survival rates were 36.8, 10.8 and 7.6%, respectively. The median progression-free survival period of CR/PR cases (n = 40) was 18.5 months and that of NC/PD cases (n = 62) was 2.0 months. The 1-, 3- and 5-year progression-free survival rates of CR/PR cases were 70.0, 21.3 and 13.3%, respectively, and those of NC/PD cases were 0, 0 and 0%, respectively.

The median survival time of CR/PR cases (n = 40) was 25 months and that of NC/PD cases (n = 62) was 6 months. The median follow-up time of survived patients was 30 months. The 1-, 3- and 5-year survival rates of CR/PR cases were 82.7, 28.6 and 18.9%, respectively, and those of NC/PD cases were 4.8, 0 and 0%, respectively. The progression-free survival and overall survival curves are shown in figures 1 and 2, respectively. There were significant differences in the progression-free survival and the overall survival between responders (CR/PR) and nonresponders (NC/PD) ($p < 0.0001$).

Adverse Effects

None of the patients developed side effects related to catheter insertion or subcutaneous implantation of the drug delivery system. However, 8.8% of patients developed grade 3 leukopenia, thrombocytopenia or anae-

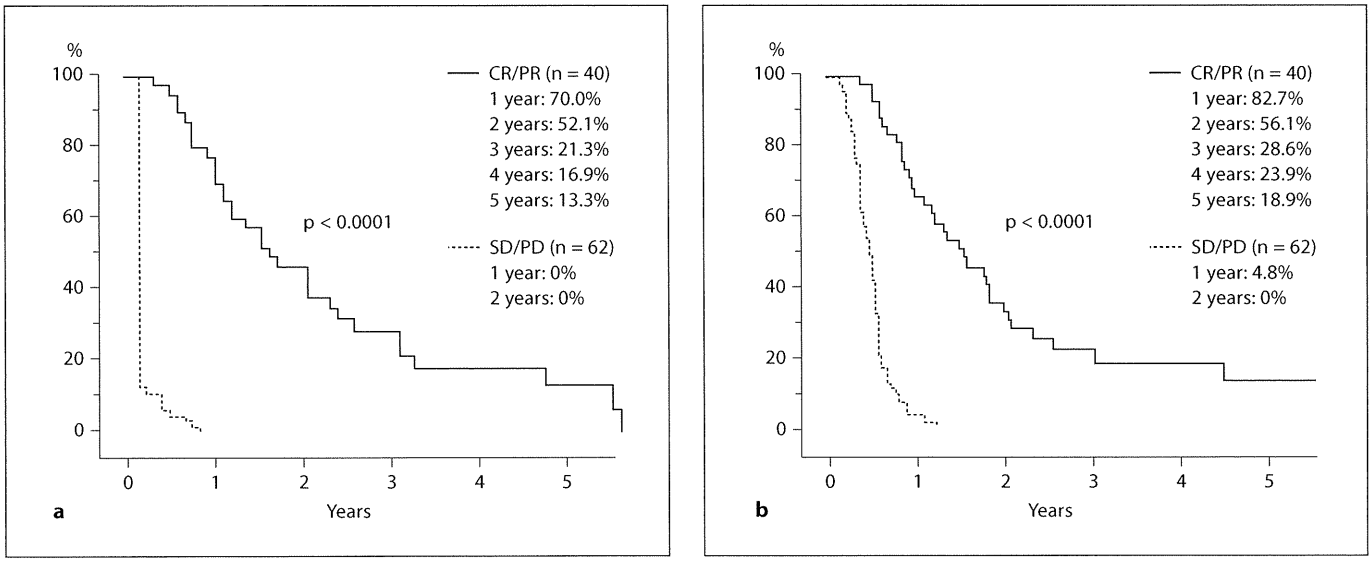

Fig. 2. Kaplan-Meyer analysis for efficiency of IFN/5-FU combination therapy. **a** Progression-free survival curves in CR/PR and NC/PD cases. The 1-, 3- and 5-year progression-free survival rates of CR/PR cases were 70.0, 21.3 and 13.3%, respectively, and those of NC/PD cases were 0, 0 and 0%, respectively. **b** Overall survival curves in CR/PR and NC/PD cases. The 1-, 3- and 5-year survival rates of CR/PR cases were 82.7, 28.6 and 18.9%, respectively, and those of NC/PD cases were 4.8, 0 and 0%, respectively. There were significant differences in the progression-free survival and the overall survival between responders (CR/PR) and nonresponders (NC/PD) ($p < 0.0001$).

mia, but drip transfusion of granulocyte colony-stimulating factors was not used during this study. Nonhematological toxicities included grade 1 or 2 fever (100% of patients), chilling sense (92.3%), nausea (6.9%), diarrhea (3.6%), gastric ulcer (2.9%), flu-like syndrome (100%), skin reaction (4.9%), general fatigue (31.3%) and depression (2.9%). The side effects are summarized in table 2.

Clinical Correlations

Finally, we compared the responders (CR/PR) (n = 10) with nonresponders (NC/PD) (n = 20) in terms of serum AFP (within normal range; <5), serum PIVKA-II (normal range; <45), Child-Pugh score, OKUDA score and CLIP score by univariate analysis. Serum AFP, PIVKA-II, Child-Pugh score, OKUDA score and CLIP score did not correlate with the response to combination therapy, similar to our previous report [19] (data not shown).

Table 2. Adverse effects

	Patients (n = 102)			
	grade 1	grade 2	grade 3	grade 4
Hematological				
Leukopenia	14	23	6	0
Anemia	0	1	3	0
Thrombocytopenia	16	20	9	0
Nonhematological				
Fever	97	5	0	0
Chilling sense	94	0	0	0
Nausea	7	0	0	0
Diarrhea	4	0	0	0
Gastric ulcer	0	3	0	0
Flu-like syndrome	102	0	0	0
Skin reaction	5	0	0	0
General fatigue	32	0	0	0
Depression	3	0	0	0

Discussion

In this study, we showed the beneficial effects of IFN-α/5-FU combination therapy in patients with multiple lesions and tumor thrombi in the major branches of the portal vein (Vp3 or 4), as our third report on this combined treatment. The efficacy of such treatment was 39.2% in our patients with highly advanced HCC, which was almost similar to the others and our previous reports of patients with the same stage HCC [15, 19, 25]. The

prognosis of such patients is extremely poor and survival is generally limited to a few months after diagnosis, despite multimodal therapies even in cases suitable for surgical resection [26]. The combination treatment IFN-α and 5-FU markedly decreased tumor size and levels of tumor markers with an encouraging response rate and prolonged survival time in the responders. Furthermore, the clinical response completely reflected the survival benefits, as shown in figures 1 and 2. There are several other reports about the possibilities as a treatment for advanced HCC with PVTT, such as intra-arterial infusion chemotherapy with 5-FU and CDDP [27–29] or transarterial chemoembolization [30]. A certain level of antitumor effect has been shown in 5-FU and CDDP intra-arterial chemotherapy for the lower stage of HCC patients, but not just for PVTT; antitumor effect for the HCC partially including PVTT patients were not significant compared to IFN-α/5-FU combined treatment in terms of median survival time, response rate and overall survival. Transarterial chemoembolization was reported as an effective treatment for advanced HCC with PVTT in RCT, but the clinical outcome was not better than IFN and 5-FU combined treatment. From these findings, the clinical result of IFN and 5-FU combined treatment was promising for the disastrous advanced HCC with PVTT patients.

On the other hand, no response to the combination therapy was seen in 60.8% (62/102) of the patients in this study. To advance the effect of IFN-α/5-FU combination therapy and to increase the response rate, it is necessary to investigate the mechanism of IFN-α/5-FU combination therapy. Among the nonresponders, there were only a few NC (8/102) in this study, in spite of the mostly chemo-resistant disease. We reasoned this finding to the following; the HCC in this series was far advanced and HCC progression was extremely rapid and aggressive. Under such conditions, almost all nonresponders died within 12 months (59/62); 40 of 62 cases (64.5%) within 6 months. For nonresponders to this treatment, however, the survival period was too short to allow receiving another treatment modality. Therefore, accurate prediction of chemosensitivity is desirable not only for loss of a limited chance for another possible treatment but also to avoid potentially serious side effects. However, there are no suitable markers that could distinguish patients who are likely to respond to this combination chemotherapy from those who are not. In this point, Obi et al. [15] recommended to start the combination therapy with close monitoring of response, preferably that of tumor biomarkers, and treatment should be continued if there is a response after the first cycle of chemotherapy.

Several mechanisms for the anticancer effects of IFN-α, with or without 5-FU, have been proposed [31–34]. We showed previously that IFN-α and 5-FU synergistically inhibit tumor cell proliferation with cell cycle arrest [35] and induced apoptosis by regulating the apoptosis-related molecules [36] as well as an antiangiogenic effect [37]. We also reported that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), its receptor pathway [38] and Fas and Fas-L pathway [39] partially contributed to the antitumor effects of IFN-α and 5-FU combination therapy. About apoptosis induction, the close involvement of P53 has been reported [40, 41]. Moreover, IFN-α suppresses the proliferation of all type I interferon receptor 2 (IFNAR2)-positive HCC cell lines in vitro through mechanisms related to apoptosis or inhibition of cell cycle [42]. The importance of IFNAR2 expression for the anticancer effect of IFN/5-FU was highlighted in a similar situation in our previous report [35, 36, 43]. In addition, we reported the significance of Ep-CAM [44] and IGFR-7 [45] as a noble biomarker to assess the antitumor effect of IFN/5-FU combined treatment. CD133 may be related to antitumor effect of IFN and 5-FU as a predictor in perspective of cancer stem cell [46].

The combination of IFN/5-FU is not effective against extrahepatic metastases. This is understandable because 5-FU, administered into the hepatic artery, will not reach extrahepatic tissues in high concentration. However, systemic administration of 5-FU or related agents may be effective against extrahepatic lesions in combination with IFN-α [47]. This possibility is highly interesting since the implantation of dwelling catheter is one of the demerits of the present combination therapy [15]. Recently, several molecularly targeting agents have been developed and applied for HCC treatment [48–51]. Especially sorafenib is the first agent leading to improved overall survival with advanced HCC, revealed in a phase III clinical trial [51]. These molecularly targeting agents are a very effective therapeutic modality, which has the different mechanism of antitumor effect from IFN/5-FU combination as an cytotoxic medicine. We reported actually that PTK/ZK, a kind of molecularly targeting medicine, enhanced the antitumor effect of IFN/5-FU in vitro [52]. After this, mutual interaction and sharing roles might be very important for the progression of the treatment for intractable advanced HCC.

In conclusion, we demonstrated the long-term outcome about the efficacy of IFN/5-FU combination therapy for advanced HCC patients with tumor thrombi in major branches of the portal vein.

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IGFBP7 downregulation is associated with tumor progression and clinical outcome in hepatocellular carcinoma

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Insulin-like growth factor-binding protein 7 (IGFBP7) functions in several cellular processes including proliferation, senescence and apoptosis. This study analyzed IGFBP7 function in hepatocellular carcinoma (HCC) cells by gene manipulation and investigated the prognostic significance of IGFBP7 expression in clinical HCC samples. In this study, we investigated changes in malignant potential such as cell growth and invasiveness in an HCC cell line, PLC/PRF/5, after transfection with shRNA against *IGFBP7*. The extent of apoptosis and cell cycle progression were examined after the transfection. The correlation between immunohistochemically determined IGFBP7 expression and long-term postoperative prognosis after curative resection was also investigated in clinical HCC specimens obtained from 104 patients. PLC/PRF/5 cells transfected with shRNA against *IGFBP7* showed significantly more rapid growth and stronger invasiveness than control cells. Annexin V assays showed that the IGFBP7-depleted cells were significantly more resistant to apoptosis than the control cells, and showed decreased expression of cleaved caspase-3 and PARP. Cell cycle progression was more rapid in the IGFBP7-suppressed cells. In clinical HCC specimens, IGFBP7 expression was judged as positive in 67 patients (64.4%) and negative in the remaining 37 patients (35.6%). The IGFBP7 downregulation correlated significantly with poor postoperative prognosis, and IGFBP7 status was identified as an independent significant prognostic factor. Our results indicated that IGFBP7 expression correlated significantly with the malignant potential in HCC cells, suggesting that the expression could be a useful prognostic marker for HCC.

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, but especially in Japan and other East Asian countries.[1,2] Although surgery plays a major role in the treatment of HCC, less than 30% of patients with HCC are surgical candidates owing to limiting factors such as severe impairment of reserve hepatic function, bilobar tumor distribution and extrahepatic metastasis. Additionally, no effective chemotherapy regimens have been established for treating HCC.[3] Thus, no effective therapy can be offered in many cases of HCC. Such dismal prognosis is not always predicted by conventional prognostic indicators such as vascular invasion, tumor multiplicity and tumor size.[4–6] New indicators are thus clearly needed.

Insulin-like growth factor binding protein 7 (IGFBP7), which is also known as IGFBP-rP1 and MAC25, has been implicated in several cellular processes such as proliferation, senescence and apoptosis. IGFBP7 also shows tumor suppressive activity through the induction of apoptosis and it is downregulated in some cancers.[7–13] In addition, several studies found a significant association between IGFBP7 and not only apoptosis, but also prognosis, in some kinds of cancers including colorectal and breast cancer.[14,15] However, the functional significance of IGFBP7 in HCC remains unclear.

This study analyzed the function of IGFBP7 in HCC cells in gene manipulation experiments, and investigated the prognostic significance of IGFBP7 expression in clinical HCC samples by immunohistochemical analysis of resected specimens.

Key words: hepatocellular carcinoma (HCC), insulin-like growth factor binding protein 7 (IGFBP7), apoptosis

Abbreviations: 95% CI: 95% confidence interval; AFP: alpha-fetoprotein; Anti-HCV Ab: anti-hepatic C virus antibody; DFS: disease-free survival; HBs-Ag: hepatitis B surface antigen; HCC: hepatocellular carcinoma; IGFBP7: insulin-like growth factor binding protein 7; OR: odds ratio; OS: overall survival; PBGD: porphobilinogen deaminase; pERK: phoshorylated ERK; PI: propidium iodide; PIVKA-II: protein induced by vitamin K absence or antagonists-II; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; shRNA: short hairpin RNA

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Material and Methods

HCC cell lines and clinical tissue specimens

Four human HCC cell lines, PLC/PRF/5, HuH7, HLE and HepG2 were obtained from the Japan Cancer Research

Resources Bank (Tokyo, Japan). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2 in air.

Surgical specimens were obtained from 104 patients with HCC who underwent curative hepatic resection in the Osaka University Hospital from 2000 to 2007 after informed consent in accordance with the institutional ethical guidelines of Osaka University. Curative resection was defined as complete removal of all macroscopically evident tumors. Patients who underwent transarterial chemoembolization preoperatively were excluded from this study. After hepatic resection, the patients were followed up at regular intervals of 3–4 months with physical examination, assaying of tumor markers including alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II), liver biochemistry testing, abdominal ultrasonography and abdominal computed tomography. The median duration of clinical follow-up after the initial hepatectomy was 3.5 ± 2.3 years.

Drugs and reagents

A polyclonal goat anti-human IGFBP7 antibody and polyclonal rabbit anti-human IGFBP7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunohistochemistry and western blot analysis, respectively. Antibodies to caspase-3, cleaved caspase-3, PARP, cleaved PARP, ERK, phoshorylated ERK (pERK), cyclin D1 and p27 were purchased from Cell Signaling Technology (Beverly, MA), antibodies to cyclin E and p21 were purchased from Santa Cruz Biotechnology, and an antibody to actin was purchased from Sigma-Aldrich Co. (Louis, MO).

Plasmids and transfection

Plasmid coding for short hairpin RNA (shRNA) against *IGFBP7* and *IGFBP7* expression plasmid were purchased from OriGene Technologies (Rockville, MD) and used to transfect HCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. After transfection of the shRNA plasmid and the *IGFBP7* expression plasmid into the HCC cells for 24 hr, stable transfectants were selected and maintained in 1.0 μg/ml of puromycin (Sigma-Aldrich, St. Louis, MO) and 600 μg/ml of G418 (Gibco-BRL, Grand Island, NY), respectively. The control vector plasmid expressing non-effective shRNA was similarly introduced into cells to establish negative control cells for the shRNA plasmid experiments. Empty vector plasmid was also similarly used to establish negative control cells for the *IGFBP7* expression plasmid for the *IGFBP7* expression plasmid experiments.

Cell proliferation assay

Cells were uniformly seeded (4×10^4/well for PLC/PRF/5 and 2×10^4/well for HuH7) in triplicates into 24-well dishes (Day 0). Cells were counted using a CellTac kit (Nihon Koden, Tokyo, Japan) on Days 1–5.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA isolated from cells was prepared using TRIzol reagent (Invitrogen), and reverse transcription was performed with SuperScript II (Invitrogen) based on the protocols supplied by the manufacturer. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the Light Cycler (Roche Diagnostics, Mannheim, Germany), and the amount of target gene expression was calculated. The expression of the target gene was normalized relative to the expression of *porphobilinogen deaminase* (*PBGD*), which was used as an internal control. The designed PCR primers were as follows; *IGFBP7* forward primer; 5′-CTGGGTGCTGGTATCTCCTC-3′; *IGFBP7* reverse primer; 5′-TATAGCTCGGCACCTTCACC-3′; *SMARCB1* forward primer; 5′-TCTGGATTTGAACCCGCTGA-3′; *SMARCB1* reverse primer; 5′-TGCTGTATGCGATGGTGGTG-3′; *BNIP3L* forward primer; 5′-CGGACTCGGCTTGTTGTGTT-3′; *BNIP3L* reverse primer; 5′-ATGGGTAGCTCCACCCA GGA-3′; *PBGD* forward primer; 5′-TGTCTGGTAACGGC AATGCGGCTGCAAC-3′; *PBGD* reverse primer; 5′-TCAA TGTTGCCACCACACTGTCCGTCT-3′

Western blot analysis

Cells grown to semiconfluence were washed and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended, and the extracts were centrifuged and the supernatant fraction was collected. Western blot analysis was carried out as described previously.[16,17] The expression of the target protein was evaluated by comparison to the expression of actin.

Annexin V assay

The binding of annexin V was used here as a sensitive assessment of apoptosis, as described previously.[17,18] Cells were stained by Annexin V-APC and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ), and analyzed on a FACS Aria (BD Biosciences).

Invasion assay

The invasion assay was performed using transwell culture chambers (BD Biosciences) according to the instructions provided by the manufacturer. The upper chamber was loaded with cell suspension and the lower chamber was loaded with 10% FBS. After incubation (48 hr for PLC/PRF/5 and 24 hr for HuH7), cells that had invaded the undersurface of the membrane were counted under a microscope. Four microscopic fields were randomly selected for cell counting.

Cell cycle analysis

Cell cycle analysis was performed based on flow cytometric analysis, as described previously.[19] Briefly, cells were washed and fixed. PI and RNase (Sigma-Aldrich) were then added, and data were acquired on the FACS Calibur (BD Biosciences). The cell cycle analysis was carried out using ModFIT software (BD Biosciences).

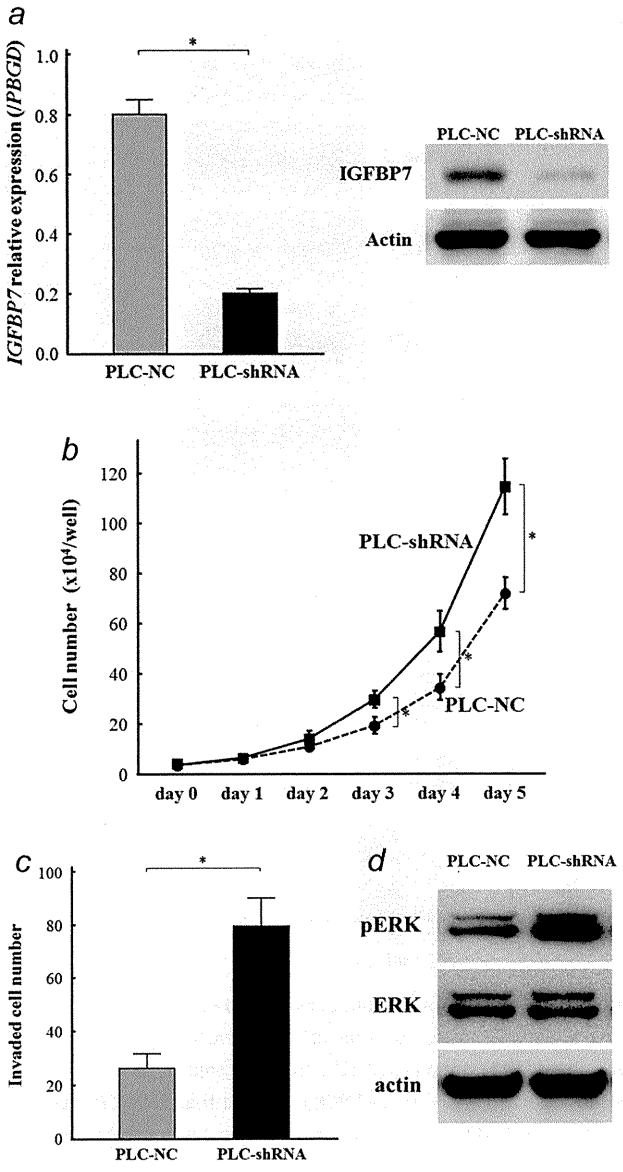

Figure 1. Characteristics of PLC/PRF/5 transfected with shRNA against *IGFBP7*. (*a*) qRT-PCR (Left panel) and western blot analysis (Right panel) indicated a significant decrease in IGFBP7 in cells transfected with shRNA compared to control cells (*$p < 0.05$). (*b*) Proliferation assays showed significantly quicker growth in the IGFBP7-suppressed cells compared with the control cells (*$p < 0.05$). (*c*) The invasion assay showed that the invasive ability of the IGFBP7-suppressed cells was significantly greater than that of the control cells (*$p < 0.05$). (*d*) Western blot analysis demonstrated significantly increased pERK expression in the IGFBP7-supperessed cells compared to the control cells. Data are mean ± SD of 3 experiments.

Immunohistochemical staining

Resected tissue samples were fixed in 10% buffered formalin and finally embedded in paraffin. Immunohistochemical staining for IGFBP7 in the same samples was performed as described previously.[17,20] Briefly, after deparaffinization and blocking, the sections were incubated overnight at 4°C with polyclonal goat anti-human IGFBP7 antibody, and then counterstained with Mayer's hematoxylin. IGFBP7 expression was defined as the presence of specific staining in the cytoplasm of cancer cells. IGFBP7 expression was evaluated as positive or negative, as previously prescribed.[17]

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were assessed using the χ^2-test, and continuous variables were compared using Student's *t*-test. Survival rates were calculated according to the Kaplan-Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0; SAS Institute, Cary, NC). A p value < 0.05 was considered statistically significant.

Results

In vitro studies

IGFBP7 downregulation promotes proliferation and invasive activity. First, *IGFBP7* expression was examined by qRT-PCR in 4 HCC cell lines, PLC/PRF/5, HuH7, HLE and HepG2. The *IGFBP7* expression levels in PLC/PRF/5 and HuH7 were the highest and lowest of the 4 cell lines, respectively. A plasmid coding for shRNA against *IGFBP7* was then transfected into PLC/PRF/5, whose IGFBP7 expression level was the highest in the 4 cell lines. The IGFBP7 expression was suppressed by the transfection, as confirmed by qRT-PCR and western blot analysis (Fig. 1*a*). The proliferation assay showed significantly more rapid growth in the IGFBP7-suppressed cells compared to control cells (Fig. 1*b*). In addition, the invasive ability of the IGFBP7-suppressed cells was significantly greater than that of the control cells (Fig. 1*c*). Based on previous studies that IGFBP7 downregulation promotes cell proliferation through ERK signaling, we analyzed the levels of total ERK and pERK in our cells.[10,21] pERK expression was significantly increased in the IGFBP7-supperessed cells, while total ERK expression was not changed, which coincided with previous reports (Fig. 1*d*). On the other hand, as we previously reported, there were no significant differences in the expression of total Akt or phoshorylated Akt between the IGFBP7-supperessed cells and the control cells.[17]

Downregulation of IGFBP7 attenuates apoptosis. The extent of apoptosis of these cells was examined by the Annexin V assay. The percentages of early apoptotic cells and late apoptotic cells defined by Annexin V-positive/PI-negative cells and Annexin V-positive/PI-positive cells respectively were significantly lower in the IGFBP7-suppressed cells than those in the control cells (Fig. 2*a*).[22] This significant difference of the extent of apoptosis was also confirmed under the condition where apoptosis is induced by some agents, which was reported previously.[17] Next, the expression of proteins related to apoptosis was examined. The result showed that cleaved caspase-3 and cleaved PARP are significantly decreased in

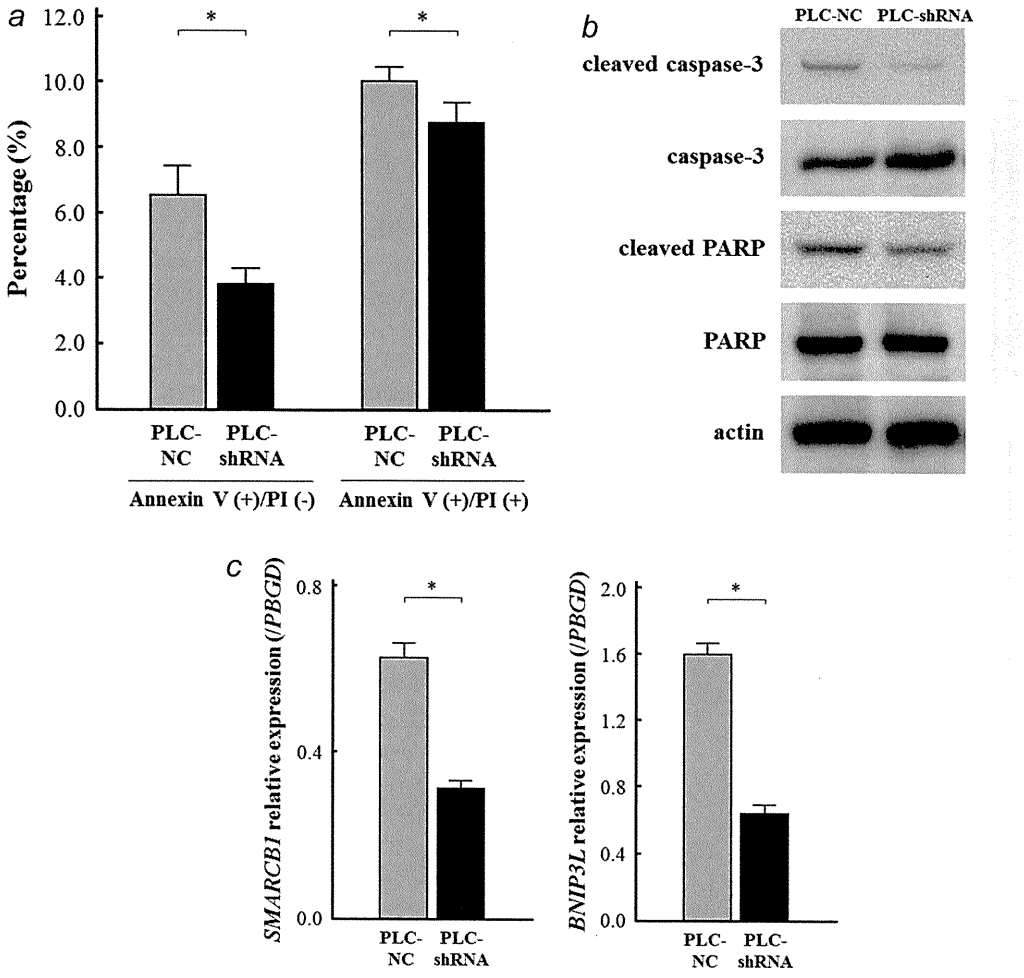

Figure 2. The extent of apoptosis evaluated by the amount of apoptotic cells and the expression of apoptosis-related molecules. (*a*) The Annexin V assay showed the percentage of cells in early apoptosis and in late apoptosis defined by Annexin V-positive/PI-negative cells and Annexin V-positive/PI-positive cells respectively were significantly lower in the IGFBP7-suppressed cells than the control cells (*$p < 0.05$). (*b*) Cleaved caspase-3 and cleaved PARP were decreased in the IGFBP7-suppressed cells. (*c*) qRT-PCR indicated that *SMARCB1* (Left panel) and *BNIP3L* (Right panel) expression levels were significantly decreased in the IGFBP7-supressed cells than the control cells (*$p < 0.05$). Data are mean ± SD of 3 experiments.

the IGFBP7-suppressed cells, while total caspase-3 and PARP expressions were not changed (Fig. 2*b*). In addition, since apoptosis induced by IGFBP7 was reported to occur *via* SMARCB1 and BNIP3L upregulation, we also evaluated the expression levels of *SMARCB1* and *BNIP3L* by qRT-PCR.[10] The results showed that *SMARCB1* and *BNIP3L* expressions were significantly decreased in the IGFBP7-supressed cells compared with the control cells (Fig. 2*c*).

Downregulation of IGFBP7 promotes cell cycle progression. The influence of IGFBP7 on cell cycle was examined by flow cytometric analysis. Prior to the examination, cells were synchronized in the G_0/G_1 phase by serum starvation for 72 hr, and then put back in the regular medium with 10% fetal bovine serum. Dynamic changes in percentage between G_0/G_1 phase and S phase are shown in Figure 3*a*. The proportion of G_0/G_1 phase and S phase on the end of the starvation (0 hr) was almost comparable between the IGFBP7-suppressed cells and the control cells. As shown in Figure 4, the time with minimum percentage of G_0/G_1 phase and maximum percentage of S phase was 24 hr in the control cells, while the time was 12 hr in the IGFBP7-suppressed cells, which suggests that the cell cycle progression was more rapid in the IGFBP7-suppressed cells than that in the control cells. Furthermore, we found that cyclin D1 and cyclin E were increased and p27 was decreased in the IGFBP7-suppressed cells than those in the control cells, and that there was no significant difference in p21 expression between the 2 cells, which was agreement with more rapid cell cycle progression in the IGFBP7-suppressed cells (Fig. 3*b*).

Transfection of IGFBP7 attenuates proliferation and invasive activity. Next, the *IGFBP7* expression plasmid was transfected into HuH7, whose IGFBP7 expression level was the lowest in the 4 HCC cell lines. The IGFBP7 expression was increased by

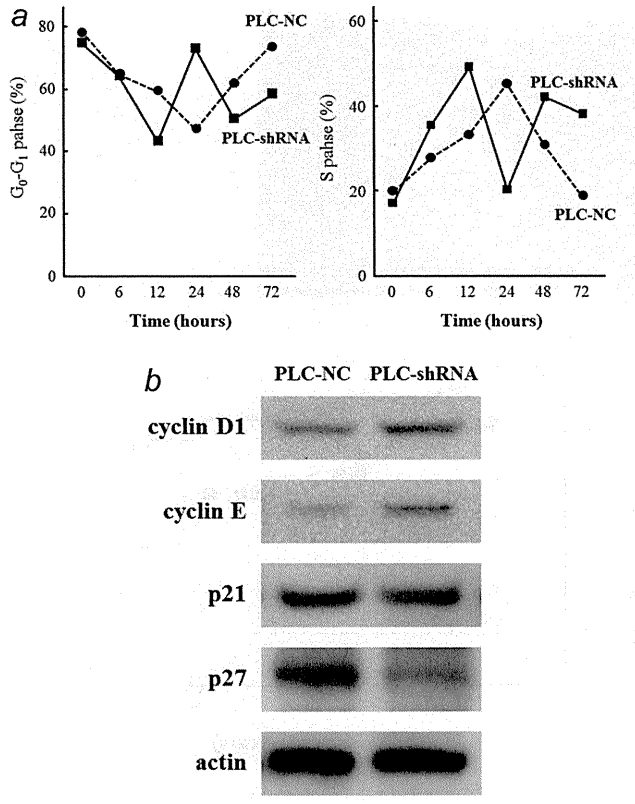

Figure 3. Cell cycle progression assessed by a flow cytometric analysis and the expression of cell cycle-related molecules. (*a*) Time with minimum percentage of G_0/G_1 phase (Left panel) and maximum percentage of S phase (Right panel) was 24 hr and 12 hr in the control cells and the IGFBP7-suppressed cells, respectively. (*b*) Cyclin D1 and cyclin E were increased and p27 was decreased in the IGFBP7-suppressed cells than the control cells, and there was no significant difference in p21 expression between the 2 cells.

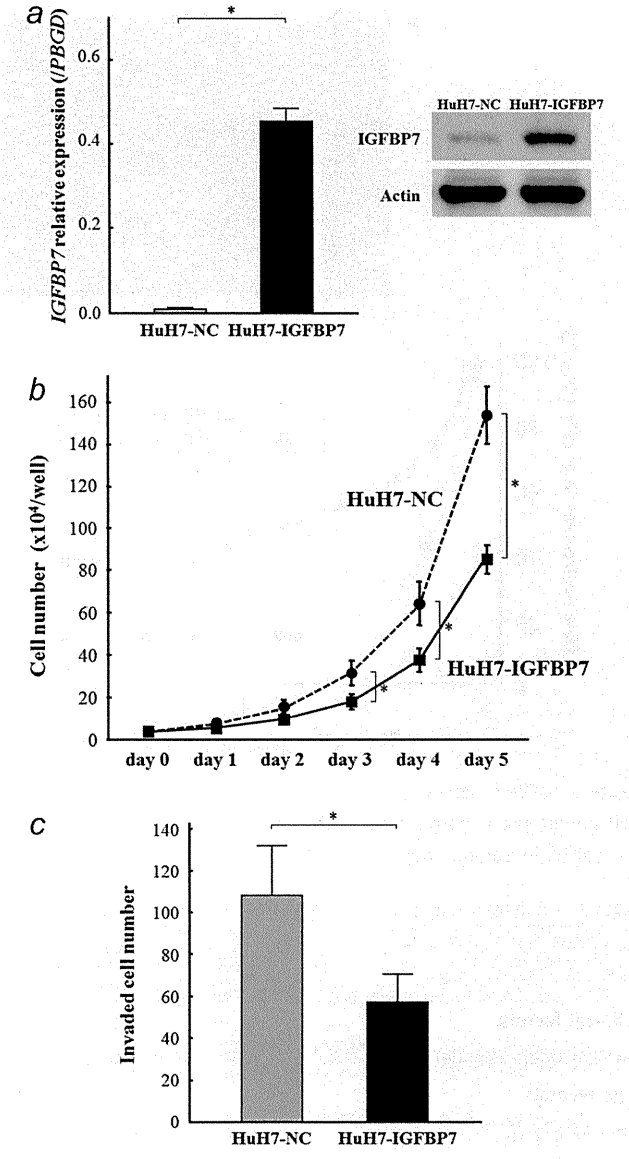

Figure 4. Characteristics of HuH7 transfected with *IGFBP7* expression plasmid. (*a*) qRT-PCR (Left panel) and western blot analysis (Right panel) indicated a significant increase in IGFBP7 in cells transfected with *IGFBP7* expression plasmid compared with control cells (*$p < 0.05$). (*b*) Proliferation assays showed significantly slower growth in the IGFBP7-overexpressing cells compared to the control cells (*$p < 0.05$). (*c*) The invasion assay showed that the invasive ability of the IGFBP7-overexpressing cells was significantly weaker than that of the control cells (*$p < 0.05$). Data are mean ± SD of 3 experiments.

the transfection, as confirmed by qRT-PCR and western blot analysis (Fig. 4*a*). The proliferation assay showed significantly less rapid growth in the IGFBP7-overexpressing cells compared to the control cells (Fig. 4*b*). In addition, the invasive ability of the IGFBP7-overexpressing cells was significantly weaker than that of the control cells (Fig. 4*c*), which were consistent to the results of the above shRNA plasmid experiments.

In vivo studies

IGFBP7 expression correlates with tumor-related factors in clinical HCC samples.. Next, IGFBP7 expression in the tumoral leison was evaluated in clinical sample by immunohistochemical staining. The immunohistochemical analysis showed that among the 104 patients examined, 67 patients (64.4%) showed positive staining for IGFBP7 and the remaining 37 patients (35.6%) were negative for IGFBP7. The immunohistochemical findings of representative cases are shown in Figure 5*a*. The clinicopathological factors related to IGFBP7 expression status of the 104 patients are summarized in Table 1. The data indicated that IGFBP7 expression was significantly associated with maximum tumor size and vascular invasion ($p < 0.0001$, $p = 0.0095$, respectively).

On the other hand, the IGFBP7 expression in nontumoral lesion was homogenously observed in the cytoplasm of cells in all the 104 patients. The immunohistochemically

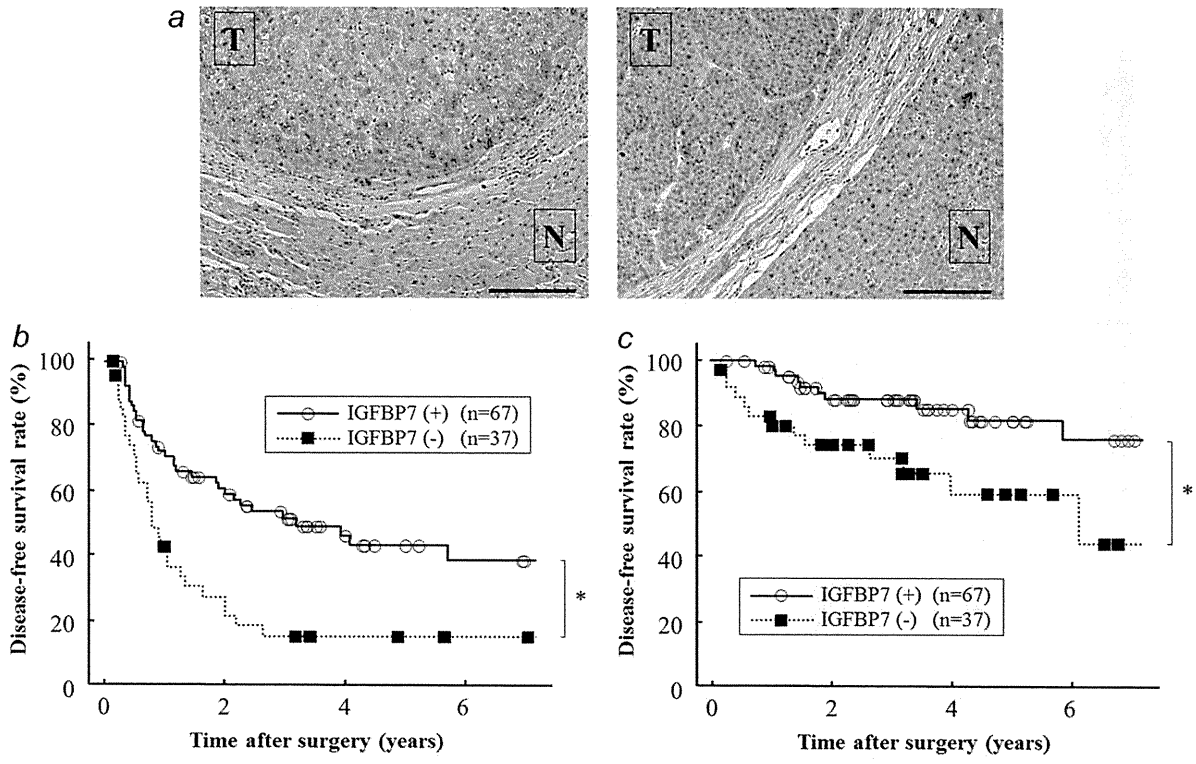

Figure 5. IGFBP7 expression and postoperative outcome in HCC patients. (*a*) Immunohistochemical findings in representative positive case (Left panel) and negative case (Right panel). T, tumoral lesion; N, non-tumoral lesion. Bar = 200 μm. Disease-free survival (*b*) and overall survival (*c*) in patients negative for IGFBP7 expression were significantly poorer than in cells expressing IGFBP7 (*$p < 0.05$).

Table 1. Clinicopathological characteristics of patients with HCC according to IGFBP7 status

	IFGFBP7 (+) ($n = 67$)	IGFBP7 (−) ($n = 37$)	*p*-value
Clinical factors			
Gender (male/female)	53/14	29/8	0.9308
Age (years)[1]	65 ± 10	63 ± 10	0.3900
HBs-Ag (−/+)	52/15	30/7	0.6783
Anti-HCV Ab (−/+)	29/38	15/22	0.7863
Child-Pugh classification (A/B)	58/9	29/8	0.2796
Liver cirrhosis (−/+)	31/36	20/17	0.4470
Tumor-related factors			
AFP (ng/ml)[1]	15,155 ± 122,143	30,243 ± 86,058	0.5075
PIVKA-II (mAU/ml)[1]	26,656 ± 87,446	151,639 ± 1,221,431	0.5365
Number of tumors (single/multiple)	47/20	21/16	0.1693
Maximum tumor size (cm)	3.2 ± 1.7	6.5 ± 4.7	<0.0001
Vascular invasion (−/+)	58/9	24/13	0.0095
Edmondson-Steiner grade (I,II/III,IV)	31/36	11/26	0.0998

[1]Data are expressed as mean ± SD.
Abbreviations: IGFBP7, insulin-like growth factor binding protein 7; HBs-Ag, hepatitis B surface antigen; Anti-HCV Ab, anti-hepatic C virus antibody; AFP:, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II.

determined IGFBP7 expression level was similar between 53 cirrhotic patients and the remaining 51 non-cirrhotic patients.

IGFBP7 downregulation is an independent significant predictor for postoperative outcome in HCC patients. The disease-free survival (DFS) in patients without IGFBP7 expression (1-/

Table 2. Statistical analysis of disease-free survival and overall survival of patients with HCC

	Disease-free survival				Overall survival			
	Univariate	Multivariate			Univariate	Multivariate		
	p-value	OR	95% CI	*p*-value	*p*-value	OR	95% CI	*p*-value
Clinical factors								
Gender (male/female)	0.2192				0.1968			
Age (years) (≤64/>64)	0.5542				0.8018			
HBs-Ag (−/+)	0.2440				0.3605			
Anti-HCV Ab (−/+)	0.9405				0.5034			
Child-Pugh classification (A/B)	0.2586				0.7501			
Liver cirrhosis (−/+)	0.1429				0.5587			
Tumor-related factors								
AFP (ng/ml) (≤400/>400)	0.0629				0.1042			
PIVKA-II (mAU/ml) (≤40/>40)	0.2912				0.1563			
Number of tumors (single/multiple)	0.0025	1.659	0.978–2.815	0.0604	0.0007	2.288	0.905–5.780	0.0801
Maximum tumor size (cm) (≤5/>5)	0.0001	1.387	0.737–2.611	0.3100	0.0290	1.512	0.579–3.949	0.3991
Vascular invasion (−/+)	<0.0001	2.681	1.400–5.135	0.0029	<0.0001	4.649	1.705–12.679	0.0027
Edmondson-Steiner grade (I,II/III,IV)	0.0392	1.520	0.894–2.574	0.1225	0.0180	5.587	1.616–19.231	0.0066
IGFBP7 status (−/+)	0.0007	1.919	1.112–3.313	0.0192	0.0063	2.659	1.102–6.418	0.0296

Abbreviations: IGFBP7, insulin-like growth factor binding protein 7; HBs-Ag, hepatitis B surface antigen; Anti-HCV Ab, anti-hepatic C virus antibody; AFP:, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; OR, odds ratio; 95% CI, 95% confidence interval.

3-/5-year: 42.9%/15.3%/15.3%) was significantly poorer than that in patients showing IGFBP7 expression (1-/3-/5-year: 70.9%/51.8%/43.6%) ($p = 0.0002$; Fig. 5*b*). Univariate analyses showed that number of tumors ($p = 0.0025$), maximum tumor size ($p = 0.0001$), presence/absence of vascular invasion ($p < 0.0001$), and Edmondson-Steiner grade ($p = 0.0392$) all significantly correlated with DFS, in addition to IGFBP7 status (Table 2). Multivariate analysis for DFS using the above 5 factors identified presence/absence of vascular invasion and IGFBP7 status as independent significant factors (Table 2).

The overall survival (OS) rate in patients without IGFBP7 expression (1-/3-/5-year: 83.4%/70.2%/59.2%) was also significantly lower than that in patients with positive IGFBP7 expression (1-/3-/5-year: 98.5%/88.4%/82.0%) ($p = 0.0063$; Fig. 5*c*). By univariate analysis, number of tumors ($p = 0.0007$), maximum tumor size ($p = 0.0290$), presence/absence of vascular invasion ($p < 0.0001$), and Edmondson-Steiner grade ($p = 0.0180$) were also significantly correlated with OS (Table 2). Multivariate analysis using the above 5 factors identified presence/absence of vascular invasion, Edmondson-Steiner grade, and IGFBP7 status as independent significant factors in OS (Table 2). Thus, IGFBP7 expression was an overall independent significant factor for postoperative prognosis in HCC patients.

Discussion

In this study, we first analyzed IGFBP7 function *in vitro* experiments. The results demonstrated that IGFBP7 downregulation was significantly associated with rapid growth and proliferation of HCC cells. In addition, the cells showed decreased apoptotic cell numbers and expression of apoptosis-related proteins, enhancement of ERK signaling, and rapid cell cycle progression. Considering the implicated tumor suppression activity of IGFBP7, the results of this study are consistent with previous similar reports.[7,9–11,14,15,21] We also previously reported a significant association of IGFBP7 downregulation with resistance to some chemotherapeutic drugs in HCC cells.[17] Taken together, it seems apparent that IGFBP7 downregulation is significantly associated with the malignant potential of cancer cells including proliferation, invasiveness, and resistance to chemotherapeutic drugs. To our knowledge, this is the first study to examine the functional role of IGFBP7 in HCC. On the other hand, the cause-and-effect relationship between the IGFBP7 downregulation and the malignant potential is still unsolved, which is expected to be elucidated by further studies in future.

This study also assessed the prognostic significance of IGFBP7 expression in resected human HCC samples. From these findings, IGFBP7 downregulation was significantly associated with tumor progression and postoperative poor prognosis in our patients group, and IGFBP7 status was identified as an independent significant prognostic factor, in addition to other well-known factors. This finding was consistent with the results of the *in vitro* experiments and serves to suggest that assessing the IGFBP7 expression status of patients with HCC could improve the prediction of prognosis.

We have reported some studies of significant prognostic predictors after surgery for HCC.[20,23–38] In one of the studies, based on cDNA microarray analysis, we identified a set of multiple genes whose expressions were significantly different

between patients with good prognosis and those with poor prognosis, and revealed that the gene set was one of the independent prognostic factors.[38] IGFBP7 was not included in the gene set because the difference of the expression level was not large between the 2 groups, but the IGFBP7 expression level examined by the microarray analysis was also significantly correlated to the prognosis. This result seems to be consistent with the result of this study. In addition, considering the significant inverse correlation of IGFBP7 expression to the extent of apoptosis and cell cycle progression confirmed by the *in vitro* experiments, our previous reports of apoptosis- and cell cycle-related molecules as prognostic factors are in agreement with the prognostic impact of IGFBP7.[25,26,34,35] Furthermore, we have reported that angiogenesis-related molecules such as angiopoietin-2 and hypoxia-induced factor-1α are significant factors for postoperative prognosis.[33] Considering that IGFBP7 was reported to block angiogenesis in human vascular endothelial cells, it may be possible that there is a significant correlation between IGFBP7 expression and the angiogenesis-related molecules, though we did not examine it in this study.[39]

IGFBP7 is a secreted protein, and recombinant IGFBP7 is commonly purified.[40,41] Indeed, previous studies of IGFBP7 function have expressed the protein exogenously using IGFBP7 viral vectors or by administering recombinant protein.[39] In addition, IGFBP7 has also been studied as a possible therapeutic agent for treatment of malignancies that are dependent on BRAF-MEK-ERK signaling.[10,42] Thus, recombinant IGFBP7 could be potentially suitable therapeutically to improve the poor prognosis of HCC patients lacking IGFBP7 expression. In addition, IGFBP7 expression is also subject to epigenetic modification, and aberrant methylation of CpG islands in the *IGFBP7* promoter region was confirmed in several kinds of cancers.[10,43] In this regard, Wajapeyee *et al.*[10] showed that treatment of melanoma cell lines with DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, restored IGFBP7 expression. Such a finding suggests a therapeutic application whereby IGFBP7 expression and thus function are restored using a DNA methyltransferase inhibitor. Exploring these therapeutic interventions against HCC was unfortunately beyond the scope of this study, and further studies are definitely needed in this regard.

In summary, we found that IGFBP7 downregulation was significantly associated with both tumor progression and clinical outcome in HCC. This result suggested that analysis of IGFBP7 expression in patients might help to predict prognosis, and that IGFBP7 could be a novel therapeutic target in HCC patients with poor prognosis.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Clinical Significance of Alpha-Fetoprotein mRNA in Peripheral Blood in Liver Resection for Hepatocellular Carcinoma

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ABSTRACT

Purpose. Detection of AFP mRNA in peripheral blood is considered a useful predictor of HCC recurrence after resection. However, its interpretation and clinical significance remains to be determined. This study was designed to evaluate the clinical significance of detecting AFP mRNA positive cells in peripheral blood.

Methods. A total of 153 patients without macroscopic vascular invasion, who underwent liver resection, were prospectively enrolled in this study. The pattern of HCC recurrence was confirmed by image studies and divided into four types: (1) no recurrence (control group, $n = 68$); (2) intrahepatic single recurrence (SR group, $n = 28$); (3) intrahepatic multiple recurrences (MR group, $n = 38$); and (4) extrahepatic HCC recurrence (EX group, $n = 19$).

Results. HCC recurrence was identified in 85 (55.6%) patients during a follow-up of 8.6 ± 6.7 (range, 0.7–36) months. Multivariate analysis identified preoperative AFP mRNA (HR = 2.54; $P = 0.006$) as an independent risk factor for HCC recurrence. Preoperative AFP mRNA expression was a significant predictor of HCC recurrence in the MR/EX group ($P = 0.029$) but not in the SR group ($P = 0.467$).

Conclusions. Detection of AFP mRNA expression in peripheral blood before surgery for HCC is a useful predictor of multiple or extrahepatic HCC recurrences.

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Hepatocellular carcinoma (HCC) is the fifth commonest malignant disease and is highly associated with viral hepatitis in up to 90% of cases. Similar to other malignant tumors, HCC has the potential of recurrence with local and distant metastasis. Liver resection has been established as the first-line treatment for HCC, although the high incidence of postoperative recurrence of HCC remains a serious problem. HCC recurrence after liver resection is recognized to have unique characteristics and is divided into three patterns of recurrence: (1) intrahepatic metastasis; (2) multicentric HCC; and (3) extrahepatic metastasis. The diagnosis of these patterns of recurrence requires close follow-up with image studies after liver resection as well as histopathological evaluation of the tumor recurrence, if available.[1]

Circulating tumor cells (CTC) in the peripheral blood or disseminated tumor cells (DTC) in the bone marrow are reported to be the cause of tumor recurrence in various malignant tumors.[2] In liver transplantation for HCC, the fact that the most common site of tumor recurrence is the transplanted allograft provides strong support for this notion and the central role of CTC and DTC in tumor recurrence.[3,4]

The mRNA level of alpha-fetoprotein (AFP) in peripheral blood is a candidate marker of CTC. We reported previously the efficacy of detecting AFP-expressing cells by quantitative RT-PCR in patients who had undergone liver resection or liver transplantation for HCC.[5,6] Despite numbers of publications on this prognostic marker of HCC recurrence, it has not been studied in reference with the patterns of HCC recurrence.

This study was designed to determine the prognostic value of detecting AFP mRNA-positive cells in peripheral blood in patients with HCC who underwent curative resection, in predicting HCC recurrence after surgery, and to clarify the correlation between AFP mRNA expression in peripheral blood and the three patterns of HCC recurrence.

PATIENTS AND METHODS

The study protocol was approved by the Human Subjects Review Committee of Osaka University. All study subjects provided written, informed consent.

Patients

Among 295 consecutive patients who underwent liver resection for HCC between December 2001 and October 2008 in our hospital, 188 patients who underwent curative resection were free of macroscopic portal or venous invasion and consented to this prospective study. Peripheral blood samples (16 ml) were obtained from each participant for analysis of AFP mRNA at the following time points: within 3 days before surgery, and postoperatively immediately after surgery. Of the 188 patients, 37 were excluded because of short follow-up period without HCC recurrence (<12 months), and thus data of 153 patients were subjected to the analysis of risk factors.

The patient demographic and operative data, tumor characteristics, preoperative serum AFP levels, serum levels of protein induced by vitamin K antagonist II (PIVKA-II), and computed tomographic (CT) scans of the abdomen and chest after surgery were collected prospectively. The standard postoperative follow-up consisted of abdominal dynamic CT scan or magnetic resonance imaging (MRI) every 3–4 months with serum AFP, PIVKA-II, and chest X-ray or chest CT scan every 3–6 months. Bone scintigraphy or brain MRI was performed whenever metastasis was suspected.

Patients with HCC > 5 cm in preoperative image studies received transcatheter arterial chemoembolization (TACE) therapy 1–2 months before liver resection. No adjuvant chemotherapy, TACE, or other anticancer treatment was provided to the study patients until HCC recurrence was confirmed.

HCC recurrence confirmed by image studies was divided based on the patterns of the recurrence into: (1) no recurrence (control group); (2) intrahepatic single recurrence after liver resection (SR group); (3) multiple intrahepatic recurrences (MR group); and (4) extrahepatic HCC recurrence (EX group).

Real-Time Quantitative RT-PCR for AFP mRNA in Peripheral Blood

Peripheral blood (16 ml) samples were obtained prospectively from each patient within 3 days before surgery (preoperative AFP mRNA) and again immediately after surgery (postoperative AFP mRNA). The method used for the detection of AFP mRNA in peripheral blood was described previously.[7,8] Briefly, blood samples were collected in a VACUTAINER CPT™ cell preparation tubes with sodium citrate (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 17,000×*g* for 20 min. The separated mononuclear cells were placed into a 15-ml centrifugation tube, suspended with 10 ml of phosphate buffered saline (PBS), and centrifuged at 2,000 rpm for 10 min. After washing with PBS again, the cells were suspended with TRIzol Reagent (Molecular Research Center, Cincinnati, OH), and stored at −80°C until RNA isolation. AFP mRNA was quantified with the LightCycler™ analysis software (Roche Diagnostics, Mannheim, Germany) using the protocol provided by the manufacturer. The level of AFP mRNA in the blood was expressed relative to that of the mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The lower limit of detection of the AFP mRNA by this method was 1.0×10^{-8}, and any value above this level was designated as positive, as described previously.[5,6]

Statistical Analysis

Continuous data were expressed as mean ± standard deviation, and group data sets were compared using the Mann–Whitney *U* test or Kruskal–Wallis test. Categorical data are presented as percentages, and differences between proportions were compared using the chi-square test. The cumulative risk of HCC recurrence and the 95% confidence intervals (CI) were computed by Kaplan–Meier analysis. Univariate and multivariate risk-factor assessments were performed using the Kaplan–Meier method (log-rank test) and Cox's proportional hazards model. Variables that correlated with the risk of HCC recurrence in the univariate analysis ($P < 0.1$) were entered into the multivariate analysis. $P < 0.05$ was considered significant.

RESULTS

The 153 patients with HCC comprised 116 men and 37 women. The underlying liver disease was HCV ($n = 90$, 58.8%), HBV ($n = 33$, 21.6%), Laennec's ($n = 4$, 2.6%), and no apparent background liver disease ($n = 32$, 20.9%). The mean follow-up duration was 13.4 ± 10.8 (range, 0.4–54.2) months. Of the 153 patients, 68 (44.4%) were recurrence-free after a follow-up period of 22.6 ± 11.3 (range, 12–54.2) months, whereas 85 patients (55.6%) developed HCC recurrence within a follow-up period of 8.6 ± 6.7 (range, 0.7–36) months. The proportion of patients showing each type of recurrence pattern was 44.4% ($n = 68$) for the control group (no recurrence), 16.3% ($n = 28$) for the SR group (intrahepatic single recurrence after liver resection), 24.8% ($n = 38$) for the MR group (multiple intrahepatic recurrences after liver

resection), and 12.4% ($n = 19$) for the EX group (extrahepatic HCC recurrence), which included pulmonary metastasis ($n = 10$, 53%), lymph node metastasis ($n = 3$, 16%), diaphragm metastasis ($n = 3$, 16%), bone metastasis ($n = 2$, 11%), and adrenal gland metastasis ($n = 1$, 5%).

Table 1 shows the demographic and clinical features of the four groups. Age, gender, and background liver disease were similar among the four groups. Tumor size tended to be smaller in the control group and largest in the MR group ($P = 0.018$ between control vs. MR groups). Tumor number was single in 54 of 68 (79.4%)

TABLE 1 Characteristics of patients and hepatocellular carcinoma

	Control group ($n = 68$)	SR group ($n = 28$)	MR group ($n = 38$)	EX group ($n = 19$)	P
Age (years)	65.2 ± 9.9	67.1 ± 9.9	66.6 ± 7.6	63.9 ± 7.8	0.515
Gender (male/female)	46/22	22/6	31/7	17/2	0.157
Primary diagnosis					
HCV	41 (60.3)	16 (57.1)	25 (65.8)	8 (42.1)	0.213
HBV	16 (23.5)	5 (17.9)	5 (13.1)	7 (36.8)	
Laennec's	2 (2.9)	1 (0.4)	0 (0)	1 (5.3)	
Non-B, non-C	14 (20.6)	9 (32.1)	9 (23.6)	5 (26.3)	
Tumor characteristics					
Size (cm)	3.74 ± 2.47	4.14 ± 2.22	5.18 ± 3.63	4.78 ± 3.75	0.055
Number	128 ± 0.67	1.57 ± 1	1.97 ± 1.46	1.8 ± 1.24	0.093
Microscopic vascular invasion (%)	25.4	26	50	26.3	0.06
Histological differentiation (Edmondson classification)					
1	1 (1.8)	1 (3.7)	0 (0)	0 (0)	0.119
2	19 (33.3)	15 (55.6)	12 (31.6)	9 (47.3)	
3	34 (59.6)	10 (37)	25 (65.8)	6 (31.6)	
4	3 (5.3)	1 (3.7)	1 (2.6)	3 (15.8)	
Preoperative TACE (%)	45.5	46.4	47.4	68.4	0.353
Hepatectomy (HR)[a]					
0	34 (50)	17 (60.7)	20 (52.6)	9 (47.4)	0.9
S	8 (11.8)	1 (3.6)	4 (10.5)	3 (15.8)	
1	16 (23.5)	6 (21.4)	7 (18.4)	6 (31.6)	
2	9 (13.2)	4 (14.3)	7 (18.4)	1 (5.3)	
3	1 (1.5)	0 (0)	0 (0)	0 (0)	
Blood loss (ml)	842 ± 1280	647 ± 595	1460 ± 2683	721 ± 454	0.075
Transfusion	6/68 (8.8)	6/28 (21.4)	6/38 (15.8)	0	0.102
Transfused RC-M.A.P. (ml)	133 ± 610	89 ± 253	302 ± 1098	0	0.769
TNM stage[a]					
1	4 (5.9)	4 (14.3)	2 (5.3)	1 (5.3)	0.096
2	50 (73.5)	13 (46.4)	19 (50)	10 (52.6)	
3	12 (17.6)	8 (28.6)	12 (31.6)	5 (26.3)	
4a	2 (2.9)	3 (10.7)	3 (7.9)	3 (15.8)	
4b	0 (0)	0 (0)	2 (5.3)	0 (0)	
AFP (median; range)	17.5 (2–206249)	36.5 (3–31310)	52 (4–179200)	38 (4–947500)	0.314
PIVKA	105 (28–61330)	300 (9–32539)	334 (20–122976)	252 (23–304000)	0.356
AFP mRNA (%)					
Preoperative	4.4	10.7	15.8	10.5	0.264
Postoperative	20.6	42.9	36.8	31.6	0.095
Preoperative and postoperative	4.4	0	5.3	5.3	0.466

Data are mean ± standard deviation or number of patients with percentages in parentheses unless otherwise indicated

RC-M.A.P. Red cell concentrates mannitol adenine phosphate, *AFP* alpha-fetoprotein, *PIVKA* protein induced by vitamin K antagonist, *TACE* transcatheter arterial chemoembolization, *SR* single recurrence, *MR* multiple recurrence, *EX* extrahepatic recurrence

[a] According to the Liver Cancer Study Group of Japan (LCSGJ)

patients of the control group and in 18 of 28 (64.3%) patients of the SR group, whereas a solitary tumor was less frequently seen in 21 of 38 (55%) patients of the MR group and 11 of 19 (58%) patients of the EX group. The number of tumors was the lowest in the control group compared with the MR ($P = 0.007$) and EX ($P = 0.035$) groups. Tumor differentiation according to Edmondson classification, HAI score in background liver, and the extent of liver resection were not different among the four groups. The estimated blood loss and transfused red cell concentrates mannitol adenine phosphate were not significantly different among the groups. AFP and PIVKA-II were not different among the four groups.

The AFP mRNA/GAPDH mRNA ratio in peripheral blood ranged from undetectable and 1.04E−4. AFP mRNA was detected in 14 (9.2%) patients before surgery, whereas 46 (30.1%) patients were positive postoperatively. Six (3.9%) patients were positive for AFP mRNA both preoperatively and postoperatively. A larger proportion of patients of the MR group were AFP mRNA-positive preoperatively and less in the control group than the SR and EX groups, whereas a larger proportion of patients of the SR, MR, and EX groups were AFP-mRNA-positive postoperatively than the control group. The status of AFP mRNA (positive/negative) did not correlate with tumor characteristics, such as microscopic vascular invasion, blood loss, blood transfusion, TNM stage, and PIVKA-II,

TABLE 2 Relationship between preoperative AFP mRNA and various clinical parameters

	Preoperative AFP mRNA		*P*
	Positive ($n = 14$)	Negative ($n = 139$)	
Age (years)	70.1 ± 6.8	65.3 ± 9.2	0.057
Gender (male/female)	11/3	105/34	0.064
Primary diagnosis (%)			
HCV	42.9	60.4	0.203
HBV	14.3	22.3	0.487
Non-B, non-C	42.9	21.6	0.187
Tumor characteristics			
Size (cm)	5.2 ± 3.5	4.2 ± 2.9	0.146
Number	2.57 ± 1.83	1.47 ± 0.92	0.070
Microscopic vascular invasion (%)	30.8	32.4	0.906
Histological differentiation (Edmondson classification)			
1	0 (0)	2 (1.4)	0.947
2	5 (35.7)	50 (36)	
3	8 (57.1)	67 (48.2)	
4	1 (7.1)	7 (5)	
Preoperative TACE (%)	57.1	48.9	0.557
Hepatectomy (HR)[a]			
s	0	16	0.094
0	11	69	
1	0	35	
2	3	18	
3	0	1	
Blood loss (median; range) (ml)	480 (20–16600)	550 (30–2400)	0.724
Blood transfusion (RC-M.A.P.) incidence (amount (ml))	14% (780 ± 736)	10.1% (1539 ± 1781)	0.571
TNM stage[a]			
1	1	10	0.527
2	6	86	
3	6	31	
4a	1	10	
4b	0	2	
AFP (median; range)	396 (4–947500)	32 (2–206249)	0.039
PIVKA	115 (31–304000)	174 (9–122976)	0.917

Data are mean ± standard deviation or number of patients with percentages in parentheses unless otherwise indicated

RC-M.A.P. Red cell concentrates mannitol adenine phosphate, *AFP* alpha-fetoprotein, *PIVKA* protein induced by vitamin K antagonist, *TACE* transcatheter arterial chemoembolization

[a] According to the Liver Cancer Study Group of Japan (LCSGJ)