the difference between the cHCC-CC and the HCC group or the cHCC-CC and the CCC group was not statistically significant due to the small number of patients in the cHCC-CC group.

The recurrence of cHCC-CC has been frequently reported. Yano et al.[2] reported a higher prevalence of intrahepatic recurrence than extrahepatic recurrence, whereas Sasaki et al.[11] reported more prevalence of extrahepatic recurrence than intrahepatic recurrence. Although both extrahepatic and intrahepatic recurrences were frequently seen in the present study, extrahepatic recurrences were more frequent than intrahepatic recurrence. It is well known that HCC can easily develop intrahepatic recurrence after hepatectomy, and local therapeutic approaches such as percutaneous radiofrequency ablation have been proved effective.[21] The extrahepatic recurrence of cHCC-CC might not be suitable for these local therapeutic modalities. Besides, the recurrence components were mainly CCC in this present study; the HCC component was pathologically proven in only one case. These phenomena indicate that the postoperative recurrence pattern of cHCC-CC was more similar to CCC than HCC. These results are in agreement with the report of Uenishi et al.[22] that the CCC component of MHC seems to determine the prognosis, because metastases are usually composed of the CCC elements. Recent trials using combination systemic chemotherapy and neoadjuvant chemoradiation have shown promise for recurrent CCC.[23] Furthermore, we have also reported one case of cHCC-CC of local recurrence, with long-term survival after a combination of reoperation and hepatic arterial infusion chemotherapy.[12] Therefore, systemic chemotherapy might also be helpful for extrahepatic recurrent cHCC-CC.

CONCLUSION

In conclusion, cHCC-CC Allen's type C is a rare type of PLC with clinicopathological features that are more similar to HCC and recurrence patterns that are more similar to those of CCC. The preoperative diagnosis is difficult; however, liver masses similar to those of HCC, together with moderately elevated serum AFP and CA19-9, are reliable cHCC-CC indicators. Surgical resection of this tumor can yield results that are intermediate, between HCC and CCC in characteristics. However, although differences were not significant due to the small number of patients in the cHCC-CC group, both extrahepatic and intrahepatic recurrences can easily occur. More cases are needed to further elucidate the characteristics of this type of tumor.

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SURGERY TODAY

Association Between Recurrence of Hepatocellular Carcinoma and α-Fetoprotein Messenger RNA Levels in Peripheral Blood

OSAKUNI MORIMOTO, HIROAKI NAGANO, ATSUSHI MIYAMOTO, YOSHIYUKI FUJIWARA, MOTOI KONDO, TAMEYOSHI YAMAMOTO, HIDEO OTA, MASATO NAKAMURA, HIROSHI WADA, BAZARRAGCHAA DAMDINSUREN, SHIGERU MARUBASHI, KEIZO DONO, KOJI UMESHITA, SHOJI NAKAMORI, MASATO SAKON, and MORITO MONDEN

Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita, Osaka 565-0871, Japan

Abstract

Purpose. Intra- and extrahepatic recurrence is common, even after curative resection for hepatocellular carcinoma (HCC), suggesting preoperative or intraoperative tumor cell dissemination. Reverse transcription — polymerase chain reaction (RT-PCR) for α-fetoprotein (AFP) is used to detect circulating liver cancer cells. We previously developed a quantitative method that allows estimation of the AFP mRNA level by real-time PCR. In the present study, we used this method to measure the AFP mRNA level before and after resection of HCC, then correlated the findings with various clinicopathological characteristics and prognosis.

Methods. We prospectively examined peripheral blood samples from 38 patients with HCC, and bone marrow aspirate from 25 of these patients. As a control, we examined bone marrow from 20 patients with benign diseases. The follow-up period ranged from 32 to 66 months. Real-time RT-PCR was used to detect AFP mRNA levels in the samples.

Results. AFP was expressed in 9 (23.7%) of the 38 peripheral blood samples. The detection of AFP mRNA was significantly correlated with extrahepatic metastasis after primary surgery, and a shorter disease-free survival time (P = 0.0245 each). Bone marrow samples were defined as positive if they expressed AFP mRNA at levels higher than the maximum expressed level in the controls, because only 1 (5%) of the 20 control bone marrow samples had low AFP mRNA expression. Using this cutoff level, 12 (48%) of the 25 patients with HCC had positivity for AFP mRNA. The results of bone marrow RT-PCR did not correlate with the clinicopathological characteristics of prognosis.

Conclusions. Using real-time PCR to measure the AFP mRNA level in blood, but not bone marrow, could be useful for predicting postoperative tumor recurrence.

Key words Hepatocellular carcinoma · Micrometastasis · α-Fetoprotein messenger RNA · Metastatic recurrence

Introduction

Despite improvements in the diagnosis and treatment of hepatocellular carcinoma (HCC), intrahepatic and extrahepatic recurrence is still frequent, even after curative surgery.[1] Two distinct mechanisms are responsible for intrahepatic recurrence; namely, multicentric carcinogenesis (MC) in the underlying chronic liver disease and intrahepatic metastasis (IM) resulting from the hematogenous spread of tumor cells.[2–7] Intrahepatic metastasis tends to occur within a relatively short period (usually within 2 years) whereas MC tends to cause intrahepatic recurrence at a constant year rate over more than 10 years.[8] Although resection is theoretically the most effective treatment for HCC, about half of all postoperative recurrences are considered to result from IM.[9] Therefore, it is important to develop an effective prediction system and oncotherapeutic regimen for IM with or without extrahepatic recurrence to improve the prognosis after surgery for HCC.

The serum levels of α-fetoprotein (AFP) and protein induced by vitamin K absence–II (PIVKA-II) are commonly measured as tumor markers of HCC, but their sensitivity and specificity are not sufficient for the detection of micrometastasis of HCC.[10,11] Furthermore, high levels of tumor markers do not indicate the presence of "cancer cells" or "micrometastasis" in the blood or bone marrow. To resolve these issues, AFP mRNA levels in the peripheral blood or bone marrow are now measured by polymerase chain reaction (PCR) tech-

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niques.[12-22] Although the PCR method for AFP mRNA is very sensitive, its specificity in hepatoma cells is still controversial, considering that low levels of AFP mRNA are often detected even in healthy subjects.[12,17] We recently established a real-time, quantitative PCR assay for AFP mRNA in peripheral blood, using the LightCycler.[23] This method allows the detection of hepatoma cells at the $1/10^5$ level and clearly differentiates persons with cancer from those without cancer. However, it is still unknown whether this method is useful for predicting postoperative intra- and extrahepatic recurrences in patients with HCC.

The detection of bone marrow micrometastases by immunocytochemical or reverse transcription–polymerase chain reaction (RT-PCR) methods is a predictor of poor prognosis in patients with breast, lung, and various epithelial cancers.[24-28] However, to our knowledge, there are few reports on using the real-time PCR assay to detect bone marrow micrometastasis of HCC.[20,21] Therefore, the clinical relevance of HCC micrometastasis in bone marrow is still unclear. We quantitatively measured the levels of peripheral blood and bone marrow AFP mRNA in patients undergoing hepatectomy for HCC to evaluate its usefulness in predicting recurrence.

Patients and Methods

Patients

Peripheral blood was collected from 38 consecutive patients who underwent curative surgery for HCC at the Department of Surgery, Osaka University Hospital, between June 1998 and April 2001. Bone marrow aspirate was obtained from the sternum of 25 of these patients during anesthesia before skin incision. Control bone marrow aspirate was also obtained from 20 patients undergoing surgery for a benign disease. Written informed consent was obtained from all patients before participation in the study.

Clinicopathological Examinations

In addition to measurement of serum AFP and PIVKA-II, abdominal echogram, computed tomography (CT), and magnetic resonance imaging (MRI), or a combination of these tests were done before and after surgery, at least every 3 months. Chest radiograms and bone scintigrams were also done if extrahepatic metastases were suspected. The duration of clinical follow-up after surgery ranged from 32 to 66 months (mean, 49 months). Tumor stage and grade of the resected specimens were classified according to the fifth edition of the TNM classification.[23]

Blood and Bone Marrow Sample Preparation

We collected 8 ml of peripheral blood from each patient just before the skin incision, then again immediately after surgery. We also withdrew 8 ml of bone marrow aspirate from the sternum just before skin incision. Blood and bone marrow samples were collected in a Vacutainer CPT cell preparation tube with sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at $17000 \times g$ for 20 min. Separated mononuclear cells were placed into a 15-ml centrifugation tube, suspended with 10 ml of phosphate-buffered saline (PBS), and centrifuged at 2000 rpm for 10 min. After washing with 1 ml PBS again, the cells were suspended with 1 ml TRIzol Reagent (Molecular Research Center, Cincinnati, OH, USA), and stored at –80°C until RNA isolation.

RNA Extraction and Real-Time PCR

RNA extraction and real-time PCR were done using the methods reported previously.[22] We analyzed quantified data with the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) using the protocol provided by the manufacturer. The amplification products of tenfold serial dilutions of total RNA extracted from cultured cells (HuH7) were monitored by the fluorescence of AFP-specific hybridization probes. The relationship between the crossing points of real-time PCR and the initial amount of total RNA was found to be linear on logarithmic scales, in a 10^9-fold range of 1×10^{-2}–1×10^6 pg. The correlation coefficient (r) was 0.99, indicating a perfect log-linear relation in this range. Likewise, log-linearity was observed in the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts (data not shown). The level of AFP mRNA in blood was expressed relative to that of GAPDH mRNA. The lower detection limit of AFP mRNA/GAPDH mRNA in this method was 1.0×10^{-2}, and any value above this threshold was designated as positive.

Statistical Analysis

The correlations between the real-time PCR results and clinical stage and other prognostic measures of HCC were tested with the χ^2 test, Fisher's Exact test, and Mann–Whitney U-test for rank data. The distribution of time to recurrence was estimated according to the Kaplan–Meier method and compared with log-rank statistics. P values of less than 0.05 were considered significant. Statistical analysis was performed using StatView (5.0 version) software (SAS, Cary, NC, USA).

Results

Blood AFP mRNA Levels Before and After Surgery

The level of AFP mRNA in the blood, relative to that of GAPDH, ranged from undetectable to 1.76×10^3 (Fig. 1). α-Fetoprotein mRNA was positive preoperative in 4 (10.5%) of the 38 patients, but became negative postoperatively in 2 of these patients. α-Fetoprotein mRNA was positive after surgery in 7 (18.4%) patients, 5 (13.2%) of whom were negative before surgery, but became positive after surgery. The remaining 29 patients were negative before and after surgery.

Correlation Between the Blood AFP mRNA Level and Clinicopathological Characteristics

The patient demographics are summarized in Table 1. To investigate the relationship between the blood AFP mRNA level and the clinicopathological characteristics, patients were classified into three groups, according to when AFP mRNA first became positive. Group A consisted of the four patients who were positive for AFP mRNA before surgery (cases 1–4); group B consisted of the five patients who became positive for AFP mRNA after surgery (cases 5–9); and group C consisted of the 29 patients who were negative for AFP mRNA before and after surgery (cases 10–38). Unexpectedly, AFP mRNA was detected in several patients with a small HCC tumor. The tumor diameter was ≤3 cm in six of the nine patients with a positive AFP mRNA (groups A and B) and only 2 cm in diameter in one patient (case 1). There was no significant relationship between tumor diameter and AFP mRNA positivity (Fig. 2). In fact, 6 (27.3%) of the 22 patients with a small tumor (diameter: ≤3 cm) were AFP mRNA-positive. There was no significant relationship between blood AFP and other features, except for intrahepatic nodules and extrahepatic recurrence (Table 2). Intrahepatic nodules were detected in significantly more AFP mRNA positive patients than AFP mRNA negative patients.

Correlation Between the AFP mRNA Level and Postoperative Tumor Recurrence

Tumor recurrence developed 19 of the 38 patients, and was detected within 2 years of surgery for the primary tumor in all except 4 patients (case 7, recurrence at 30 months; case 25, recurrence at 27 months; case 30, recurrence at 25 months; and case 34, recurrence at 32 months, Table 1).

Extrahepatic recurrence was detected in six patients, all except one of whom (case 14) were AFP mRNA-positive (Table 1). Tumor recurrence was detected in 7 (77.8%) of the 9 patients from groups A and B, but in only 12 (41.3%) of the 29 patients from group C ($P < 0.05$, Table 2). In four patients (cases 1, 3, 5, 6), both extrahepatic and intrahepatic recurrences were detected within 1 year of their initial operation. In case 7, recurrence was detected in the residual liver 15 months

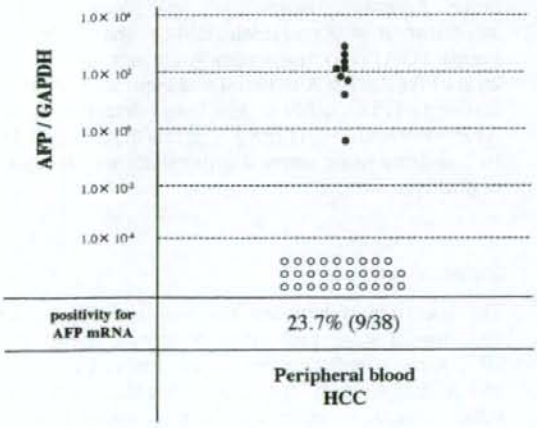

Fig. 1. α-Fetoprotein (*AFP*) mRNA levels in the peripheral blood. The amount of AFP transcript in blood was estimated by quantitative real-time polymerase chain reaction (PCR). Results are expressed as the ratio of the AFP transcript to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in each sample. *HCC*, hepatocellular carcinoma

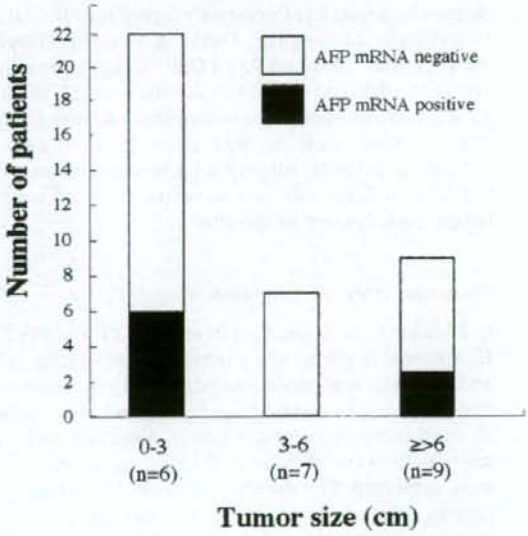

Fig. 2. Relationship between tumor diameter and positive expression of α-fetoprotein (AFP) mRNA in the peripheral blood

Table 1. α-Fetoprotein (AFP) mRNA levels and clinicopathological factors

			AFP/GAPDH Peripheral blood										
		Case	Pre-operative	Post-operative	Bone marrow	Tumor size (cm)	Serum AFP (ng/mL)	Differentiation	Stage	Portal invasion	Intrahepatic nodules	Interval (months)	Location of recurrence
A	1	42/M	1.09×10^{1}	7.81×10^{3}		2.0	39	Poor	2	−	+	9	Liver, adrenal, bone
	2	63/M	1.25×10^{1}	1.65×10^{0}	0	2.3	15	Moderate	4	−	+	10	Liver
	3	76/M	5.81×10^{-1}	0		10.5	1475	Poor	4	+	+	4	Liver, lung, bone
	4	47/M	1.06×10^{2}	0	0	9.0	38000	Moderate	4	+	+	15	Liver
B	5	63/M	0	1.76×10^{3}		11.0	312000	Poor	2	+	+	1	Liver, lung, brain
	6	61/M	0	1.34×10^{2}		2.2	31	Moderate	4	−	+	10	Liver, brain
	7	60/M	0	4.55×10^{2}		2.5	80	Moderate	3	−	+	30	Liver
	8	70/M	0	2.12×10^{2}	0	3.0	5	Poor	2	+	−	21	No recurrence
	9	72/M	0	6.35×10^{1}	2.58×10^{-5}	2.2	9	Moderate	2	−	−	39	No recurrence
C	10	64/M	0	0	0	7.8	30	Moderate	3	+	+	1	Liver
	11	54/M	0	0	0	1.5	6	Poor	1	+	−	4	Liver
	12	66/M	0	0	8.91×10^{-3}	2.5	3255	Poor	2	+	−	7	Liver
	13	68/M	0	0	8.35×10^{-8}	2.3	9	Moderate	2	−	−	8	Liver
	14	71/M	0	0	0	8.0	5	Poor	3	+	+	9	Liver, lung
	15	70/M	0	0	5.53×10^{-6}	1.3	15	Well	1	−	−	13	Liver
	16	68/M	0	0	0	3.6	203	Moderate	2	−	−	16	Liver
	17	69/M	0	0		4.5	39	Poor	2	+	+	17	Liver
	18	70/M	0	0		2.2	13	Moderate	2	+	−	50	No recurrence
	19	50/M	0	0		6.5	19	Poor	2	+	−	34	No recurrence
	20	66/M	0	0		1.5	55	Poor	1	+	−	36	No recurrence
	21	51/M	0	0		7.5	28	Moderate	4	+	+	37	No recurrence
	22	64/M	0	0		3.4	75	Poor	2	−	+	12	Liver
	23	64/M	0	0	0	7.5	3124	Undifferentiated	3	+	−	38	No recurrence
	24	55/M	0	0	0	1.0	5	Moderate	1	−	−	39	No recurrence
	25	67/M	0	0	2.16×10^{-7}	2.5	36	Poor	3	+	+	27	Liver
	26	54/M	0	0	0	4.5	5	Poor	2	−	−	43	No recurrence
	27	59/M	0	0	0	2.1	95	Poor	2	+	−	43	No recurrence
	28	76/M	0	0		1.0	29	Moderate	1	−	−	43	No recurrence
	29	56/M	0	0	2.16×10^{-7}	4.6	5	Poor	3	−	+	44	No recurrence
	30	50/M	0	0	2.01×10^{-2}	2.7	71	Moderate	4	−	+	25	Liver
	31	59/M	0	0	1.40×10^{-6}	1.9	5	Moderate	1	+	−	47	No recurrence
	32	63/M	0	0	0	4.2	11	Poor	2	+	−	47	No recurrence
	33	66/M	0	0	1.73×10^{-5}	5.0	7	Moderate	2	−	−	46	No recurrence
	34	54/M	0	0	0	1.5	8	Moderate	1	+	−	32	Liver
	35	57/M	0	0	4.97×10^{-3}	1.1	726	Moderate	1	−	−	48	No recurrence
	36	70/M	0	0	6.36×10^{-3}	2.8	5	Moderate	2	−	−	48	No recurrence
	37	72/M	0	0	1.01×10^{-3}	1.5	32	Well	1	−	−	48	No recurrence
	38	49/M	0	0		6.8	1340000	Moderate	2	−	−	53	No recurrence

Table 2. Relationship between α-fetoprotein mRNA in the peripheral blood and clinicopathological characteristics

Variable	Group A ($n = 4$)	Group B ($n = 5$)	Group A + B ($n = 9$)	Group C ($n = 29$)
HBs-Ag (+/–)	1/3	1/4	2/7	3/26
HCV-Ab (+/–)	3/1	3/2	6/3	22/7
Tumor size (cm)	6.0 ± 4.4	4.2 ± 3.8	5.0 ± 3.9	3.6 ± 2.3
Stage (1,2/3,4)	1/3	3/2	4/5	22/7
Histology (good, moderate/poor, undiff)	2/2	3/2	5/4	16/13
Intrahepatic nodules (yes/no)	4/0†	3/2	7/2†	8/21
Portal invasion (yes/no)	2/2	2/3	4/5	15/14
Serum AFP level (≧200/<200 ng/ml)	2/2	1/4	3/6	5/24
Extrahepatic recurrence (yes/no)	2/2†	2/3	4/5†	1/28

HB, hepatitis B; HCV, hepatitis C virus
Group A, B, A and B vs C: † $P < 0.05$

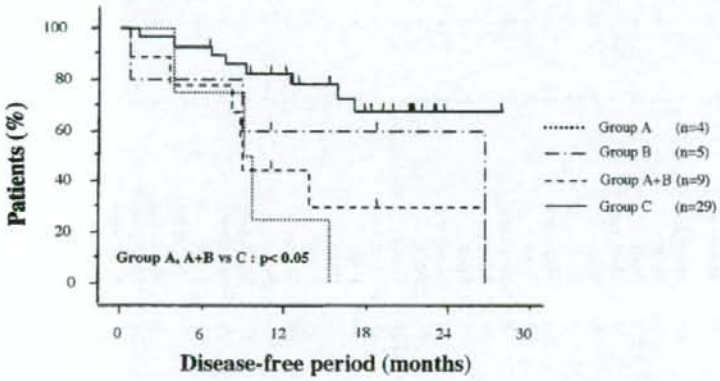

Fig. 3. Comparison of disease-free survival rates in patients with positivity and those with negativity for AFP mRNA in the peripheral blood

after surgery, and lung metastasis was found 20 months later. The intrahepatic recurrences developed within 15 months in two of the remaining three patients (cases 2, 4) and after 30 months in one (case 7). The specificity of tumor recurrence was 89.5% (number of group C patients without tumor recurrence/total number of patients without tumor recurrence, 17/19), and the sensitivity was 36.8% (number of group A and B patients with tumor recurrence/total number of patients with tumor recurrence, 7/19).

Disease-free survival was significantly correlated with the positivity of AFP mRNA. The group C patients had significantly better disease-free survival than those in group A or groups A + B ($P < 0.05$; Fig. 3). The disease-free survival rates after 2 and 3 years were 68% and 60%, respectively, in group C, and 31% and 23%, respectively, in group A + B.

Bone Marrow AFP mRNA Levels Before Surgery

Fluorescence signals were detected in 12 (48%) out of 25 bone marrow specimens from the patients with HCC. The levels of AFP mRNA varied from 5.00×10^{-2} to 2.01×10^{4} pg; however, fluorescence was detected in 1 (5%) of the 20 bone marrow samples from patients with benign diseases. The levels of AFP mRNA in patients with benign diseases were lower (5.32×10^{-2} pg) than those in the patients with HCC. Therefore, we defined bone marrow samples with AFP mRNA levels exceeding 6.0×10^{-2} pg as AFP mRNA-positive. The results were standardized by the ratio of the quantity of AFP to that of GAPDH (Fig. 4).

Bone Marrow AFP mRNA Levels, Clinicopathological Characteristics, and Prognosis

Table 1 lists the clinical characteristics, data, and AFP mRNA values of patients with HCC. There was no significant correlation between AFP mRNA expression in bone marrow and various clinicopathological characteristics or prognostic relevance except for portal invasion (Table 3). Multiple intrahepatic recurrences developed in 5 (41.7%) of the 12 patients with positive AFP mRNA expression in their bone marrow. Intra-

Table 3. Relationship between the detection of α-fetoprotein mRNA in bone marrow and clinicopathological characteristics

Variable	AFP mRNA negative cases ($n = 18$)	AFP mRNA positive cases ($n = 15$)	P value
HBs-Ag (+/–)	2/16	2/13	0.8456
HCV-Ab (+/–)	14/4	10/5	0.6968
Tumor size (cm)	4.3 ± 2.7	2.9 ± 1.6	0.0773
Stage (1,2/3,4)	12/6	12/3	0.4585
Histology (good, moderate/poor, undiff)	11/7	9/6	0.9481
Intrahepatic nodules (yes/no)	6/12	3/12	0.4585
Portal invasion (yes/no)	11/7	3/12	**0.0329**
Serum AFP level (≧200/<200 ng/ml)	6/12	4/11	0.7220
Extrahepatic recurrence (yes/no)	2/16	1/14	0.6583

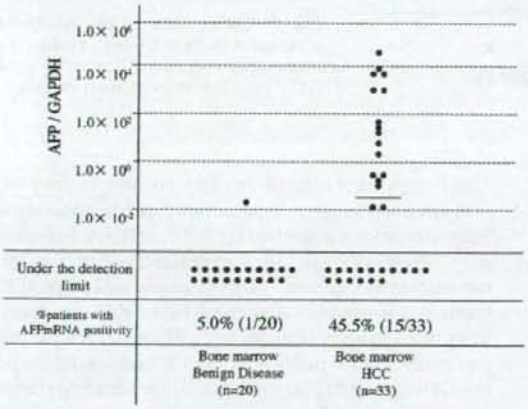

Fig. 4. α-Fetoprotein (*AFP*) mRNA in bone marrow samples

hepatic recurrence developed in 7 (53.8%) of the 13 patients with negative AFP mRNA expression in their bone marrow. One patient had distant organ metastasis (case 14, Table 1).

Disease-free survival did not correlate with the positivity of AFP mRNA in the bone marrow cells. Disease-free survival was similar in the patients with AFP mRNA-negative and AFP mRNA-positive bone marrow ($P = 0.82095$; Fig. 5). The disease-free survival rates at 2 and 3 years were 56% and 44%, respectively, for patients with bone marrow negative for AFP mRNA, and 60% and 46%, respectively, for patients with bone marrow positive for AFP mRNA.

Comparison Between Blood AFP mRNA and Bone Marrow AFP mRNA

Paired blood and bone marrow samples were obtained from 25 patients. Four (16%) of these 25 patients had positive AFP mRNA blood samples and 12 (48%) patients had positive AFP mRNA bone marrow samples. Only one (4%) patient (Case 8) had AFP mRNA positivity in both their blood and bone marrow samples (Table 4). This patient was still alive 39 months after surgery with no sign of recurrence. There was no relationship between disease-free survival and blood or bone marrow AFP mRNA in these 25 patients.

Discussion

This study showed that positive expression of AFP mRNA in the blood was closely correlated with early postoperative tumor recurrence. Since not only extrahepatic recurrences, but also most early hepatic recurrences are thought to result from the hematogenous spread of cancer cells,[9] our results strongly suggest that a positive AFP mRNA might reflect the presence of circulating cancer cells in the peripheral blood. This assumption is consistent with the finding that intrahepatic nodules were more frequently detected in AFP mRNA-positive patients. Thus, patients with an AFP mRNA/GAPDH mRNA ratio exceeding 1.0×10^{-2}, being positive for AFP mRNA, are thought to be at high risk of metastatic recurrence, indicating a need for adjuvant chemotherapy.

The specificity and sensitivity of our real-time RT-PCR method for detecting tumor recurrence were 89.5% and 36.8%, respectively, which suggests that using the real-time PCR method for blood AFP mRNA may not be sensitive enough to accurately predict tumor recurrence. Multicentric carcinogenesis is also involved in intrahepatic recurrence although its frequency is estimated to be constant, at around 3%–4%/year in surgical patients.[29-31] Therefore, the development of intrahepatic recurrence in patients negative for AFP mRNA may be partly attributable to MC. However, the annual rate of early tumor recurrence, defined as within 2 years, in patients negative for AFP mRNA was estimated at

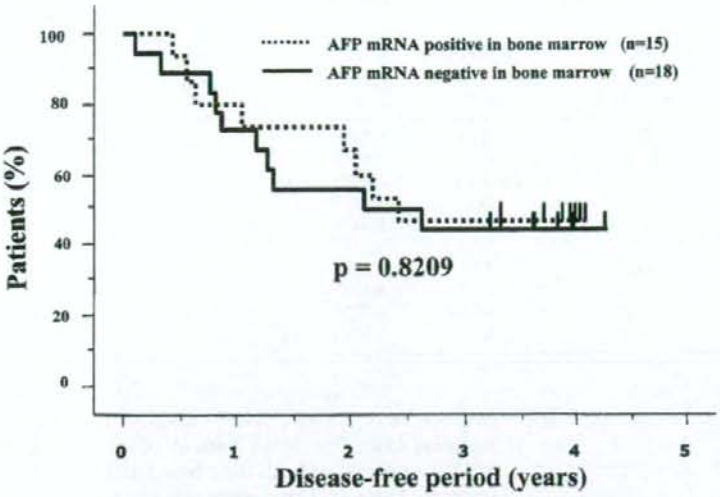

Fig. 5. Comparison of disease-free survival rates in patients with positivity and those with negativity for α-fetoprotein (*AFP*) mRNA in bone marrow cells

Table 4. Comparison of α-fetoprotein mRNA in blood and bone marrow samples from 25 patients with hepatocellular carcinoma

	Blood	
	Positive (4/25; 16%)	Negative (21/25; 84%)
Bone marrow-positive (12/25; 48%)	1 (4%)	11 (44%)
Bone marrow-negative (13/25; 52%)	3 (12%)	10 (40%)

around 16% per year (Fig. 3); almost fivefold the frequency of MC. The disease-free survival rate at 31 months was 60% and no tumor recurrence was observed thereafter. Therefore, using the real-time RT-PCR method for blood AFP mRNA might be useful to predict postoperative IM, but not MC.

Despite the significant correlation between preoperative positivity for AFP mRNA and intrahepatic nodules, there was no relationship with other clinicopathological factors, including tumor diameter. Because intrahepatic nodules are uncommon in patients with small hepatomas,[32] it would be difficult to predict tumor recurrence by these factors, especially in this group of patients. In this study, the proportion of patients with small tumors who were positive for AFP mRNA was 27.3%, and four of these six patients were later found to have tumor recurrence. Thus, the detection of AFP mRNA in blood by real-time RT-PCR is useful for the prediction of tumor recurrence in patients undergoing surgery for HCC, even if the tumor is small.

α-Fetoprotein mRNA became positive in the peripheral circulation after hepatectomy in five patients who were otherwise negative for AFP mRNA before surgery. Since AFP mRNA is expressed not only in HCC, but also in noncancerous liver tissue and normal liver tissue,[18] it is possible that the AFP mRNA we detected originated from noncancer cells. However, in our previous study,[23] AFP mRNA did not become positive postoperatively in ten patients who underwent hepatectomy for metastatic adenocarcinoma. Considered together, these results suggest that AFP mRNA originating from hepatoma cells could become detectable in the peripheral blood after surgery. Wong et al.[11] recently reported a similar increase in blood AFP mRNA in 13 patients who underwent hepatic resection for HCC. This indicates that hepatoma cells may be released from cancer tissue into the circulation as the result of surgical manipulation.[6,33] Intraoperative tumor cell dissemination might be prevented by alternative surgical strategies without liver manipulation, such as the liver-hanging maneuver or clamping the hepatic hilum during hepatectomy.

The positivity (10.5%) of preoperative AFP mRNA in this study was lower than that in previous studies (27%–51%).[22,34–37] Moreover, lung metastasis developed after primary surgery in two of our patients with AFP mRNA-negative bone marrow samples (cases 18, 21). There are two possible reasons for this discrepancy. First, the sample preparation might be accountable. In a previous study, we used Ficoll (Pharmacia, Uppsala, Sweden) as the method of cell extraction, but in the present study, we used the Vacutainer CPT cell preparation tube. This method may have lower sensitivity

than techniques using Ficoll cell extraction. Second, a sampling error may have caused the discrepancy because detecting and comparing tumor cells in blood at different times is always associated with statistical sampling errors. Moreover, only 8 ml of blood was examined and the release of tumor cells into the bloodstream may not be a continuous process. However, this has yet to be elucidated completely.

No fluorescence was detected in any of the peripheral blood samples from healthy volunteers, patients with liver cirrhosis, or patients who underwent hepatectomy for diseases other than HCC because this method adopts fluorescent probes that specifically bind to amplified products.[23] In contrast, 1 of 20 bone marrow control samples was positive for AFP mRNA. This result probably reflects the fact that a subset of bone marrow cells originally expresses AFP mRNA, but the level of expression is lower than that in hepatocytes.[38] In this assay, we defined the cutoff level of expression for AFP mRNA in control bone marrow samples.

There was no correlation between AFP expression in bone marrow and any clinicopathological features except for portal invasion. Furthermore, disease-free survival did not correlate with the positivity of AFP mRNA in bone marrow cells, probably because of the limited number of patients tested. Interestingly, local recurrence was detected soon after resection in 5 of the 12 AFP-positive patients, all of whom had early stage HCC. This might reflect the fact that the systemic spread of circulating tumor cells occurred more often in the early stage, whereas malignant hepatocyte cells metastasized more easily intrahepatically than to distant organs. Moreover, according to the original "seed and soil" hypothesis of Paget,[39] the dissemination of single cancer cells is only a prerequisite for the formation of solid metastases. Local environment factors, including adhesion molecules, growth factors, the ability of disseminated tumor cells to respond to them, and the effectiveness of immunologic antitumor defense mechanisms, also determine the development and pattern of clinically overt metastasis. The biological characteristics and the fate of disseminated tumor cells are largely unknown. In this study, we investigated AFP mRNA in both blood and bone marrow in 25 paired samples and found positivity for AFP mRNA in both blood and bone marrow samples in only one patient. Moreover, there was no correlation between the rate of AFP mRNA positivity in bone marrow and blood in terms of clinicopathological characteristics and prognosis. All of the patients with AFP mRNA positivity in the bone marrow had negative preoperative peripheral blood AFP mRNA. This discrepancy may be attributed to the chance of false positivity because not only cancer cells but also bone marrow cells in patients with benign disease express AFP mRNA. In this study, AFP mRNA was detected in the bone marrow from only 1 of the 20 patients with benign diseases and the level of AFP mRNA expression was low. However, the number of AFP mRNA-positive cases might increase with a larger sample of patients with benign diseases, and the cutoff level of expression for AFP mRNA may rise. A sampling error might also have contributed to this discrepancy. Heparin was not used as an anticoagulant in this study, so there is a possibility that cancer cells were trapped when part of the bone marrow coagulated. These factors may have also influenced the result that disease-free survival did not correlate with the positivity of AFP mRNA in bone marrow cells.

In conclusion, our results showed an association between blood AFP mRNA levels measured by real-time RT-PCR and the incidence of postoperative HCC recurrence, with high specificity. Although its sensitivity is still not ideal, this assay may be useful for some patients with small tumors and no known clinicopathological risk factors. We did not find a significant correlation between bone marrow AFP mRNA and clinicopathological features or prognosis. Thus, a long–term follow-up study of a larger patient population is required to confirm the clinical usefulness of this assay.

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治療法の進歩—効果，成績—

肝癌—FAIT(FU Arterial Infusion and Interferon Therapy)—

永野浩昭　門田守人

FAIT(FU Arterial Infusion and Interferon Therapy) for hepatocellular carcinoma

Hiroaki Nagano, Morito Monden
Department of Surgery, Graduate School of Medicine, Osaka University

Abstract

We summarised the beneficial effects of FAIT(FU Arterial Infusion and Interferon Therapy) for advanced hepatocellular carcinoma(HCC). In the 55 patients of HCC with portal venous tumour thrombi(PVTT) treated with FAIT alone, 24(43.6%) showed objective response, 4(7.3%) showed no response, and 27(49.1%) showed progressive disease. In the 15 patients of HCC with PVTT treated with FAIT and surgery, all of the cases(100%) survived over 1 year; without FAIT and surgery, 10 patients(67%) were died within 1 year. Concerning the mechanism of FAIT, we reported the synergistic effects of IFNα and 5-FU, in terms of the influence to cell cycle progression leading into the S phase via $p27^{Kip1}$, an apoptosis-inducing effect through a reduction of Bcl-xl, and an immuno-modulatory effect via the TRAIL/TRAIL-receptor pathway. FAIT is a promising modality for advanced HCC with tumour thrombi in the major portal branches.

Key words: hepatocellular carcinoma, portal vein thrombosis, arterial infusion chemotherapy, interferon, surgery

はじめに

肝細胞癌は早期の段階であれば，RFAなどのlocal ablation therapyや肝切除により，その治療効果を期待し得る．しかしながら，門脈内腫瘍栓などを伴うような既存治療が無効である進行癌症例も少なからず存在する．これらの症例は，極めて予後不良であり，有効な治療が施されなければ，ほぼ1年以内に癌死する[1,2]．このような場合は，一般的に化学療法が選択されるが，肝細胞癌は抗癌剤の感受性が低く，その奏効率は20%以下である[3]．また門脈内腫瘍栓を伴うような進行肝細胞癌では，たとえ肉眼的治癒切除を施行し得ても，術後早期に肝内再発を来し，その治療成績は極めて不良である．最近，当科においては，門脈内腫瘍栓(Vp3以上)合併進行肝細胞癌に対してインターフェロン(IFN)-α併用(5-FU肝動注)化学療法(Fluorouracil Arterial infusion and Interferon Therapy: FAIT)を施行することにより良好な成績を得，報告してきた[4-6]．

本稿では，これらIFN-α併用5-FU肝動注化学療法(FAIT)の成績およびその作用機序について概説する．

大阪大学大学院医学系研究科外科学講座消化器外科学

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1. 肝細胞癌に対するIFN併用化学療法

IFN単独による肝細胞癌に対する臨床的治療成績については必ずしも満足のいくものではない[7]．その一方で，IFN単剤ではなく，種々の抗癌剤との併用が試みられ，IFN-αに5-FUやCDDPなどの薬剤を併用することによる抗腫瘍効果についての報告がされつつある．Pattらは，肝細胞癌43症例に対して，IFN-αと5-FU持続静脈内投与による25%の奏効率について報告した[8]．更に抗癌剤を動脈内投与することによりその治療成績は上昇する．肝動注化学療法は，薬物到達濃度を高濃度で保ち，全身の副作用を軽減する点において，有用であり[9]，最近では，皮下埋め込み式リザーバーの開発により，頻回の薬剤投与と持続投与が可能となった．Urabeら[10]は，進行肝細胞癌症例に対し，IFN-αと5-FU，CDDP，methotrexateの3剤の肝動注化学療法，leucovorinの全身投与の併用による50%の奏効率を，また，同グループのKanekoら[11]は更に，門脈内腫瘍栓を伴った肝細胞癌29例に対して，同様のプロトコールによる45%の奏効率について報告した．Chungらも，IFN-αとCDDPの肝動注療法との併用で33%の奏効率を報告[12]した．著者らも，術後肝内多発再発と肺，骨の肝外転移を伴う肝細胞癌に対してIFN-αの投与とUFTの内服によりCRを得られた症例の経験[13]から，1997年より既存の治療法では全く治療効果の期待できない門脈内腫瘍栓を伴う進行肝細胞癌症例に対して，IFN-αと5-FU持続肝動注化学療法を併用し，後述するような極めて良好な結果[4-6]を得ている．

2. FAITの対象および方法

当科における本療法の対象は，門脈一次分枝または本幹侵襲(Vp3以上)を伴う高度進行肝細胞癌症例であり，3群に分類し切除不能症例のみならず肝機能良好で肝切除可能症例においては，肉眼的治癒切除後もしくは減量肝切除後の補助療法として本療法を組み入れている．適応は，副作用や抗癌剤投与による肝障害を考慮して，70歳未満，総ビリルビン値が正常範囲内で，AST，ALTがともに100 IU/*l*未満，血小板80,000/mm^{3}以上，血清クレアチニン値が1.5 mg/d*l*以下で，外来通院が可能なperformance statusが0，1としている．全肝多発病変を伴う症例や耐術が不可能と考えられる切除不能症例では，Seldinger法にて肝動注カテーテルを挿入する．肝切除可能例では，術中にカテーテルを留置し，肝切除後の補助療法として本療法を施行している．治療スケジュールは，皮下埋め込み式動注リザーバーより5-FUを300 mg/m^2/dayで2週間持続投与を行い，2週間休薬を1クールとする．同時にIFN-αを500万単位/回，週3回投与，4週間を1クールとして皮下投与する．

3. 切除不能症例に対するFAITの治療成績

1997年から現在までに切除不能な門脈内腫瘍栓を伴う高度進行肝細胞癌症例(Vp3以上，IM3)55例に対してFAITを施行した[5]．治療回数は2クール以上で，効果の得られた症例には繰り返し治療を行った．治療効果は，CR: 8例，PR: 16例と，その奏効率は43.6%であり，奏効した24例の生存期間の中央値は12カ月であった．また，全55症例の生存率は，1，2，3年生存率が，それぞれ62.0%，28.8%，16.4%であり，これらの治療成績は，既存の治療法がなくbest supportive careのみの場合ほとんどの症例が6カ月以内に死亡することと比較すると，極めて有効な治療法であると考えられる(**表1**)．副作用では，発熱，悪寒といったGrade 2以下の非血液毒性をほぼ全例に認めるが，Grade 3以上の非血液毒性の副作用はなく，Grade 3の血液毒性の発現率は，9/55例(16.4%)であった．治療の中断を要するような重篤な副作用を伴う症例は極めてまれであり，全例が外来通院のみで反復治療が可能であった．

4. 肝切除および術後FAITの治療成績

肝機能良好でVp3以上の門脈内腫瘍栓を伴う高度進行肝細胞癌症例に対して，肝切除後に補助療法としてFAITを45例に施行した．

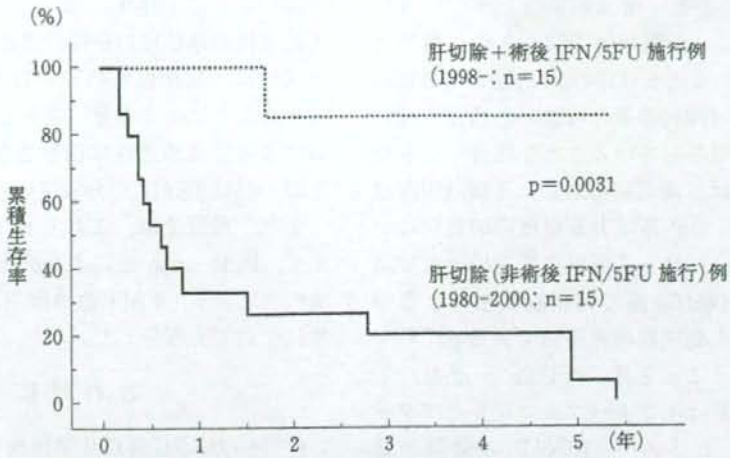

図 1　切除症例(根治 B)に対する術後補助化学療法としての FAIT の累積生存率(文献[6]より改変)

表 1　非切除症例 55 例に対する FAIT の治療成績

	全　例 (55 例)	有効例 (CR/PR) (24 例)	無効例 (NC/PD) (31 例)
無増悪生存期間・中央値(月)	5.2	12	2.2
無増悪生存率(%)			
1 年	11.3	49.3	0
2 年	3.8	20.6	0
3 年	3.8	20.6	0
累積生存期間・中央値(月)	11.8	24.4	5.4
累積生存率(%)			
1 年	62	82.9	13.1
2 年	28.8	54.2	0
3 年	16.4	30.9	0

(文献[5]より改変)

このうち，門脈内腫瘍栓と片葉に主腫瘍を伴い，肉眼的に癌遺残のない肝切除を施行したのちに，術後補助療法として FAIT を 3 クール以上施行した症例が 15 例であった．これらの 15 例の現時点での成績は，無再発生存 11 例(12-53 カ月)，再発生存 2 例(22，36 カ月)，癌死 2 例(18 カ月：肺転移，60 カ月：残肝再発)，他病死 2 例(22，68 カ月)であり，1 年生存率は 100%であった[6]．これら 15 例の予後は，当科において肝切除のみ施行した Vp3 以上の肝細胞癌症例 15 例と比較して有意に良好であった(図 1)．

また，門脈内腫瘍栓に片葉の主腫瘍と全肝多発病巣を伴う 30 例に対して，減量肝切除と術後に FAIT を施行した．肝内病巣に関しては，7 例の CR を含む 10 例に効果を認め，その奏効率は 33.3%であった．CR/PR 症例 10 例の 1，2，3 年生存率は，それぞれ 100，75，37.5%であり，NC/PD 症例より有意に良好であった．しかし，肝内病巣に奏効したものの肺への遠隔転移を 3 例に認めた．このように，本療法は，肝内病巣には有効であるものの，肝外病変の制御は困難であり，今後の検討すべき課題である．

5. FAIT の作用機序に関する基礎的検討

FAIT の抗腫瘍効果は，IFN-α，5-FU それぞれ単独による効果と相互作用による相乗・相加効果がある．しかし，臨床報告における肝細胞癌に対する IFN-α 単独もしくは 5-FU 単剤投与の治療成績を考えるとそれぞれ単独での治療効果は乏しく，主には IFN-α と 5-FU の相加・相乗効果による抗腫瘍効果であると考えられる[10]．

その相加・相乗効果について，IFN-α が 5-FU の代謝調節に作用し，TP(thymidine phosphorylase)を活性化し中間代謝産物である FdUMP(5-fluoro-2′-deoxyuridine 5-monophosphate)の細胞内濃度を上昇させる効果や TS(thymidylate

synthetase)阻害率の増強効果などが報告されている[15]．更に，両薬剤併用による作用機序として，IFN-αによる5-FUのDNA傷害の増強以外にも，①増殖抑制効果の増強，②宿主免疫の賦活作用，が関与していることを報告してきた．

①については，両薬剤併用による細胞周期遅延やapoptosisの誘導による増殖抑制効果について検討を行い，ヒト肝細胞癌株を用いた併用治療により，G0/G1期での細胞集積による細胞増殖遅延と細胞周期関連蛋白であるp27^{Kip1}の発現増強を伴うことを見いだした[16]．また，この増殖抑制効果はインターフェロンレセプター(IFN-α/βレセプター：IFNAR)の発現が強い細胞株で顕著に認められ，IFNARの発現が，STAT1(signal transducer and activator of transcription)のリン酸化による活性化，apoptosisの頻度およびapoptosis関連蛋白であるBcl-2 familyの発現調節に相関することを確認した[17,18]．

また②のIFN-αによる宿主免疫作用として，IFN-α投与により進行肝細胞癌患者の末梢血中の単核球にTRAIL(tumor necrosis factor-related apoptosis-induced ligand) mRNAの発現が誘導され，*in vitro*においても同様にIFN-α添加によってTRAIL mRNAの発現を確認した．更に末梢血単核球の肝細胞癌株に対する細胞障害活性は，末梢血単核球にIFN-αの前刺激を加えることにより有意に増加し，TRAIL中和抗体によってその活性は阻害されること[19]から，その一部はTRAILを介していると考えられる．

また，最近では，これら*in vitro*の検討に加えて，PCR-array法による網羅的遺伝子解析の施行[20]による，FAITの治療前効果予測の可能性についても報告した．

おわりに

IFN-α/5-FU併用化学療法(FAIT)は高度進行肝細胞癌に対して，極めて有効な治療法であるとともに，進行肝細胞癌に対する集学的治療の一基軸として，肝切除や肝移植などと組み合わせることにより，その治療成績の飛躍的な向上が期待できる．しかし，無効例が約半数存在すること，肝外病変の制御は困難であることなどが問題点であり，これらの克服のためには，更なる作用機序の解明による本療法の効果増強を可能とする分子の同定などについても，今後は考慮せねばならない．

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第5章 肝癌の治療

2．肝癌治療の実際

(5) 肝動注化学療法

②インターフェロン α・5-FU 併用動注化学療法

和田 浩志* 永野 浩昭*
門田 守人*

〈Key point〉

はじめに

本邦における肝細胞癌は，HBV，HCV 感染によるウイルス性肝炎や肝硬変を背景とする症例がそのほとんどを占め[1]，これら慢性肝障害を有する患者に対する定期的な follow up に加えて，近年の画像診断の進歩により，比較的早期の段階に発見される症例が増加してきた．このような症例は，単発で腫瘍径も小さく，内科的な経皮的エタノール注入療法(PEI)，マイクロ波凝固療法(MCT)，ラジオ波焼灼療法(RFA)などの local ablation therapy や肝機能が良好であれば根治的肝切除の適応であり，かなりの治療効果を期待しうる[2]．しかし，その一方で，門脈内腫瘍栓や肝内転移を伴い，発見時には既存治療の適応外となる症例も少なくなく，加えて，早期肝癌に対して局所療法や肝動脈塞栓術(TAE)を反復施行するなかで，門脈内腫瘍栓や多発肝内転移の出現により制御不能となる症例も存在する．これらの予後はきわめて不良であり，有効な治療が施されなければ，その生命予後は1年に満たない[3),4)]．

Key words：肝細胞癌，肝動注化学療法，インターフェロン，5-FU，IFN 併用化学療法

Subcutaneous Interferon-alpha and Intra-arterial Infusion of 5-Fluorouracil Combined Therapy for Advanced Hepatocellular Carcinoma

Hiroshi Wada／Hiroaki Nagano／Morito Monden
*大阪大学大学院医学系研究科外科学講座消化器外科学（〒565-0871 大阪府吹田市山田丘2-2）

従来，このような進行例に対しては化学療法が選択されるが，一般的に肝細胞癌は抗癌剤の感受性が低く，全身化学療法の奏効率は20%以下であった[5]．また門脈内腫瘍栓(Vp 3以上)を伴う肝細胞癌では，たとえ肉眼的治癒切除を施行しえても，術後早期に肝内再発をきたし，その治療成績は十分とはいえない．そこで，当科においては，門脈内腫瘍栓(Vp 3以上)合併高度進行肝細胞癌に対してインターフェロンα(IFN-α)併用フルオロウラシル(5-FU)肝動注化学療法(Fluorouracil Arterial infusion and Interferon Therapy；FAIT)を施行することにより良好な結果を得ている[6)~8)]．本稿では，これらFAITの成績およびその作用機序について概説する．

インターフェロンα(IFN-α)併用5-FU肝動注化学療法

Ⅰ．肝細胞癌に対するIFN併用化学療法

IFN単独療法

肝細胞癌に対するIFN単独療法について，諸家の報告では奏効率0~7%であり，そのIFN単独療法による抗腫瘍効果は乏しいと考えられる．一方で，大腸癌患者に対してIFN-αと5-FUの併用投与により，奏効率76%(13/17例)と高い抗腫瘍効果を認めたとの報告[9)]がされている．このことより，肝細胞癌に対してもIFN単独ではなく，種々の抗癌剤との併用が，試みられてきた．IFN-αにアントラサイクリン系の薬剤の全身投与を併用した報告では，いずれも化学療法単剤と比較しても，予後改善効果，奏効率ともに満足いくものではなかった．しかし，IFN-αに5-FUやシスプラチン(CDDP)などの薬剤を併用することによって，その効果を確認した報告がされつつある．Pattらは，9症例のfibrolamellar carcinomaを含む43症例に対して，IFN-αと5-FU持続静脈内投与によって，25%の奏効率を報告している[10)]．

また，肝細胞癌の血行動態の特徴である肝動脈血流支配を考慮し，薬物到達濃度を高濃度で保つことと全身の副作用を軽減する点において肝動注化学療法は，きわめて有用な治療法であると考えられる[11),12)]．さらに，肝動脈内留置カテーテルの進歩と皮下埋め込み式リザーバーの開発により，頻回の薬剤投与と持続投与が可能となり，その治療法を適応した報告は飛躍的に増加している．これら肝動注化学療法とIFN-αの併用では，奏効率30~60%と全身化学療法とIFN-αの併用よりも，比較的良好な治療成績が報告されている．Urabeらの報告[13)]では，進行肝細胞癌症例に対し，IFN-αと5-FU，CDDP，メトトレキサートの3剤の肝動注化学療法，ロイ

肝動注化学療法

肝動注化学療法とIFN-α併用

コボリンの全身投与を併用し，50%の奏効率を得ている．また，同グループのKanekoらの報告[14]では，門脈内腫瘍栓を伴った肝細胞癌29例に対して，同様のレジメンにより，45%の奏効率と良好な結果を示している．Chungらも，IFN-αとCDDPの肝動注療法との併用で33%の奏効率を報告[15]した．

われわれは，術後肝内多発再発と肺，骨の肝外転移を伴う肝細胞癌に対してIFN-α投与とテガフール・ウラシル配合剤(UFT)内服により著効(CR)を得られた症例の経験[16]から，1997年より，既存の治療法では十分な治療効果の期待できない門脈内腫瘍栓を伴った高度進行肝細胞癌症例に対して，IFN-αと5-FU持続肝動注化学療法を併用してきわめて良好な結果[6)~8)]を得ている．また骨髄抑制，発熱，うつ状態などの副作用は認められるものの，治療を中断しなければならないような重篤な副作用を伴う症例はきわめてまれであり，外来通院のみで反復治療が可能である．

II. IFN-α併用5-FU動注化学療法(FAIT)の対象および方法

本療法の対象

当科における本療法の対象は，門脈一次分枝または本幹侵襲(Vp3以上)を伴う高度進行肝細胞癌症例であり，図1に示すように3群に分類し，切除不能症例のみならず肝機能良好で肝切除可能症例においては，肉眼的治癒切除後もしくは減量肝切除後の補助療

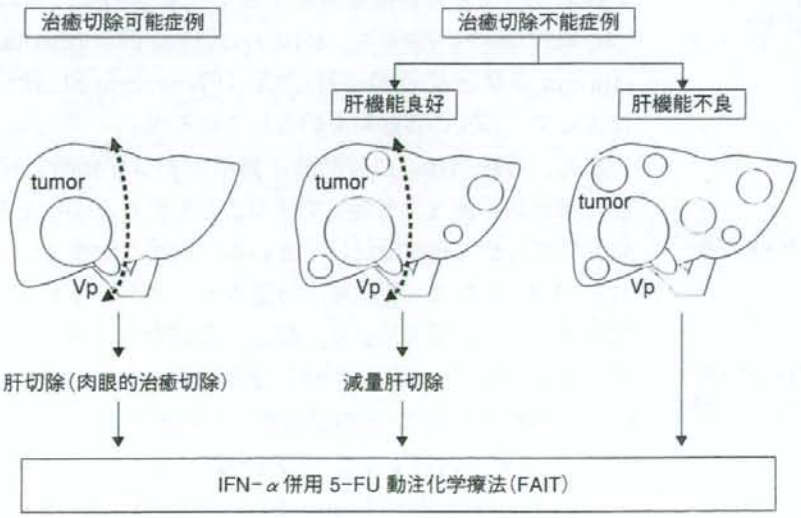

図1　当科における高度進行肝細胞癌(Vp3~4)に対する治療方針

表1　IFN-α 併用 5-FU 動注化学療法(FAIT)の適応基準

肝細胞癌	門脈内腫瘍栓	Vp 3 以上
	肝外転移	なし
年齢		70 歳未満
肝機能	AST	＜100 IU/l
	ALT	＜100 IU/l
	T-Bil	正常(閉塞性黄疸は除く)
血液検査	血小板	80,000/mm³以上
腎機能	血清 Cr	＜1.5 mg/dl
PS		0, 1

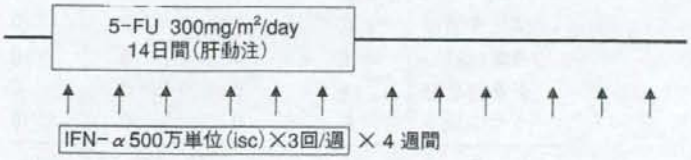

図2　IFN-α 併用 5-FU 肝動注化学療法のプロトコール

法として本療法を組み入れている.

適応は，副作用や抗癌剤投与による肝障害を考慮して，70 歳未満，総ビリルビン値が正常範囲内で，AST，ALT がともに 100 IU/l 未満，血小板 80,000/mm³以上，血清クレアチニン値が 1.5 mg/dl 以下で，外来通院が可能な performance status(PS)が 0, 1 としている(**表1**)．全肝多発病変を伴う症例や耐術が不可能と考えられる肝切除不能症例では，Seldinger 法にて肝動注カテーテルを挿入する．肝切除可能例では，術中にカテーテルを留置し，肝切除後の補助療法として本療法を施行している.

治療スケジュール(**図2**)は，皮下埋め込み式動注リザーバーより 5-FU を 300 mg/m²/day で 2 週間持続投与を行い，2 週間休薬を 1 クールとする．同時に IFN-α を 500 万単位/回，週 3 回投与，4 週間を 1 クールとして皮下投与する.

III. 切除不能症例に対する FAIT の治療成績

1997 年から現在までに，切除不能な門脈内腫瘍栓を伴う高度進行肝細胞癌症例(Vp 3 以上，IM 3)55 例に対して FAIT を施行した．治療回数は 2 クール以上で，効果の得られた症例には繰り返し治療を行った．治療効果は，CR；8 例，有効(PR)；16 例と，その

治療効果