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α-Fetoprotein Messenger RNA in the Blood Predicts Poor Prognosis of the Patients with Hepatocellular Carcinoma

Manri Kawakami, Masahiko Koda*, Yoshikazu Murawaki*, Hironaka Kawasaki* and Shiro Ikawa

*Department of Clinical Laboratory Medicine and *Second Department of Internal Medicine, Faculty of Medicine, Tottori University, Yonago 683-0826, Japan*

α-Fetoprotein (AFP) messenger RNA (mRNA) in the peripheral blood of patients with hepatocellular carcinoma (HCC) may indicate hematogenous spread of HCC. This study examined the presence of AFP mRNA in the blood of 148 patients, in terms of clinical parameters, tumor metastasis and survival rate. For the prospective study, 109 patients with HCC were followed in the period between March 1996 and March 1999. AFP mRNA in the blood was examined by means of nested reverse transcription polymerase chain reaction. AFP mRNA was detected in the blood in 23 (15.5%) of 148 patients with HCC. AFP mRNA in the blood was significantly correlated with protein induced by vitamin K absence or antagonist II level, higher AFP level (200 IU/mL or more) and extrahepatic metastases, but not with tumor size, number of tumor nodules or tumor-nodule-metastasis stage. This prospective study confirmed that intra- and extra-hepatic metastases developed more frequently in the 22 AFP mRNA-positive patients than in the 87 AFP mRNA-negative patients ($P < 0.01$). The cumulative survival rate was significantly lower in the former than in the latter ($P < 0.01$). In conclusion, AFP mRNA in the blood is closely related to hematogenous spread and might be a good predictor of metastasis and poorer survival rate in HCC patients.

Key words: α-fetoprotein messenger RNA; hepatocellular carcinoma; metastasis; tumor marker

Hepatocellular carcinoma (HCC) is a common malignancy throughout the world, especially in Asian countries. Despite advances in diagnostic tools and therapeutic options, intra- or extrahepatic tumor metastases are frequently found after surgical and medical treatments such as hepatic resection, transcatheter arterial embolization , percutaneous ethanol injection and liver transplantation (Yokoyama et al., 1990; Behghiti et al., 1991; Okuda, 1992; Nagasue et al., 1993; Adachi et al., 1995; Zhou, et al, 1996). HCC metastases include intrahepatic metastases and/or de novo tumors (Chen et al., 1989; Heu et al., 1991; Sheu et al., 1993; Nakano et al., 1994; Takenaka et al., 1994; Tarao et al., 1994; Toyosaka et al., 1996; Kumada et al., 1997; Kubo et al., 1998). Therefore, it is important to detect micrometastases in order to choose effective therapeutic approaches.

α-Fetoprotein (AFP), a serum protein produced in large amounts during fetal life, rapidly reduces from late fetal life and is essentially scarce in normal adults. The synthesis of AFP is often associated with the development of HCC and yolk sac tumors (Gitlin and Boesman, 1966; Adinolfi et al., 1975). The detection of serum AFP provides a useful marker for diagnosis and prognosis of these tumors (Adinolfi et al., 1975). However, the serum AFP level does not always correspond to the clinical stage of HCC (Okuda et al., 1975; Nomura et al., 1989; Inoue et al., 1994; Suehiro et al., 1994; Nakagawa et al., 1999). Recent molecular biological techniques have provided a method for detecting

Abbreviations: AFP, α-fetoprotein; bp, base pair; CT, computed tomography; HCC, hepatocellular carcinoma; mRNA, messenger RNA; PCR, polymerase chain reaction; PIVKA-II, protein induced by vitamin K absence or antagonist II; RT-PCR, reverse transcription-PCR; TNM, tumor-nodule-metastasis

Table 1. Profiles of HCC patients

Number of patients	148	
Age (year)	66 ± 9	
Gender (male/female)	94/54	
Etiology of liver disease		
HBV-related	33	
HCV-related	96	
HBV- and HCV-related	4	
Alcoholic	5	
Cryptogenic	10	
Underlying liver disease		
Chronic hepatitis	19	
Cirrhosis		
Child-Pugh grade A	67	
Child-Pugh grade B	36	
Child-Pugh grade C	26	
TNM stage*		
I	10	
II	30	
III	27	
IVA	54	
IVB	27	
Tumor differentiation†		
Well	40	(34.5%)
Moderate	65	(56.0%)
Poor	11	(9.5%)
Serum AFP (ng/mL)		
Median	159	
Range	1 – 332,500	
Serum PIVKA-II (m*A*U/mL)‡		
Median	80	
Range	4 – 1,270,000	

AFP, α-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; TNM, tumor-nodule-metastasis.

* Clinical staging of the tumor was assessed according to the TNM system of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan on the basis of imaging studies.

† Tumor biopsies were performed in 116 lesions and classified according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan.

‡ Serum PIVKA-II levels were measured in 126 patients.

malignant cells in the peripheral blood by amplification of messenger RNA (mRNA) of various genes specific to a particular cell type from peripheral blood mononuclear cells (Smith et al., 1991; Mattano et al., 1992; Moreno et al., 1992; Tada et al., 1993; Brandt et al., 1996; Krüger et al., 1996; Nomoto et al., 1996; Ishikawa et al., 1998). AFP mRNA has been demonstrated to be one of the candidate molecules for detecting HCC cells in the blood (Matsumura et al., 1994, 1995, 1999; Komeda et al., 1995; Jiang et al., 1997; Lemoine et al., 1997; Louha et al., 1997; Nambu et al., 1997; Omichi-Funaki et al., 1997, 1998; Wong et al., 1997; ; Luo et al., 1999; Okuda et al., 1999). With this background, this study examined AFP mRNA in patients with HCC using nested reverse transcription polymerase chain reaction (RT-PCR), in terms of clinical parameters, tumor metastasis and survival rate.

Subjects and Methods

Patients and cell line

All patients were admitted to Tottori University Hospital between March 1996 and March 1999. Samples were obtained from the 148 patients with HCC. Informed consent was obtained from each patient or from family members if the patient could not make a decision. Table 1 summarized the patients' profiles. In 116 of 148 patients, HCC was histologically classified according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan (Liver Cancer Study Group of Japan, 1989) by tumor biopsies under the guidance of ultrasonography. The remaining patients were diagnosed by several imaging modalities such as ultrasonography, computed tomography (CT), magnetic resonance imaging or angiography. On the basis of these findings, the tumors were classified according to the tumor-nodule-metastasis (TNM) classification of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan (Liver Cancer Study Group of Japan, 1989) when the sample was obtained. Extrahepatic metastases were

detected by means of interviews, physical examinations and imaging techniques such as chest X-ray, scintigraphy or CT. The mean diameters of the main tumors were 1.7 ± 0.4 cm in stage I, 2.5 ± 1.3 cm in stage II, 3.7 ± 1.6 cm in stage III, 3.3 ± 1.8 cm in stage IVA and 4.6 ± 4.1 cm in stage IVB. No HCC patients underwent treatment in the 2 months before the blood samples were obtained.

Samples were also obtained from healthy volunteers, who agreed to donate blood. A human hepatic carcinoma cell line, HepG2 cells known to produce AFP, was kindly supplied by Prof. Kenzo Sato of the Dept. of Molecular Biochemistry, School of Life Sciences, Faculty of Medicine, Tottori University. For the prospective study, 109 patients (72 males and 37 females) with a median age of 66 ± 9 years and a range of 34–80 years were enrolled. Four (3.7%) of 109 patients underwent hepatic resection, 71 (65.1%) were treated medically by transcatheter arterial embolization and/or percutaneous ethanol injection and 33 (30.3%) remained untreated because of advanced liver dysfunction, advanced liver cancer or the decision of each patient. These patients were examined every 1 or 2 months at our outpatient clinic for the detection of metastasis by the measurement of tumor markers, AFP and protein induced by vitamin K absence or antagonist II (PIVKA-II), every 1 or 2 months, ultrasonography every 3 months and CT every 6 months. The patients who developed a diffuse, infiltrative HCC were not treated. The patients were followed up for a mean period of 16.2 ± 11.2 months.

Extraction of RNA

Five milliliters of heparinized blood were collected from each patient. RNA was extracted from blood nuclear cells and HepG2 cells. Peripheral mononuclear cells that may have contained tumor cells were separated by discontinuous gradient centrifugation using a Ficoll-Paque (Amersham-Pharmacia-Biotech, Buckinghamshire, United Kingdom). Total RNA from mononuclear cells was extracted with an RN easy Mini Kit (QIAGEN, Hilden, Germany), which was based on the acid-guanidium phenol-chloroform method and stored at –80°C until use.

Synthesis of complementary DNA (cDNA)

One microgram of RNA was heated at 65°C for 10 min and cooled rapidly on ice, diluted to 13-mL buffer with 45-mmol/L Tris (pH 8.3), 68-mmol/L KCl, 9-mmol/L $MgCl_2$, 15-mmol/L 1,4-dithiorethreitol, 0.2-mg random hexadeoxynucleotides, RNA guard (porcine), 0.8-μg/mL RNase/DNase-free bovine serum albumin, 1.8-mmol/L each dNTP, and Moloney Murine Leukemia Virus reverse transcriptase (First-Strand cDNA synthesis kit, Amersham-Pharmacia-Biotech). cDNA was synthesized by means of incubation at 37°C for 60 min. It was then heated at 94 °C for 5 min for inactivation of reverse transcriptase, cooled rapidly and stored at –20°C until use for the 1st PCR.

Sequence of primers used in nested PCR

The sequences of primers used in the experiment were as follows. The primers for the AFP gene were i) 5'-ACA GCA GCC ACT TGT TGC CAA-3' (nucleotides 1413 to 1433) and ii) 5'-CTC TTC AGC AAA GCA GAC TTC-3' (nucleotides 1809 to 1829) for the outer primers; and iii) 5'-GCT GAC ATT ATT ATC GGA CAC-3' (nucleotides 1473 to 1493) and iv) 5'-AGC CTC AAG TTG TTC CTC TGT-3' (nucleotides 1734 to 1754) for the inner primers.

The PCR amplification of the β-globin gene was done to confirm the successful extraction of mRNA from blood nuclear cells and HepG2 cells. The primer sequences for the β-globin gene were v) 5'-TGG TCT CCT TAA ACC TGT CTT G-3' and vi) 5'-ACA CAA CTG TGT TCA CTA GC-3' for the outer primers; and vii) 5'-GTC TCC TTA AAC CTG TCT TG-3' and viii) 5'- ACA ACT GTG TTC ACT AGC-3' for the inner primers. The sense and antisense primers described above were selected from different exons to distinguish amplification of RNA from contamination of DNA (Saiki et al., 1988).

Nested RT-PCR

One microliter of cDNA solution was mixed with 9 μL of the PCR reaction mixture containing 20-mmol/L Tris-HCl (pH 8.0), 100-mmol/L KCl, 0.1-mmol/L EDTA, 0.5% Tween 20, 1-mmol/L 1,4-dithiothreitol, 50% glycerol, 15-mmol/L Mg^{2+}, 1 mmol of each primer (Nos. i, ii, v and vi) and 0.25-unit Taq polymerase (Gene Taq; Wako Pure Chemical Ind., Osaka, Japan). The reaction mixture was overlaid with mineral oil (Perkin Elmer, Foster City, CA) and heated at 95°C for 5 min. It was subjected to a total of 35 cycles of heat at 94°C for 30 s, 54°C for 30 s and 72°C for 1 min with a thermal cycler (Gene Amp PCR System 9600-R, Perkin-Elmer). The reaction terminated by heating at 72°C for 7 min and cooling at 4°C. One microliter of the amplified product was then used in the 2nd PCR.

One microliter of the amplified sample was mixed with the same buffer as in the 1st PCR for the 2nd PCR, except that the primers were inner primers (Nos. iii, iv, vii and viii). The amplification program was also the same as for the 1st PCR. Three microliters of the above secondary amplified products were electrophoresed on a 1.8% agarose gel containing ethidium bromide and photographed on a UV transilluminator (ATTO densitograph, ATTO Corporation, Tokyo, Japan). The amplified products of AFP and β-globin were 240 and 167 base pair (bp), respectively.

Assay for AFP and PIVKA-II

Serum AFP and PIVKA-II concentrations were measured with a chemiluminescence assay (Wako Pure Chemical) and an electrochemiluminescence assay (Eizai Pharmaceutical Co., Tokyo), respectively.

Statistics

Statistical analysis was performed with the Mann-Whitney *U* test. The χ^2 test was used for statistical analysis between group frequencies. The cumulative overall and metastasis-free survival rates were calculated by the Kaplan-Meier method. Survival curves were compared with a generalized Wilcoxon test. A *P* value less than 0.05 was considered significant.

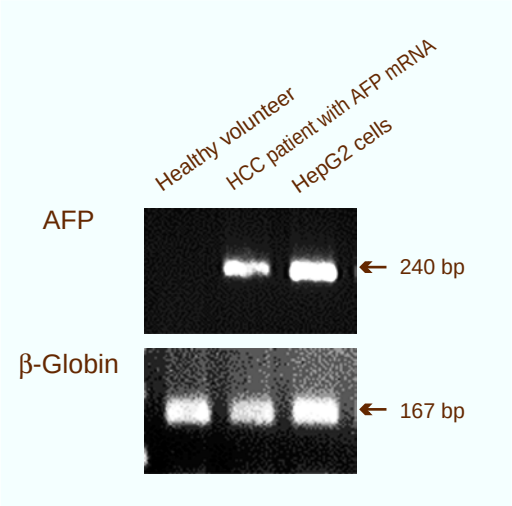

Fig. 1. Nested reverse transcription-polymerase chain reaction of α-fetoprotein (AFP) messenger RNA (mRNA). bp, base pair; HCC, hepatocellular carcinoma.

Results

A specific band for the amplified AFP gene (240 bp) was noted in the samples obtained from HepG2 cells and an HCC patient with AFP mRNA, but not in the sample from a healthy volunteer (Fig. 1).

AFP mRNA in the peripheral blood was detected in 23 (15.5%) of 148 patients with HCC. The frequency of positive patients in each TNM stage was as follows: 1 of 10 patients (10.0%) in stage I, 4 of 30 patients (13.3%) in stage II, 4 of 27 patients (14.8%) in stage III, 7 of 54 patients (13.0%) in stage IVA and 7 of 27 patients (25.9%) in stage IVB (Table 2). Although the frequency tended to be higher in stage IVB, there was no statistical significance.

HCC patients with AFP mRNA in the peripheral blood were analyzed in relation to tumor size, number of tumor nodules, the presence of portal vein thrombosis, portocaval shunt, intrahepatic metastasis, extrahepatic

Table 2. AFP mRNA in the blood of 148 HCC patients

		Number of patients	AFP mRNA in blood			*P* value
			Positive [23]	Negative [125]	Positive rate (%)	
Tumor diameter	≥ 5 cm	26	5	21	19.2	NS
	< 5 cm	122	18	104	14.8	
Number of tumors	≥ 3	90	17	73	18.9	NS
	< 3	58	6	52	10.3	
AFP	≥ 200 ng/mL	64	15	49	23.4	< 0.05
	< 200 ng/mL	84	8	76	9.5	
PIVKA-II*	≥ 40 mAU/mL	72	10	62	13.9	NS
	< 40 mAU/mL	54	8	46	14.8	
TNM stage†	I	10	1	9	10.0	
	II	30	4	26	13.3	NS
	III	27	4	23	14.8	
	IVA	54	7	47	13.0	
	IVB	27	7	20	25.9	NS
Portal thrombosis	Present	27	7	20	25.9	
	Absent	121	16	105	13.2	
Portocaval shunt	Present	37	3	34	8.1	NS
	Absent	111	20	91	18.0	
Extrahepatic metastasis	Present	27	8	19	29.6	< 0.05
	Absent	121	15	106	12.4	

[ ], number of patients.
AFP, α-fetoprotein; HCC, hepatocellular carcinoma; mRNA, messenger RNA; NS, not significant; PIVKA-II, protein induced by vitamin K absence or antagonist II; TNM, tumor-nodule-metastasis.
* Serum PIVKA-II levels were measured in 126 patients.
† Clinical staging of the tumor was assessed according to TNM system of the General Rules for Clinical and Pathological Study of Primary Liver Cancer in Japan on the basis of imaging studies.

metastasis and serum tumor markers (AFP and PIVKA-II) (Tables 2 and 3). The largest tumor was described in cases with two or more tumors in the liver. Tumor diameter was not significantly different between the HCC patients with AFP mRNA in the blood and those without AFP mRNA. The incidence of AFP mRNA in the blood was not correlated with tumor size.

The number of tumor nodules in the liver was not significantly different between the HCC patients with AFP mRNA in the blood and those without AFP mRNA. The incidence of AFP mRNA in the blood was not correlated with the number of tumor nodules (Table 2).

Serum AFP concentration was higher in the HCC patients with AFP mRNA (median: 1209 ng/mL, range: 9–319,900 ng/mL) than in the patients without AFP mRNA (median: 125 ng/mL, range: 1–332,500 ng/mL), the value being not statistically significant (Fig. 2, Table 3). When the patients were divided into 2 groups according to serum AFP concentration, the inci-

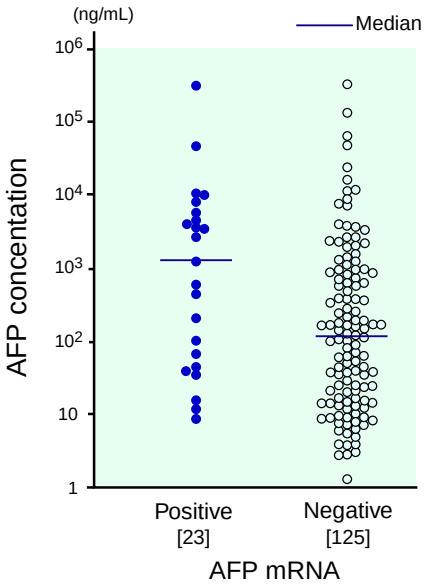

Fig. 2. Serum α-fetoprotein (AFP) concentrations in 23 patients with AFP messenger RNA (mRNA) in the blood (median: 1209 ng/mL, range; 9–319,900 ng/mL) and 125 patients without AFP mRNA (median: 125 ng/mL, range; 1–332,500 ng/mL). [ ], number of patients.

Table 3. Profiles of 148 patients with or without AFP mRNA

		AFP mRNA in blood		*P* value
		Positive	Negative	
Number of patients		23	125	
Age (year)		65.7 ± 8.8	66.1 ± 8.6	NS
Gender (male/female)		14/9	80/45	NS
Etiology of liver disease	HBV-related	7	26	
	HCV-related	15	81	
	HBV- and HCV-related	0	4	NS
	Alcoholic	0	5	
	Cryptogenic	1	9	
Underlying liver disease	Chronic hepatitis	4	15	
	Cirrhosis			
	Child-Pugh grade A	6	61	< 0.05
	Child-Pugh grade B	4	32	
	Child-Pugh grade C	9	17	
Tumor diameter (cm)		3.3 ± 2.9	3.3 ± 2.3	NS
Number of tumors		8.9 ± 8.4	7.2 ± 7.8	NS
TNM stage*	I, II	5	35	NS
	III , IV	18	90	
Tumor differentiation†	Well	8 (40.0%)	32 (33.3%)	NS
	Moderate or poor	12 (60.0%)	64 (66.7%)	
Serum AFP (ng/mL)	Median	1209	125	NS
	Range	9 – 319,900	1 – 332,500	
Serum PIVKA-II (m*A*U/mL)‡	Median	151	73.5	< 0.05
	Range	10 – 1,270,000	4 – 409,000	

AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus; mRNA, messenger RNA; NS, not significant; PIVKA-II, protein induced by vitamin K absence or antagonist II; TNM, tumor-nodule-metastasis.

* Clinical staging of the tumor was assessed according to the TNM system of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan on the basis of imaging studies.

† Tumor biopsies were performed in 116 lesions (20 cases in the AFP mRNA-positive group and 96 cases in the AFP mRNA-negative group) and classified according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan.

‡ Serum PIVKA-II was measured in 126 patients (18 in the AFP mRNA-positive group and 108 in the AFP mRNA-negative group).

dence of AFP mRNA in the blood was significantly higher in the patients with 200 ng/mL or more (23.4%) than those with less than 200 ng/mL (9.5%; $P < 0.05$; Table 2). PIVKA-II concentration in the serum was significantly higher in HCC patients with AFP mRNA in the blood (76,443 ± 298,178 m*A*U/mL) than in the patients without AFP mRNA (12,209 ± 50,415 m*A*U/mL; $P < 0.05$). However, when the patients were divided into 2 groups according to PIVKA-II concentration, the incidence of AFP mRNA was not different between the patients with PIVKA-II concentrations of 40 m*A*U/mL or more and those with less than 40 m*A*U/mL (Table 2).

The presence of portal thrombosis was found in 7 (30.4%) of the 23 patients with AFP mRNA in the blood and in 20 (16.0%) of the 125 patients without AFP mRNA (Table 2). The presence of a portocaval shunt was found in 3 (13.0%) of the 23 patients with AFP mRNA in the blood and in 34 (27.2%) of the 125 patients without AFP mRNA (Table 2). Thus, AFP mRNA in the blood did not correlate with portal thrombosis or a portocaval shunt.

Extrahepatic metastases were noted in 8 (34.8%) of the 23 patients with AFP mRNA in the blood and 19 (15.2%) of the 125 patients without AFP mRNA, the value being significantly higher in the former than in the latter ($P < 0.05$; χ^2 test). When the patients were classified according to the presence or absence of extrahepatic metastases, AFP mRNA in the blood was detected in 8 (29.6%) of the 27 patients with extrahepatic metastases and in 15 (12.4%)

of the 121 patients without extrahepatic metastases, the value being statistically significant ($P < 0.05$; Table 2).

Aspartate aminotransferase and alanine aminotransferase concentrations in the serum were significantly higher in the 23 HCC patients with AFP mRNA in the blood (aspartate aminotransferase; 203 ± 297 IU/L, alanine aminotransferase; 113 ± 134 IU/L) than in the 125 patients without AFP mRNA (104 ± 116, $P < 0.01$,72 ± 61 IU/L, $P < 0.05$).

Table 4 summarizes the profiles of 109 patients undergoing follow-up studies which disclosed the cause of death in 51 patients (46.8%); 35 died from HCC progression, 9 from hepatic failure, 3 from gastrointestinal bleeding and 4 from other diseases. AFP mRNA was noted in 8, 4, 0 and 0 patients, respectively.

Extrahepatic metastasis was observed in 13 (11.9 %) patients (7 in the AFP mRNA-positive and 6 in the AFP mRNA-negative group). Tumor metastases were found in the lung in 6 patients, the bone in 3, the adrenal glands in 4, the spleen in 1 and the pharynx in 1.

Next, the following parameters were analyzed: the cumulative rate of the extrahepatic metastasis, the cumulative rate of intra- and extra-hepatic metastases and the cumulative survival rate for each AFP mRNA status. The cumulative rate of extrahepatic metastasis was similar between the 2 groups (data not shown). However, the cumulative rate of intra- and

Table 4. Profiles of the 109 HCC patients with or without AFP mRNA

		AFP mRNA in blood		*P* value
		Positive	Negative	
Number of patients		22	87	
Age (year)		65.4 ± 8.9	65.6 ± 8.9	NS
Gender (male/female)		13/9	59/28	NS
Etiology of liver disease	HBV-related	7	18	
	HCV-related	14	54	
	HBV- and HCV-related	0	3	NS
	Alcoholic	0	4	
	Cryptogenic	1	8	
Underlying liver disease	Chronic hepatitis	3	12	
	Cirrhosis			
	Child-Pugh grade A	6	48	< 0.05
	Child-Pugh grade B	4	21	
	Child-Pugh grade C	9	6	
Tumor diameter (cm)		3.3 ± 3.0	3.5 ± 2.5	NS
Number of tumors		9.0 ± 8.6	6.1 ± 7.3	NS
TNM stage*	I, II	5	28	NS
	III , IV	17	59	
Tumor differentiation†	Well	7 (36.8%)	23 (36.5%)	NS
	Moderate or poor	12 (63.2%)	40 (63.5%)	
Serum AFP (ng/mL)	Median	1951	63	NS
	Range	9 – 319,900	1 – 332,500	
Serum PIVKA-II (m*A*U/mL)‡	Median	101	30	NS
	Range	10 – 1,270,000	4 – 409,000	

AFP, α-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; mRNA, messenger RNA; NS, not significant; PIVKA-II, protein induced by vitamin K absence or antagonist II; TNM, tumor-nodule-metastasis.

* Clinical staging of the tumor was assessed according to the TNM system of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan on the basis of imaging studies.

† Tumor biopsies were performed in 82 lesions (19 cases in the AFP mRNA-positive group and 63 cases in the AFP mRNA-negative group) and classified according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan.

‡ Serum PIVKA-II was measured in 94 patients (17 in the AFP mRNA-positive group and 77 in the AFP mRNA-negative group).

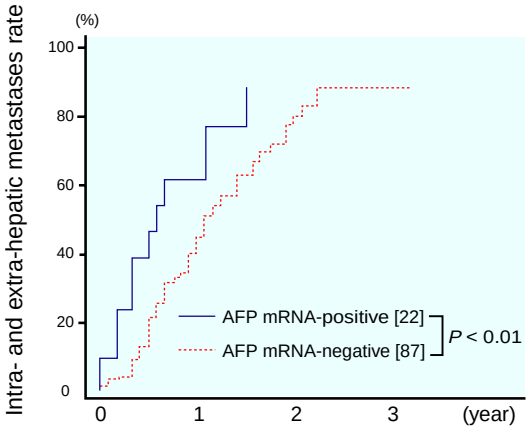

Fig. 3. Rates of intra- and extra-hepatic metastases calculated by the Kaplan-Meier method for 22 patients with positive α-fetoprotein (AFP) messenger RNA (mRNA) and 87 patients with negative AFP mRNA. The difference is statistically significant ($P < 0.01$). [ ], number of patients.

extra-hepatic metastases was significantly higher in the 22 AFP mRNA-positive patients than that in the 87 AFP mRNA-negative patients ($P < 0.01$; Fig. 3). The cumulative survival rate was significantly lower in the 22 AFP mRNA-positive group than in the 87 AFP mRNA-negative group ($P < 0.01$; Fig. 4).

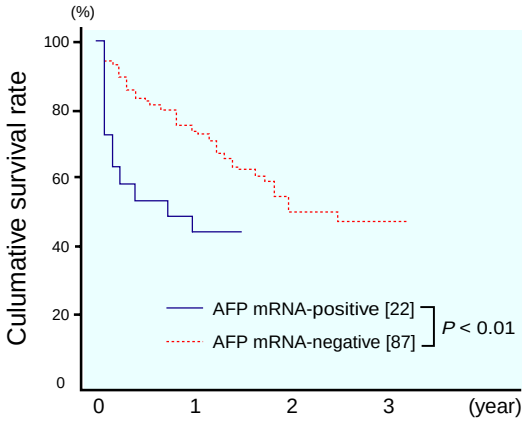

Fig. 4. Cumulative survival curve calculated by the Kaplan-Meier method for 22 patients with positive α-fetoprotein (AFP) messenger RNA (mRNA) and 87 patients with negative AFP mRNA. The difference is statistically significant ($P < 0.01$). [ ], number of patients.

Discussion

In this study, AFP mRNA in the blood was detected in the 23 (15.5%) of the total 148 HCC patients. Previous studies showed the positive rate of AFP mRNA in the peripheral blood to be from 25 to 95% of HCC patients (Matsumura et al., 1994, 1999; Komeda et al., 1995; Jiang et al., 1997; Lemoine et al., 1997; Louha et al., 1997; Nambu et al., 1997; Luo et al., 1999; Okuda et al., 1999). The lower frequency in the present study might be partly due to the selection of patients, in whom extrahepatic metastases were noted only in 27 patients. Furthermore, there were more patients with AFP non-producing HCC in the present study than in the previous reports **(**Matsumura et al., 1994; Jiang et al., 1997**)**. The next possible reason is the difference in the timing of the examination of blood samples. Louha et al. (1997) reported that AFP mRNA producing cells in the blood were detected after locoregional therapy such as transcatheter arterial embolization or percutaneous ethanol injection. Matsumura et al. (1994) and Okuda et al. (1999) also showed that tumor cells do not always circulate in the blood, but may appear intermittently. We analyzed the blood sample before therapy or at least 2 months after therapy.

Next, we investigated the relationship between AFP mRNA in the blood and the clinical parameters of HCC. The AFP mRNA in the blood did not correlate with tumor size, number of tumor nodules, presence of portal thrombosis and portocaval shunt, or TNM stage. Clinical significance of AFP mRNA in the blood is controversial even now (Matsumura et al., 1994, 1999; Komeda et al., 1995; Lemoine et al., 1997; Louha et al., 1997; Nambu et al., 1997; Luo et al., 1999). What is interesting is that this study confirmed a significant correlation between serum aspartate aminotransferase or alanine aminotransferase concentration and AFP mRNA in the blood. The present study also demonstrated that the HCC patients with high levels of serum AFP showed higher detectable rates of AFP mRNA in the blood than those with low levels of serum AFP. The frequency of AFP mRNA

in the blood, however, was not so much higher in the HCC patients with a high AFP level (23.4%). This result suggests that the serum AFP level does not necessarily reflect the presence of circulating cells with AFP mRNA. In other words, the serum AFP level is not a useful test to indicate the presence of circulating HCC cells. Nambu et al. (1997) and Luo et al. (1999) showed that the PIVKA-II value may be a good indicator of AFP mRNA in the blood. The positive rate of AFP mRNA in the blood was not different between PIVKA-II-positive patients (40 m*A*U/mL and more) and -negative patients (less than 40 m*A*U/mL) in this study, although HCC patients with AFP mRNA-positive in the blood had higher PIVKA-II values than those with lower PIVKA-II. It is conceivable that the positivity of AFP mRNA did not correlate with the serum PIVKA-II value, because PIVKA-II-positive and AFP-negative HCCs are commonly experienced and the nested RT-PCR in the present study does not detect AFP non-producing cells.

In agreement with some previous research (Matsumura et al., 1994, 1999; Komeda et al., 1995; Louha et al., 1997), we found that the positivity of AFP mRNA in the blood was associated with extrahepatic metastasis. The detection of AFP mRNA was clearly more frequent in the patients with extrahepatic metastasis than those without. This suggests that circulating AFP producing cells might be responsible for extrahepatic metastasis.

The present study compared the survival rate and intra- and extra-hepatic metastases, in which there were no differences in serum AFP levels, PIVKA-II levels or TNM stages. Matsumura et al. (1999) reported that the incidence of extrahepatic metastasis was significantly higher in the AFP mRNA-positive group than that in the AFP mRNA-negative group. However, the extrahepatic metastasis was not significantly different between the 2 groups in the present study. The cumulative rate of intra- and extrahepatic metastases was significantly higher in the patients positive with AFP mRNA in the blood than in the patients without. This result may indicate that tumor cells in the systemic circulation are responsible not only for extrahepatic metastasis but also intrahepatic metastasis, which have been considered to be caused mainly by the spreading of cancer cells via the portal vein (Toyosaka et al., 1996). Funaki et al. (1997) reported that cancer cells released from the primary tumor to the systemic circulation might participate in intrahepatic metastasis. Since the liver provides an essentially favorable environment for HCC cells, the incidence of intrahepatic metastasis through the systemic circulation might be more frequent than previously considered. Therefore, it is reasonable to assume that HCC patients who undergo liver transplantation do not always have good prognoses and often experience metastases in the transplanted livers (Zhou et al., 1996).

In the present follow-up study, we found that the cumulative survival rate was significantly poorer in the AFP mRNA-positive group than that in the AFP mRNA-negative group. Matsumura et al. (1999) found that overall survival was similar between patients in AFP mRNA-positive and -negative groups, but the survival rates in continuous AFP mRNA-positive patients was poorer than that in continuous AFP mRNA-negative patients. This result shows that the presence of AFP mRNA cells in the blood might be a predictive factor in HCC patients. Since the present AFP mRNA-positive group had more severe underlying liver disease than the AFP mRNA-negative group, this might affect the survival rate. However, it is indisputable that the high frequency of intra- and extra-hepatic metastases in the AFP mRNA-positive group was responsible for their poor prognoses.

In conclusion, AFP mRNA in the blood of HCC patients related to PIVKA-II levels and was significantly detectable in patients with an AFP level of 200 IU/mL or more, or with extrahepatic metastases. Thus, the detection of AFP mRNA in the blood might be a predictor for tumor metastasis and survival of HCC patients.

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