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ORIGINAL ARTICLE

Detection of AFP mRNA-expressing cells in the peripheral blood for prediction of HCC recurrence after living donor liver transplantation

Shigeru Marubashi, Keizo Dono, Hiroaki Nagano, Yukari Sugita, Tadafumi Asaoka, Naoki Hama, Atsushi Miyamoto, Yutaka Takeda, Koji Umeshita and Morito Monden

Department of Surgery, Osaka University Graduate School of Medicine Suita, Osaka, Japan

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Correspondence
K. Dono MD, PhD, Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. Tel.: +81 6 6879 3251; fax: +81 6 6879 3259; e-mail: kdono@surg2.med.osaka-u.ac.jp

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Summary

The aim of this study was to evaluate the hypothesis that the detection of alpha-fetoprotein (AFP) mRNA-expressing cells could be a novel, direct and accurate method for predicting tumor recurrence after living donor liver transplantation (LDLT) in patients with hepatocellular carcinoma (HCC). The test group consisted of 32 patients who underwent LDLT for end-stage liver disease with HCC. Quantitative real-time reverse transcription polymerase chain reaction was used for the detection of AFP mRNA-expressing cells in the peripheral blood. Nine (28.1%) of the 32 patients developed tumor recurrences during the follow-up period (mean, 27.9 months). The test for the presence of AFP mRNA in the peripheral blood was positive either preoperatively or postoperatively in 11 (34.3%) of the 32 patients, and positive preoperatively in three patients (9.4%). Univariate analysis revealed that a positive preoperative test for peripheral blood AFP mRNA, as well as exceeding Milan criteria and microscopic evidence of vascular invasion were significant predictors for the recurrence of HCC ($P = 0.002$, 0.049, and 0.001, respectively). Multivariate analysis using Cox's proportional hazards model revealed that a positive preoperative test for peripheral blood AFP mRNA was an independent risk factor for the recurrence of HCC. We concluded that the presence of AFP mRNA-expressing cells preoperatively could be a useful predictor of the recurrence of HCC in liver transplant patients.

Introduction

Liver transplantation is the only radically curative treatment for hepatocellular carcinoma (HCC), especially in patients with viral hepatitis and cirrhosis. However, recurrence of HCC after liver transplantation poses a major problem. The risk factors for tumor recurrence after liver transplantation have been reported by many studies, and include large tumor size, bilobar tumor spread, vascular invasion, poor differentiation of HCC, and the serum alpha-fetoprotein (AFP) level [1–3]. The finding that HCC can recur even in the graft liver as well as distant sites after apparently complete surgical removal of the tumor, suggests that circulating cancer cells might be responsible for the tumor recurrence in patients of HCC. A peripheral blood test for AFP mRNA is often used to detect the presence of circulating cancer cells. Ijich *et al.* [4] used a nested-polymerase chain reaction (PCR) technique and reported that the presence of AFP mRNA-expressing cells in the peripheral blood might be a useful predictor of postoperative recurrence of HCC.

We previously reported the usefulness of quantitative reverse transcription (RT)-PCR for detecting AFP mRNA-expressing cells in the peripheral blood [5]. Subsequently, we also reported that it might be possible to predict the recurrence of HCC after surgical resection by perioperative

testing of the peripheral blood for the presence of AFP mRNA-expressing cells [6]. The present study, an extension of the above studies, was designed to assess the value of peripheral blood testing for AFP mRNA for predicting HCC recurrence after living donor liver transplantation (LDLT). We used real-time RT-PCR, as described in our previous study, to quantitate AFP mRNA in the peripheral blood of patients who were scheduled to undergo LDLT for end-stage liver disease (ESLD) with HCC.

Patients and methods

Among 77 consecutive adult patients who underwent LDLT at our institution between 1998 and December 2006, all 32 adult patients who had HCC prior to the LDLT were enrolled in this prospective study. As a control group, we also examined 48 live donors and 16 patients without HCC who underwent LDLT for ESLD during the same period. Standard post-LDLT immunosuppression consisted of a calcineurin inhibitor, mycophenolate mofetil, and a steroid. The latter two were tapered and discontinued by 3 months after the liver transplantation.

The criteria for LDLT for the patients with HCC at our institution were: patients with ESLD, and absence of extrahepatic malignancies, macroscopic portal venous involvement, or extrahepatic metastasis of HCC. The number and size of the HCC tumors are not considered to be relevant criteria for judging the suitability for LDLT. One patient had liver failure and HCC with a tumor thrombus in the portal vein which was a contraindication to liver transplantation. However, this patient and his spouse had a strong desire for LDLT despite the extremely poor prognosis. This case was discussed by the institutional ethics committee, which then approved of LDLT for this patient.

The patient demographic and operative data, the tumor characteristics, postoperative biweekly serum AFP, serum levels of protein induced by vitamin K antagonist II (PIVKA-II), computed tomographic (CT) scans of the abdomen and chest at 3, 6, 12, 18 and 24 months after the surgery and annually thereafter, and bone scintigraphy annually after the surgery were prospectively collected. The size and number of HCC tumors, presence/absence of vascular invasion, and the grade of tumor differentiation were confirmed histopathologically after the LDLT.

Hepatocellular carcinoma recurrence was confirmed by clinical examination, laboratory and radiological tests, and histopathological examination of the resected specimens.

Real-time PCR for detecting AFP mRNA in the peripheral blood

Peripheral blood (16 ml) samples were obtained with the informed consent of the study participants for analysis of AFP mRNA at the following time-points: pretransplant within 3 days prior to the surgery, intraoperatively during the anhepatic period, and postoperatively immediately after the surgery. Peripheral blood samples were also obtained at the time of HCC recurrence in patients with HCC recurrence. In the control live donor group, peripheral blood (16 ml) samples were obtained with the informed consent of the subjects before and immediately after the surgery.

The method used for the detection of AFP mRNA in the peripheral blood has been described previously [5]. 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].

Definition of the preoperative and postoperative AFP mRNA status

We defined AFP mRNA-positive patients in two ways. *Postoperative* positivity for AFP mRNA was defined as peripheral blood positivity for AFP mRNA either during the anhepatic period or immediately postoperatively. Negativity for postoperative AFP mRNA was defined as the absence of detectable AFP mRNA in the peripheral blood during both the anhepatic and the immediately postoperative periods. *Preoperative* positivity for AFP mRNA was defined as peripheral blood positivity for AFP mRNA preoperatively, regardless of the AFP mRNA status during the anhepatic and immediate postoperative periods.

Statistical analysis

The cumulative risk of HCC recurrence and the 95% CIs were estimated by the Kaplan–Meier analysis. Univariate and multivariate risk factor assessment was performed using the Kaplan–Meier method (log-rank test) and Cox's proportional hazards model. Variables that showed associations with the risk of HCC recurrence in the univariate analysis ($P < 0.15$) and the preoperative AFP mRNA status were included for the multivariate analysis. *P*-values of <0.05 were considered to be significant.

Results

The 32 liver recipients with HCC comprised 24 males and eight females. The primary diagnoses were HCV cirrhosis ($n = 17$), HBV cirrhosis ($n = 10$), Laennec's ($n = 3$), autoimmune hepatitis ($n = 1$), and the Budd-Chiari syndrome ($n = 1$). The mean follow-up duration was 27.9 months (0.9–65.1). The HCCs in 14 patients satisfied

the Milan criteria, whereas they exceeded the Milan criteria in 18 patients based on the size and number of tumors (17 patients) or the presence of macroscopic vascular invasion of the portal vein (one patient). Twenty-one patients (65.6%) had received treatment for HCC, such as RFA or TACE, from 7 days to 39 months (median 6 months) prior to the liver transplantation, while the remaining 11 patients had not received any treatment. The preoperative model for the end-stage liver disease (MELD) score ranged from 7 to 33 (median, 14), and the Cancer of the Liver Italian Program (CLIP) score ranged from 1 to 4 (median, 2). HCC recurred in nine patients (28.8%) at 5.3–21.0 months (median 12.2 months) after LDLT, and the sites of recurrence included the lung ($n = 4$), liver ($n = 1$), peritoneum ($n = 1$), bone ($n = 2$), and adrenal gland ($n = 1$). The case with peritoneal recurrence underwent surgical removal 13 months after the LDLT and was still alive at 39 months after the LDLT. Successful resection of the recurrent tumor was conducted in two of the four patients with lung metastases. The patient with multiple bone metastases died of HCC 2 years and 6 months after the LDLT (Table 1).

Alpha-fetoprotein mRNA was positive either preoperatively or postoperatively in 11 patients (34.3%), while the anhepatic AFP mRNA status could not be determined in two patients because of inadequate blood samples (Table 2). The test for AFP mRNA was positive preoperatively in three patients (9.4%), and postoperatively in 10 patients (31.3%) (Table 2). In comparison, none of the 48 live donors and 16 liver transplant recipients without HCC had AFP mRNA-expressing cells in the peripheral blood preoperatively ($P = 0.017$), and two (4.2%) of the 48 live donors and five (31.3%) of the 16 liver transplant recipients without HCC had AFP mRNA-expressing cells in the peripheral blood postoperatively ($P = 0.005$, 0.999, respectively).

All the three patients with preoperative AFP mRNA positivity showed HCC recurrence postoperatively, while three (50%) of the six patients with positive AFP mRNA during the anhepatic period and two (29%) of the seven patients with positive AFP mRNA in the immediate operative period showed HCC recurrence. AFP mRNA at the time of HCC recurrence was positive in one patient, while it was negative in five patients with HCC recurrence (Table 2).

Table 1. Patients' characteristics (demography and HCC profiles, $n = 32$).

Recipient age	54.5 ± 8.1
Recipient gender (M/F)	24/8
Model for end-stage liver disease score	16.6 ± 7.3
Cause of disease	
HCV	17 (53.1%)
HBV	10 (31.3%)
Laennec's	3 (9.4%)
Autoimmune	1 (3.1%)
Budd-Chiari syndrome	1 (3.1%)
Donor age	35.1 ± 12.9
Donor gender (M/F)	25/7
Graft-to-recipient standard liver volume ratio (%)	48.3 ± 9.5
Type of graft (right/left/posterior)	20/7/5
Immunosuppression (tacrolimus/cyclosporine)	21/11
HCC profiles	
Milan criteria	
Within	14 (44%)
Beyond	18 (56%)
Cancer of the Liver Italian Program score	
1	9 (28%)
2	11 (34%)
3	10 (31%)
4	2 (6%)
Size (cm)	3.0 (0.7–7.5)
Number	6.3 (1–22)
Vascular invasion	
Negative	27 (84%)
Positive	5 (16%)
Differentiation	
Well	1 (3.1%)
Moderately	14 (44%)
Poorly	17 (53%)
Preoperative locoregional treatment	
No	11 (34%)
Yes	21 (66%)
Serum AFP (ng/ml)	693 (<5 to 8110)

Data are expressed as mean ± SD, mean (range), or *n* (%).
HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.

Risk factor analyses

We excluded two patients, in whom the anhepatic AFP mRNA status was indeterminate, from our univariate analysis of the significance of the postoperative AFP mRNA status. Univariate analyses of the risk factors for HCC recurrence using the log-rank test showed that the presence of AFP mRNA-positive cells in the peripheral blood in the preoperative period was correlated with HCC recurrence ($P = 0.002$; Table 3). The presence of microscopic vascular invasion and Milan criteria were also associated with HCC recurrence ($P = 0.001$ and 0.049, respectively, Table 3). On the other hand, the risk of HCC recurrence between the two groups divided based on tumor size ≥3/<3 cm, tumor number >10/≤ 10, and the degree of tumor differentiation tended to differ, although the differences did not reach statistical significance (Table 3).

In the patients with HCC exceeding the Milan criteria ($n = 18$), the presence of AFP mRNA-positive cells in the

Table 2. Patients' characteristics (AFP mRNA and HCC recurrence, $n = 32$).

Case	AFP mRNA			HCC recurrence [location, time after LDLT (month)]	AFP mRNA at the time of HCC recurrence	Follow-up period (months)
	Preoperative	Anhepatic	Postoperative			
1	–	–	–	–		65.1
2	–	NA	–	Lung, 21.0	NA	59.5
3	+	+	–	Lung, 18.0	–	43.8
4	–	+	–	–		54.7
5	–	NA	–	Bone, 12.2	–	30.1
6	–	–	–	–		51.3
7	–	+	–	Peritoneum, 11.7	–	49.1
8	–	–	–	–		48.7
9	–	–	–	–		42.5
10	–	–	–	–		40.6
11	–	–	–	–		40.3
12	–	–	–	Liver, 17.8	NA	27.0
13	+	–	–	Lung, 12.8	–	33.8
14	–	–	–	–		34.2
15	–	–	–	–		33.2
16	–	–	+	–		30.6
17	–	+	+	–		30.3
18	–	+	+	Adrenal gland, 10.4	–	28.3
19	–	–	–	–		18.1
20	–	–	–	–		17.6
21	–	–	–	–		16.9
22	+	–	+	Lung, 5.0	+	12.3
23	–	–	–	Bone, 5.3	NA	14.9
24	–	–	+	–		13.0
25	–	–	–	–		2.5
26	–	+	+	–		10.8
27	–	–	–	–		9.4
28	–	–	–	–		8.9
29	–	–	–	–		8.6
30	–	–	–	–		8.2
31	–	–	–	–		6.2
32	–	–	+	–		0.9

NA, not available; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.

peripheral blood correlated significantly with HCC recurrence (preoperative AFP mRNA, $P = 0.041$, Fig. 1).

The multivariate analysis using Cox's proportional hazards model revealed preoperative AFP mRNA and vascular invasion as independent risk factors for HCC recurrence (HR 10.8, 95% CI: 1.53–76.9, $P = 0.017$, and HR 21.7, 95% CI: 2.92–166.7, $P = 0.003$, respectively; Table 4).

Discussion

Liver transplantation for HCC has become widespread as more adult-to-adult LDLT using right lobe grafts are performed in Asian and western countries [7,8]. The Milan criteria reported by Mazzaferro *et al.* [9] have been widely adopted and are the cornerstone of pretransplantation evaluation of patients with HCC. Nevertheless, patients with HCC exceeding the Milan criteria are still considered to have a chance of a substantial survival benefit by liver transplantation [10]. In fact, due to the nature of the direct partial liver donation from relatives, most centers in Japan do not exclude these patients with HCC exceeding the Milan criteria, and consider these patients as appropriate candidates for LDLT as long as they have no extrahepatic metastasis or macroscopic vascular invasion [2]. Under these circumstances, it would be important to be able to predict the risk of HCC recurrence accurately.

To date, many reports have described the risk factors predictive of HCC recurrence after liver transplantation, such as the degree of tumor differentiation, the tumor spread, the serum AFP level, as well as the Milan criteria [1–3]. However, all these risk factors are indirect methods of predicting HCC recurrence, based on the clinical findings of the HCCs. On the other hand, many studies have

Table 3. Risk factors for HCC recurrence after liver transplantation.

	Number of cases	Number of cases with HCC recurrence	Recurrence rate (%)	*P*-value
Milan criteria				
Within	14	1	7	0.049
Beyond	18	8	44	
Tumor size (cm)				
≤3.0	21	4	19	0.067
>3.0	11	5	45	
Tumor number				
<10	23	4	17	0.072
≥10	9	5	56	
Differentiation				
Well/moderately	15	4	27	0.439
Poorly	17	5	29	
Vascular invasion				
–	27	5	19	0.001
+	5	4	80	
AFP				
<400	23	7	30	0.871
≥400	9	2	22	
Preoperative AFP mRNA				
–	29	6	21	0.002
+	3	3	100	
Postoperative AFP mRNA				
–	20	3	15	0.112
+	10	4	40	

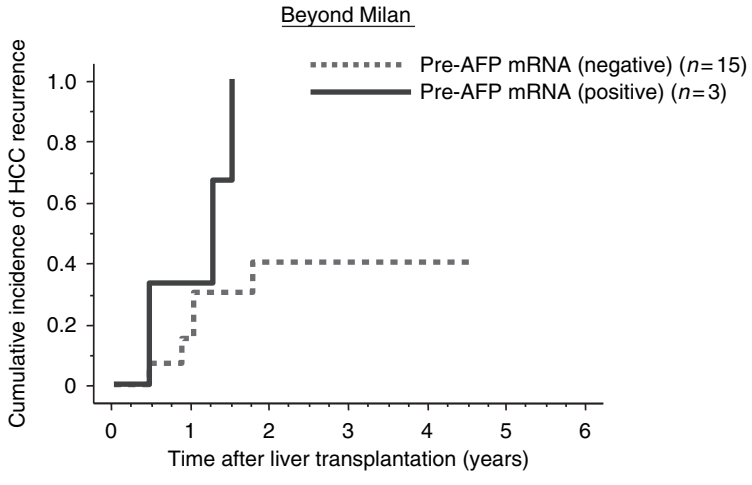
P-value from log-rank test.
HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.

reported the usefulness of the detection of HCC cells in the systemic circulation, as a direct method to predict HCC recurrence after liver resection [4,6,11,12]. It sounds theoretically reasonable to consider the detection of circulating HCC cells as being predictive of HCC recurrence after liver transplantation, but there has been no report as yet of applying this method for liver transplantation.

We have established an accurate and sensitive method for the detection of circulating HCC cells by a real-time RT-PCR method to quantify AFP mRNA [5]. We also confirmed that the positivity of this test is associated with HCC recurrence after liver resection in nontransplant patients [6]. In the present study, we applied the same technique for LDLT patients with HCC.

Univariate analysis showed that microvascular invasion, preoperative peripheral blood positivity for AFP mRNA and the Milan criteria were associated with the risk of HCC recurrence after LDLT. Although the multivariate risk factor analysis using Cox's proportional hazard model might not be a suitable method to study such a small sample size, the results of multivariate analysis together with those of the univariate analysis could be interpreted as supporting the possibility that two factors, namely, microvascular invasion and preoperative peripheral blood positivity for AFP mRNA, were independently associated with the risk of HCC recurrence. Vascular invasion has been reported to be associated with HCC recurrence in the literature [1,2]. The reason why other factors, such as the Milan criteria and the degree of differentiation, were not confirmed to be predictive factors for HCC recurrence is probably because the sample size in the study was relatively small to allow appreciation of any significant difference in the frequency of HCC recurrence the groups.

With regard to the Milan criteria, our data suggest that AFP mRNA detection could differentiate patients with



Number of the patients at risk

AFPmRNA negative	**15**	**10**	**6**	**5**	**2**	**0**
AFPmRNA positive	**3**	**2**	**0**	**0**	**0**	**0**

Figure 1 Cumulative incidence of hepatocellular carcinoma (HCC) recurrence according to the alpha-fetoprotein (AFP) mRNA status. In the cohort of patients with HCC exceeding the Milan criteria also, the presence of preoperative AFP mRNA-expressing cells in the peripheral was significantly correlated with tumor recurrence ($P = 0.041$, log-rank test).

Table 4. Multivariate analysis for HCC recurrence after liver transplantation.

	Hazard ratio (95% CI)	*P*-value
Milan criteria		
Beyond	6.33 (0.42–90.9)	0.189
Tumor size (cm)		
>3.0	0.56 (0.10–3.06)	0.507
Tumor number		
≥10	0.92 (0.17–5.08)	0.922
Vascular invasion		
Positive	21.7 (2.92–166.7)	0.003
Preoperative AFP mRNA		
Positive	10.8 (1.53–76.9)	0.017

P-value from Cox proportional hazards regression analysis.

HCC recurrence after LDLT from those without HCC recurrence even in the cohort of patients with HCC exceeding the Milan criteria (Fig. 1). This result was compatible with the results of the multivariate risk factor analysis showing that the positive AFP mRNA status was an independent risk factor of HCC recurrence.

Preoperative treatments for HCC, such as RFA or TACE, prior to the liver transplantation could be related to the preoperative AFP mRNA positivity either because of the spread of the HCC cells or because of the disruption of hepatocytes into the systemic circulation. However, all the three patients who were positive for AFP mRNA preoperatively had received preoperative treatment for 4 months, 8 months, and 26 months, respectively, prior to the liver transplantation, while the two patients who had received preoperative treatment within 4 weeks prior to the liver transplantation were not positive for AFP mRNA preoperatively, suggesting that the preoperative positivity for AFP mRNA in this study was not likely to be related to the preoperative treatment for HCC.

From the standpoint of the timing of AFP mRNA detection, only preoperative AFP mRNA detection was associated with HCC recurrence, and was an independent risk factor for HCC recurrence, according to the results of both univariate and multivariate analyses. Thus, preoperative AFP mRNA detection seemed to be useful for the prediction of HCC after LDLT. The frequency of detection of preoperative AFP mRNA-expressing cells in the peripheral blood was significantly different between the recipients and the control patients ($P = 0.017$), while a similar incidence of postoperative AFP mRNA positivity was observed in non-HCC patients. Surgical manipulation might have squeezed normal hepatocytes into the systemic circulation, resulting in a positive test result for AFP mRNA in the postoperative phase, regardless of the presence of the circulating HCC cells. Analyses of more cases of HCC may clarify the optimal timing of the test for AFP mRNA detection for predicting HCC recurrence after liver transplantation.

A limitation of this study was the small number of study subjects. Only three patients were positive for AFP mRNA preoperatively, and all of these three patients had HCCs exceeding the Milan criteria. Therefore, these results cannot be generalized to all transplant recipients. Thus, careful interpretation of the positivity of AFP mRNA is necessary at this point, and data from more cases with longer follow-up durations should be accumulated for validation of the preliminary results of this study.

In patients with HCC recurrence, there seemed to be no association between the HCC recurrence and the AFP mRNA detection at the time of the HCC recurrence, while the tumor markers (AFP and/or PIVKA-II) were monotonically increasing in those patients (data not shown). This could be interpreted that the recurrent tumors did not grow enough to release HCC cells into peripheral circulation at the time of HCC recurrence, although they had already produced AFP and/or PIVKA-II, which were, therefore, sensitive markers for recurrent HCC in LDLT patients.

Based on these results, we conclude that the detection of AFP mRNA-expressing cells in the peripheral blood preoperatively could be associated with the HCC recurrence and might serve as a useful predictor of the HCC recurrence after LDLT. More data must be accumulated to confirm these findings.

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